The role of sperm morphology in assisted reproduction (ART)

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Declaration

I, the undersigned, declare that the work contained in this dissertation is an overview of research by me and co-workers over the past two and a half decades, with a central focus point. The results of research stimulated by the original studies, as well as several doctoral theses, are included in the dissertation.

.........................
Theunis Frans Kruger
December 2012

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This DSc thesis is dedicated to my wife, Sanderina, who always inspired me and allowed me to “play” in God’s “micro cosmos”.

Hierdie DSc tesis dra ek graag op aan my vrou, Sanderina, wat my altyd inspireer en ruimte gegee het om te kon “speel” in God se “mikro-kosmos”.
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### Chapter 1

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#### i. Clinical impact at post-graduate level nationally and internationally:


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Verwondering oor God se “mikro-kosmos”:

Die 30 jaar se werk in hierdie veld vul my steeds met verwondering oor die wonder van God se skepping. Saam met die Psalmdigter van Psalm 139, wil ek elke keer uitroep as ons ons verdiep in die fysiologie van bevragting en die ontwikkeling van ’n embrio.

“U het my gevorm, my aanmekaargeweef in die skoot van my moeder. Ek wil U loof, want U het my op ’n wonderlike wyse geskep. Wat U gedoen het, vervul my met verwondering. Dit weet ek seker: geen been van my was vir U verborge toe ek gevorm is waar niemand dit kan sien nie, toe ek aanmekaargeweef is diep in die moederskoot.”

Ps. 139:13-15.
In awe of God’s “Micro Cosmos”

After 30 Years of work (“play”) in the field of reproductive medicine, I am still in awe about the amazing detail in this “micro cosmos”. I want to cry out with the poet of Psalm 139:13-15:

“For You did form my inward parts; You did knit me together in my mother’s womb.

I will confess and praise You for You are fearful and wonderful and for the awful wonder of my birth! Wonderful are Your works, and that my inner self knows right well.

My frame was not hidden from You when I was being formed in secret [and] intricately and curiously wrought [as if embroidered with various colors] in the depths of the earth [a region of darkness and mystery].

Ps. 139:13-15 (AMP)
Voorwoord
Hierdie reis het sekerlik begin in my ouerhuis. Net ’n paar gedagtes bly voorop staan:

Geluk as kind en baie speel, lê fondasies vir kreatiwiteit. Dit was my gegun.

*Dankbaarheid* en *genade* is werklik die oorheersende gedagtes terwyl ek besig was om hierdie stuk te skryf.

Die vraag wat dikwels gevra word is, of dit geluk is wat jou op jou lewenspad neem in ’n sekere rigting? My persoonlike antwoord is *nee* – oorheersend is die gedagte van ’n Groter Mag wat my bestuur en begenadig het. Daarom die woorde *dankbaar* en *genade* wat my vul as ek ook dink aan my loopbaan as “n reis en die DSc-tesis, wat dié verhaal vertel.

*Netwerke* – „dinge” wat presies reg verloop het en my op dié besondere baan geplaas het. Dit was ’n voorreg om in die “mikro-kosmos” te kon werk en droom en ja, *speel*.

*Soli Deo Gloria.*
Julie 2011

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Foreword
This journey started as a child in a very blessed and happy environment. I believe that a child in this type of environment has no excuse but to excell and to be creative. Life is a network of contacts, and influences and opportunities. I am filled with gratitude towards my Creator for the opportunity to work in this field which to me is a neverending micro-cosmos in the field of reproductive biology. When I look back over my career, it was a journey with so much joy and excitement. The DSc is the story of this 30-year journey.

*Soli Deo Gloria.*
July 2011
Acknowledgements:

Numerous people have contributed to this DSc journey which is in a way a life journey, a history of my and our group’s research. Perhaps it all started with my parents who created a loving home environment where we could grow up unscathed. While in Hoogenhout High School in Bethal, Mr Wim de Klerk stimulated us to look beyond the known borders, to be inquisitive and he encouraged us to dream. I later realized that research is first dreaming and then asking a simple question to be answered. Research is about team work.

I want to thank:

- My wife Sanderina who was and still is my inspiration. On a professional level she set an academic career in Law aside and was always there with encouragement to me and our children. She created an environment in our home that stimulated academic work. The later we could burn the academic candle the better.

- My children, Wilma, Pieter, Gerrit and Jana who gave me so much joy through the years and whom I must encourage with one simple principle that I learned from my dear Mother: “Always give your utmost best – only the best is good enough and discover the joy of giving”.

- All of my co-authors who contributed directly and indirectly to the work presented in this thesis. I know that one is as strong as your team. Thank you again for the privilege to have played in an exceptional team.

- Ms. Erna Vos for excellent editorial assistance as well as with design and layout of the manuscript. Without her help the endproduct would not have met the high standards that she has achieved.

- Thanks to Prof Gerhard Theron, who sowed the seed and encouraged me to embark on this DSc road. Thanks for his guidance from chapter to chapter to deliver a quality product. Also scientific Promotor Prof Ralf Henkel for his invaluable and meticulous guidance.
The late Prof. Willie van Niekerk brought so much to our department – expertise, innovative thinking, and research skills. We all build on this foundation.

Prof. Hein Odendaal a respected international researcher. He was the promoter to my MD and guided me on the first steps of this fascinating research path.

Dr. Howard Jones my USA mentor who will always be a role model to me. He embarked on a second career when he was 67 years of age and the next 25 years were crowned with so much success. He set an example for many to follow.

Prof. Roelof Menkveld for his role in defining the „normal form” and I want to honor him for his contribution to the field of Andrology and the foundation he laid in the field of sperm morphology on which we all could build.

Prof. Danie Franken for sharing so much creative moments in the dreaming phases of our combined research. He is a very creative person. Our dreaming however also lead to a large number of completed projects.

Mr. Frik Stander for the hours of work that we have spent in the IVF laboratory and our quest for answers in the beginning years of IVF brought so many happy moments.

Dr. Marie-Lena Windt de Beer who was my PhD student. A pillar of strength in the IVF world. Her PhD led to the first successful ICSI baby in SA. She is also the cornerstone of teaching in our MSc program. Also a true teacher and „mother” for her students.

Mr. Greg Tinney for his meticulous work and total honesty on a day to day basis in the IVF laboratory at Vincent Palotti Hospital.

Prof. Igno Siebert for his friendship, enthusiasm about research and new ideas. Also for his willingness to play a roll in this project. It was indeed a privalage to have worked with him over the last ten years.
Dr. Kobie van der Merwe for his friendship over 35 years. We have shared so much at clinical level and personal level, but also about the miracle of God’s creation. I honor him for his pioneering work in the field of endoscopic surgery, bringing yet another area of reproductive medicine on par with international standards.

Ms. Madeleine du Toit for her invaluable editorial assistance during and at the end of the project. Her help was much appreciated by myself and my secretary, Erna Vos.

Prof. Marietjie de Villiers for her idea about the educational aspect of research. This led to the development of chapter 6.

Prof. Sergio Oeningher for his friendship and the many exiting projects that we have completed during the last 25 years.

Dr. Anibal Accosta for his assistance in our combined research efforts during the post doctoral visit to the Jones Institute in Norfolk, Virginia and afterwards.

Dr. Johan Steytler, a friend and colleague who suggested the format of the last pages of chapter 6 (chronological approach).

Various publishers for permission to be able to use the articles and chapters from text books in this thesis.
Summary

The role of sperm morphology in assisted reproduction (ART)

Chapter 1

With the first publication in 1986 (Kruger et al., 1986) on the impact of sperm morphology on fertilization and pregnancy rate in assisted reproduction, it was not realized how this observation would impact on the research and clinical practice.

To reiterate the conclusion of the 1986 study: „It was clearly shown that sperm morphology is an important factor in predicting fertilization and pregnancy outcome in In vitro fertilization (IVF). In a logistic regression model it was outlined that the number of oocytes are also playing an important role in outcome and that the male factor must not be viewed in isolation.” In this first publication, the importance of the strict criteria and how it differed from the then WHO criteria was highlighted.

In an article published in 1988 (Kruger et al., 1988) 3 morphology patterns were described. The P-pattern (0 to 4% normal forms) was described as the poor prognosis pattern group. A fertilization rate of 7.6% was reported for the P-pattern group compared to the 63.9% for the G-pattern group (5 to 14% normal forms). The G-pattern was defined as those with a good prognosis. The term N-pattern was used for those with normal forms above 14% normal forms and in previous publications had a fertilization rate of more than 80%. This publication and the 1986 publication mentioned above became the 2\textsuperscript{nd} and 3\textsuperscript{rd} most quoted publications in the history of Fertility and Sterility as reported in 2006 (Yang and Pang 2006).

Chapter 2

Based on a structured literature review new semen thresholds for the subfertile male were suggested (van der Merwe et al., 2005). One important inclusion criteria was the use of strict morphology criteria (Menkveld et al., 1990) in the studies on fertile and infertile
couples in the *In vivo* situation. The following semen values were suggested for the sub-fertile male: Morphology < 5% normal forms, concentration below 10 million per ml. and motility below 30%. It was emphasized that by using the sperm morphology parameter in combination with the other semen values, the clinical value of the semen analysis should be improved.

**Chapter 3**

In the light of the findings discussed in Chapters one and two, an interest developed to understand the pathophysiological mechanisms involved in the low fertilization rates in men with severe teratozoospermia (P-pattern sperm morphology). For that reason, a study was undertaken to correlate the different morphological patterns with the hamster penetration assay (SPA test) (Kruger *et al*., 1987). A good correlation was observed between poor penetration of the hamster oocytes (Kruger *et al*., 1987) as well as low binding in the hemi-zona assay (HZA) (Franken *et al*., 1990) and P-pattern sperm morphology. It was also reported that poor morphology correlates with abnormal calcium influx (Oehninger *et al*., 1994) and abnormal acrosome reaction (Bastiaan *et al*., 2003). The hemi-zona assay (HZA) was found to be a good predictor of fertilization *In vitro* (Oehninger *et al*., 2000). The enzyme acrosin was also studied in 1988 but it could not be shown that a correlation exist between sperm morphology and the enzyme acrosin (Kruger *et al*., 1988) although other researchers did find a correlation (Menkveld *et al*., 1995).

The hemi-zona test (HZA) became an important clinical tool and functional assay. An example of laboratory research with clinical application is the article about preservation of oocytes to assist laboratories in using this test on a day to day basis as fresh oocytes were not always readily available (Kruger *et al*., 1991). This article assisted the laboratories interested in the HZA, to be able to provide the test as a routine test to the clinician or scientist.

**Chapter 4**

The publications on computerised strict sperm morphology were novel in this field. In 1993, the first publication in the international literature followed after research at the
Stellenbosch University and Tygerberg Hospital on the computer and correlation with the manual method (Kruger et al., 1993).

The aim of the first research was to correlate strict criteria (manual method) with strict criteria evaluated by computer using image analysis (computer method). What was interesting was the finding of an excellent level of agreement between the human and the computer. The correlation was the same as if two experienced human readers were evaluating the sperm morphology slides. Using various statistical methods it was reported that an excellent measure of agreement was reached. These observations set the stage for studies between the computer method and the manual method.

The first publication on sperm morphology not only dealt with technical aspects of sperm morphology evaluation but also with clinical outcome (Kruger et al., 1993). Studying fertilization rates in patients with sperm morphology < 14% and > 14 % normal forms in 2 centers, a significantly lower fertilization rates in the group below 14% normal forms highlighted the fact that a new diagnostic tool to predict fertilization in vitro was now available.

**Chapter 5**

The value of sperm selection has been studied extensively over the last 10 years in an attempt to improve fertilization and pregnancy rates in intra cytoplasmic sperm injection (ICSI) programs.

It was the work of Bartoov (Bartoov et al. 2001) that brought the concept of sperm selection to the forefront. The selection of spermatozoa with high magnification, selecting the ideal form if possible, solved poor pregnancy rates in couples with repeated failures. This method was called intracytoplasmic morphology selected sperm injection (IMSI). If no normal sperm could be found for sperm injection, Berkovitz observed a poorer embryo quality and lower pregnancy rates with higher abortion rates (Berkovitz et al. 2006).

Recently, in a randomized controlled trial Antinori reported a significant improved pregnancy rate in the IMSI group versus the ICSI group (Antinori et al. 2008). With annexin
V binding it was observed that non-apoptotic sperm. This observation confirmed the early finding that the random selection of the ideal form will assist in obtaining a „healthier“ sperm to be used for ICSI (Hoogendijk et al., 2009).

Huzar have shown that mature spermatozoa selectively bind to solid state Hyaluronic acid (HA) (Huzar et al., 2007). These spermatozoa are devoid of cytoplasmic retention, persistent histones, DNA fragmentation with lower frequency of chromosomal aneuploidy. The normal oval shape spermatozoa were also significantly richer in hyaluronic acid compared to the amorphous forms. A single blind collaborative study between Huzar at Yale and the Stellenbosch group followed. After hyaluronic acid selection took place of spermatozoa, with a non selected control group, the percentage normal forms were read. This led to the publication by Prinosilova (Prinosilova et al., 2009) confirming that a significant improvement in selection of the ideal forms took place in the test samples (hyaluronic acid) compared to control.

In this chapter, a new classification for spermatozoa to be used for IMSI is proposed based on physiological (Garolla et al., 2008; Huzar et al., 2007) and electron microscopical principles (Chemes et al., 2007).

To summarize: The original research reported on the normal form and its impact on fertilization and pregnancy rates in In vitro (Kruger et al., 1986; Kruger et al., 1988). In addition physiological defects were reported in the patients with severe abnormalities in sperm shape (P-pattern). Twenty years later the same observations were made for individual spermatozoa and the importance realized of selection of the ideal form for improved results in ICSI programs (Bartoov et al., 2001; Berkovitz et al., 2001; Huzar et al., 2007; Prinosilova et al., 2009).

❖ Chapter 6

The research on sperm morphology had an impact on the interpretation of the semen analysis by students in training and also general practitioners country wide. As the research progressed, the chapter on male infertility was updated. The medical community thus knew how to handle a male factor and when to refer to a tertiary centre for fertility treatment.
At postgraduate and international level, the textbooks and sperm morphology atlases updated knowledge and gave more practical information on how to handle a patient with an abnormal semen analysis. The basis of acceptance by the WHO in 1999 (WHO 1999) and more so in 2010 (WHO 2010) of strict criteria was also highlighted and discussed in this section.

❖ Conclusion

Since the first publication in 1986 (Kruger et al., 1986), the international scientific community’s understanding of sperm morphology has changed. Not only were new morphology patterns described with clinical application, but it was also shown that the P-pattern group had more DNA abnormalities (Garolla et al., 2008), sperm binding/function was defective (Franken et al., 1990) and the potential to fertilize In vitro or In vivo was reduced (Kruger et al., 1988; van Waart et al., 2001). New semen fertility thresholds were suggested which was accepted in 2010 by the WHO (Van der Merwe et al., 2005; WHO 2010). The computerised method (Kruger et al., 1993) to evaluate sperm morphology brought a new objective tool to evaluate the male factor.

Initially an attempt to overcome the low fertilization chance was to increase the sperm insemination concentration (Franken et al., 1990). With the development of the ICSI technique this problem was partially overcome but with better sperm selection (strict evaluation) the prognosis of severe male factor patients and those with P-pattern morphology was further improved (Antinori et al., 2008).

The finding that sperm morphology affects fertilization In vivo and In vitro stimulated research in this field tremendously which brought new insight in male factor infertility.
Opsomming

❖ Hoofstuk 1

Die eerste publikasie in 1986 oor sperm morfologie (Kruger et al., 1986) het ‘n verreikende effek op navorsing en kliniese praktyk gehad in jare wat gevolg het. In 1988 word die effek van sperm morfologie verder bestudeer en word die 3 patrone beskryf naamlik die P-patroon, G-patroon en N-patroon. In die studie (Kruger et al., 1988) word rapporteer dat in die P-patroon, 0-4% normale vorme, die bevrugting syfer slegs 7.6% was teenoor die G-patroon, 5-14% normale vorme, 63.9 % en in die N-patroon, >14% normale vorme, meer as 80% bevrugting.

Die twee artikels word belangrike artikels in die veld van diagnostiese andrologie en volgens Fertility and Sterility in 2006 die tweede en derde mees aangehaalde publikasies in die geskiedenis van die joernaal (Yang and Pang, 2006).

❖ Hoofstuk 2

Studies wat handel oor die fertiele en subfertiele populasies word bestudeer en ‘n artikel volg wat die afsnypunte vir subfertiele pasiënte voorstel: Morfologie < 5% normale vorme, ‘n semen konsentrasie onder 10 miljoen per ml. en motiliteit onder 30% (van der Merwe et al., 2005). Dit word beklemtoon dat alle parameters in berekening gebring moet word in die evaluasie van die man en nie net byvoorbeeld sperm morfologie alleen nie.

❖ Hoofstuk 3

In hierdie hoofstuk word die sperm patofisiologie bestudeer veral in die P-patroon groep in vergelyking met die ander 2 patrone (G- en N-).

Daar word aangetoon dat die sperm binding op die zona pellucida betekenisvol laer is in die P-patroon as in die G- en N-patroon (Franken et al., 1990) Die hamster penetrasie toets wys ook laer penetrasie teenoor die ander patrone (Kruger et al., 1987), so ook is die akrosoom reaksie defektief in die P-patroon groep (Bastiaan HS et al., 2003). Verder word
gesien dat die P-patroon korreleer met abnormale kalsuim invloei teenoor kontrole groep (G- en N-patroon). Dit word dus duidelik deur hierdie waarnemings waarom die bevrugtingskoers so laag is in die P-patroon groep en die fisiologiese mekanismes betrokke by die bevrugtingsproses kan nou beter verklaar word.

❖ **Hoofstuk 4**

Die publikasies in 1993 oor die gerekenariseerde sperm morfologie beoordeling, was uniek en die eerste oor streng morfologie beoordeling internasionaal met behulp van "n rekenaar (Kruger et al., 1993).

Daar word aangetoon dat die rekenaarlesings en die mikroskopiese lesings (manueel) uitstekend korreleer en dat die rekenaar soos die manuele metode bevrugting *In vitro* kan voorspel (Kruger et al., 1993). Die gevolgtrekking was duidelik, dat hier nou "n nuwe objektiewe metode was om te gebruik om die man se vrugbaarheid te bepaal en ook bevrugting *In vitro* te voorspel.

❖ **Hoofstuk 5**

Sperm seleksie word belangrik in die era van ICSI (mikro inspuiting van sperme in ova). In twee studies word dit aangetoon dat die normale vorm korreleer met hialironidase seleksie van sperme (Prinosilova et al., 2009) en ook met non-apaptotiese sperm (Hoogendijk et al., 2009).

Hierdie werk sluit ook aan by die rapporte van Bartoov oor "n beter swangerskap uitkoms met ICSI as die sperm onder hoëveld vergroting geselekteer word en die ideale vorm ingespuit word (Bartoov et al., 2001).

Die skrywer (TFK) stel "n nuwe spermklassifikasie metode voor, gegrond op fisiologiese en elektronmikroskopiese data in "n poging om kans op swangerskap verder te verbeter.
Hoofstuk 6

In hierdie hoofstuk word daar aangetoon dat die navorsing oor subfertiele mans voordurend opdateer was in handboeke aan voor- en nagraadse studente, om sodoende geneeshere en studente die nuutste inligting in hierdie veld te kon gee. Verder is ook klem gelê op verwysingsriglyne, gebasseer op die subfertiele afsnypunte van die semenanalise.


Ten slotte:

Die internasionale wetenskaplike gemeenskap se interpretasie van sperm morfologie het verander sedert die eerste publikasie in 1986 (Kruger et al., 1986). Nuwe sperm morfologie patrone is beskryf met duidelike kliniese toepassing. Dit is ook aangedui dat die P-patroon groep meer DNA abnormaliteite (Garolla et al., 2008) getoon het en die spermfunksie wat betref binding op die zona pellucida (Franken et al., 1990), was defekter. Verder was die bevrugtingspotensiaal in vitro en in vivo verlaag in die P-patroon groep (Kruger et al., 1988; van Waart et al., 2001). Nuwe semen afsnypunte vir subfertilité is voorgestel en aanvaar deur die WGO (Van der Merwe et al., 2005; WGO 2010). Die gerekenariseerde evaluasie van sperm morfologie het “n nuwe objektiewe instrument navore gebring om manlike infertiliteit te evalueer (Kruger et al., 1993).

Aanvanklik was gepoog om die lae bevrugtingskans in die P-patroon te oorkom deur die inseminasiekonsentrasie te verhoog (Franken et al., 1990). Met die ontwikkeling van die mikro-inspuitingstegniek (ICSI) is hierdie probleem grootliks oorkom. Met beter spermseleksie het die prognose van ernstige manlike infertiliteit (P-patroon) verder verbeter (Antinori et al., 2008).
Die bevinding dat sperm morfologie bevrugting *In vitro* en *In vivo* affekteer, het navorsing in hierdie veld geweldig gestimuleer. Hierdie werk het nuwe insig in die veld van manlike infertiliteit na vore gebring.
Literature cited in the Foreword and Summary


Van der Merwe FH, Kruger TF, Oehninger SC, Lombard CJ. The use of semen parameters to identify the subfertile male in the general population. Gynecol Obstet Invest 2005;59:86-91


Yang H, Pan B. *Citation classics in Fertility and Sterility 1975 – 2004*. Fertil Steril 2006;86(4)795-797
Chapter 1
## Detailed Index – Chapter 1

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<td>Kruger TF, Coetzee K. The role of sperm morphology in assisted reproduction. Hum Reprod 1999;5:172-178.</td>
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</table>
c. **To explore the influence of sperm morphology on IUI pregnancy rate**

### i. *Data on intra uterine insemination (IUI)*


### ii. *The male factor and IUI*


### Conclusion

### References
With the first publication in 1986 (Kruger et al., 1986) on the impact of sperm morphology on fertilization and pregnancy rate in assisted reproduction, it was not realized how this observation would impact on the research and clinical practice.

To reiterate the conclusion of the 1986 study: ‘It was clearly shown that sperm morphology is an important factor in predicting fertilization and pregnancy outcome in in vitro fertilization (IVF). In a logistic regression model it was outlined that the number of oocytes are also playing an important role in outcome and that the male factor must not be viewed in isolation.’ In this first publication, the importance of the Strict Criteria and how it differed from the then WHO criteria (WHO, 1980) was highlighted.

In an article published in 1988 (Kruger et al., 1988) 3 morphology patterns were described. The P-pattern (0 to 4% normal forms) was described as the poor prognosis pattern group. A fertilization rate of 7.6% was reported for the P-pattern group compared to the 63.9% for the G-pattern group (5 to 14% normal forms). The G-pattern was defined as those with a good prognosis. The term N-pattern was used for those with normal forms above 14% normal forms and in previous publications had a fertilization rate of more than 80%. This publication and the 1986 publication mentioned above became the 2nd and 3rd most quoted publications in the history of Fertility and Sterility as reported in 2006 (Yang and Pang, 2006).
Sperm morphology in assisted reproduction (ART) \{In vitro fertilization (IVF) and intrauterine insemination (IUI)\}

a. To explore the influence of sperm morphology on ART pregnancy outcome

i. In vitro fertilization (IVF)

With the first publication in 1986 on the impact of sperm morphology on fertilization and pregnancy rate in assisted reproduction (Kruger et al., 1986), it was not realized how this observation would impact on the research in this field and the clinical arena in ART in the next 30 years. Initially it was thought that an observation was made that was unique to South Africa, but with my Post Doctoral visit in 1986 to The Eastern Virginia Medical School, Norfolk Virginia, USA, it was realized that the same sperm morphology defects were visible in those male patients in the USA. At the Jones Institute, patients often presented with non fertilization but the scientists did not recognize that there was a sperm morphology problem in many of these men and that this sperm defect could explain the non-fertilization. They were made aware of this abnormality and this awareness led to a second prospective observational study on the impact of sperm morphology on fertilization rate and pregnancy rate in their ART program. The data correlated with that of the Tygerberg publication of 1986 in Fertility and Sterility and a second publication followed in 1987 with data obtained at the Jones Institute in Norfolk (Kruger et al., 1987). The information on sperm morphology was also shared for the first time with the international community by Dr Anibal Acosta at the World Conference on IVF in 1987 which was hosted by the Jones Institute in Norfolk in the autumn of 1987. This lecture and the publications in 1986, 1987 and 1988 stirred a renewed interest in the field of male infertility worldwide and especially sperm morphology. Numerous publications followed on this topic with the majority confirming the initial observations on fertilization and pregnancy in In vitro fertilization.

It was stated in the first publication in 1986 that different means of assessing normal morphologic features have been described. However, to answer the question of whether there is a prognostic value in this parameter regarding the fertilization and pregnancy rate in human In vitro fertilization (IVF) a prospective study was conducted using Strict Criteria (Menkveld et al., 1990) to evaluate the sperm morphology.
The following article reflects the scientific basis that supports the above argument:

Sperm morphologic features as a prognostic factor in in vitro fertilization

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Roelof Menkved, M.Sc.∗
Frik S.H. Stander, Cytotechnician∗
Carl J. Lombard, Ph.D.†
Jacobus P. Van der Merwe, M.Med. (O & G)∗
Johannes A. van Zyl, M.D.∗
Karen Smith, Technician∗

Tygerberg Hospital, and Medical Research Council, Tygerberg, Republic of South Africa

To determine whether there is a prognostic value in the percentage normal sperm morphologic features in a human in vitro fertilization (IVF) program, the authors conducted a prospective study in women with bilateral tubal damage. Based on the percentage of morphologically normal spermatozoa, the patients were divided into four groups: group I, normal morphologic features between 0% and 14%; group II, 15% to 30%; group III, 31% to 45%; and group IV, 46% to 60%. One hundred ninety successful laparoscopic cycles were evaluated. In group I, 104 oocytes were obtained, of which 37% fertilized, but no pregnancy resulted; in group II, 81% of 324 oocytes were fertilized, with a pregnancy rate per embryo transfer (ET) of 22%; in group III, 82% of 309 oocytes were fertilized, with a 31% pregnancy rate; and in group IV, 91% of 69 oocytes were fertilized, with a pregnancy rate of 12%. Probability models indicated that there was a clear threshold in normal sperm morphologic features at 14%, with high fertilization and pregnancy rate in the groups with normal sperm morphologic features > 14%. Fertil Steril 46:1118, 1986

Sperm count, motility, and the percentage normal morphologic features have been the traditional criteria for semen quality. In 1976 Van Zyl et al. proposed a reclassification of the criteria for "normal" semen parameters. The in vitro penetration of zona-free hamster eggs by human spermatozoa has become a valuable new tool in the assessment of human semen, but this test is not easy to perform, and the results have not always been consistent between laboratories. Rogers et al. analyzed sources of variability in the assay and stressed quality control.

Evaluation of the percentage normal sperm morphologic features is subjective and difficult to compare between different laboratories throughout the world. Different means of assessing normal sperm morphologic features have been described. Although it is difficult to compare the morphologic features, the critical issue is what the morphologic features actually tells us in a specific laboratory or clinic. To answer the question of whether there is a prognostic value in this parameter regarding the fertilization and preg-
nancy rate in a human in vitro fertilization (IVF) program, we conducted a prospective study.

MATeRIALS AND METHODS

PATIENTS

The women accepted into the program had bilateral tubal damage, diagnosed with the use of laparoscopy and hysterosalpingography. Their male partners had four semen analyses before the women were accepted into the IVF program.

The semen analysis methods used in our laboratory were described in detail by Van Zyl. The methods used for determination of the three variables used in this study are, in short, as follows. The semen samples were obtained after 3 to 4 days of abstinence by masturbation at the laboratory. Immediately after liquefaction, a drop of the well-mixed specimen was placed on a clean and prewarmed glass slide at 37°C, covered with a cover slip, and left for a few minutes. The microscope was provided with a hot stage to keep the slides at 37°C. The preparation was examined under a magnification of both ×10 and ×40 objectives. The quantitative motility or percentage of motile spermatozoa and qualitative motility or speed of forward progression was assessed in at least ten separate randomly selected high-power fields, as described by MacLeod. At the same time, presence of agglutination and particulate debris was observed and an estimate of the sperm concentration made. The viability (i.e., percentage of live and dead spermatozoa) was determined with the use of supravital staining.

Depending on the estimated sperm concentration, a 1/10, 1/20, or 1/100 dilution of the semen sample was made with the use of a glass tuberculin syringe, instead of a white blood cell pipette. An improved, double-ruled Neubauer hemocytometer (Assistent, FRG) was used for counting the spermatozoa. Two dilutions were made for every sample. The difference between the two dilutions for each sample was not > 10% for low concentrations and not > 20% for concentrations of > 60 × 10⁶/ml.

The following procedures were used for the assessment of the morphologic characteristics of the spermatozoa. The slides were thoroughly cleaned, washed in alcohol, and dried before use. For a good, reliable, and repeatable assessment, a thin and well-spread smear was made so that each spermatozoon could be clearly and individually visualized. The smears were air-dried and on the following day fixed and stained according to the Papanicolau method. The morphologic classification used in the Tygerberg hospital unit is based on a modification of the methods described by MacLeod and Eliasson. This system takes the whole spermatozoon, as well as the presence of germinal epithelial cells, into consideration.

In this laboratory, a spermatozoon is considered normal when the head has a smooth, oval configuration with a well-defined acrosome comprising about 40% to 70% of the spermhead. Also, there must be no neck, midpiece, or tail defects and no cytoplasmic droplets of more than one-half the size of the spermhead. In contrast with other authors, we consider borderline forms abnormal. At least 100, but preferably 200, spermatozoa with tails were classified into one of seven groups: normal (head and tail normal), normal head but with an other abnormality present, large heads, small heads, tapering heads, duplicated heads or amorphous heads all with or without tail, neck or midpiece defects. Tail, neck, and midpiece defects, loose head, immature germinal cells, and unknown cells were recorded separately and reported per 100 spermatozoa. The size of the spermatozoon were evaluated in five different areas to ensure a more randomized evaluation.

All of the men had a normal spermatozoon concentration of ≥ 20 × 10⁶/ml, normal motility of ≥ 30%, and a normal forward progression of ≥ 2.0. In some of the patients, the percentage normal sperm morphologic features was < 20%. On the basis of previous experience, we prospectively divided all of the men into four groups based on the percentage normal morphologic features evaluated on the day of insemination in the IVF cycle. In group I the percentage normal morphologic features was 0% to 14%; in group II, 15% to 30%; in group III, 31% to 45%, and in group IV, 45% to 60%.

The semen samples were obtained 2.5 hours before insemination and prepared as follows: 1 ml semen was diluted with 2 ml of Ham’s F-10 medium (GIBCO, Grand Island, NY) and washed twice with centrifugation at 200 × g for 10 minutes. After the final wash, the supernatant was discarded and 1 ml of medium was layered over the pellet. The tube was placed in the incubator at 37°C for 30 minutes. A count was performed after 30 minutes and the motility recorded.

All of the women received a combination of clomiphene citrate (CC) and human menopausal

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Table 1. Number of Cycles, Cycles with No Fertilized Oocytes, Cycles with Embryo Transfer, Cycles with Pregancies, Pregnancy Rate per Successful Laparoscopy, and Pregnancy Rate per Embryo Transfer

<table>
<thead>
<tr>
<th></th>
<th>Group I (0%–14%)</th>
<th>Group II (15%–30%)</th>
<th>Group III (31%–49%)</th>
<th>Group IV (46%–60%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles observed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>22</td>
<td>83</td>
<td>67</td>
<td>18</td>
<td>190</td>
</tr>
<tr>
<td>%</td>
<td>12</td>
<td>44</td>
<td>35</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Cycles with 0 fertilized oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>11</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>%</td>
<td>50</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Cycles with embryo transfer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>10</td>
<td>72</td>
<td>62</td>
<td>17</td>
<td>161</td>
</tr>
<tr>
<td>%</td>
<td>45</td>
<td>87</td>
<td>93</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Cycles with pregnancies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16</td>
<td>19</td>
<td>28</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Pregnancy rate per successful laparoscopy (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy rate per embryo transfer (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22</td>
<td>31</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*Percentage of cycles in group with no fertilized oocytes.
+aPercentage of cycles in group with embryo transfer.

gonadotropin (hMG) as outlined previously.16

The oocyte recovery took place 36 hours after 10,000 U of human chorionic gonadotropin (hCG) was injected. Each oocyte was incubated in 1.5 ml of Ham's F-10 medium with 10% patient's serum in a Petri dish (Falcon Plastics 3037, Oxnard, CA) for 5 to 6 hours. Insemination took place with 100,000 spermatozoa/ml of insemination medium. Fertilization was recorded after 12 to 16 hours if two pronuclei could be detected and, finally, if cleavage occurred. A pregnancy was defined as a β-hCG, which doubled from day 10 to 12 and had to be confirmed at 7 to 8 weeks with the use of ultrasound examination. The pregnancy rate was computed by dividing the number of pregnancies by the number of successful laparoscopies and embryo transfers (ET).

RESULTS

Two hundred five laparoscopies were performed, and 190 successful laparoscopic cycles in 129 patients were evaluated. (In these cycles, oocytes were obtained.) Eighty-six patients had only one cycle, 30 had two cycles, 9 had three cycles, 3 had four cycles, and 1 had five cycles repeated.

In group I (morphologic features 0% to 14%) 22 cycles, in group II 83 cycles, in group III 67 cycles, and in group IV 18 cycles were observed (Table 1). In group I, 104 oocytes were obtained; of these, 37% fertilized. In group II there were 324 oocytes, with a fertilization rate of 81%; in group III, 309 oocytes, with a fertilization rate of 82%; and in group IV, 69 oocytes, with a fertilization rate of 91% (Table 2).

In group I, 45% of patients with a successful laparoscopy reached the ET stage; in group II 87%; in group III 93%; and in group IV 94% (Table 1).

The pregnancy rate per ET was 0% in group I; 22% in group II; 31% in group III; and 12% in group IV (Table 1). In five couples with repeated cycles, the man was noted to have values both below and above the threshold of 14% normal sperm morphologic features (Table 3). The mean sperm concentrations and motility are shown in Table 4.

Logistic regression was used to investigate the associations of certain variables with pregnancy outcome.

Table 2. Fertilization Rate per Oocyte

<table>
<thead>
<tr>
<th></th>
<th>Group I (0%–14%)</th>
<th>Group II (15%–30%)</th>
<th>Group III (31%–49%)</th>
<th>Group IV (46%–60%)</th>
<th>Total</th>
</tr>
</thead>
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<tr>
<td>Total oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>104</td>
<td>324</td>
<td>309</td>
<td>69</td>
<td>806</td>
</tr>
<tr>
<td>%</td>
<td>13</td>
<td>40</td>
<td>38</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Oocytes fertilized</td>
<td>38</td>
<td>264</td>
<td>232</td>
<td>63</td>
<td>617</td>
</tr>
<tr>
<td>Fertilization rate/oocyte (%)</td>
<td>37</td>
<td>51</td>
<td>82</td>
<td>91</td>
<td>77</td>
</tr>
</tbody>
</table>

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Fertility and Sterility
Table 3. Patient Couples with Normal Sperm Morphologic Features Measurements Below and Above the Threshold of 14% Having Repeated Laparoscopies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>% Normal sperm morphologic features</th>
<th>No. of oocytes</th>
<th>No. fertilized</th>
<th>No. transferred</th>
<th>Pregnant*a</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>38</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>16</td>
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<tr>
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<td></td>
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<td>3</td>
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<tr>
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<td>20</td>
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<td>13</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*aPregnancy = 1, no pregnancy = 0.

Spearman correlations between the first and second cycle for morphologic features and number of oocytes for the 43 patients who had first and second laparoscopies were $r = 0.223$, $P = 0.15$ and $r = 0.052$, $P = 0.74$, respectively.

Because the correlations between the repeated cycles were not significant, we considered the 190 cycles independent observations in the probability analysis.

The percentage normal sperm morphologic features and the number of oocytes representing the female factor were the two variables used to investigate the associations with the probability of pregnancy. The (0-1) outcome of pregnancy was considered in the following way: 0 represented failure and 1 represented success.

MODEL A

The logistic regression showed that the male factor, percent normal sperm morphologic features, had a significant nonlinear association and the female factor, number of oocytes, a significant linear association with the probability of pregnancy (model chi-square = 12.95 with 3 degrees of freedom, $P = 0.0047$).

The nonlinear association of morphologic features is of interest if one considers the plot of outcomes in Figure 1. There is a clear threshold in percent normal sperm morphologic features at 14%. In the interval of 0% to 14% normal sperm morphologic features, the number of oocytes varied between 1 and 13, but no pregnancy was obtained in this group. This threshold, together with the absence of any pregnancies in the 50% to 60% interval in normal sperm morphologic features, is the reason for the significant nonlinear association.

What happens between the male and female factors above the natural threshold? To investigate this we modeled a subset of 168 cycles, all falling above the threshold of 14% normal sperm morphologic features.

MODEL B

Logistic regression with a backward elimination procedure was used. All of the variables of model A were presented to the modeling procedure and those that were no longer significantly associated with the probability of pregnancy were eliminated. The result was that the number of oocytes was the only variable that still had a significant linear and positive association with the probability of pregnancy (model chi-square = 5.34 with a 1 degree of freedom, $P = 0.0208$).

Table 4. Mean Sperm Concentration Count and Motility

<table>
<thead>
<tr>
<th>Group</th>
<th>Group I (9%-14%)</th>
<th>Group II (15%-30%)</th>
<th>Group III (31%-45%)</th>
<th>Group IV (46%-60%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count (x 10^6/ml)</td>
<td>mean ± SD</td>
<td>mean ± SD</td>
<td>mean ± SD</td>
<td>mean ± SD</td>
<td>mean ± SD</td>
</tr>
<tr>
<td>53 ± 22.3</td>
<td>78.2 ± 55</td>
<td>84.3 ± 33.6</td>
<td>86.5 ± 39</td>
<td>75.2 ± 44.7</td>
<td></td>
</tr>
<tr>
<td>Motility (% motile)</td>
<td>41.8 ± 11.4</td>
<td>49.0 ± 9.3</td>
<td>50.4 ± 9.9</td>
<td>55 ± 8.8</td>
<td>49.3 ± 10.3</td>
</tr>
</tbody>
</table>

Vol. 46, No. 6, December 1986

Kruger et al. Sperm morphology in IVF
There is a clear threshold in the percent normal sperm morphologic features at 14% in this study (Fig. 1). The fertilization rate per oocyte was 37% in group I, in which the percent normal sperm morphologic features was ≤ 14%; in groups II, III, and IV combined, the rate was 84.6% per oocyte. Mahadevan and Trounson\(^\text{17}\) also indicated that the percentage of abnormal sperm forms was significantly related to the fertilization rate. The chances of patients with successful laparoscopies reaching the ET stage in group I was also reduced to 45%, whereas in the other groups combined, 91% reached the ET stage.

The zona-free hamster egg test is used to test the ability of the human sperm to penetrate the ooplasm. Rogers et al.\(^\text{2}\) indicated that the morphologic factor plays an important role in the fertilization process; 73.7% of fertile men can have a normal count and motility but have lower-than-normal morphologic features. This was also the experience in this unit. If the importance of abnormal morphologic features is not appreciated, these patients can be considered fertile or as cases of unknown infertility. The ability to evaluate morphologic features is subject to experience and a strict protocol in the laboratory, as outlined above. Morphologic features are often judged by laboratory personnel without sufficient experience or background.

The observed proportions of pregnancies given by the results of this study indicate that in the group in whom the percentage normal sperm morphologic features is ≤ 14%, irrespective of the number of oocytes obtained, no pregnancy resulted (the 95% confidence limits are 0% to 16%). In the groups with normal sperm morphologic features > 14%, the female factor (e.g., the number of oocytes obtained) plays an important role in the chances of a pregnancy. If only one to two oocytes are obtained, the chance of a pregnancy

---

**DISCUSSION**

In the Tygerberg Hospital Unit, > 20% normal sperm morphologic features is considered normal, and if all semen parameters are normal such patients are probably fertile.\(^\text{1}\) Van Zyl et al.\(^\text{1}\) observed this tendency when they analyzed the pregnancy rate in the Infertility Clinic at Tygerberg Hospital. One of the reasons why the results in this unit differ from the World Health Organization’s criteria could be the very strict analysis of morphologic features where borderline forms are considered abnormal.

In only five couples with repeated cycles was the percentage normal sperm morphologic features below and above the threshold. The chance that this intrasubject variation in the percentage of the normal sperm morphologic features could be due to a laboratory fault must be regarded as small. Studies have shown that with the strict criteria used in this laboratory our evaluation of morphologic features has a high degree of accuracy and precision (Menkveld, unpublished data). This will be discussed in another publication. In three couples (23, 51, and 55) in whom the percentage was ≤ 14%, no fertilization occurred (Table 3).

---

Table 5. Observed Proportion of Pregnancies Below and Above the Threshold of Percentage Normal Sperm Morphologic Features

<table>
<thead>
<tr>
<th>% Normal sperm morphologic features</th>
<th>No. of oocytes</th>
<th>Observed proportion of pregnancies No.</th>
<th>% 95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 14%</td>
<td>1–13</td>
<td>0/22</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 14%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–2</td>
<td>5/44</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>≥ 3</td>
<td>32/124</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Fertility and Sterility
per successful laparoscopy is 11.4% (with 95% confidence limits [2% to 20%]). However, if more than two oocytes are obtained, the pregnancy rate per successful laparoscopy is 25.8% (with 95% confidence limits [18.1% to 33.5%]).

The implication of the lower fertilization rate in the group with ≤ 14% normal sperm morphological features is of practical relevance for the clinician and the patient. It is important to give the patients in an IVF program a realistic view of their prognosis. There is an excellent correlation with the percentage normal sperm morphological features and fertilization and pregnancy rates. This correlation was also pointed out by Rogers et al.18 and Aitken et al.19 in a group of patients with unexplained infertility. Aitken et al. do not agree that there is a good predictive value, in spite of the statistical association.19 However, we are convinced that the percentage normal sperm morphological features has an important role in the fertilization and pregnancy rate in the human in vitro model. The evaluation of normal sperm morphological features is a routine laboratory procedure at Tygerberg Hospital, and it has a high precision and prognostic value.

Acknowledgments. We thank the Medical Superintendent for permission to publish, the Medical Research Council for financial assistance, Sister Helletjie Rosich for her role as research assistant, and Mrs. Helena Krüger for preparing the manuscript.

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To reiterate the conclusion of the 1986 study, it was clearly stated the sperm morphology is an important factor in predicting fertilization and pregnancy outcome. In a logistic regression model it was also outlined that the number of oocytes are also playing an important role in outcome and that the male factor must not be viewed in isolation. Already in the first publication the importance of the Strict Criteria and how it differed from the then WHO criteria (WHO, 1980) was outlined. At that time Menkveld (unpublished data) was quoted saying that our laboratory have a high degree of accuracy and precision in evaluation. This was later published in detail (Menkveld et al., 1990).

The publication in 1986 was the beginning of a scientific journey that opened exciting and interesting new avenues in the field of male infertility nationally and internationally.

The next articles were published as a result of the post doctoral studies at the Jones Institute in Norfolk Virginia. The first (1987-study) was a prospective study to evaluate the impact of sperm morphology on fertilization rate and pregnancy outcome in the *In vitro* program at the Jones Institute, Norfolk, Virginia, USA.
The following article reflects the scientific basis that supports the above argument:

NEW METHOD OF EVALUATING SPERM MORPHOLOGY WITH PREDICTIVE VALUE FOR HUMAN IN VITRO FERTILIZATION

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ANIBAL A. ACOSTA, M.D. LUCINDA L. VEECK (A.S.C.P.)
KATHRYN F. SIMMONS, M.S. MAHMOOD MORSHEDI, Ph.D.
R. JAMES SWANSON, Ph.D. SANTIAGO BRUGO, M.D.

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ABSTRACT—A prospective study was planned to evaluate sperm morphology as a parameter to predict the fertilization outcome in an in vitro fertilization program. Couples applying to in vitro fertilization were admitted into this project when the sperm concentration was >20 million per mL and motility >30 per cent. Based on new strict criteria for evaluating normal sperm morphology, patients were divided prospectively into 2 groups. In group I (25 patients) normal sperm morphology was <14 per cent, and in group II (71 patients) normal sperm morphology was >14 per cent, using a threshold established previously. Multiple regression analysis was used to evaluate different parameters: concentration, motility, and morphology against the dependent variables, fertilization, and cleavage. The only factor which was significantly correlated with fertilization and cleavage was normal sperm morphology (P < 0.0001). The fertilization rate (per oocyte) and the cleavage rate were 49.4 per cent and 47.6 per cent in group I and 88.3 per cent and 87 per cent in group II (P < 0.0001). The ongoing pregnancy rate per laparoscopy and per embryo transfer was 4 per cent and 5.5 per cent, respectively, in group I and 18.3 per cent and 18.5 per cent, respectively, in group II (no significant difference). This study demonstrates the value of analyzing sperm morphology using the criteria recommended in terms of predicting fertilization and perhaps pregnancy outcome. Patients can be better counseled and the probability of fertilization or no fertilization can be more accurately established. Furthermore a trend is shown in the pregnancy rate that may indicate the importance of the male genome in establishing a pregnancy.

The role of one of the traditional semen parameters, namely sperm morphology, as a prognostic indicator of sperm-fertilizing capacity in the sperm zona free oocyte penetration assay (SPA), as well as in the human in vitro fertilization programs (IVF), is questioned by various workers in this field. However, some evidence suggests that if this parameter is evaluated using strict criteria, it is more valuable than any of the other traditional semen characteristics. A prospective study was planned to correlate this parameter with the fertilization rate in the in vitro fertilization program at the Jones Institute for Reproductive Medicine.
Material and Methods

Ninety-six patients were accepted into this study. Based on a previous project, the patients were divided into two groups, those with normal morphology of <14 per cent (group I) and those with normal morphology of >14 per cent (group II). There were 25 patients in group I and 71 patients in group II. Female partners of the couples selected for this project had either a tubal factor, endometriosis, or unexplained infertility (Table I). Female or male patients with antisperm antibodies were excluded from this study.

All male patients selected were required to have a concentration of more than 20 million per mL and a normally motile sperm fraction of 30 per cent or more in the basic semen analysis to try to minimize the impact of these two variables in the fertilization rate.

The basic semen evaluation was performed after liquefaction of the specimen delivered for IVF insemination using computer analysis (Celssoft Semen Analysis System, Lab-soft Division of Cryo Resources Ltd., New York). The following parameters were evaluated: sperm concentration, percentage of normal motility, mean velocity, mean linearity, and morphology. Evaluation was made by two independent observers unaware of the results of IVF. Two morphology slides were prepared on each patient on the day of laparoscopy directly after liquefaction and were stained by the quick-stain technique.

It is important to point out that special care was taken to clean the slides thoroughly with 70% ethyl alcohol before using them, and no more than 5 µL of semen were used to make the smears very thin. Care was taken to insure an even distribution of the semen drop along the entire interface between the staining and smearing slides. Slides were air dried at either 37°C or ambient temperature. Air-dried slides were fixed for 15 seconds with Diff-Quik Fixative (1.8 mg/L triaryl methane in methyl alcohol) prior to staining with Diff-Quik Solution 1 (1 g/L xanthene in sodium azide-preserved buffer) for 10 seconds and then with Solution 2 (0.625 g/L azure A and 0.625 g/L methylene blue in buffer) for 5 seconds. In between the fixing step and each of the staining steps, the excess solutions were drained from the slides by blotting the slide edges on bulbulous paper. After the slides were removed from Solution 2, excessive stain was removed immediately by applying a gentle stream of deionized water to the end of the slides.

The slides were read on the same day, and the results were documented. The morphology was evaluated as outlined in detail by Kruger et al. Spermatozoa were considered normal when the head had a smooth oval configuration with a well-defined acrosome involving about 40 per cent to 70 per cent of the sperm head, as well as an absence of neck, midpiece, or tail defects. No cytoplasmic droplets of more than half the size of the sperm head should be present. Furthermore, in contrast with other authors, borderline forms were counted as abnormal. At least 200 cells per slide were evaluated.

The human IVF procedures used for sperm preparation, insemination, and culture in the Norfolk program have been described previously. Only mature metaphase I and metaphase II oocytes were used in this study, and 50,000 to 100,000 sperms per mL per egg were used for oocyte insemination in a total of 3 mL. The percentage of normal morphology, concentration, and motility were carefully noted in each case, as well as the fertilization and cleavage rates and pregnancy outcome.

Relationships between the male factors and fertilization and cleavage rates were examined using multiple regression analysis and the SAS general linear model procedure. The multiple regression analysis allows the examination of the contribution of all the independent variables to the variation in the dependent variable fertilization and cleavage. Fertilization and cleavage were standardized for the number of pre-ovulatory eggs by dividing the number of eggs exhibiting fertilization and cleavage by the number of pre-ovulatory eggs. Pregnancy rate per laparoscopy was calculated by dividing the total number of laparoscopies by the number of pregnancies in the study. Pregnancy rate per embryo transfer was calculated by dividing the number of patients who reached the transfer stage by the number of pregnancies.

| Table I. Sperm morphology as predictor of human IVF: clinical diagnosis |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Factor                      | Group I (%)                | Normal Forms                | Group II (%)                | Normal Forms                |
|                             | < 14 %                     |                             | > 14 %                      |                             |
| Tubal                       | 12 (48)                    | 38 (53.5)                   |                             |                             |
| Endometriosis               | 4 (16)                     | 13 (18.3)                   |                             |                             |
| Unexplained                 | 9 (36)                     | 20 (28.2)                   |                             |                             |
| TOTALS                      | 25                         |                             | 71                          |                             |
Results

The normal morphology in group I ranged from 1 to 13 per cent (mean 7.5%, S.D. 3.84%). For group II, the morphology ranged from 15 to 43 per cent normal forms (mean 20.79%, S.D. 5.50). The sperm concentration in group I ranged from 22 to 261 million per mL (mean 82.12 million/mL, S.D. 59.08). Sperm concentration in group II ranged from 20 to 364 million (mean 135.11, S.D. 79.37). Motility in group I ranged from 31 to 92 per cent (mean 56.48%, S.D. 16.79). Motility in group II ranged from 30 to 96 per cent (mean 64.23%, S.D. 17.59). No significant difference was found between the two groups in terms of motility.

The fertilization rate in group I was 49.4 per cent, and the cleavage rate was 47.6 per cent. The fertilization and cleavage rates in group II were 88.3 and 87 per cent, respectively, which corresponds well with those rates in the IVF population at large in the Norfolk program. The rate of fertilization and cleavage was significantly different (P < 0.0001) between the two groups.

Sperm concentration, motility, and normal morphology were evaluated for their effect on the dependent variable fertilization and cleavage (standardized for the number of pre-ovulatory eggs) utilizing stepwise regression analysis. The only sperm parameter which was significant was the per cent normal morphology (P < 0.0001).

Of the 25 patients in group I, 28 per cent did not fertilize any egg. 36 per cent fertilized less than half of the eggs retrieved, while the remaining 36 per cent fertilized more than half of the eggs retrieved. In group II, 11.2 per cent fertilized less than half of the eggs retrieved, and 87.4 per cent fertilized more than half the eggs retrieved. In only 1 patient (1.4%) there was no fertilization at all.

The pregnancy rate per laparoscopy in group I was 12 per cent (3 of 25), and in group II, 33.8 per cent (24 of 71). The pregnancy rate per transfer was 16.6 per cent in group I (3 of 18) and in group II, 34.25 per cent (24 of 70). One patient aborted in first trimester in group I and one had an ectopic pregnancy, while 11 of 24 patients (45.8%) aborted in group II. The ongoing pregnancy rate per laparoscopy in group I was 4 per cent, and in group II it was 18.3 per cent, while the pregnancy rates per embryo transfer were 5 per cent in group I and 18.5 per cent in group II, respectively. No significant difference was found using this parameter because of the small number of cases, but a trend seems to be emerging.

Of the patients within group I who did not fertilize at all, 6 had severe amorphous head abnormalities, with an average normal morphology of 3 per cent, and an amorphous head morphology of 73 per cent. Of those individuals in group I who did fertilize, 17 of 18 had the same morphology pattern with average normal morphology of 8.6 per cent and amorphous head abnormalities of 60 per cent.

Comment

The value of normal morphology as an indicator of a patient’s fertilizing ability was pointed out by Rogers et al. in the hamster system and in human IVF by other workers in the field. A threshold was established previously based on data from a human IVF program, indicating that in the group where normal morphology is 14 per cent or below, there is a statistically significant lower fertilization rate. This study also demonstrated that normal sperm morphology is a good indicator of fertilization and cleavage in the human IVF system in patients with a concentration above 20 million sperm per mL and a motility above 30 per cent (P < 0.0001).

To select an accurate threshold, stricter criteria are used to evaluate the normal morphology than the criteria laid down by the World Health Organization. Borderline forms are classified as abnormal, contributing to the “low per cent” normal morphology. The SPA and, more important, the human IVF model are valuable end points which assist us in developing these criteria for sperm morphology. With the possibility of evaluating egg-sperm interaction in vitro, a much better understanding of the meaning of “normal sperm morphology” can be obtained. Therefore, we propose to review our concept of sperm normality based on fertilization and cleavage rates and on pregnancies and pregnancy outcome in the IVF patients.

In centers not able to run SPA, careful evaluation of normal morphology using the aforementioned criteria will be a valuable tool in predicting a patient’s ability to fertilize.

In the IVF program, the fertilization rate was 49.4 per cent and cleavage was 46.6 per cent in group I and 88.3 per cent and 87 per cent, respectively, in group II. Although there is
a statistically significant difference between the two groups, the question arises as to how to differentiate those patients in group I who still can fertilize an egg. There is a difference between these two subgroups with a predominance of amorphous forms in the group that did not fertilize, and with more normal forms in the group that did fertilize. Group I with its subgroups needs further study. However, it is still of great benefit for both the physician and the patient to know that the prognosis, as far as success in the IVF program is concerned, is much lower if the percentage of normal morphology is 14 per cent or lower. Although the figures are small in group I, there is a difference in the ongoing pregnancy rate with 5 per cent transfer in group I and 18.5 per cent in group II, although the difference is not statistically significant.

We are in total agreement with Rogers et al.\(^4\) that abnomal sperm morphology is a reflection of poor testicular physiology and an important factor in infertility. Often a patient will present with a sperm concentration well above 20 million per mL and a motility of >30 per cent. If the morphology is not evaluated with care, a diagnosis of unexplained infertility can be made incorrectly and lead to much frustration for both the patient and the physician.

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References

This study demonstrated the value of analyzing sperm morphology using the Strict Criteria in terms of predicting fertilization. It was reported that patients can be better counseled and the probability of fertilization can be more accurately established. A trend was shown in pregnancy rate (5.5% per embryo transfer in the group <14% vs. 18.5% per embryo transfer in the group > 14% normal forms) that may indicate the importance of the male genome in establishing a pregnancy.

During this time attention was also given to the staining of spermatozoa. Numerous methods were described, taking at least an hour or 3 before slides can be evaluated. This often led to delays in reporting in semen analysis results. A novel staining method (Diff-Quik [Dade Diagnostics, Miami, Fla.]) was researched and reported on (Kruger et al., 1987b). This staining technique was initially developed as a rapid staining method for hematology determinations. Duplicate semen smears were used using Diff-Quik and Papanicolaou staining and the morphology results were compared. No significant differences were observed when comparing the 2 staining methods. The advantages of Diff-Quik were staining to reading time, in 7 minutes. The product consists also of commercially prepared reagents that is easily available and will save time in a busy Andrology laboratory. The golden standard Papanicolaou was time consuming compared to this new technique. The Diff-Quik technique did not affect accuracy and quality as will be outlined in the following publication.
The following article reflects the scientific basis that supports the above argument:

Kruger TF, Ackerman SB, Simmons KF, Swanson RJ, Brugo S, Acosta AA. **A quick reliable staining technique for sperm morphology.** Arch Androl 1987;18:275-277.
A QUICK, RELIABLE STAINING TECHNIQUE FOR HUMAN SPERM MORPHOLOGY

T. F. KRUGER, S. B. ACKERMAN, K. F. SIMMONS, R. J. SWANSON, S. S. BRUGO, and A. A. ACOSTA

The evaluation of sperm morphology is still an important parameter in the diagnosis of the infertile male. Most techniques used for staining human sperm are very time-consuming. A routine stain used for determining differential count of leucocytes (Diff-Quik stain) was evaluated against the standard Papanicolaou stain. Morphology results from 20 duplicate semen smears using both staining methods were determined separately by 2 technicians using a blind protocol. No significant differences were observed when comparing the two staining methods (paired Student’s t-test). The advantages of the Diff-Quik stain are: a) complete staining-to-reading time under 7 min, b) commercially prepared reagents, and c) ease of staining procedure.

Key Words: Sperm morphology; Staining methods; Papanicolaou staining; Spermatozoa.

INTRODUCTION

Evaluation of the percentage normal and abnormal spermatozoa in human semen is still an extremely important parameter in the diagnosis of the infertile male patient in spite of recent advances in clinical andrology. The percentage of morphologically normal sperm forms in semen along with sperm concentration, motility, and semen volume generally constitutes the initial screening parameters evaluated in the basic semen analysis [3, 4]. Numerous methods have been described for staining human sperm. Some of these, such as the Papanicolaou stain, yield excellent results [1]. However, this commonly used staining method takes approximately 60 min to perform. Although volume, motility, and concentration values are determined shortly after the specimen liquefies, the extensive amount of time consumed in morphology staining often produces delays in reporting the semen analysis results. In clinical andrology laboratories that perform semen analyses on many men each day, this delay may result in an undesirable backlog. A novel staining method is reported for determining sperm morphology determinations of semen from men presenting for infertility evaluation. The stain used in this study, Diff-Quik (Dade Diagnostics, Miami, Fla.), was developed as a rapid staining method for hematology determinations [2]. Duplicate semen smears were stained using Diff-Quik and the Papanicolaou method, and the morphology results were compared.

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MATERIALS AND METHODS

To avoid nonspecific adherence of the stains, microscope slides were cleaned with 70% ethyl alcohol immediately prior to use. When specimens had normal sperm concentrations only 2–3 μL were applied to each slide. For oligozoospermic specimens 5–8 μL per slide were used. Care was taken to insure an even distribution of the semen drop along the entire interface between the staining and smearing slides. Slides were air dried at either 37°C or ambient temperature. Air-dried slides were fixed for 15 sec with Diff-Quik fixative (1.8 mg/L triarylmethane in methyl alcohol) prior to staining with Diff-Quik solution 1 (1 g/l xanthene in sodium azide-preserved buffer) for 10 sec and then with solution 2 (0.625 g/l azure A and 0.625 g/l methylene blue in buffer) for 5 sec. Between the fixing step and each of the staining steps the excess solutions were drained from the slides by blotting the slide edges on bibaluous paper. After the slides were removed from solution 2, excessive stain was removed immediately by applying a gentle stream of dionized water to the ends of the slides. Duplicate slides from 20 specimens were stained with either Diff-Quik or Papanicolaou staining methods and evaluated by 2 technicians without their prior knowledge of the identity of any specimen. Between 100 and 400 sperm were evaluated on each slide and the percentage of normal and abnormal forms calculated. Percentages were transformed using an arcsine transformation, and transformed and untransformed data were analyzed using the paired Student’s t-test.

RESULTS

No significant differences were observed between the two staining methods or between parallel determinations by the 2 laboratory technicians (Table 1). When evaluated by the same technician, values for percent normal forms in the same specimen stained by two techniques never differed by more than 5%. The mean difference between the same sample in this study stained with the two staining methods was only 0.1% for technician 1 and 0.6% for technician 2.

DISCUSSION

There are a few critical steps in this staining procedure. Best results were obtained with Diff-Quik when slides were thoroughly cleaned and, even more important, if a very thin slide was prepared. Otherwise, background staining could negatively influence the results. Over-staining with Diff-Quik 2 was also a problem when the slides were not rinsed after 5 sec. It was observed that freshly prepared slides as well as previously prepared and stored air-dried slides stain equally well. Differences in morphologic studies between the two staining methods were minimal. (Mean difference 0.1% and 0.6%.) A mean difference of 2.31% has been reported by others if the same slide is examined twice by the same observer [5].

We have recently begun to use a very strict set of criteria for classifying a sperm as

<table>
<thead>
<tr>
<th></th>
<th>Pap Stain Mean % Normal</th>
<th>Diff-Quik Stain Mean % Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technician 1</td>
<td>11.3</td>
<td>11.4 ns</td>
</tr>
<tr>
<td>Technician 2</td>
<td>13.3</td>
<td>12.7 ns</td>
</tr>
</tbody>
</table>

ns, not significant.
Staining Technique for Human Sperm Morphology

Our laboratory’s recommended reference value for “normal” sperm morphology is >20%. Preliminary evaluation of sperm morphology using this methodology indicates that this procedure provides excellent predictive data for both the hamster ova penetration test and human in vitro fertilization. In this study the mean percent normal sperm forms was approximately 12% with a range of 0–29%. Only 4 specimens in this group of 20 possessed normal morphology values over 20%. This low percentage of morphologically normal specimens is not unusual for our patient population, since many of our laboratory’s patients are second and third “male factor” referrals.

Sperm stained with this Diff-Quik method exhibited a pale purple acrosomal region and dark purple postacrosome, midpience, and tail. Although the quality of the staining is slightly better with the Papanicolaou, the morphology of the Diff-Quik-stained sperm were readily apparent. The advantages of the Diff-Quik stain include: a) complete stain to reading time within 7 min; b) commercially prepared reagents with manufacturer’s insured quality control; c) ease of performance that can be duplicated by even the novice technician. Using this system the clinician can receive a reliable semen analysis report very rapidly and the laboratory can reduce technician time thus increasing productivity.

Acknowledgments: The authors thank the technicians of the Andrology Laboratory, Rosita Acosta, Anne Bogert, Mary Hamilton, and Mahmood Morshed, for their devoted work as well as Sharon Durio for secretarial assistance and the M.R.C. and Tygerberg Hospital, which financially assisted the first author during his stay at the Jones Institute for Reproductive Medicine.

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2. Insert Diff-Quik Stain Set (1984): For the rapid, differential staining of hematological smears yielding qualitative results similar to Wright-Giemsa stain, 7
ii. **Sperm morphology patterns**

The next step in the quest to understand the low morphology group (<14%) better, was to study this group with lower fertilization potential. In this article it was clearly established, for the first time, that 3 patterns in morphology to be used clinically [P-pattern, G-pattern and N-pattern] (Kruger et al., 1988). The P-pattern, 0 to 4% normal forms, is the poor pattern group. In this article a fertilization rate of 7.6% was reported for the P-pattern group compared to the 63.9% for the G-pattern group (5 to 14% normal forms). The G-pattern group was defined as those with a good prognosis. The term N-pattern was used for those with normal forms above 14% normal and in previous publications had a fertilization rate of more than 80%. This publication and the 1986 publication became the 2nd and 3rd most quoted publications in the history of Fertility and Sterility as reported in 2006 Yang and Pan, 2006 (Table 1). The use of these patterns in clinical practice followed worldwide, with a number of publications to test the validity of the suggested sperm morphology thresholds.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>Fertility and Sterility articles most frequently cited in the science citation index, 1975 through 2004.</strong></td>
</tr>
<tr>
<td><strong>No. of citations</strong></td>
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</tbody>
</table>

Note: The complete table is available online.

Yang, Qing; Ovadia; in Fertility and Sterility. Fertil Steril 2005.
The following article reflects the scientific basis that supports the above argument:

Predictive value of abnormal sperm morphology in in vitro fertilization

Thinus F. Kruger, M.D.* R. James Swanson, Ph.D.§
Anibal A. Acosta, M.D.‡‡ James F. Matta, Ph.D.§
Kathryn F. Simmons, M.S.§ Sergio Oehninger, M.D.‡

Tygerberg Hospital, University of Stellenbosch, Parow, South Africa, and Eastern Virginia Medical School, Norfolk, Virginia

Although there is still extensive debate about the role of sperm morphology in in vitro fertilization (IVF), the human model has greatly improved the understanding of the significance of this parameter for fertilization and pregnancy outcome. In previous publications, it was noted that if evaluation of normal sperm morphology is done using strict criteria, this parameter has an excellent predictive value of fertilization. In patients with a sperm concentration > 20 × 10⁶/ml and a motility of >30% with a normal sperm morphology of <14%, the fertilization rate was markedly impaired (37% to 47% per oocyte), as opposed to a high fertilization rate (85% to 88%) when normal morphology was >14%.

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Although there was severe impairment in the fertilization rate, some of these patients still fertilized the human egg; in these cases, a pregnancy was possible.

The purpose of this study was to evaluate patients with normal sperm morphology < 14% to try to establish a morphologic pattern which can differentiate the subgroup that fertilized from the subgroup that did not.

MATERIALS AND METHODS

Forty-five couples were allocated to the study group in a prospective way. All female partners in these couples had tubal infertility, and the males had either been considered normal or had some abnormal parameters by other laboratories evaluations. Twenty-eight patients were stimulated with a combination of hFSH/hMG/hCG (human follicle-stimulating hormone/human menopausal gonadotropin/human chorionic gonadotropin; 62.2%), 13 with hFSH/hCG (28.8%), and 4 with hMG/hCG (8.8%) following protocols previously published. In the Norfolk experience, all of these protocols have demonstrated provision of preovulatory oocytes with identical fertilization rates. All male patients had to have a sperm concentration > 20
× 10⁶/ml and a progressive motility of >30% in the basic semen analysis to try to minimize the impact of these two variables on the fertilization rate. The basic semen evaluation was performed after liquefaction of the specimen delivered for IVF insemination using computer analysis (Cellsoft Semen Analysis System, Labssoft Division of Cryo Resources Ltd., NY). Sperm concentration and percentage of normal motility were assessed in this fashion. Two morphology slides were prepared for each patient from the specimen delivered on the day of laparoscopy for in vitro insemination after liquefaction and were stained by the quick-stain technique. Special care was taken to clean the slides thoroughly with 70% ethyl alcohol before using them, and no more than 5 μl of semen were used in order to make the smears as thin as possible. The slides were air-dried at room temperature, were fixed for 15 seconds with Diff-Quik fixative (Diff-Quik AHS del Caribe, Inc. Aguada, PR 00602) (1.8 mg/l triarylmethane methyl alcohol) prior to staining with Diff-Quik solution 1 (1 gm/l xanthene in sodium azide-preserved buffer) for 10 seconds, and then with solution 2 (0.625 gm/l azure A and 0.625 gm/l methylene blue in buffer) for 5 seconds. In between the fixing step and each of the staining steps, the excess solutions were drained from the slides by blotting the slide edges on bibulous paper. The slides were read on the same day and documented. The morphology was evaluated, as outlined in detail by Kruger et al, by two independent observers, each unaware of the results obtained by the other. This method of evaluation has an inter-technician coefficient of variation and an intra-technician variability that are not significant (Spearman’s rank correlation coefficient, r = 0.8695 and 0.9630, respectively). Spermatozoa were considered normal when the head had a smooth oval configuration with a well-defined acrosome involving about 40% to 70% of the sperm head, as well as an absence of neck, midpiece, or tail defects. No cytoplasmic droplets of more than half the size of the sperm head should be present. The length of a normal sperm head was 5 to 6 μm and the diameter 2.5 to 3.5 μm (Fig. 1). A micrometer eyepiece of the microscope was used to do the routine measurements. In contrast to other methods, borderline forms were counted as normal. At least 200 cells per slide were evaluated. The amorphous-head group was divided into two categories: slightly amorphous and severely amorphous. Slightly amorphous forms were those sperm with a head diameter of 2.0 to 2.5 μm, with slight abnormalities in the shape of the head, but with a normal acrosome (Fig. 1). Severe amorphous head abnormalities were defined as those with no acrosome at all or those with an acrosome smaller than 30% or larger than 70% of the sperm head (Fig. 1). Completely abnormal shapes also were put into this category (Fig. 1). Neck defects were also classified into two categories: slightly amorphous and severely amorphous neck defects. The slight neck defects referred to those sperm with debris around the neck or a thickened neck, but with a normally shaped head (Fig. 1). The severe defects referred to those sperm with a bend in the neck or midpiece of more than 30% or a severely amorphous head shape, as described. All other abnormal sperm forms—round, small, large, tapered, double head, double or coiled tail, cytoplasmic droplets—were classified following the World Health Organization classification. Female or male patients with antisperm antibodies were excluded from this study.

The human IVF procedures used for sperm preparation, insemination, and culture in the Norfolk program have been described previously. Only mature oocytes with an extruded polar body were used in this study; 50,000 to 100,000 sperm/μl were used for oocyte insemination in a total of 3.0 ml of insemination medium.

Kruger et al. Abnormal sperm morphology and IVF 113
After completion of the study, the results of the hamster tests in patients that had the assay performed at least 8 weeks before the IVF procedure were evaluated. The hamster test was performed as outlined by Swanson et al. Penetration > 20% was considered good, between 11 and 19% doubtful, and <10% poor. The donors used as control always penetrated above the 20% level.

The percentage of normal morphology, the concentration, and motility were noted carefully in each case, as were the fertilization and cleavage rates and pregnancy outcome. The relationships between the sperm parameters and the fertilization rates were examined using multiple regression analysis in the Statistical Analysis System (SAS) general linear model (GLM) procedure. The SAS GLM procedure allows examination of all submodels of the complete multiple regression model. The multiple regression analysis examines the contribution of all the independent variables to the variation in the dependent variable fertilization.

Fertilization was standardized for the number of preovulatory eggs by dividing the number of eggs exhibiting fertilization by the number of preovulatory eggs. Pregnancy rate per laparoscopy was calculated by dividing the total number of laparoscopies by the number of pregnancies in the study. Pregnancy rate per embryo transfer was calculated by dividing the number of patients who reached the transfer stage by the number of pregnancies. The following variables were evaluated: percent normal sperm morphology, amorphous head abnormalities (slight and severe), neck abnormalities (slight and severe), small, large, round, tapered, and double heads, cytoplasmic droplets, and tail abnormalities (double and coiled).

**RESULTS**

Of the 45 patients included in this study, 13 (28.9%) did not fertilize any oocytes at all, 17 (37.8%) fertilized <50% of the oocytes obtained, and 15 (33.3%) fertilized >50% of oocytes obtained. This should be compared with a fertilization rate for patients with tubal infertility of 89% to 92% in our laboratory.

The patients were divided into two groups: group I, 14 patients (no fertilization) and group II, 32 patients (fertilization of at least one oocyte). The mean sperm concentration in group I was 63.3 ± 42.8 × 10^6/ml (mean ± standard deviation) and in group II, 83.3 ± 57.8 × 10^6/ml (no significant difference) (Table 1). The mean motility in group I was 45.6 ± 13.2% and in group II, 55.3 ± 18.6% (no significant difference) (Table 1). By eliminating abnormal concentration and abnormal motility, we tried to individualize and define the effect of morphology and its abnormalities in the process of fertilization. There was a significant difference between the percent of normal morphology (1.8 ± 2.4% in group I and 7.7 ± 3.3% in group II; P < .0001) and the percentage of slightly amorphous abnormalities (head and neck), which was 18.0 ± 10.9% in group I and 34.3 ± 6.7% in group II (P < .0001; Table 2). None of the other variables showed a significant difference between the two groups.

The predictive value of normal morphology (r² = 0.44) was better than that of slightly amorphous forms (r² = 0.38). When we added the percent of normal morphology and slightly amorphous abnormalities (morphology index) and performed regression analysis, there was a highly significant correlation between that index and fertilization (P < .0001; Table 1), with an even better predictive value (r² = 0.56). The mean for morphology index was 19.7 ± 11.7% in group I and 42 ± 7.8% in group II (Table 1).

The SAS general linear model was used with the number of embryos as the dependent variable to determine a threshold to indicate where the chances of fertilization were significantly impaired. A threshold of 4% was indicated for normal morphology and 30% for the combination of normal morphology and slightly amorphous forms (morphology index). The fertilization rate per oocyte in group I (morphology index <30%, normal morphology <4%) was 7.6% and in group II (morphology index >30%, normal morphology >4%) was 63.9% (Table 2).

The mean number of embryos in the 13 patients in group I was 0.4 and for the 32 patients in group II...
Table 2 Abnormal Morphology as a Predictor of Human IVF Fertilization Rate

<table>
<thead>
<tr>
<th></th>
<th>Group I (n = 15)</th>
<th>Group II (n = 32)</th>
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</thead>
<tbody>
<tr>
<td>Fertilization rate (%) (per oocyte)</td>
<td>7.5</td>
<td>63.9*</td>
</tr>
<tr>
<td>Mean no. embryos (per patient)</td>
<td>0.4</td>
<td>2.6*</td>
</tr>
</tbody>
</table>

* P = 0.0001.

was 2.6; these means were significantly different (P < 0.0001; Table 2). Patients were followed with β-hCG, estradiol, and progesterone determinations on a weekly basis, pelvic ultrasound starting on the seventh week after the last menstrual period, and clinical evaluation to determine pregnancy status and type of gestation. The pregnancy rate in group I was 1 out of 13 patients (7.6%) and in group II was 10 out of 32 patients (31.2%). The ongoing pregnancy rate in group I was 1 out of 13 patients (7.6%) and in group II, 6 out of 32 patients (18.7%), with three clinical miscarriages and one ectopic pregnancy. The differences between these two groups in terms of reproductive performance did not reach statistical significance because of the small number of patients.

Of the 45 patients studied, 14 had a hamster test performed prior to the IVF procedure. All 14 had a penetration rate below 10%. Four out of the 14 (28.6%) patients with poor penetration rates did not fertilize any oocytes in vitro, but 10 of 14 patients (71.4%) did fertilize in vitro. Five of 10 patients (50%) fertilized >50% of the eggs, and 5 (50%) fertilized <50% of the eggs.

**DISCUSSION**

Normal morphology evaluated by strict criteria is a valuable tool to predict a patient’s chance to fertilize and to reach the transfer stage. In a previous study performed at the Jones Institute,⁴ 70 of 71 patients with normal morphology >14% reached the transfer stage, reflecting a high fertilization rate in this group. If the normal morphology is <14%, the fertilization rate per oocyte is markedly impaired.⁴ This study was designed to evaluate the sperm morphology in this group and to try to define morphologic patterns in patients with and without fertilization. Our results indicate that severe impairment of fertilization will take place at a level of <4% normal morphology, based on the strict criteria explained previously (Table 2). Results also indicate that by adding the slightly amorphous forms to the normal forms, a “morphology index” can be established with a cutoff figure at the 30% level. Patients with a value of <30% morphology index will have a severe reduction in fertilization as compared with patients having an index >30% (P < 0.0001) (Table 2). None of the other semen parameters evaluated were of any help to predict a patient’s chance to fertilize.

The advantage of strict morphology evaluation is the fact that it is reproducible between patients and between different technicians performing the test.¹⁸ It also allows the clinician to classify the patient into one of two specific groups (<14% and >14% normal morphology), giving a reliable criterion that can be used to counsel the patient and to plan the approach in future IVF cycles.

Based on the significant differences between normal morphology and the slightly amorphous forms in groups I and II, we propose that two patterns can be observed in the <14% normal morphology group. The P pattern (poor prognosis pattern) has a mean normal morphology of 1.8% and mean slightly amorphous forms of 18%, with a morphology index <30% (Table 1). The G pattern (good prognosis pattern) gives the patient a significantly better chance to fertilize (P < 0.0001) than the P pattern (Table 2). The mean normal morphology in the G pattern was 7.7%, the mean slightly amorphous forms were 34.3%, with a morphology index >30%. Based on these patterns, predictions on chances of fertilization can be done with much more accuracy in the group with <14% normal morphology.

The fertilization rate for all patients with a normal morphology <4% and morphology index <30% (P pattern) was 7.6%; when the normal morphology was >4% and the morphology index >30% (G pattern), the fertilization rate was 63.9%. Only one pregnancy was established in patients with a P pattern; in patients with a G pattern, the ongoing pregnancy rate was 60%, which compares favorably with the ongoing pregnancy rate previously reported in our overall population.⁴ This observation again confirms previous reports¹⁷,¹⁶ that if fertilization occurs, the performance of the embryos, as well as the transfer and pregnancy rates, are no different from the general IVF population.

The question now arises whether the fertilization rate and prognosis of patients with normal morphology <14% can be improved, especially in those with a P pattern, but also in those with a G pattern who fertilized <50% of the oocytes. Can they per-

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haps benefit by simply increasing the concentration of sperm per milliliter of the insemination medium at the time of IVF from 50,000/ml to 500,000/ml? There have been several reports warning against a significant decrease in fertilization rates in vitro in mice and hamsters when the sperm concentration was increased. This decrease can be due to excessive numbers of antifertilization factors or proteases near the oocyte. Nevertheless, in the beginning of our own program, insemination was done routinely with 500,000 sperm/ml/egg and the fertilization rate was no different. It also was demonstrated in studies with mice, using suboptimal concentrations of sperm, that as the sperm density is reduced, fertilization rates also are reduced. To answer these questions, a prospective study is being conducted.

Another important point in male factor cases is the timing of insemination. In Norfolk the extrusion of the polar body is used as an indicator of oocyte maturity, at which time insemination takes place.

The correlation of the sperm penetration assay (SPA) and IVF was not good in this study, with 28.6% no fertilization and 71.4% fertilization rate per patient with <10% SPA penetration in all 14 cases. In a previous study this group indicated that there is a good correlation between normal morphology and SPA penetration rate. If normal morphology was <14%, 85% of cases did not penetrate above the 10% level. If normal morphology was >14%, the penetration rate above the 10% level was 86% (P < 0.0001). We conclude from these observations that the SPA is giving us the same information as normal morphology greater or lesser than 14% in the population studied. A SPA <10% and a normal morphology <14% are parameters warning the clinician of potential problems in IVF due to the male factor. Without identifying the different patterns (P and G), valuable predictive information will be lost.

It is worth emphasizing that these criteria are useful only in IVF with the techniques used in Norfolk. The significance of these abnormalities in clinical practice remains to be demonstrated.

The evaluation of sperm morphology is a controversial issue. Results in fertilization rates in IVF units differ. Do we look at the same spectrum of abnormalities, explaining the differences in results, or is our classification of abnormally and normally shaped sperm in need of revision? It is our opinion that the latter is true and thus needs the attention of those involved in the field.

Acknowledgments. We thank the scientists in the In Vitro Fertilization Laboratory, Ms. Simona Simonetti, Dr. Jake Mayer, and Ms. Mary Maloney for making the slides daily, and Ms. Debbie Jones for retrieval of data. We also thank the technicians in the Andrology Laboratory, Mrs. Rosita Acosta, Ms. Anne Bogaert, and Ms. Mary Hamilton, for their devoted work, and Mrs. Sharon Durio and Mrs. Myra Waters for secretarial assistance. Last, but not least, we thank the SA Medical Research Council and Tygerberg Hospital who financially assisted the first author during his stay at the Jones Institute for Reproductive Medicine.

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13. Swanson RJ, Mayer JF, Jones KH, Lanzendorf SE, McDowell J: Hamster ova/human sperm penetration: correla-
iii. *Increase in sperm concentration*

It is obvious from the publication on the patterns in sperm morphology that a solution was necessary to improve the fertilization rate in the P-pattern group. Oehninger suggested the increase in sperm concentration in this group with a clear improvement in fertilization rate (Oehninger *et al.*, 1988).

A publication followed where the concept of increased insemination concentration in a 'normal environment', using the fallopian tube, was used. It was proved that by increasing the semen concentration, the fertilization rate was improved in *In vitro*. No significant improvement in the pregnancy rate in the gamete intra fallopian transfer (GIFT) program at Tygerberg Hospital was observed.
The following article reflects the scientific basis that supports the above argument:

EFFECT OF SEMEN CHARACTERISTICS ON PREGNANCY RATE IN A GAMETE INTRAFALLOPION TRANSFER PROGRAM

T. F. KRUGER, D. R. FRANKEN, E. STANDER, Y. SWART, and J. P. VAN DER MERWE

The aim of this study was to evaluate the influence of sperm morphology, swim-up concentration, and insemination volume on pregnancy outcome in patients undergoing gamete intrafallopian transfer (GIFT) treatment in whom the male partner had a morphology of less than 14%. Only patients who received four oocytes were entered into this study (n = 103). In all cases the swim-up procedure time was standardized to 1 h and the insemination concentration was standardized to 500,000 per oviduct. There was no significant difference in pregnancy rate when normal morphology, swim-up concentration, or insemination volume were used as predictors. In the P pattern group (<4% normal forms) only four of the 28 (14%) patients had ongoing pregnancies, whereas in the G pattern group (5% to 14% normal forms) 16 out of 75 (21%) had ongoing pregnancies (P < G, p > 0.05; not significant). The fertilization rate among excess oocytes in the P pattern group was 18% but was 39% (p < .0001) in the G pattern group. When an attempt was made to compensate for low morphology by increasing insemination concentration no significant difference in the pregnancy rate between the P and G pattern groups in the GIFT program was recorded. This was not the case when the in vitro fertilization rate was used as an endpoint. Insemination volume and swim-up concentration played no role in pregnancy rate.

Key Words: Semen; Sperm; Gamete intrafallopian tube (GIFT); Pregnancy; Swim-up.

INTRODUCTION

The recognition in the current literature that sperm morphology can be used as a predictor of fertilization and pregnancy is encouraging [2, 6, 8]. Two sperm morphology patterns were described, namely the P pattern (normal sperm morphology 0% to 4%) and the G pattern (normal sperm morphology between 5% and 14%). Among patients with less than 14% normal forms, the chance of in vitro fertilization (IVF) is reduced [4] and this reduction is more marked in cases where sperm morphology is less than 5% normal forms (only 8% per oocyte) [5]. If corrective measures are taken the fertilization rate can be improved but the pregnancy outcome in vitro does not improve [8].

The principle aim of this study was to evaluate the influence of different semen parameters on the pregnancy rate in a GIFT program among the P and G pattern morphologic groups. The semen...
parameters studied were the percentage of normal forms, the volume of semen transferred at insemination, and the sperm concentration/mL retrieved after swim-up.

MATERIALS AND METHODS

One hundred three patients whose female partners suffered from unexplained infertility were randomly selected for the study [9]. Testing by both the tray agglutination test (TAT) [9] and the sperm immobilization test (SIT) [9] had to reveal the absence of antibodies in all patients. The men had to have sperm morphologic values of 0% to 14% normal forms, a sperm concentration/mL between 2 and 300 million/mL, a motility of between 30% and 70%, and forward progression that varied between 1 and 4 (scale, 1 to 4) [4, 7, 9]. In all patients the swim-up tie was standardized to exactly 1 h on the day of the procedure. The sperm concentration/mL, percent motility, forward progression, as well as morphology (percentage normal forms) before and after swim-up were evaluated.

The semen samples were obtained 2.5 h before insemination and prepared as follows: 1 mL of semen was diluted with 2 mL of Ham’s F-10 medium (GIBCO, Grand Island, NY) and washed twice by centrifugation at 200 × g for 10 min. After the final wash, the supernatant fluid was discarded and 1 mL of medium was layered over the pellet [4]. The tube was then placed in an incubator at a 45°C angle and a 30°C for 1 h [4].

Ovulation was induced using a combination of clomiphene citrate and Humegon. Human chorionic gonadotropin (hCG) was administered when the dominant follicle was greater than 18 mm in diameter and at least two other follicles of 16 mm or more in diameter were present [4]. Oocyte retrieval was performed by standard procedure. Two mature oocytes together with 500,000 were transferred into each fallopian tube [8, 9]. Patients producing less than four oocytes on the day of the procedure were excluded from the study. Any excess oocytes were inseminated 6 h after retrieval and if possible the embryos were frozen [1]. The fertilization rate for the group of patients with normal morphology between 0% and 4% normal forms (P pattern) and those with morphology between 5% and 14% normal forms (G pattern) was carefully recorded. The pregnancy outcome was assessed by confirming the presence of or absence of an ongoing pregnancy by ultrasound at 8 wk after the GIFT procedure. The fertilization rates in both the P and G pattern group were also compared with patients with normal semen parameters seen during the duration of the study.

Following GIFT, the patients were divided into categories according to the percentage normal morphology, the semen insemination volume that had to be transferred into the oviduct in order to reach a concentration of at least 500,000 per oviduct, the seminal sperm concentration/mL prior to swim-up, and the swim-up concentration before the GIFT procedure. Logistic regression was used to study the predictive ability of the above factors. The chi square test was used to compare the IVF results as well as pregnancy rate in different groups.

RESULTS

None of the factors studied were predictors of pregnancy outcome based on logistic regression. One hundred three patients were studied in the group of patients with sperm morphology of below 14% normal forms. Twenty eight of the patients had 0 to 4% normal sperm morphology and 75 had 5% to 14% normal sperm morphology. The fertilization rate of excess oocytes in the 0% to 4% normal morphology group was 18% (P pattern) and 39% in the 5% to 14% normal morphology group (G pattern). In the control group of patients the fertilization rate was 73% (normal). P versus G = p = 0.0005; P versus N (normal morphology) = p < 0.0001 (Table 1).

Sperm morphology. The ongoing pregnancy rate in the morphology group with 0% to 4% normal forms was 14% and 21% in the 5% to 14% normal group (Table 2).
Semen Characteristics and the GIFT Program

TABLE 1  Fertilization Rate of Oocytes in Patients with Normal Morphology 0%–14%

<table>
<thead>
<tr>
<th>Sperm Morphology (%)</th>
<th>Fertilization Rate (%)</th>
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<tr>
<td>0–4</td>
<td>18*</td>
</tr>
<tr>
<td>5–14</td>
<td>39*</td>
</tr>
<tr>
<td>&gt;14</td>
<td>73*</td>
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* vs * p = 0.0005.
* vs † p = 0.0001.
* vs ‡ p = 0.0001.

Semen volume. Semen volume inserted into the oviduct at time of transfer was also evaluated. The patients were divided into three groups according to semen volume. In those who received 0.01 to 0.09 mL the pregnancy rate was 20%; in those who received 0.1 to 0.19 mL the pregnancy rate was 14%; and in those who received 0.2 to 0.9 mL the pregnancy rate was 19% (not significant) (Table 2).

Seminal sperm concentration/mL. Patients were also divided into three groups according to the seminal sperm concentration/mL prior to the swim-up. If the sperm concentration was between 3 and 10 million/mL then the ongoing pregnancy rate was 17%; while if sperm concentration was between 11 and 19 million/mL the ongoing pregnancy rate was 8%. If it was above 20 million/mL the ongoing pregnancy rate was 22%. There was no significant difference between the different groups (Table 1).

Swim-up concentration (before GIFT). The number of sperm/mL retrieved after swim-up ranged among the 103 patients from between 100,000 to 18 million sperm. Patients were therefore divided into three groups. In those with 0.1 to 4.9 million sperm/mL the pregnancy rate was 18%; in those with 5.0 to 9.9 million/mL the pregnancy rate was 11%; and in those

TABLE 2  The Effect of Different Semen Parameters on Pregnancy Rate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ongoing Pregnancies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm morphology (%)</td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td>14</td>
</tr>
<tr>
<td>5–14</td>
<td>21</td>
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<tr>
<td>Insemination semen volume in oviduct</td>
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<tr>
<td>0.01–0.09</td>
<td>20</td>
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<tr>
<td>0.1–0.19</td>
<td>14</td>
</tr>
<tr>
<td>0.2–0.9</td>
<td>19</td>
</tr>
<tr>
<td>Seminal sperm concentration/mL</td>
<td></td>
</tr>
<tr>
<td>3–10</td>
<td>17*</td>
</tr>
<tr>
<td>11–19</td>
<td>8</td>
</tr>
<tr>
<td>&gt;20</td>
<td>22*</td>
</tr>
<tr>
<td>Swim-up concentration</td>
<td></td>
</tr>
<tr>
<td>0.1–4.9</td>
<td>18*</td>
</tr>
<tr>
<td>5–9.9</td>
<td>11</td>
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<tr>
<td>&gt;10</td>
<td>30*</td>
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</table>

*No significant difference.
with above 10 million/mL the pregnancy rate was 30%. Again the difference between the groups was not significant.

DISCUSSION

The aim of this study was to assess whether or not in the group of patients with morphology less than 14% normal forms swim-up concentration and semen volume transferred have any effect on the pregnancy outcome in a GIFT program. In a retrospective study at our institution the pregnancy rate was very poor when the swim-up concentration/mL was less than 5 million. However, in this prospective controlled study no significant difference in pregnancy outcome was observed in the different groups of swim-up concentrations and semen volumes transferred into the fallopian tube. Thus we found that the pregnancy rate is not affected if too large a semen volume is used per oviduct. In the Tygerberg program where more than 500 µL is needed to reach a concentration of 500,000 sperm per fallopian tube, semen is first transferred and then the oocytes are deposited into the oviduct.

The reason for transferring 500,000 sperm per oviduct is based on a study by Oehninger et al. [8] showing that the chances of fertilization can be improved by increasing the concentration of semen. Whether the same improvement in pregnancy outcome can be obtained by increasing the sperm concentration is not clear. Van der Merwe (unpublished data) observed in 19 successive cases of patients with teratozoospermia (<14% normal forms) that no pregnancy resulted when 100,000 sperm per oviduct were transferred, but when the concentration was increased to 500,000 per oviduct, pregnancies followed.

In this study the ongoing pregnancy rate in the P pattern group (normal morphology ≤4%) was 14% and was 21% in the G pattern group (Table 2). Using the same strict criteria to evaluate morphology, Oehninger et al. [8] reported a pregnancy rate in the P pattern group in their IVF program of 4% per cycle, compared with 19% in the G pattern group and 32% in the group above 14% normal forms [4, 5].

Hinting et al. [2] have also proved a markedly decreased fertilization rate if the proportion of sperm with normal morphology is less than 14% and very severely decreased if it is less than 4%. It was also observed that the ability to predict the outcome of GIFT according to the sperm characteristics was poor, and comparison of the pregnancy rate in the P and G pattern groups in our study confirms this. In 437 studies, Van der Merwe et al. showed that there is a significant difference in pregnancy rate between patients with a normal sperm morphology both less than and greater than 14% [10]. Of the four ongoing pregnancies reported in the P pattern group, three did not fertilize any oocytes in vitro. As a result of this finding it must be assumed that the fallopian tube environment must in some way enhance fertilization and thus the chance of a pregnancy in the group with less than 14% normal morphology.

Although we used 500,000 sperm/mL, the IVF rate of the leftover metaphase II oocytes was 18% in the P pattern group and 37% in the G pattern group, while in a control group of GIFT patients (sperm morphology >14% normal forms), the fertilization rate was 73%. In the study by Oehninger et al. the IVF rate could be affected by increasing the concentration. A significantly higher fertilization rate in the G pattern group and in the group with greater than 14% normal morphology has also been observed by other workers using strict criteria in their IVF programs [2, 3, 6].

In our patients the initial concentration in the basic semen analysis ranged between 3 and 101
Semen Characteristics and the GIFT Program

million/mL. Even patients with oligozoospermia can be regarded as good candidates for the GIFT procedure, eg, the group with a semen concentration of between 3 and 10 million sperm/mL where the pregnancy rate was 17%.

If at least 500,000 sperm can be retrieved, irrespective of the basic semen concentration or volume transferred, the achievement of an ongoing pregnancy becomes a possibility when the GIFT procedure is used.

REFERENCES

b. To access the international literature’s acceptance of the initial observations and scientific reports

i. **A structured literature review after 10 years**

It took longer than 10 years after the first publication on sperm morphology in 1986, with numerous publications and debates at international conferences to settle the sperm morphology ‘predictive’ issue for *In vitro* fertilization. A PhD student in the Department of Reproductive Medicine, Dr Kevin Coetzee used a structured literature review to evaluate the question: Is there a universal predictive value of normal morphology in the IVF situation? These findings were published in 1998 (Coetzee et al., 1998). The fertilization rate were 59.3% (1979/3337; per oocytes) in the P-pattern group compared to 77.6% (10345/13327; per oocyte) in the G/N-pattern groups. The overall pregnancy rates were 15.2% per cycle (60/395) (P-pattern) and 26% (355/1368) per cycle in the G/N-pattern groups. Of importance was the fact that 24% of patients had no transfer in the P-pattern group compared to 7.4% in the G and N-pattern groups. It was concluded that accurately evaluated normal sperm morphology as integral part of the standard semen analysis makes this the most cost-effective means of evaluating the male with predictive ability assisting the clinician and scientist in decision making.

In a publication in 1999, the role of sperm morphology in *In vitro* fertilization was summarized and explained in more detail (Kruger et al., 1999). There was also reflection in this article, on the correlation of sperm functional tests as well as the computer assisted sperm evaluation with manual sperm morphology. These aspects will be discussed in other sections of this thesis.
The following article reflects the scientific basis that supports the above argument:

Predictive value of normal sperm morphology: a structured literature review

Kevin Coetzee1,3, Thinus F. Kruger1 and Carl J. Lombard2

1Reproductive Biology Unit, Obstetrics and Gynaecology Department, Tygerberg Hospital, University of Stellenbosch, Tygerberg 7505 and 2Division Epidemiology and Biostatistics, CERSA, Medical Research Council, Tygerberg 7505, South Africa

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</tbody>
</table>

The aim of the study was to conduct a structured review of the literature published on the use of normal sperm morphology, as an indicator of male fertility potential in the in-vitro fertilization (IVF) situation, and to establish the universal predictive value of this semen parameter. Published literature in which normal sperm morphology was used to predict fertilization and pregnancy, during the period 1978–1996, was reviewed. A total of 216 articles were identified by the sourcing methodology, but only 49 provided data that could be tabulated and analysed. Of these, only 18 provided sufficient data for statistical analysis.

Fifteen studies used the strict criteria to evaluate sperm morphology, two used World Health Organization (WHO) guidelines and one used both the strict criteria and the WHO guidelines. All the studies (n = 10) using the 5 and 14% normal sperm morphology thresholds (strict criteria) produced positive predictive values for IVF success. In the prediction of pregnancy, 82% (9/11) and 75% (6/8) of the studies produced positive predictive values when using the 5% and 14% thresholds respectively. Aggregating the data produced around the 5% normal sperm morphology threshold (strict criteria), the overall fertilization rates were 59.3% (1979/3337; per oocyte) for the ≤4% group and 77.6% (10345/13327; per oocyte) for the >4% group, and the overall pregnancy rates were 15.2% (60/395; per cycle) and 26.0% (355/1368; per cycle) respectively. The no-transfer rates across the 5% threshold were 24.0% (86/359; per cycle) in the ≤4% group compared to 7.4% (80/1088; per cycle) in the >4% group.

...an accurately evaluated normal sperm morphology count as an integral part of the standard semen analysis makes this analysis still the most cost-effective means of evaluating the male factor.

**Key words:** human/in-vitro fertilization/normal sperm morphology/pregnancy/structured review

**Introduction**

With the realization that male fertility was an important contributor to the conception potential of a couple, establishment of the fertility potential of men became a subject of intense research. This included the close examination (light microscopy, computerized analysis etc.) of the conventional sperm parameters (motility, forward progression and concentration) (Liu et al., 1990; Wang et al., 1991; Enginsus et al., 1992a,b) and the use of functional assays (Coetzee et al., 1989; Franken et al., 1989; Chan et al., 1990; Henkel et al., 1993) to distinguish between fertile and infertile men and correlate these parameters and outcomes with in-vivo conception and in-vitro fertilization (IVF), implantation and pregnancy. Understandably, no single test or sperm parameter was found to be absolute in its prediction of male fertility or infertility, as no single sperm feature or function could truly represent the ability of spermatozoa to accomplish the complex sequence of events leading to a clinical pregnancy.

IVF provides the best means of examining sperm–egg interaction and determining fertilization probability for diagnostic purposes, but obviously cannot be used as a routine screening assay. Therefore, due to the cost, time and ethical constraints of IVF and functional assays, the correct evaluation of the basic semen parameters still remains the most cost-effective diagnostic tool for male fertility.

Even though the basic semen parameters are descriptive in nature, several studies have obtained good correlations between IVF and motility (Alper et al., 1983; Ron-El et al.,...
1991; Robinson et al., 1994), concentration (Biljan et al., 1994; Calvo et al., 1994; Robinson et al., 1994) and normal/abnormal sperm morphology (Kruger et al., 1986; Enginsa et al., 1992a; Grow et al., 1994). Of all the semen parameters, sperm morphology has consistently been the best indicator of male fertility. Many authors have gone as far as to argue that sperm morphology is a reflection of sperm functional competence. The main shortcomings of this parameter have been the large number of classification systems used to describe what factors constitute a morphologically normal/abnormal spermatozoon, the various staining procedures employed and the subjective nature of the evaluation. The following are some of the major classification systems used to classify normality: Eliasson (1971), World Health Organization (WHO; 1980, 1987, 1992), Williams (1964), Tygerberg strict criteria (Kruger et al., 1986, 1988; Menkved et al., 1990), David et al. (1975), Freund (1966), Fredricsson (1979) and Düsseldorf (Hofmann et al., 1985).

The aim of this study was to establish the universal predictive value of normal sperm morphology, in the IVF situation, by means of a structured literature review.

**Materials and methods**

The articles included in the review were primarily found by means of a computerized Medline search using specific criteria (key words: IVF, pregnancy and normal sperm morphology; limitations: English, human and within the period 1978–1996). Our unit’s data bank of articles was also searched using the same criteria; finally, the references of the articles obtained were cross-checked. Articles were only analysed further if certain criteria were met: (i) statistical associations were investigated between sperm morphology and IVF and/or pregnancy, (ii) abnormal/normal sperm morphology fertility thresholds were identified and (iii) whether descriptive data (per oocyte fertilization, per cycle/transfer pregnancy rates and pregnancy outcome) were presented.

A total of 216 articles were identified by the initial search, of which only 49 satisfied more than one of the above selection criteria. The 49 selected articles are chronologically listed in Table 1. These selected articles were independently analysed by two of the authors (K.C. and T.F.K.) and the results tabulated by consensus.

**Table 1.** Studies that analysed the association between seminal parameters and in-vitro outcomes

<table>
<thead>
<tr>
<th>Authors</th>
<th>Classification</th>
<th>Stain method</th>
<th>Best predictor(s)/best classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mahadevan &amp; Trouson 1984</td>
<td>Eliasson (1971)</td>
<td>Eosin yellow</td>
<td>Abnormal sperm forms and motility</td>
</tr>
<tr>
<td>Apor et al. 1985</td>
<td>WHO (1980)</td>
<td>Formalin and haematoxylin</td>
<td>Sperm count and motility</td>
</tr>
<tr>
<td>Hirsh et al. 1986</td>
<td>WHO (1980)</td>
<td>Not given</td>
<td>Sperm density and motility</td>
</tr>
<tr>
<td>Jeulin et al. 1986</td>
<td>David et al. (1975)</td>
<td>Shorr</td>
<td>Acrosomal morphology and amplitude of lateral head displacement</td>
</tr>
<tr>
<td>Jeyendran et al. 1986a</td>
<td>Williams (1964)</td>
<td>Papanicolaou</td>
<td>Normal acrosome</td>
</tr>
<tr>
<td>Jeyendran et al. 1986b</td>
<td>nWilliams &amp; WHO (1980)</td>
<td>Papanicolaou</td>
<td>nWilliams</td>
</tr>
<tr>
<td>Kruger et al. 1986</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Kruger et al. 1987</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Talbert et al. 1987</td>
<td>Freund &amp; Petersen (1976)</td>
<td>Not given</td>
<td>Forward progression and white blood cell count</td>
</tr>
<tr>
<td>Kruger et al. 1988</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Oehninger et al. 1988</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Chan et al. 1990</td>
<td>WHO (1987)</td>
<td>Not given</td>
<td>Normal sperm morphology insemination concentration and normal intact acrosomes</td>
</tr>
</tbody>
</table>
### Table I. Continued

<table>
<thead>
<tr>
<th>Authors</th>
<th>Classification</th>
<th>Staining method</th>
<th>Best predictor(s)/best classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosenberg et al. (1990)</td>
<td>Fredericsson (1979)</td>
<td>Doxycycline &amp; metronidazole</td>
<td>Not given</td>
</tr>
<tr>
<td>Sevenstor et al. (1990)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Kebayashi et al. (1991)</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Ron-El et al. (1991)</td>
<td>Strict criteria</td>
<td>Eosin–nigrosin</td>
<td>Normal sperm morphology and mobile spermatozoa</td>
</tr>
<tr>
<td>Coates et al. (1992)</td>
<td>Strict criteria</td>
<td>Not given</td>
<td>None of the semen parameters</td>
</tr>
<tr>
<td>Enginsu et al. (1992a)</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology and progressive mobile spermatozoa</td>
</tr>
<tr>
<td>Enginsu et al. (1993)</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Calvo et al. (1994)</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology and sperm concentration</td>
</tr>
<tr>
<td>Grow et al. (1994)</td>
<td>Strict criteria</td>
<td>Diff-Quik</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Ombelet et al. (1994)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Robinson et al. (1994)</td>
<td>Strict criteria</td>
<td>Testesimplet</td>
<td>Sperm concentration and motility</td>
</tr>
<tr>
<td>Hofmann et al. (1995)*</td>
<td>Ideally normal, strict normal sperm and acrosomal morphology, strict criteria &amp; Düsseldorf</td>
<td>Papanicolaou</td>
<td>Düsseldorf</td>
</tr>
<tr>
<td>Yue et al. (1995)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Yang et al. (1995)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Al-Hasani et al. (1996)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Figueiredo et al. (1996)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Harrison &amp; Harrison (1996)*</td>
<td>WHO (1992) &amp; strict criteria</td>
<td>Diff-Quik</td>
<td>Strict criteria</td>
</tr>
<tr>
<td>Hernandez et al. (1996)</td>
<td>Strict criteria</td>
<td>Haematoxylin &amp; Brilliant Green</td>
<td>Normal sperm morphology</td>
</tr>
<tr>
<td>Minkveld et al. (1996)</td>
<td>Strict criteria</td>
<td>Papanicolaou</td>
<td>Normal sperm morphology and acrosomal index</td>
</tr>
<tr>
<td>Vawda et al. (1996)</td>
<td>Strict criteria</td>
<td>Papanicolaou, Spermac,</td>
<td>Normal sperm morphology</td>
</tr>
</tbody>
</table>

\*Comparison of classification systems.

Where possible, odds ratio (OR) and 95% confidence interval (CI) analysis was performed on the number of oocytes fertilized and on the number of pregnancies obtained per cycle within certain normal sperm morphology thresholds (Lau and Chalmers, 1995). Pregnancy per cycle was chosen in preference to ongoing pregnancy rate, even...
though the latter represents greater consensus in pregnancy definition, because a greater number of studies have published this figure. Studies with 0 counts (fertilization or pregnancy rate) were given the value of 0.5 to enable the estimation of OR. The studies included in the review were all observational and therefore no global estimates of the associations were made.

We do not contend that this review is complete, but only that the articles reviewed constitute a representative sample of studies published on the predictive value of sperm morphology in the IVF situation.

Results

Of the 49 articles analysed (Table I), 43 statistically compared the predictive value of a single sperm morphology classification system, while six articles statistically compared the predictive value of more than one normal sperm morphology classification system. The majority (81.4%; 35/43) of the articles concluded in their closing remarks that normal sperm morphology, including acrosomal morphology, had a role to play in the diagnosis of male fertility potential (Table II). Statistical analysis could, however, only be performed on 18 studies, due to the lack of adequate descriptive data (Tables III, IV and V).

Table II. The proportion of articles that obtained good (GPV) or poor prediction values (PPV), with regard to fertilization in vitro, using the different classification systems

<table>
<thead>
<tr>
<th>Classification system</th>
<th>GPV</th>
<th>PPV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO (1980)</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>WHO (1987)</td>
<td>10</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>WHO (1992)</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Strict criteria</td>
<td>19</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>8</td>
<td>43</td>
</tr>
</tbody>
</table>

The largest proportion (48.8%; 21/43) of the articles evaluated the association between the strict criteria normal morphology outcomes and fertilization and/or pregnancy (Table II); 90% (19/21) of these studies obtained a positive association with fertilization and/or pregnancy (Table II). Seventy-six percent (13/17) of studies using the WHO classifications (1980, 1987, 1992) also obtained a useful association. In the six studies comparing the predictive value of different normal sperm morphology classification systems, three preferred the strict criteria, one a modified Williams (1964) classification, one the WHO (1992) criteria and one the Düsseldorf classification (Table I).

The articles (with data) statistically analysed for the predictive value of normal sperm morphology with regards
to fertilization and pregnancy form a very heterogeneous group, due to the different materials and methods used (i.e. stimulation protocols, sperm cell staining procedures, insemination concentrations, sperm preparation procedures, number of embryos transferred, embryo transfer technique, pregnancy validation, etc.). Fifteen of the 18 articles with data used the strict criteria as the sperm morphology classification system, while two used WHO (1980, 1992) guidelines and one used both the WHO and the strict criteria systems.

Using a 5% threshold (strict criteria), 10 studies provided data that could be analyzed for the prediction of fertilization and 11 studies for the prediction of pregnancy (Table III). All the studies showed a positive predictive value for fertilization in vitro, with only one [Figueiredo et al., 1996; OR = 1.42 (CI: 0.90–2.25)] not reaching significance (Figure 2). In the prediction of pregnancy (per cycle), nine studies obtained a positive predictive value. The predictive value of the studies by Oehninger et al. (1988), Enginsu et al. (1992a) and Grow et al. (1994) reached significance (Figure 1). Using a 14% threshold (strict criteria), five studies provided data that could be analyzed for the prediction of fertilization and eight studies for the prediction of pregnancy (Table IV). Similar to the 5% analysis, all these studies showed positive and significant predictive value with regards to fertilization in vitro (Figure 2). In the prediction of pregnancy, two studies (Yue et al., 1995; Figueiredo et al., 1996) did not obtain a positive predictive value, while the studies of Oehninger et al. (1988) and Kruger et al. (1987) were both positive and significant (Figure 1).

The overall fertilization rates using the 5% normal sperm morphology threshold were 59.3% (1979/3337) for the ≤4% group and 77.6% (10 345/13 327) for the >4% group. The overall pregnancy rates around this threshold were 15.2% (60/395) for the ≤4% group compared to 26.0% (355/1368) for the >4% group. The overall fertilization rates using the 14% normal sperm morphology threshold were 72.7% (451/6209) for the ≤14% group and 83.6% (2780/3325) for the >14% group. The overall pregnancy rates around this threshold were 24.3% (130/534) for the ≤14% group compared to 25.2% (164/651) for the >14% group.

### Table III. Studies with data in which the 5% strict criteria threshold could be used to evaluate the predictive value (fertilization and pregnancy) of normal sperm morphology

<table>
<thead>
<tr>
<th>Reference</th>
<th>≤5% n</th>
<th>FR (%)</th>
<th>P/C (%)</th>
<th>≥2% n</th>
<th>FR (%)</th>
<th>P/C (%)</th>
<th>Fertilization Odds ratio</th>
<th>Pregnancy/cycle Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oehninger et al. (1988)</td>
<td>47</td>
<td>47.0 (71/151)</td>
<td>8.5 (4/47)</td>
<td>185</td>
<td>87.7 (642/732)</td>
<td>35.7 (66/185)</td>
<td>8.04 (5.45–11.85)</td>
<td>5.06 (2.05–17.34)</td>
</tr>
<tr>
<td>Kruger et al. (1988)</td>
<td>13</td>
<td>7.9 (5/76)</td>
<td>7.7 (1/13)</td>
<td>32</td>
<td>63.8 (83/130)</td>
<td>31.2 (10/32)</td>
<td>25.06 (9.46–66.48)</td>
<td>5.45 (0.62–47.90)</td>
</tr>
<tr>
<td>Severnster et al. (1990)</td>
<td>13</td>
<td>Not given (0/13)</td>
<td>0.0 (0/13)</td>
<td>138</td>
<td>Not given (1/13)</td>
<td>13.6 (19/138)</td>
<td>4.41 (0.25–77.16)</td>
<td>0.02 (0.00–1.25)</td>
</tr>
<tr>
<td>Enginsu et al. (1992a)</td>
<td>39</td>
<td>25.9 (105/406)</td>
<td>5.1 (2/39)</td>
<td>181</td>
<td>71.2 (1240/1741)</td>
<td>25.5 (41/161)</td>
<td>7.10 (5.55–9.07)</td>
<td>6.32 (1.46–27.39)</td>
</tr>
<tr>
<td>Enginsu et al. (1993)</td>
<td>33</td>
<td>32.3 (104/322)</td>
<td>15.2 (5/33)</td>
<td>152</td>
<td>69.6 (1103/1594)</td>
<td>23.0 (35/152)</td>
<td>4.81 (3.72–6.22)</td>
<td>1.68 (0.60–4.86)</td>
</tr>
<tr>
<td>Grow et al. (1994)</td>
<td>172</td>
<td>88.0 (1076/1332)</td>
<td>17.4 (5/27)</td>
<td>179</td>
<td>91.3 (1224/1340)</td>
<td>30.7 (57/172)</td>
<td>2.51 (1.99–3.17)</td>
<td>2.35 (1.41–3.89)</td>
</tr>
<tr>
<td>Ombelet et al. (1994)</td>
<td>12</td>
<td>37.9 (22/58)</td>
<td>0.0 (0/12)</td>
<td>88</td>
<td>79.1 (367/464)</td>
<td>30.7 (27/88)</td>
<td>6.19 (3.48–11.01)</td>
<td>11.18 (3.64–195.64)</td>
</tr>
<tr>
<td>Robinson et al. (1994)</td>
<td>86</td>
<td>68.7 (43/638)</td>
<td>Not given</td>
<td>724</td>
<td>61.3 (4329/5328)</td>
<td>Not given</td>
<td>1.98 (1.65–2.37)</td>
<td></td>
</tr>
<tr>
<td>Yue et al. (1995)*</td>
<td>25</td>
<td>46.4 (77/166)</td>
<td>52.0 (13/25)</td>
<td>172</td>
<td>63.6 (744/1166)</td>
<td>37.2 (84/172)</td>
<td>2.02 (1.45–2.81)</td>
<td>0.55 (0.24–1.27)</td>
</tr>
<tr>
<td>Hernandez et al. (1996)</td>
<td>17</td>
<td>22.6 (21/93)</td>
<td>0.0 (9/17)</td>
<td>95</td>
<td>76.7 (299/390)</td>
<td>18.9 (18/95)</td>
<td>11.27 (6.57–19.33)</td>
<td>8.35 (0.48–145.38)</td>
</tr>
<tr>
<td>Figueiredo et al. (1996)</td>
<td>14</td>
<td>82.1 (59/55)</td>
<td>35.7 (5/14)</td>
<td>63</td>
<td>89.9 (314/449)</td>
<td>11.1 (7/60)</td>
<td>1.42 (0.90–2.28)</td>
<td>0.24 (0.06–0.91)</td>
</tr>
<tr>
<td>Vawda et al. (1996)</td>
<td>10</td>
<td>Not given</td>
<td>0.0 (0/10)</td>
<td>110</td>
<td>Not given</td>
<td>10.0 (11/110)</td>
<td>2.43 (0.13–44.25)</td>
<td></td>
</tr>
</tbody>
</table>

95% confidence intervals; n = number of cycles; FR = fertilization rate (per oocyte); P/C = pregnancy per cycle rate.
*After Percoll preparation.
Table IV. Studies with data in which the 14% strict criteria threshold could be used to evaluate the predictive value (fertilization and pregnancy) of normal sperm morphology

<table>
<thead>
<tr>
<th>Reference</th>
<th>≤14%</th>
<th>&gt;14%</th>
<th>Odds ratio</th>
<th>Pregnancy/cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>FR (%)</td>
<td>P/C (%)</td>
<td>n</td>
</tr>
<tr>
<td>Kruger et al. (1986)</td>
<td>22</td>
<td>36.5</td>
<td>0.0</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>(36/104)</td>
<td>(0/22)</td>
<td></td>
<td>(579/702)</td>
</tr>
<tr>
<td>Kruger et al. (1967)</td>
<td>25</td>
<td>Not given</td>
<td>12.0</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>(3/25)</td>
<td></td>
<td></td>
<td>(24/71)</td>
</tr>
<tr>
<td>Oshininger et al. (1988)</td>
<td>191</td>
<td>77.9</td>
<td>27.2</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>(566/727)</td>
<td>(52/191)</td>
<td></td>
<td>(147/156)</td>
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<td>Sevenster et al. (1990)</td>
<td>90</td>
<td>Not given</td>
<td>8.8</td>
<td>61</td>
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<td></td>
<td>(8/90)</td>
<td></td>
<td></td>
<td>(11/61)</td>
</tr>
<tr>
<td>Kobayashi et al. (1991)</td>
<td>13</td>
<td>Not given</td>
<td>7.7</td>
<td>110</td>
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<tr>
<td></td>
<td>(1/13)</td>
<td></td>
<td></td>
<td>(32/110)</td>
</tr>
<tr>
<td>Robinson et al. (1993)</td>
<td>556</td>
<td>75.9</td>
<td>Not given</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>(323/4257)</td>
<td></td>
<td></td>
<td>(153/1709)</td>
</tr>
<tr>
<td>Yue et al. (1995)</td>
<td>132</td>
<td>59.7</td>
<td>43.9</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(534/895)</td>
<td>(58/132)</td>
<td></td>
<td>(287/440)</td>
</tr>
<tr>
<td>Figueiredo et al. (1995)</td>
<td>33</td>
<td>61.9</td>
<td>21.2</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>(140/226)</td>
<td>(7/33)</td>
<td></td>
<td>(233/318)</td>
</tr>
<tr>
<td>Al-Hassani et al. (1996)*</td>
<td>28</td>
<td>Not given</td>
<td>3.6</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>(1/28)</td>
<td></td>
<td></td>
<td>(18/91)</td>
</tr>
</tbody>
</table>

95% confidence intervals; n = cycles; FR = fertilization rate (per oocyte); P/C = pregnancy per cycle rate.

*Actual cut-off 12%.

†After Percoll preparation.

Table V. Studies with data in which ‘other’ criteria thresholds could be used to evaluate the predictive value (fertilization and pregnancy) of normal sperm morphology

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>FT</th>
<th>FR (%)</th>
<th>P/C (%)</th>
<th>n</th>
<th>Fertile</th>
<th>FR (%)</th>
<th>P/C (%)</th>
<th>Odds ratio</th>
<th>Fertilization</th>
<th>Pregnancy/cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mahadevan and Trouson (1984)</td>
<td>66</td>
<td>≥60% Not given</td>
<td>58.7</td>
<td>4.5</td>
<td>292</td>
<td>73.5</td>
<td>13.7</td>
<td></td>
<td>1.95 (1.39–2.73)</td>
<td>3.33 (0.10–11.17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(101/172)</td>
<td>(3/66)</td>
<td></td>
<td>(696/847)</td>
<td>(40/292)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yovich and Stanger (1984)</td>
<td>10</td>
<td>≥60%</td>
<td>79.5</td>
<td>10.0</td>
<td>27</td>
<td>85.0</td>
<td>14.8</td>
<td></td>
<td>1.46 (0.51–4.19)</td>
<td>1.57 (0.15–15.97)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(31/39)</td>
<td>(1/10)</td>
<td></td>
<td>(51/60)</td>
<td>(4/27)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yue et al. (1995)*</td>
<td>76</td>
<td>≥30% Not given</td>
<td>57.1</td>
<td>52.6</td>
<td>121</td>
<td>64.1</td>
<td>30.6</td>
<td></td>
<td>1.34 (1.07–1.68)</td>
<td>0.40 (0.22–0.72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(285/499)</td>
<td>(40/76)</td>
<td></td>
<td>(536/836)</td>
<td>(37/121)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

95% confidence intervals; FT = fertility thresholds; n = cycles; FR = fertilization rate (per oocyte); P/C = pregnancy per cycle rate.

*After Percoll preparation.

Of the three studies (Table V) using ‘other’ (Eliasson, 1971; WHO, 1980) normal sperm morphology classification criteria, all produced positive outcomes with regards to fertilization in vitro and two with regards to pregnancy outcome. Two of the studies reached significance in the prediction of fertilization (Mahadevan and Trouson, 1984; Yue et al., 1995), while none reached significance in the prediction of pregnancy.

**Discussion**

The debate on the role of normal sperm morphology in IVF has been continued by this article in the hope of promoting understanding of its value in the management of the infertile couple. To ensure that the basis of our arguments was unbiased we reviewed all the literature available on the subject for the period 1978–1996. The greatest disappointments of
this review were the low number \((n = 18)\) of studies presenting their descriptive data for analysis [three studies using the WHO (1980, 1987, 1992) classification systems and 16 studies using the strict criteria] and the heterogeneity of the studies, which prevented the performance of a meta-analysis.

The simplicity of sperm morphology evaluation is simultaneously its greatest advantage and disadvantage. While it only requires standard laboratory equipment and between 10 min (Diff-Quik) and 2 h (Papanicolaou) processing time, it is difficult to perceive how something as simple and abstract as the form of a spermatozoon can represent its functional capacity, i.e. its ability to complete the complex sequence of events leading to normal fertilization and embryo development. Nevertheless, the majority of the studies reviewed (35/43; 81.4%) showed that the percentage of normal sperm morphology was positively associated with IVF outcome. This association was not restricted to any one particular classification system and/or evaluation procedure. Some of these studies also showed that this association was independent of any of the other semen parameters (Mahadevan and Trouson, 1984; Kruger et al., 1986; Oehninger et al., 1988; Liu and Baker, 1990; Grow et al., 1994).

No study has, however, found normal sperm morphology to be absolute in its prediction, which is understandable considering the complex sequence of events leading to fertilization. Numerous covariates exist that are essential to successful IVF. It would therefore be ill-advised to consider the normal sperm morphology percentage of a man in isolation from the other parameters. A number of the studies reviewed found other semen parameters, such as motility (Mahadevan and Trouson, 1984; Alper et al., 1985; Hirsh et al., 1986; Ron-E1 et al., 1991; Barlow et al., 1991; Robinson et al., 1994), motility characteristics (Jeulin et al., 1986; Cornaire et al., 1988; Chan et al., 1989; Engius et al., 1992a; De Geyter et al., 1992; Duncan et al., 1993) and concentration (Yovich and Stanger, 1984; Alper et al., 1985; Liu et al., 1988; Liu and Baker, 1988, 1990; Biljan et al., 1994; Calvo et al., 1994; Robinson et al., 1994) also to be positively associated with fertilization in vitro and/or pregnancy. Oehninger et al. (1988) clinically substantiated the covariation of normal sperm morphology and the insemination concentration, and found that, by increasing the insemination concentration of severe teratozoospermic patients from 100 000 to 500 000 spermatozoa per oocyte, fertilization could be significantly improved.

From this review it is evident that the normal sperm morphology classification system and evaluation procedures used may not be the overriding factors for accurately predicting outcome, as a high proportion of studies using the strict criteria (90.5%; 19/21) as well as the WHO (1980, 1987, 1992) criteria (76.5%; 13/17) obtained positive association with fertilization and/or pregnancy. The most important factors may rather be the level of commitment to use sperm morphology in male factor diagnosis, good inter- and intra-observer and laboratory quality control and the establishment and use of clinically based normal sperm morphology descriptive guidelines and fertility thresholds. Adherence to these basic principles has helped to establish the Tygerberg strict criteria as a dependable diagnostic tool. While the classification system has been refined to include the poor-prognosis (p-pattern (4% normal sperm morphology) and the good-prognosis (g-pattern, 5–14% normal sperm morphology; Kruger et al., 1988) groups, the physiologically based criteria (Menkveld et al., 1990) and clinically based thresholds (Kruger et al., 1986) have remained constant since 1986. The classification system has now been adopted and used successfully by authors world-wide. The majority of the studies (Oehninger et al., 1988; Engius et al., 1992a; Ombelet et al., 1994; Hernandez et al., 1996) have confirmed the predictive value of normal sperm morphology within the established thresholds (54% and ≤14%). In comparison, the WHO guidelines, which are another of the major classification systems in use world-wide, have changed dramatically since their inception in 1980, becoming ‘stricter’ with each revision (1987 and 1992). The result has been a high level of subjectivity and a lack of consensus, especially with regards to their clinical value and corresponding fertility thresholds. In a recent publication by Ombelet et al. (1997b), a similar demographic distribution of methodologies was obtained from the analysis of questionnaires sent to different laboratories world-wide. In the article they make a plea for the urgent need to standardize sperm morphology methodology to extract maximum value from this important semen parameter.

In all the studies \((n = 18)\) presenting sufficient data for OR evaluation, positive OR (1.27–25.08) were obtained for IVF, with only 16.7% (3/18) not reaching significance. When the data were analysed according to the particular classification system and threshold used, the following did not reach significance: Figueiredo et al. (1996) using a 5% (strict criteria) threshold, Yue et al. (1995) using a 14% (strict criteria) threshold and Yovich and Stanger (1984) using a 60% (WHO, 1980) threshold. The reasons for the good association between normal sperm morphology and IVF have been shown by studies demonstrating the selective properties of the zona pellucida (Franken et al., 1989; Menkveld et al., 1991) and the oocyte oolemma (Liu and Baker, 1994b). The selection process performed by these physiological agents helped in the initial formulation of the strict criteria, the aim of which is to identify those
spermatozoa with the greatest probability of fertilizing an oocyte.

A lower percentage of the analysed studies produced significant predictive value outcomes when predicting pregnancy than when predicting IVF outcome. This outcome was in no way influenced by the normal sperm morphology threshold used. The reasons for lower number of significant OR outcomes in the prediction of pregnancy may be two-fold: additional variables may have decreased the importance of sperm morphology and/or it may be due to statistical formulation, i.e. relatively small sample sizes and small percentage differences. An important additional variable controlled for by the clinician and having a major influence on pregnancy outcome is the number of embryos transferred. Ten of the 18 studies analysed provided their protocol or the mean number of embryos transferred. All the protocol values and the means given in the studies were equal to or below four embryos transferred. Whereas the later premise may be correct for individual studies, the differences between the combined pregnancy rates for this study should reach significance. The mean combined pregnancy rate for patients with ≤4% normal forms is 15.2% (60/395) compared to 26.0% (355/1368) for patients with >4% normal forms, as calculated from all studies providing pregnancy data (Table III). Another important factor influencing the pregnancy rate per cycle obtained is the number of cycles that produce no embryo transfers. In the 5% (strict criteria) threshold analysis, the no-transfer rate was 24.0% (86/359) in the ≤4% group compared to 7.4% (80/1088) in the >4% group. A similar outcome was obtained when the 14% threshold was used: a 26.4% (73/276) rate was obtained in the ≤14% group, while a 7.3% (35/480) rate was obtained in the >14% group. Patients suffering from severe teratozoospermia may therefore have a one in three chance of not having a transfer.

The question is, how can the percentage of normal forms in a semen ejaculate project its influence to the stage of conception, as we have shown that higher percentages of normal forms can be equated with higher pregnancy rates. The importance of the spermatozoa’s contribution to embryo genesis, haploid genome, the centrosome, and the signal to initiate oocyte activation, cannot, however, be underestimated. Three of the studies reviewed (Yovich and Stanger, 1984; Ron-El et al., 1991; Parinaud et al., 1993) concluded from their analyses that the presence of increased levels of sperm head abnormalities resulted in delayed fertilization and poor embryo quality. In a review, Grow and Oehninger (1995) also speculated that higher incidences of head abnormalities lead to embryos with a lower pregnancy potential. Although the fertilization rate can be enhanced by increasing the insemination concentration, a lower pregnancy rate is obtained for severe teratozoospermic patients. In a retrospective cohort study, Oehninger et al. (1996) compared intracytoplasmic sperm injection (ICSI) with high insemination concentration (HIC) in the severe teratozoospermic (<5%) group and found that HIC produced a higher fertilization rate, but that the percentage of high quality embryos was lower, and the implantation rates were lower. Oehninger et al. (1996) speculated that this may be attributed to a ‘toxic effect’ as a result of the presence of high concentrations of spermatozoa and seminal debris, and/or the presence of large numbers of immotile spermatozoa may influence embryo quality and consequently implantation. Dumoulin et al. (1992) also showed that embryonic growth was retarded when greater numbers of spermatozoa were used for insemination.

The advancement of infertility treatment with the introduction of the ICSI procedure has made the correct classification of male fertility paramount, to ensure the best cost–benefit ratio. This is especially true in cases of severe male infertility. The ICSI procedure has been shown to produce consistently fertilization rates of between 50 and 70% in severe male factor cases. The mean IVF rates for patients with a normal morphology percentage <5% ranged from 7.9 to 80.8%. This underscores the importance of being able to identify these severe cases so that they can be given the option of ICSI or at least a diagnostic cycle, i.e. a cycle in which half the oocytes are fertilized by ICSI and the other half inseminated.

In conclusion, standard semen analysis with an accurately evaluated normal sperm morphology count still remains an important screening procedure for male fertility. Although normal sperm morphology may be the most significant indicator of male fertility, the other parameters are essential for an accurate diagnosis. The subjective nature of normal sperm morphology evaluation and its consequential variability, even with the ‘strict’ approach, requires certain measures to be implemented worldwide. Consensus has to be obtained on what constitutes a functionally normal spermatozoon and which preparation methods are essential for the accurate evaluation of sperm morphology. Laboratories that commit themselves to the evaluation of sperm morphology must ensure that they adhere to these basic principles and implement the necessary training programme and quality control procedures. Computer-aided sperm analysis systems may be able to play an active role in this process of standardization, as a tool to complement the manual evaluation of sperm morphology and as a training tool. The importance of this role will, however, be determined by the development of computer technology and clinical trials to assess the accuracy of computer-generated
normal sperm morphology assessments. The clinical application of normal sperm morphology requires the performance of a study on a reference population to determine the normal sperm morphology threshold points distinguishing fertile and infertile groups. Omelet et al. (1997a) performed just such a study by prospectively comparing a fertile and a subfertile population to define normal values for different semen parameters. Sperm morphology was found to be the most significant indicator for subfertility, with a cut-off value of 10% according to receiver operating characteristic (ROC) analysis and 5% using the 10th percentile of the fertile population. This reaffirmed the possibility of two subfertile populations and the 5% threshold as the lowest point of fertility.

Although a true meta-analysis was not performed, the OR analyses clearly showed the advantage in accurately evaluating sperm morphology. Normal sperm morphology may not be absolute in its prediction of fertilization and pregnancy, but remains the most cost-effective means of diagnosing male fertility and assisting in the formulation of a treatment regimen. The selection of the correct treatment regimen will help to maximize fertilization, transfer and, ultimately, probability of pregnancy.

References


82  K.Coeetze, T.F.Kruger and C.J.Lombard


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The following article reflects the scientific basis that supports the above argument:

The role of sperm morphology in assisted reproduction

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This article attempts to evaluate the value of sperm morphology in assisted reproduction by summarizing a recent structured literature review covering the topic. New developments in the field of sperm morphology with emphasis on computer evaluation of morphology and its latest clinical application are highlighted, as well as the correlation between sperm functional tests and sperm morphology. Based on the correlation between the sperm functional tests and sperm morphology, as well as the latter’s proven value as a predictor in in-vitro fertilization, one can assume that sperm morphology reflects function, although based on definition it is not a sperm functional test per se. The evaluation of sperm morphology by strict criteria is a simple, cost-effective method and can be used to guide the clinician and scientist on a day-to-day basis to make sound clinical decisions.

Key words: computer evaluation/sperm functional test/sperm morphology

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Introduction

There is no single semen test that will assist the clinician totally in his ability to predict the potential of a given couple to achieve success in in-vitro fertilization (IVF) or in an assisted reproduction programme. Clinicians and scientists are still confronted with the question of whether a given laboratory test or battery of tests can predict the outcome in assisted reproduction. No simple solution to these problems is available. There is no wide consensus on the value of the different tests available in clinical practice today. In this article the following are discussed: (i) a structured literature review looking at the influence and value of sperm morphology in IVF programmes, (ii) new developments in the field of sperm morphology with emphasis on computer evaluation of spermatozoa, and (iii) the correlation between sperm functional tests and sperm morphology.

Structured literature review of sperm morphology and its value in assisted reproduction

IVF provides the best means of examining sperm-egg interaction and determining fertilization probability for diagnostic purposes, but, obviously, it cannot be used as a routine screening assay. Therefore, due to the cost, time and ethical constraints of IVF and functional assays, correct evaluation of basic semen analysis would be a useful diagnostic tool in male fertility.

The basic semen parameters, e.g. motility concentration and morphology (Kruger et al., 1986; Enginsu et al., 1992a,b; Grow et al., 1994), have been correlated with IVF success. The problem with the different semen parameters is that in the literature there is not a consistent threshold indicating fertility and subfertility, especially when using motility and concentration. There have, however, been attempts to establish thresholds for sperm morphology (Kruger et al., 1986, 1988). By means of a structured literature review of the IVF situation, Coetzee et al. studied the impact of sperm morphology on fertilization and pregnancy rates (Coetzee et al., 1998).

A total of 216 articles were identified by the initial search, of which only 49 satisfied the selection criteria. The selection criteria were (i) statistical associations between sperm mor-
Role of sperm morphology in assisted reproduction

Phylogeny and IVF and/or pregnancy, (ii) abnormal/normal sperm morphology fertility thresholds, and (iii) whether descriptive data (per oocyte fertilization, per cycle/transfer pregnancy rates and pregnancy outcome) were presented. Odds ratio (OR) and 95% confidence interval (CI) analysis were performed on the number of oocytes fertilized and on the number of pregnancies within certain morphology thresholds (Coetzee et al., 1998).

The majority of the articles (3643 = 81.4%) concluded in their closing remarks that normal sperm morphology, including acrosomal morphology, had a role to play in the diagnosis of male fertility potential. Statistical analysis, however, could only be performed on 18 studies due to a lack of adequate descriptive data.

Using a 5% threshold (strict criteria), 10 studies provided data that could be analysed for the prediction of fertilization and 11 studies for the prediction of pregnancy. All the studies showed a positive predictive value for fertilization, with only one [Figueiredo et al., 1996; OR = 1.42 (CI: 0.90–2.25)] not reaching significance. In the prediction of pregnancy (per cycle), nine studies obtained a positive predictive value with predictive value association. The studies of Oehninger et al., Enginsu et al. and Grow et al. reached significance (Oehninger et al., 1988; Enginsu et al., 1992a; Grow et al., 1994). Using a 14% threshold (strict criteria), five studies provided data that could be analysed for the prediction of fertilization and eight studies for the prediction of pregnancy. Similarly, all the studies analysed showed positive and significant predictive value with regard to fertilization. In the prediction of pregnancy, two studies (Yue et al., 1995; Figueiredo et al., 1996) did not obtain a positive predictive value, while the studies of Oehninger et al. (1988) and Kruger et al. (1987) were both positive and significant.

When studying all the data in the 5% (strict criteria) threshold analysis, the no-transfer rate was 24.0% (86/359) in the ≤54% group compared to 7.4% (80/1088) in the >4% group.

Of the three studies using ‘other’ [Eliasson et al., 1971; World Health Organization (WHO), 1980] normal sperm morphology classification criteria, three were positive with regard to fertilization and two with regard to pregnancy. Two of the studies reached significance in the prediction of fertilization (Mahadevan and Trouson, 1984; Yue et al., 1995), while none reached significance in the prediction of pregnancy.

When looking at all the studies available, 92% of the articles evaluated showed a positive association between sperm morphology and IVF success. The association was not restricted to any particular classification system and/or evaluation procedure. Some of these studies also showed that this association was independent of any of the other semen parameters (Mahadevan and Trouson, 1984; Kruger et al., 1986; Oehninger et al., 1988; Liu and Baker, 1990; Grow et al., 1994).

It is of utmost importance to obtain good quality control in a laboratory evaluating sperm morphology. Adherence to these principles has helped to establish the strict criteria as a dependable diagnostic tool. While the strict morphology classification system has been refined with time [P (poor prognosis) pattern ≤54%, G (good prognosis) pattern 5–14% and N (normal) pattern >14%] (Kruger et al., 1988), the physiologically based criteria (Menkeveld et al., 1990) and clinically based threshold (Kruger et al., 1986) have remained constant since 1986. This classification system has now been adopted and used successfully by authors world-wide. The majority of the studies (Oehninger et al., 1988; Enginsu et al., 1992b; Obreut et al., 1994; Hernandez et al., 1996) have confirmed the predictive value of normal sperm morphology within the established thresholds. In comparison, the WHO (1987, 1992) guidelines, another of the major classification systems in use world-wide, have changed dramatically since 1980, becoming stricter with each revision. The result has been a high level of subjectivity and a lack of consensus, especially with regard to the clinical value and corresponding fertility thresholds of this classification (Coetzee et al., 1998). Recently, new publications (Donnelly et al., 1998; Hammadhe et al., 1998; Lim et al., 1998) dealing with strict morphology criteria and IVF outcome supported the conclusions of Coetzee et al. (Coetzee et al., 1998). However, others did not get such clear thresholds and clinical help using this approach.

The advancement of infertility treatment with the introduction of the intracytoplasmic sperm injection (ICSI) procedure has made the correct classification of male fertility paramount, to ensure the best cost–benefit ratio. This is especially true in cases of severe male infertility. The ICSI procedure has been shown to consistently produce fertilization rates of between 50 and 70% in severe male factor cases. This underlines the importance of being able to identify these severe cases so that they can be given the option of ICSI, or at least a diagnostic cycle (a cycle in which half the oocytes are fertilized by ICSI and the other half inseminated).

The importance of standard semen analysis, especially with reference to sperm morphology, is highlighted in the review by Coetzee et al. (1998). If laboratories adhere to the basic principles, do sperm morphology carefully, and if they are consistent in their evaluations, this parameter will be of use in the clinical arena on a day-to-day basis.

New developments in the field of sperm morphology, with emphasis on computer evaluation of spermatozoa, and its predictive ability in IVF

The lack of objectivity in evaluating human sperm morphology, the difficulty of standardizing, implementing and controlling manual methods, and the high degree of variation within and between laboratories and technicians have provided the incentives for the development of instruments for automated sperm morphology analysis (ASMA; Lau and Chalmers, 1995). Although the instruments available are able
to supply quantitative data rapidly, objectively and repetitively, their clinical value still remains unproven (Coetzee et al., 1997). In this section an attempt is made to evaluate the precision of ASMA, its clinical value and future prospects.

**Automated sperm morphology analysis systems: how computer morphology analysers work**

Conventional pattern recognition image analysis systems (Kruger, 1995). To obtain more detailed information regarding the differences between the CellForm-Human instrument and the integrated visual optic system (IVOS; dimension system) from Hamilton-Thorn Research, we refer the reader to the following two articles: Davis et al. (Davis et al., 1992) and Kruger et al., (Kruger et al., 1995). Both systems take measurement of length and width of spermatozoa into consideration. A clear difference between the two systems is the evaluation of acrosomal size and shape of spermatozoa by the IVOS (dimension system), which was shown in previous studies to be of importance in clinical practice (Grow et al., 1994; Kruger et al., 1988).

Automated sperm morphology analysis (ASMA) instruments: These work much like current versions of instruments for computer-aided sperm analyses for motion, except that no movement information is required (Wang et al., 1991a,b; Kruger et al., 1993, 1995; Davis 1993; Garrett and Baker, 1995). The system consists of a microscope, a video camera, a computer frame grabber and morphology software. The video camera delivers the image to the computer's frame grabber which stores it for analysis (Wang et al., 1991a; Davis et al., 1992; Kruger et al., 1993). The image is evaluated by the software to determine whether spermatozoa are present. Sperm recognition is based on software specifications for size, shape, colour intensity and other characteristics. Once spermatozoa have been recognized and segregated from debris and other objects, metric measurements are performed on the head, midpiece, acrosome and other cytoplasmic features. These measurements are the basis for the sperm morphological classification. The accuracy and precision of ASMA instruments depend on (i) the microscope optics, magnification and focusing capabilities; (ii) video camera quality; (iii) array size of the frame grabber; (iv) image processing techniques; (v) definitions of metric measurements (Wang et al., 1991a; Kruger et al., 1993, 1995) and (vi) staining methods used (Kruger et al., 1993; Menkveld and Kruger, 1995; Lacquet et al., 1996).

**Slide preparation and staining**

Slide preparation and staining, as with the manual assessment of sperm morphology, can have a significant influence on the precision of recognition and evaluation achieved with automated systems. The possible variation that can arise from these steps must therefore be minimized, by selecting methods which optimize automated analysis. The production of reproducible and readable slides to ensure reliable results is for obvious reasons more critical for computerized evaluations than for manual evaluations of sperm morphology (Coetzee and Kruger, 1997).

Davis and Gravance found that the percentage of normal spermatozoa detected by the CellForm-Human instrument was not different for washed specimens compared with unwashed controls, but technical variability arising from semen preparation and slide staining methods could be reduced when specimens were washed and resuspended to a standard concentration (150–200 × 10⁶) before smearing (Davis and Gravance, 1993).

No statistical difference in outcome was found between five different Diff-Quik staining procedures (Lacquet et al., 1996). They did, however, prefer washing the semen sample once by centrifugation and resuspending the pellet to a concentration of 100 × 10⁶ cells/ml. Thin, evenly spread smears were made from this solution to ensure that approximately five cells were available per screen for analysis. This approach gave the best results. In their study, Menkveld et al., who were investigating the effect of washing and staining methods (Papanicolaou, Shorr, Diff-Quik and Spermac) on automated evaluation, obtained results comparable with manual evaluation by washing the semen samples once and staining with Diff-Quik stain (Menkveld et al., 1997).

**Accuracy and repeatability**

In a study dealing with this topic, the computer’s ability to classify normal morphology per slide was reported to be promising (Kruger et al., 1995). The computer gives excellent repeatability on normal and abnormal cells. Based on results obtained, it was postulated that this system can be of clinical value in both IVF units and andrology laboratories, but more clinical data are required in this field.

**Clinical studies in assisted reproduction**

In our initial work (Kruger et al., 1993), computer evaluation was also compared with manual evaluation in a clinical study using fertilization rate as an end point. The computerized system identified the <14% of normal forms very well and showed a significant difference in fertilization rate in the groups with ≤14% and >14% normal forms as well as at the 10% level. It was concluded that this new development holds promise for clinical practice.

The ability of the IVOS to predict fertilization *in vitro* was evaluated in a prospective study (Kruger et al., 1995). In all, 80 patients from the Tygerberg gamete intra-Fallopian transfer (GIFT) programme were evaluated in a prospective manner. The same semen sample was analysed on a day-to-day basis by both the laboratory (manual method) and the computerized system for percentage normal morphology, concentration/ml, motility and forward progression. Only patients with more than two oocytes available after GIFT was
performed were included in the study. In all cases, an insemination concentration of 500 000 spermatozoa per oocyte was used where normal morphology was ≥14%. Logistic regression analysis was used to study predictors of fertilization in vitro on excess oocytes. A total of 338 oocytes were obtained from the 80 patients, of which 239 fertilized.

The logistic regression analysis of the manual method (% normal morphology) and IVOS (dimensions) indicated that both were predictors of fertilization. Logistic regression analysis also showed that sperm morphology as evaluated by the IVOS in patients with <10 x 10⁵ spermatozoa/ml after swim-up was a significant predictor of fertilization. The influence of the number of oocytes was also pointed out as significant by this model. Thus, the more oocytes obtained from the partners of the men with spermatozoa in the lower morphological groups, the better the chance of fertilization (Kruger et al., 1995). It was shown that, in patients from whom ≤10 x 10⁵ spermatozoa/ml were obtained, the role of morphology (evaluated by IVOS) as well as the number of oocytes were significant. These factors (% normal morphology, spermatozoa retrieved and number of oocytes obtained) can be considered predictors of fertilization in patients with retrieval of <10 x 10⁵ spermatozoa/ml and <10% normal. The computer can be of great help in identifying the poor prognosis group as far as fertilization is concerned (Kruger et al., 1995).

By using a more simplistic approach on the same data set, it was noted that the overall fertilization rate for IVOS in the group 0-4% normal forms (P pattern) was 45.6% (37/81); 5-9% normal morphology group, 72.5% (87/120); 10-14% normal morphology group, 82.1% (46/56) and in the group >14%, 85.2% (69/81) (P = 0.0001 for P pattern versus other groups).

Recently, K.Coezee (unpublished data) investigated 206 GIFT cycles where sperm morphology was prospectively analysed by computer (IVOS). The pregnancy rate per cycle in the group with ≤5% normal forms was 15.4% (4/26), compared to 35.6% (64/180) in the group with ≥5% normal morphology. Bearing in mind that the ICSI pregnancy rate in our unit was 31% per cycle for 1997 (unpublished), the computer can be used as a screening method to select patients for this procedure.

It is thus obvious from the above-mentioned data from different sections that the computer can become a helpful clinical tool in andrology laboratories and IVF centres. If careful slide preparation is adhered to, computerized analysis can bring more objectivity into morphology evaluation worldwide. More research in this field in the next few years is, however, mandatory to obtain definitive answers.

Correlation between sperm functional tests, other tests on semen and sperm morphology

The aim in this section is to focus on specific tests and their relationships with sperm morphology and, when available, IVF outcome.

Role of sperm morphology in assisted reproduction

The hemizona assay (HZA)

Zona binding and subsequent fertilization in vitro are known to be impaired when spermatozoa from men with teratozoospermia are used (Franken et al., 1996). In an article by Franken et al. it was clearly shown that spermatozoa from teratozoospermic men do not bind at the same level as those from normozoospermic men (Franken et al., 1990). In a group of patients studied who had no fertilization in vitro compared to a control group showing good fertilization in vitro, the hemizona assay (HZA; tightly bound spermatozoa per hemizona) in the group with fertilization was 36 ± 7, and in the group with no fertilization 10 ± 3. It was also shown, by increasing the number of spermatozoa, that for each teratozoospermic man there was a specific sperm concentration necessary to achieve zona binding parity with the number of spermatozoa bound in the control sample (normozoospermic men). This observation in the HZA model fits the observation by Oehninger et al., where an increased fertilization rate per oocyte was observed in men with severe teratozoospermia when the insemination concentration was increased from 50 000 spermatozoa/ml to 500 000 spermatozoa/ml (Oehninger et al., 1988).

Oehninger et al. recently reviewed sperm–zona pellucida binding assays (Oehninger et al., 1998). The two most common zona binding tests currently utilized are the HZA and a competitive intact-zona binding assay. Although different in their methodologies, they both use assessment of tight sperm binding to the zona as the primary end point in an independent comparison within a controlled assay. Significantly, both assays have been demonstrated to have a high predictive value for fertilization results under in-vitro conditions in prospectively designed studies comparing fertile controls and patient samples with a diverse in order spectrum of sperm abnormalities (Burkman et al., 1988; Liu et al., 1988, 1989; Oehninger et al., 1989, 1992, 1997; Liu and Baker, 1992; Franken et al., 1993a,b; Coddington et al., 1994; Gamzu et al., 1994).

Further analyses by Oehninger et al. indicated that there were three sets of homogeneous studies (Oehninger et al., 1998). The largest set consisted of seven studies (Liu et al., 1988; Oehninger et al., 1989, 1992, 1997; Franken et al., 1993a,b; Coddington et al., 1994). The estimated combined correlation for these studies was 0.643 (95% CI = 0.60–0.645) and was, of course, significantly different from zero. The second set consisted of two studies (Liu et al., 1989; Liu and Baker, 1992). The estimated correlation was 0.470 (95% CI = 0.465–0.475) and was also significantly different from zero. The last study (Gamzu et al., 1994) stood alone with an unusually high correlation of 0.96.

From these analyses, it can be concluded that the results of sperm–zona pellucida binding tests (expressed as hemizona index and sperm–zona binding ratio) and fertilization rate are significantly correlated. The best estimate of the correlation, based on the largest set of homogeneous studies, is ~0.64.
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Oehninger et al. also showed that the hemizona index provided the highest discriminatory power for fertilization success/failure compared with sperm morphology (Oehninger et al., 1997). These authors’ approach reveals the value of the HZA as a functional test that is valuable for clinical decision making.

**Acrosome reaction**

The acrosome reaction is an exocytic process involving fusion of sperm plasma membrane and outer acrosomal membrane. Only acrosome-reacted spermatozoa can penetrate the zona pellucida (Koehler et al., 1982). Oehninger et al. studied the acrosome reaction and its prerequisite, a calcium influx, in spermatozoa of infertile men with a high incidence of abnormal sperm forms (Oehninger et al., 1994). They concluded that infertile patients with a high incidence of abnormal sperm forms as diagnosed by strict criteria have a low incidence of spontaneous acrosome reaction and a diminished progesterone-stimulated acrosome reaction, whereas the non-specific response to a calcium ionophore is conserved.

In a study by Bastiaan, sperm samples from 29 men randomly selected from the andrology laboratory were used to evaluate the acrosome reaction response to solubilized human zona pellucida (Bastiaan, 1997). Capacitated sperm samples were exposed to a solution containing 2ZP per μl for 60 min, whereafter acrosomal status was recorded using a PSA-FITC (pisum sativum agglutinin-fluorescein isothiocyanate) technique. After completion of the acrosome reaction studies, patient samples were divided according to percentage of morphologically normal spermatozoa. Three basic groups were identified, namely, fertile donors and teratozoospermic (normal sperm morphology 5–14%, G pattern; n = 25), and severely teratozoospermic (normal sperm morphology ≤4%; n = 4) men. Results were analysed and expressed as correlations between sperm morphology and acrosomal response to solubilized zona pellucida and spontaneous and calcium ionophore-induced reactions. Predictive values for acrosome responsiveness were depicted with ROC (receiver operating characteristic) curve analysis. Sperm morphology evaluated by strict criteria correlated positively and highly significantly with the responsiveness of the acrosome reaction (r = 0.91; P = 0.0001). At a morphologically cut-off value of 4%, the ROC curve analysis showed sperm morphology to be highly predictive of zona pellucida-induced acrosome responsiveness. Of importance was the observation that spontaneous and calcium ionophore-induced acrosome reactions revealed no correlation with sperm morphology. It was concluded that (i) morphological features of human spermatozoa are indicative of specific functional characteristics and (ii) zona pellucida induction of the acrosome is superior as a predictor of sperm morphology compared to calcium ionophore-induced and spontaneous acrosome reactions. This observation could lead to the development of a valuable diagnostic functional test.

Liu and Baker also reported on patients with severe teratozoospermia and the inability of these patients’ spermatozoa to undergo the acrosome reaction in the presence of zona pellucida, in contrast to those from controls (Liu and Baker, 1994). This observation is important and helps to explain the poor fertilization potential of the spermatozoa of some of these patients in vitro.

Using calcium ionophore in patients with teratozoospermia, the acrosome reaction was significantly related to fertilization in vitro (Liu and Baker, 1998). This observation on the acrosome reaction, morphology and motility was also supported by the study of Parinaud et al. (Parinaud et al., 1996).

**Creatine kinase**

A cellular marker of sperm quality, creatine kinase, has been found to be a key enzyme in synthesis of energy transport factors. Higher concentrations seem to indicate a defect in sperm cytoplasmic extrusions (Huszar et al., 1990). Motile sperm fractions from oligozoospermic samples enriched by the swim-up method were found to have lower creatine kinase concentrations than the original samples. Furthermore, when IVF was performed in oligozoospermic men, the group that would prove to be fertile could be predicted on the basis of their sperm creatine kinase activity. However, more work is necessary to make this observation valuable for routine clinical use.

Huszar et al. (1994), using immunocytochemistry for creatine kinase, found that mature sperm selectively bind to the zona pellucida. Spermatozoa with immature creatine kinase-staining patterns seem to be deficient in oocyte recognition and binding capabilities. This corroborates the report (Menkveld et al., 1991) that morphologically superior spermatozoa have a higher binding capacity than abnormally shaped sperm forms. Furthermore, a good relationship has been found between the sperm biochemical parameters of creatine kinase concentration, lipid peroxidation and abnormal sperm morphology (Huszar and Vigue, 1994).

In a recent study, (Rolf et al., 1998) a correlation between total creatine kinase activity and creatine isoenzyme distribution and IVF outcome could not be found. They did, however, notice a correlation between these measurements and sperm morphology.

**Wheat germ agglutinin binding sites**

Wheat germ agglutinin receptors on the sperm head are an interesting discovery in the field of diagnostic andrology (Gabriel et al., 1994). Gabriel et al. indicated a correlation between wheat germ agglutinin receptors at the equatorial region and semen parameters, specifically, morphology. This observation could potentially be of clinical value as well as part of the evaluation of the male partner. Gabriel et al. observed the percentage of wheat germ agglutinin receptor localization on human sperm membrane domain in spermatozoa with P and G patterns and normal semen samples. In the P pattern group,
6.46 ± 14% was observed, compared to 32.91 ± 21 in the normal group.

**Relationship between poor morphology and nuclear DNA**

A number of articles have covered this subject recently (Claassens et al., 1992; Liu and Baker, 1992a; Sanchez et al., 1994; Haid and Schill, 1994; Henkel et al., 1994; Du-doune et al., 1988). Liu and Baker (1992b) reported on sperm nuclear normality (Aniline Blue stain) and its correlation with morphology, sperm zona pellucida binding and fertilization rates in *vitro*. They observed that the number of spermatozoa bound to the zona pellucida, the percentage of spermatozoa with normal morphology and the percentage of spermatozoa with normal DNA were the most significant factors related to fertilization in *vitro*. In another clinical study (Claassens et al., 1992) using Acriodine Orange stain to investigate nuclear maturity, a moderate positive correlation (r = 0.38) between results of the Acriodine Orange test and normal sperm morphology was observed. Sperm morphology, however, proved to be a more significant predictor of fertilization in *vitro* compared to the Acriodine Orange test. In a recent publication (Angelopoulos et al., 1998), a correlation between sperm morphology and nuclear protein maturation (using Acriodine Orange staining) was observed. Acriodine Orange staining, however, did not predict fertilization outcome in *vitro*.

**References**


**Role of sperm morphology in assisted reproduction**


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Received on May 19, 1998; accepted on December 1, 1998.
c. To explore the influence of sperm morphology on IUI pregnancy rate

i. Data on intra uterine insemination (IUI)

IUI is simpler, cheaper and more available to the general public as a treatment for infertility, may it be used for idiopathic infertility or male factor infertility. The question was asked, in a study by Dr Johannes van Waart (Van Waart et al., 2001): Is sperm morphology an indicator of pregnancy outcome in the P-pattern group vs. the G- and N-patterns in an IUI program? After a careful structured literature review was performed it was shown in a meta-analysis that there is a significant improvement in pregnancy rate above the 4% threshold for Strict Criteria. This was an important publication showing for the first time that the observations made In vitro were repeatable in the In vivo situation, in this case IUI programs. The pregnancy rate in the P-pattern vs. the G-pattern showed a 7% difference per treatment cycle.
The following article reflects the scientific basis that supports the above argument:

Predictive value of normal sperm morphology in intrauterine insemination (IUI): a structured literature review

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The aim of the study was to conduct a structured review of the literature published on the use of normal sperm morphology, as an indicator of male fertility potential in intrauterine insemination (IUI) programmes. Published literature in which normal sperm morphology was used to predict pregnancy outcome in IUI during the period 1984–1998 was reviewed. In total, 421 articles were identified via Medline searches. Eighteen provided data that could be tabulated and analysed. Eight of the analysed studies provided sufficient data for statistical analysis, six studies used the Tygerberg ‘strict’ criteria, and two the WHO guidelines (1987, 1992). A meta-analysis of the six studies in the strict morphology group yielded a risk difference (RD) between the pregnancy rates achieved in the patients below and above the 4% strict criteria threshold of −0.07 (95% CI: −0.11 to −0.03; P < 0.001). The WHO criteria group (1987, 1992) had insufficient data to be analysed. Meta-analysis showed a significant improvement in pregnancy rate above 4% threshold for strict criteria. Accurate evaluation of normal sperm morphology results should be an integral part of evaluating the male factor.

Key words: intrauterine insemination/meta-analysis/morphology/strict criteria/structured literature review

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Introduction

Intrauterine insemination (IUI) is a widely utilized method for treating distinct types of infertility. IUI is cheaper, simpler and less invasive than the more sophisticated assisted reproductive techniques of IVF, intracytoplasmic sperm injection (ICSI) and gamete intra-Fallopian transfer (GIFT). For these reasons, it is often the first line of treatment offered to infertile couples with a male factor, hostile cervical mucus, anti-sperm antibodies or idiopathic infertility.

Sperm parameters have been correlated with success in IVF by a large number of studies, but of all the semen parameters, sperm morphology has consistently been the best indicator of male fertility (Coetzee et al., 1998). The main shortcomings of this parameter were the large number of classification systems used to describe which factors constitute a morphologically normal/abnormal spermatozoa, and the subjective nature of the evaluation. Universally, the most common accepted classification systems used to classify sperm morphology are World Health Organization (WHO) (1987, 1992) and Tygerberg ‘strict’ criteria (Kruger et al., 1986, 1988; WHO, 1999).

The aim of this study was two-fold: (i) a structured literature review using Medline, covering articles dealing with sperm morphology and IUI outcome published in the English language from 1984–1998; and (ii) the establishment of normal sperm morphology using Tygerberg ‘strict’ criteria (Kruger et al., 1986, 1988; WHO, 1999) and WHO guidelines (1987, 1992) as an indicator of male fertility potential in IUI programmes.

Structured literature review

In this review, articles were located by means of a computerized Medline search using only the keyword ‘intrauterine insemination’. Limitations were English, human, and 1984–1998. Reviews were also searched in the Cochrane Library using the keywords

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'sperm morphology and intrauterine insemination'. The databank of our infertility unit was searched by hand using the same criteria. Data were also used from the article of Montanaro-Gauci (2001). Cross-references identified during the review research were also included if they were not included initially. The searches were carried out by two authors (J. Van W. & T. C.) independently, and the results compared. Two authors were contacted to obtain data not published in order to make the analysable data as complete as possible. Abstracts of the 421 articles sourced were evaluated and analysed further if sperm parameters were measured against IUl outcome. Fifty-one articles met these criteria. No reviews meeting our criteria were found in the Cochrane Library.

Further analysis yielded 20 articles measuring normal sperm morphology against IUl outcome. Of these articles, 18 stated a definite predictive value of normal sperm morphology; but only eight included sufficient data to be analysed statistically. All data published as pregnancy rate per cycle above and below a given/indicated threshold were included. In one study (Karabinus and Geley, 1997), pregnancy rates were documented as percentage and not as numbers, which made analysis difficult. Percentages were converted to real numbers and rounded off to the lower end in both the ≤4% and >4% morphology groups in order to include this useful dataset. Where possible, pregnancy rates when only a male factor (idiopathic) could be identified (Matorras et al., 1995; Montanaro-Gauci and Geley, 1997) were used in the meta-analysis. If the absence of female pathology was not clearly stated, the pregnancy rates as given in the articles were used (whole population). Meta-analyses were conducted separately for the idiopathic and whole population groups (Matorras et al., 1995 supplied data in both groups). A combined meta-analysis was performed using idiopathic data available (Matorras et al., 1995; Montanaro-Gauci et al., 2001) and whole population data in the balance (Toner et al., 1995; Lindheim et al., 1996; Karabinus and Geley, 1997; Omebelet et al., 1997) (Figure 1). The statistical measure used was the risk difference (RD) between the pregnancy rates of the groups below and above the strict criteria. 4% threshold. Confidence intervals (CI) were calculated as well as probability of the chi-square test under the null hypothesis of a zero risk difference (P-value). A random effects model (Der Simonian and Laird, 1986) was used that acknowledges the presence of heterogeneity. As the Tygerberg 'strict' criteria (Kruger et al., 1986, 1988; WHO, 1999) and WHO criteria (1987, 1992) have been the most commonly used criteria for sperm morphology world-wide during the past few years, only articles using these criteria were analysed.

We do not contend that this review is complete, but only that the articles reviewed constitute a representative sample of studies published on the predictive value of normal sperm morphology in the IUl situation.

Predictive value of normal sperm morphology in IUl

The 18 articles that stated a definite predictive value of normal sperm morphology were divided into a Tygerberg 'strict' criteria group (Kruger et al., 1986, 1988; WHO, 1999) and a WHO (1987, 1992) group. Of the nine articles that used the Tygerberg 'strict' criteria (Kruger et al., 1986, 1988; WHO, 1999) (Table I), six stated a positive predictive value for sperm morphology (Trianni et al., 1993; Toner et al., 1995; Lindheim et al., 1996; Omebelet et al., 1996, 1997; Montanaro-Gauci et al., 2001), and three stated no predictive value at all (Matorras et al., 1995; Karabinus and Geley, 1997; Schulman et al., 1998) (Table I). The nine articles that used the WHO criteria (WHO, 1987, 1992) for normal sperm morphology (Table II) yielded six with a positive predictive value (Bostoff et al., 1990; Francavilla et al., 1990; Johnston et al., 1994; Comhaire et al., 1995; Toner et al., 1995; Chung et al., 1997), and three with no predictive value at all (Bolton et al., 1989; Milingsos et al., 1996; Tomlinson et al., 1996). Eight of the reports included sufficient data to be re-analysed statistically. Six used the Tygerberg 'strict' criteria (Table III), and two (Burr et al., 1996; Tomlinson et al., 1996) the WHO criteria (1987, 1992). Using 4% as a threshold (strict criteria), the results are shown in Table III.

Predictive value of Tygerberg 'strict' criteria

In the strict criteria group, significant differences in pregnancy rate per cycle were found when using a 4% normal morphology as a cut-off (Lindheim et al., 1996; Montanaro-Gauci et al., 2001) (Figure 1). A definite trend was seen towards better pregnancy rates per cycle in the >4% normal morphology group in some studies (Karabinus et al., 1997; Omebelet et al., 1997; Toner et al., 1995), while others (Matorras et al., 1995) found no difference in pregnancy rate in the two groups when using 4% morphology as a cut-off. Idiopathic infertility (pure male factor) was used in two of

Table I. Tygerberg strict criteria

<table>
<thead>
<tr>
<th>Reference value</th>
<th>Cycles</th>
<th>Predictive Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanaro-Gauci et al. (2001)</td>
<td>495</td>
<td>Positive</td>
</tr>
<tr>
<td>Schulman et al. (1998)</td>
<td>544</td>
<td>None</td>
</tr>
<tr>
<td>Karabinus and Geley (1997)</td>
<td>538</td>
<td>None</td>
</tr>
<tr>
<td>Omebelet et al. (1997)</td>
<td>792</td>
<td>Positive</td>
</tr>
<tr>
<td>Lindheim et al. (1996)</td>
<td>172</td>
<td>Positive</td>
</tr>
<tr>
<td>Omebelet et al. (1996)</td>
<td>283</td>
<td>Positive</td>
</tr>
<tr>
<td>Toner et al. (1995)</td>
<td>395</td>
<td>Positive</td>
</tr>
<tr>
<td>Matorras et al. (1995)</td>
<td>271</td>
<td>None</td>
</tr>
<tr>
<td>Trianni et al. (1993)</td>
<td>208</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table II. World Health Organization (1987,1992) criteria

<table>
<thead>
<tr>
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<th>Cycles</th>
<th>Predictive Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chung et al. (1997)</td>
<td>56</td>
<td>Positive</td>
</tr>
<tr>
<td>Burr et al. (1996)</td>
<td>326</td>
<td>Positive</td>
</tr>
<tr>
<td>Tomlinson et al. (1996)</td>
<td>260</td>
<td>None</td>
</tr>
<tr>
<td>Milingsos et al. (1996)</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>Comhaire et al. (1995)</td>
<td>367*</td>
<td>Positive</td>
</tr>
<tr>
<td>Johnston et al. (1994)</td>
<td>10796</td>
<td>Positive</td>
</tr>
<tr>
<td>Francavilla et al. (1990)</td>
<td>441</td>
<td>Positive</td>
</tr>
<tr>
<td>Bostoff et al. (1990)</td>
<td>1086</td>
<td>Positive</td>
</tr>
<tr>
<td>Bolton et al. (1989)</td>
<td>Unknown</td>
<td>None</td>
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</table>

*Couple/months
### Pregnancy rate per cycle

<table>
<thead>
<tr>
<th>Study</th>
<th>≤4%</th>
<th>&gt;4%</th>
<th>Risk diff (95% CI)</th>
<th>Weight</th>
<th>Risk diff (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IDIOPATHIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montanaro-Gauci et al. (2001)</td>
<td>1/38</td>
<td>35/274</td>
<td></td>
<td>77.9</td>
<td>-0.10 (-0.17 to -0.04)</td>
</tr>
<tr>
<td>Matorras et al. (1995)</td>
<td>13/130</td>
<td>3/23</td>
<td></td>
<td>22.1</td>
<td>-0.02 (-0.17 to 0.13)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>-0.08 (-0.16 to -0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$X^2 = 1.22 (df = 1)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WHOLE POPULATION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toner et al. (1995)</td>
<td>6/96</td>
<td>35/309</td>
<td></td>
<td>20.9</td>
<td>-0.04 (-0.11 to 0.02)</td>
</tr>
<tr>
<td>Ombelet et al. (1997)</td>
<td>40/135</td>
<td>76/460</td>
<td></td>
<td>24.7</td>
<td>-0.05 (-0.09 to 0.00)</td>
</tr>
<tr>
<td>Karabinus &amp; Gelety (1997)</td>
<td>3/53</td>
<td>44/485</td>
<td></td>
<td>20.3</td>
<td>-0.03 (-0.10 to 0.03)</td>
</tr>
<tr>
<td>Lindheim et al. (1996)</td>
<td>1/99</td>
<td>15/77</td>
<td></td>
<td>15.5</td>
<td>-0.19 (-0.28 to -0.09)</td>
</tr>
<tr>
<td>*Matorras et al. (1995)</td>
<td>18/172</td>
<td>10/99</td>
<td></td>
<td>18.6</td>
<td>-0.00 (-0.07 to 0.08)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td>$X^2 = 10.74 (df = 4)$</td>
<td>100</td>
<td>-0.06 (-0.11 to -0.01)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td>$X^2 = 11.79 (df = 5)$</td>
<td>100</td>
<td>-0.07 (-0.11 to -0.03)</td>
</tr>
</tbody>
</table>

*Figure 1. Risk difference for pregnancy rate (strict criteria. 4% threshold). Value not included (whole population) in final meta-analysis.*

the studies (Matorras et al., 1995; Montanaro-Gauci et al., 2001) and whole population infertility (female factors were not clearly ruled out) in the rest of the studies (Figure 1). It must be noted that in one study (Matorras et al., 1995), the data were described for idiopathic and whole population infertility. In that study, the whole population data included the idiopathic data. A meta-analysis of the idiopathic infertility data indicated a significantly higher pregnancy rate in the >4% morphology group with a RD of −0.084 (95% CI = −0.158 to −0.010; $P=0.013$). A meta-analysis of the whole population infertility data also indicated a significantly higher pregnancy rate in the >4% morphology group with a RD of −0.055 (95% CI = −0.105 to −0.006; $P=0.014$). Combining these two sets of data in a meta-analysis (as described in the previous section), a RD of −0.07 (95% CI = −0.11 to −0.03; $P<0.001$) was calculated (Figure 1).

Ovulation induction methods used in the six studies that were included in the final analysis were: mostly clomiphene citrate (CC) (maximum number of cycles not stated) (Montanaro-Gauci et al., 2000); human menopausal gonadotrophin (HMG) (maximum number of cycles = 6) (Montanaro-Gauci et al., 1995); HMG + human chorionic gonadotrophin (HCG) (maximum number of cycles = 4) (Lindheim et al., 1996); CC and HMG as well as
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Table III. Studies with data in which 4% strict criteria threshold could be used to evaluate predictive value of normal sperm morphology (all numbers are given as pregnancy rate per cycle).

<table>
<thead>
<tr>
<th>Reference</th>
<th>≤4%</th>
<th>&gt;4%</th>
<th>P</th>
<th>Risk difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanaro-Gauci et al. (2001)</td>
<td>2.6%</td>
<td>15.6%</td>
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<td>Omelet et al. (1997)</td>
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<td>16.5%</td>
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<tr>
<td>Karabinus and Gelety (1997)</td>
<td>6.5%</td>
<td>9.0%</td>
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<tr>
<td>Lindheim et al. (1996)</td>
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<td>10.5%</td>
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<tr>
<td>Matorras et al. (1995)</td>
<td>10.5%</td>
<td>13.0%</td>
<td>–</td>
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<td>Total</td>
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<td>–</td>
<td>&lt;0.001</td>
<td>–0.07</td>
</tr>
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</table>

both in combination (maximum number of cycles not stated) (Karabinus and Gelety, 1997); CC and HMG (maximum number of cycles ≥ 30) (Omelet et al., 1997); and CC as well as HMG and HMGC (maximum number of cycles not stated) (Toner et al., 1995).

Using higher cut-off values in the strict criteria group, a pregnancy rate of 11.4% (17/149) per cycle was found in the G-pattern group (5-14% normal morphology) compared with 24% (18/75) in the N-pattern group (≥14% normal morphology) (Montanaro-Gauci et al., 2001). Others (Toner et al., 1995) had similar findings, with a 7% (12/159) pregnancy rate per cycle in the G-pattern group compared with a 15% (23/150) success rate in the N-pattern group.

Predictive pattern of WHO criteria

Using the WHO morphological evaluation (World Health Organization, 1987, 1992), one group (Burr et al., 1996) found no difference in pregnancy rate per cycle when 30% normal morphology was used as a cut-off. The pregnancy rate in the <30% group was 16.0% (33/206) compared with 16.7% (20/120) in the >30% normal morphology group (P = NS). Others (Tomlinson et al., 1996) had similar findings when using 30% normal morphology as a cut-off. In their study the pregnancy rate per cycle was 21% (3/14) in the <30% morphology group compared with 20% (48/246) in the >30% morphology group (P = 1.000). When using 10% as a cut-off, pregnancy rates of 4.3% (2/46) were found in the <10% morphology group, and 18.2% (51/280) in the >10% morphology group (P = 0.0559) (Burr et al., 1996).

Discussion

Ovulation induction with HMG and IUI as treatment modality was studied to compare to IVF. GIFT and zygote intra-Fallopian transfer (ZIFT) and comparable with IVF or ZIFT, two cycles were comparable with IVF or ZIFT, and inferior to GIFT, three cycles were superior to IVF or ZIFT and comparable with GIFT, and four cycles were theoretically superior to all techniques. In a structured literature review (Coetzee et al., 1998), sperm morphology was shown to be the most useful parameter when evaluating the male factor in the IVF setting. In this review, taking all the data into consideration, an ongoing pregnancy rate of 15.2% (60/396) per cycle was calculated with morphology ≤4%. With morphology >4%, an ongoing pregnancy rate of 26% (355/1368) per cycle was calculated. The findings of these authors (Petersen et al., 1994; Coetzee et al., 1998) prompted us to evaluate sperm morphology as a predictor of pregnancy outcome in an IUI programme, as IUI is a non-invasive procedure (no anaesthetic required) and is less costly than other infertility treatment modalities.

As the Tygerberg ‘strict’ criteria (Kruger et al., 1986, 1988; WHO, 1999) and WHO criteria (1987, 1992) have been the most commonly reported criteria for sperm morphology evaluation worldwide during the past few years, only articles using these criteria were used in the current study. In our search, 18 articles evaluated the definite predictive value of normal sperm morphology against IUI outcome. Of the nine articles that used the Tygerberg ‘strict’ criteria (Table I), five stated a positive predictive value for sperm morphology, while three authors found no predictive value at all. Six of the nine articles in this group had sufficient data to be analysed statistically; four of the six articles stated that sperm morphology had a positive predictive value (Lindheim et al., 1996; Omelet et al., 1997; Toner et al., 1995; Montanaro-Gauci et al., 2001), while two stated no predictive value at all (Matorras et al., 1995; Karabinus et al., 1997). Our overall impression following the literature search (421 articles) was that few data were available for statistical analysis. More raw data would be very helpful in order to make a stronger conclusive statement in a study of this nature. The approach of having important data available in an article should be encouraged amongst prospective researchers/authors in the reproductive field.

The six analysable articles were divided into two groups according to male factor infertility only (idiopathic) and whole population (if female pathology was not clearly ruled out). Idiopathic infertility was investigated separately because, in the absence of female pathology, the impact of sperm parameters can be studied in a better model. Only one group (Matorras et al., 1995) evaluated pure male infertility as well as whole-population infertility pregnancy rates after IUI. Similar trends were seen in both instances. Meta-analyses of both groups were carried out separately, and eventually combined (Figure 1). Pregnancy rates in the idiopathic group were significantly higher in the >4% group, with a RD of -0.084 (95% CI = -0.158 to -0.010; P = 0.013). Pregnancy rates in the whole population group were also significantly higher in the >4% group, with a RD of -0.055 (95% CI = -0.105 to -0.006; P = 0.014). Combining these two sets of data, as described earlier, an RD of -0.07 (95% CI = -0.11 to -0.03; P < 0.001; Figure 1) was calculated. This analysis, although heterogeneous, provides a clear message that the 4% threshold can be used to establish the prognosis of treatment.

Although not subject to systematic review, further analysis of the analysed articles highlighted some interesting findings. One group (Omelet et al., 1997) showed that an inseminating motile count (IMC) of <1 x 10^5 was highly predictive of IUI failure if the morphology was ≤4%. No pregnancies were achieved when parameters were unfavourable. Other authors also evaluated the
Sperm morphology and IUI: meta-analysis

Although the data presented in this study are heterogeneous, the tendency to fail pregnant when sperm morphology is ≤4% is significantly decreased, especially when other factors having an impact are also unfavourable.

References


Montanaro-Gauci, M., Kruger, T.F., Coutee, K. et al. (2001) Stepwise IMC cut-off as 1×10^6 (Horvath et al., 1989; Dodson and Haney, 1991). When using <1×10^6 motile spermatozoa, the latter authors reported no pregnancies (0/17) in their study, while the former group reported only one pregnancy (1/38). Furthermore, success rates of IUI increased linearly when more than one follicle could be induced with follicle stimulation (Plosker et al., 1994; Tomlinson et al., 1996; Montanaro-Gauci et al., 2001). Controlled ovarian stimulation thus always improves the outcome of infertility procedures. This principle must always be kept in mind when dealing with the male factor. Other semen parameters highlighted in the articles were percentage motility where motility >50% had a three-fold higher success rate in IUI when compared with motility <50%, with all other parameters comparable (Montanaro-Gauci et al., 2001). Linearity of movement was also noted to enhance the predictive value of morphology significantly (Toner et al., 1995). The findings of two groups (Toner et al., 1995; Montanaro-Gauci et al., 2001) regarding motility and linearity of movement correlates well with the findings of others (Horvath et al., 1989; Dodson and Haney, 1991; Ombelet et al., 1997) with regard to IMC. All of the latter three groups used standard swim-up techniques for sperm preparation. Thus, the evaluation of IMC in conjunction with sperm morphology might represent an important consideration for clinical decision making. A systematic review of the other parameters stated should be carried out before any clear-cut recommendations regarding the predictive value of these parameters can be made, however. One point of criticism on morphology evaluation is the difficulty of teaching strict criteria and of obtaining uniform consistency. Based on a recent article (Franken et al., 2000), we feel confident that training, followed by consistent quality control, will bring uniform evaluation to clinics.

Using the WHO morphological evaluation (1987, 1992), no difference was found in pregnancy rates when using 30% as a morphological cut-off point (Burr et al., 1996; Tomlinson et al., 1996). The former authors also calculated pregnancy rates using 10% morphology as a cut-off; the difference was not significant (see earlier), but a trend was seen towards better pregnancy rates. The 10% threshold (Burr et al., 1996) for morphology, however, has a tendency towards stricter criteria. The disadvantages of the WHO (1987, 1992) morphology criteria is lack of consistency and uniform threshold values by which to compare data from different authors. Threshold values (when indicated) varied from 8% (Comhaire et al., 1995) to 50% (Francavilla et al., 1990) (Table II), making the WHO (1987, 1992) criteria almost impossible to be used practically/clinically world-wide, even if sufficient raw data are supplied for statistical analysis.

Sperm morphology evaluation is an integral part of male factor evaluation. In the IUI setting, morphology (by strict criteria) proves to be a good predictor of IUI outcome. On the basis of this review, we recommend that if morphology is ≤4%, then IUI should be performed irrespective of other parameters. However, if morphology is ≤4% and other parameters are adequate (IMC >1×10^6, motility >50%, two or more follicles available), then four IUI cycles could be performed (Chaffkin et al., 1991; Petersen et al., 1994; Burr et al., 1996). If morphology is ≤4%, IMC <1×10^6 and motility ≤50%, then other treatment modalities should be considered, for example ICSI/IVF or combined ICSI/IVF cycles. The basic semen analysis can thus be used in infertility practice to develop a cost-effective plan of treatment for
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ii. **The male factor and IUI**

The studies on the male factor and its impact on pregnancy outcome in an IUI program were continued. In a study by a visiting clinician from Malta it was suggested to study male and female factors and the possible effect on pregnancy outcome in the IUI program at Tygerberg Hospital (Montanaro *et al*., 2001). It was concluded in this large study of 522 treatment cycles that the number of follicles was of importance in pregnancy outcome especially in the case of a male factor. As far as sperm morphology was concerned we reported a 2.6% pregnancy rate in the P-pattern group, 11.4% in the G-pattern group and 24% in the N-pattern group. Other female factors that impacted negatively on outcome were endometriosis and tubal factor infertility. It is thus important to realize that male and female factors contribute to pregnancy outcome but the clinician can influence prognosis by increasing the number of follicles especially in severe male factor infertility. In a combined review from the Jones Institute and the Tygerberg group, different factors affecting pregnancy outcome in IUI programs were studied and reported on (Duran *et al*., 2002).
The following article reflects the scientific basis that supports the above argument:

Stepwise regression analysis to study male and female factors impacting on pregnancy rate in an intrauterine insemination programme

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Key words. Female factor—intrauterine insemination—idiopathic infertility—male factor—sperm morphology

Summary. The aim of this study was to evaluate the impact of male and female factors on the pregnancy rate in an intrauterine insemination (IUI) programme. Data on 522 cycles were retrospectively studied. All patients 39 years or younger were included in the study where data were available on male and female diagnosis, as well as on ovulation induction methodology. Regression analysis was possible on 495 cycles to study different factors affecting the pregnancy rate per treatment cycle. Logistic regression identified variables which were related to outcome and were subsequently incorporated into a statistical model. The number of follicles was found to have a linear association with the risk ratio (chance) of pregnancy. The age of the woman was also found to have a linear (negative) association with pregnancy. The percentage motility and percentage normal morphology (by strict criteria) of spermatozoa in the fresh ejaculate were the male factors that significantly and independently predicted the outcome. Percentage motility ≥50 was associated with a risk ratio of pregnancy of 2.95 compared to percentage motility <50. Percentage normal sperm morphology >14% was associated with a risk ratio of pregnancy of 1.8 compared to percentage normal morphology ≤14%. Female patients with idiopathic infertility were divided into three groups according to normal sperm morphology. The pregnancy rate per cycle was 2.63% (1/38) for the P (poor) pattern group (0–4% normal forms), 11.4% (17/149) for the G (good) pattern group (3–14%), and 24% (18/75) for the N (normal) pattern group (>14% normal forms). A female diagnosis of endometriosis or tubal factor impacted negatively on the probability of pregnancy (risk ratio of 0.17), compared with other female diagnoses. Male and female factors contribute to pregnancy outcome, but the clinician can influence prognosis by increasing the number of follicles, especially in severe male factor cases.

Introduction

Some reports claim cumulative pregnancy rates for intrauterine insemination (IUI) with the controlled ovarian hyperstimulation (COH) approach to be equal to the results obtained from more sophisticated assisted reproductive techniques such as in vitro fertilization (IVF) or gamete intrafallopian transfer (GIFT) (Chaifkin et al., 1991; Peterson et al., 1994). IUI is a cheaper (Peterson et al., 1994), simpler and less invasive treatment option, which is more readily available. For these reasons it is often the first line of treatment offered to subfertile couples.

Success rates in IUI have improved since the introduction of sperm preparation techniques such as swim-up or Percoll gradient centrifugation. Better results can also be obtained by combining IUI with COH (Chaifkin et al., 1991; Di Marzo et al., 1992).

It is useful to have prognostic variables to help identify couples who would do well or, more importantly, who might fail to conceive with IUI. Attention has focused on the different semen parameters, as semen analysis results are
often used to make clinical decisions. Many laboratories follow the World Health Organization (WHO) guidelines for semen analysis and utilize the 1987/1992 criteria for normal values (WHO, 1992). However, a number of laboratories are now choosing stricter criteria for defining normal sperm morphology. The Tygerberg strict criteria (Kruger et al., 1986; Menkveld et al., 1990) have a biological basis for its definition and have proved helpful in predicting IVF outcome. This is also true in IUI programmes (Toner et al. 1995; Lindheim et al. 1996). Apart from morphology (Toner et al. 1995, Lindheim et al. 1996), other semen parameters, particularly motility and the inseminating motile count [IMC], also were shown to be useful in predicting the outcome of IUI. (Horvarth et al., 1989; Brasch et al., 1994; Tomlinson & Amisah-Arthur, 1996; Ormeleki et al., 1997).

The aim of this retrospective study was to determine whether sperm morphology (strict criteria) and/or other semen parameters, as well as female factors, can assist in predicting the outcome of IUI.

**Materials and methods**

**Subjects**

This retrospective study included 522 cycles of IUI selected from a larger number of cycles performed at the Reproductive Biology Unit of Tygerberg Hospital between February 1993 and November 1997. Cycles were selected on the basis of the availability of all the relevant data in women of 39 years or younger. Donor spermatozoa were utilized in some cases of severe teratozoospermia or azoospermia or hereditary genetic disorders.

**Infertility evaluation**

All patients had been investigated for infertility prior to IUI, according to the Tygerberg Hospital protocol (Grobler et al., 1990; Hulme et al., 1990). Routine semen analysis was performed according to the Tygerberg standard method (Menkveld & Kruger, 1990). For sperm morphology assessment, the Tygerberg strict criteria were used (Kruger et al., 1986; Menkveld et al., 1990). Aetiologies were classified into the following groups: idiopathic; male factor only; female factor only (including ovulatory dysfunction, cervical factor, endometriosis, others); and mixed factors (male and female).

Male factor was defined as subnormal semen parameter/s, namely: oligozoospermia—sperm count of \(<10 \times 10^6\) ml\(^{-1}\); teratozoospermia—normal morphology of \(<15\%\); asthenozoospermia—motility of \(<30\%\) (with forward progression equal to 2) (Kruger et al., 1986).

In the case of teratozoospermia, patients were divided into three groups: P (poor) pattern (0–4% normal forms); G (good) pattern (5–14% normal forms); and N (normal) pattern (>14% normal forms) (Kruger et al., 1988).

**Ovarian stimulation**

For the stimulated cycles in this study various stimulation protocols were employed. They could be grouped into three categories: those stimulated with clomiphene citrate (CC), those stimulated with human menopausal gonadotrophin (hMG), and those stimulated with hMG and CC.

In a stimulated cycle 5000 or 10000 units human chorionic gonadotrophin (hCG), was given in mid-cycle to trigger ovulation. Luteal phase support was not employed in any treatment cycles.

**Timing of insemination**

In natural cycles or stimulated cycles where hCG was not used, timing of insemination was by serum LH levels (\(\geq 2 \times\) basal level) and also transvaginal ultrasound to assess follicle size (\(\geq 18\) mm) and endometrial thickness (\(\geq 8\) mm) and by cervical mucus evaluation (\(\geq 8\) modified Inslcr score, Van Zyl 1980). Insemination was performed 24–30 h after the start of the LH surge. Many cycles involved double insemination with the second insemination taking place approximately 24 h after the first. In these cases the semen parameters utilized in the study were those of the first insemination sample. In cycles where hCG was given, a single insemination was performed 36–40 h after hCG injection.

**Sperm preparation**

The methodology used to evaluate semen parameters (morphology, motility and concentration) was as described in previous publications (Kruger et al., 1986; Menkveld et al., 1990; Coetzee et al., 1993). The majority of samples were subjected to a double wash and swim-up technique using Ham’s F10 medium supplemented with natural serum (10% v/v).

**Method of insemination**

Inseminations were performed with the 4½ inch open-ended Tomcat catheter (Sherwood).
Stellenbosch University

PREGNANCY RATE IN IUI

St Louis, USA.). The inseminate (in 1 ml of medium) was slowly injected, high up within the uterine cavity. The patient rested for 10 min before rising and resuming normal activity.

**Diagnosis of pregnancy**

Serum levels of β-hCG were tested 12 days after insemination and again 4 days later. Pregnancy was diagnosed if the first result was positive (>10 IU l⁻¹) and was followed by a significant rise (doubling every 48 h) over the next 4 days. Outcome in this study was measured in terms of rate of total pregnancies. This included early miscarriages, pregnancies confirmed by ultrasound at 7 weeks gestation but subsequently lost, ongoing pregnancies and pregnancies taken to term.

**Data analysis**

Outcome was expressed in terms of total pregnancy rate per cycle. The effect on outcome of a number of factors was examined, namely semen parameters, female diagnosis, age of the woman, number of pre-ovulatory follicles and ovarian stimulation (or its omission). Logistic regression analysis was carried out and a statistical model created where the risk ratio (or chance) for achieving pregnancy was reported for the variables incorporated into the model. In addition, the results were also stratified by sperm morphology to help determine if this was a valid prognostic indicator.

**The logistic regression model**

For the continuous variables cut-off points were first identified by preliminary analysis of the data and the continuous variables were then categorized accordingly. Each variable was cross-tabulated with pregnancy outcome. The variables were thus individually investigated and from these results a multivariate model was tested. The cycles performed on a particular couple are not truly independent and the generalized estimation equations methodology (GEE) was used with a binomial link function in order to calculate pregnancy outcome. The correlation between cycles of the same couple was, however, very small (0.02-0.03) and this, together with the small cluster size, meant that the modelling could be done using a standard and logistic regression approach (Kruger et al., 1995).

In the final regression model, the pregnancy outcome is modelled on the following variables: number of pre-ovulatory follicles; percentage sperm motility (in the fresh ejaculate); percentage normal sperm morphology (in the fresh ejaculate); woman’s age; use of stimulation (or its omission); and diagnosis of endometriosis or tubal factor (compared with other female diagnoses). The statistical measure used in reporting associations is the risk ratio.

The one-sided t-test was used in the tables to compare pregnancy rates in different groups.

**Results**

Five hundred and twenty-two heterogenous cycles of IUI were entered into the study. From these cycles, 57 conceptions resulted, giving an overall total pregnancy rate per cycle of 10.9%. IUI was performed on 273 couples with the number of cycles per couple ranging from one to nine. The cycles were classified according to aetiology of infertility into four groups, namely: idiopathic infertility; female factor; male factor; and mixed male and female factors. The total pregnancy rate per cycle was 24% (18/75) for couples with idiopathic infertility, 5.13% (2/39) for couples with a female factor, 9.62% (18/187) for couples with a male factor, and 9.28% (18/194) for couples with mixed male and female factors.

The mean age of the male patients was 34.01 (3.1) and 31.05 (3.8) for the female patients, respectively. Average duration of infertility in the study group was 3 years, with a range of 1–11 years. The descriptive data of semen parameters (concentration ml⁻¹, motility and teratozoospermia) are shown in Table 1. Sixty-six patients had a concentration between 1 and 10 million ml⁻¹ (12.9%), 108 between 10 and 20 million ml⁻¹ (20.6%) and 348 had a concentration >20 million ml⁻¹ (20.6%). The descriptive statistics of the motility and morphology groups are also depicted in Table 1. Six variables were identified and incorporated into the statistical model, namely: number of

<table>
<thead>
<tr>
<th>Concentration (ml⁻¹)</th>
<th>Motility (%)</th>
<th>Morphology (%) normal forms</th>
</tr>
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<tr>
<td>1–10 x 10⁶</td>
<td>≤30</td>
<td>0–4</td>
</tr>
<tr>
<td>10–20 x 10⁶</td>
<td>30–49</td>
<td>3–14</td>
</tr>
<tr>
<td>108 (20.6%)</td>
<td>115 (22.0%)</td>
<td>315 (60.3%)</td>
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<tr>
<td>&gt;20 x 10⁶</td>
<td>≥50</td>
<td>&gt;14</td>
</tr>
<tr>
<td>548 (60.6%)</td>
<td>159 (68.7%)</td>
<td>116 (92.2%)</td>
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</table>

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follicles; female age; endometriosis; tubal factor; motility; and sperm morphology. The two factors most significantly related to outcome were the number of follicles and the woman's age. Both variables showed a linear association with outcome. The age of the woman was similarly categorized into three different groups, namely age <25 years, age 25–33 years and age >35 years. The chance of pregnancy increased with an increase in the number of follicles (Table 2) and decreased with rising age of the woman. There is a three times higher chance for a pregnancy at 25 years of age compared to >35. The number of follicles was a continuous variable that was categorized into two different groups, namely cycles with one follicle and cycles with ≥2 follicles. In 429 (86.67%) of these cycles, some form of ovarian stimulation was employed, whereas 66 (13.33%) of the cycles were unstimulated. The pregnancy rate for unstimulated cycles was 13.2% (9/68). Without taking the number of follicles into consideration, the pregnancy rate in the CC group was 10.36% (40/390), in the hMG group 19.2% (5/26) and in the combination of hMG and CC group 13.33% (2/15). When taking the number of follicles into consideration, the pregnancy rate per cycle was 7.75% (19/245) for those with one follicle, and 14.8% (37/250) where two or more follicles were obtained (Table 2) (P = 0.007). The pregnancy rate per cycle and multiple pregnancy rate are noted in Table 2.

Sperm motility before preparation emerged as a significant predictor of IUI outcome within the statistical model. A cut-off value of 50% motility was established from preliminary analysis of the data. With sperm motility of >50%, the chance of achieving pregnancy was almost three times the chance with motility ≤50% (95% confidence interval, 1.3–6.6) (Table 3).

Sperm morphology was also found to be a significant predictor of IUI outcome within the statistical model. When the normal sperm morphology (according to strict criteria) was >14%, the chance of achieving a pregnancy was 1.6 times greater than when the percentage normal sperm morphology was ≤14% (95% confidence interval, 0.31–0.96). To control for the female factor the role of sperm morphology on pregnancy outcome in women with idiopathic infertility was studied. Sperm morphology was divided into three categories: P pattern group (0–4% normal morphology), G pattern group (3–4% normal morphology) and N pattern group (>14% normal morphology). Pregnancy rate in the P pattern group was 2.63% (1/38), in the G pattern group 11.4% (17/149) and in the N pattern group 24% (18/75) (P group vs. N group, P = 0.005).

To study the impact of multiple factors on pregnancy outcome we categorized the cycles in Table 3, based on the logistic model developed. The categories were motility (<50% and >50%), normal sperm morphology [P, G and N patterns], and number of follicles (1 and ≥2). In the <50% motility category the pregnancy rate showed no improvement with an increase in the

| Table 2. Total pregnancy rate per cycle according to number of follicles |
|--------------------------|--------------------------|--------------------------|
| Number of follicles      | Pregnancy rate per cycle (%) | Multiple pregnancies (%) |
| 1                        | 19/245 (7.75)             | 0/19                     |
| 2                        | 29/188 (15.6)             | 1/29 (3.4)               |
| 3                        | 6/33 (11.3)               | 2/6 (33)                 |
| 4                        | 2/3 (22.2)                | 0/2                     |

1 follicle vs. ≥2 (P = 0.087).

| Table 3. The impact of sperm morphology groups, percentage motility and number of follicles on pregnancy rate |
|--------------------------|--------------------------|--------------------------|
| Percentage motility      | Number of follicles      | Sperm morphology groups |
|                          | P group PR/c (%)         | G group PR/c (%)         | N group PR/c (%)         |
| <50                      | 1                        | 0/18 (0%)                | 3/41 (7.3%)              | 3/19 (15.8%)             |
|                          | ≥2                       | 0/15 (0%)                | 1/57 (1.8%)              | 1/12 (8.3%)              |
| Combined cycles           | 1                        | 0/31 (0%)                | 4/104 (3.8%)             | 4/31 (12.9%)             |
| ≥50                      | 2/31 (6.5%)              | 8/101 (7.9%)             | 4/47 (8.5%)              |
| Combined cycles           | ≥2                       | 6/29 (20.7%)             | 17/100 (17.0%)           | 12/38 (32.6%)            |
|                          | 8/60 (13.3%)             | 23/211 (11.8%)           | 16/35 (46.0%)            |

P group, poor prognosis; G group, good prognosis; N group, normal prognosis; PR/c = pregnancy rate per cycle.

\( ^* \) vs. \( ^* \), \( P = 0.5 \); \( ^* \) vs. \( ^* \), \( P = 0.002 \); \( ^* \) vs. \( ^* \), \( P = 0.001 \); \( ^* \) vs. \( ^* \), \( P = 0.112 \); \( ^* \) vs. \( ^* \), \( P = 0.069 \); \( ^* \) vs. \( ^* \), \( P = 0.011 \); \( ^* \) vs. \( ^* \), \( P = 0.273 \); \( ^* \) vs. \( ^* \), \( P = 0.004 \); \( ^* \) vs. \( ^* \), \( P = 0.228 \).
number of follicles. The categorization according to normal sperm morphology, within both the follicle categories, showed an increasing pregnancy tendency with an increase in normal sperm morphology. In the >50% motility category the increasing pregnancy tendency according to normal sperm morphology was lost. In this category (motility >50%) the number of follicles, however, had a significant impact. Comparing the two motility categories (combined cycles) we found that the pregnancy rates in the P and G pattern groups of the category >50% motility were significantly higher.

With regard to female causes of infertility it emerged from the analysis that endometriosis and tubal factor were associated with a poorer prognosis than other female factors within the model. Endometriosis or tubal factor is associated with a 0.17 chance of pregnancy compared to other female factors (e.g. anovulation, cervical factor) \(P=0.0756\).

**Discussion**

Many factors are known to affect the outcome of IUI. Logistic regression analysis identified six variables for incorporation into a statistical model. These were: number of follicles, the woman’s age, sperm motility (in the fresh ejaculate), normal sperm morphology (strict criteria), endometriosis or partial tubal factor (compared to other female factors), use of controlled ovarian hyperstimulation (or its omission).

Sperm morphology was assessed using the strict criteria. Data analysis showed this parameter to be an independent, significant prognostic indicator in IUI. The value of 14% normal forms separated patients with a good prognosis (>14% normal forms) from those with a poorer prognosis (≤14% normal forms). We then considered the group of patients where the female was normal, stratified the male patients by morphology groups and compared them with the female group having idiopathic infertility. Here the pregnancy rates were significantly different in the various morphology categories. In our study the pregnancy rate per cycle in the P pattern group (0–4% normal morphology) was 2.63% (1/38) compared to 11.41% (17/149) in the G group, and 24% (18/75) in the N group. These findings underline the importance of sperm morphology assessment in predicting the outcome of IUI.

The predictive value of the percentage of morphologically normal spermatozoa was first assessed in the IVF setting. Using the strict criteria a threshold of 14% was similarly established to identify patients with different prognoses (Kruger et al., 1986). In addition, patients with ≤4% normal forms (by strict criteria) were found to have a significantly worse prognosis in IVF (Kruger et al., 1988; Grow et al., 1994). More recently other studies have assessed the predictive power of this same parameter for the outcome of IUI. A study undertaken by Toner et al. (1995), utilizing strict criteria, also described a threshold of 14% to distinguish between prognostic groups but failed to identify a worse prognosis for patients with <5% normal sperm morphology. Studies by Lindhein et al. (1996) and by Ombelet et al. (1997) identified lower thresholds for sperm morphology that are predictive of a poor outcome in IUI. Ombelet et al. (1997) concluded that the percentage of normal forms (by strict criteria) was only a valuable prognostic parameter when the inseminating motile count was <1 × 10⁶ as in these cases a value of ≤4% normal forms represented a very poor prognosis. Lindheim et al. (1996) described a similar threshold for normal morphology (by strict criteria) below which the outcome was significantly worse in IUI.

Of importance was the finding that the pregnancy rate per cycle could be influenced by increasing the number of follicles (Plosker et al., 1994; Tomlinson & Amissah-Arthur, 1996). The current data indicate a good pregnancy chance for the P pattern group if the motility is >50%, but not in the <50% motility group. The pregnancy chance can be improved significantly if the number of follicles can be increased in the >50% motility group (Table 3). This was not the case in the P and G pattern groups with motility ≤50% (in the P pattern group no pregnancy resulted in 31 treatment cycles). Where motility parameters are poor with poor morphology, patients should be considered for intracytoplasmic sperm injection (ICSI) treatment (Table 3).

If adhered to the principles outlined by Peterson et al. (1994), four insemination (IUI) cycles will give a superior chance to either IVF or GIFT. Based on the data from our study (Table 3), all the morphology groups in the motility group >50% as well as the N pattern group with <50% motility should be offered four insemination cycles. The chance of pregnancy should be discussed with the patient prior to embarking on IUI as a treatment option. In contrast to all the above studies, Mataresi et al. (1995) did not find sperm morphology (by strict criteria) at all useful in predicting IUI outcome in male factor infertility.
The multiple regression analysis model identified a linear decrease in fertility with age in the IUI cycles studied. This is in agreement with the findings of Wichman et al. (1994) and Elmers et al. (1994) relating to infertile couples in general. Thus in our study women under 20 years of age had a three times higher chance of pregnancy compared to women aged 35–38 years. However, Tomlinson & Amishah-Arthur (1996) looked at prognostic indicators for IUI and found that pregnancy rates did not decline with increasing female age and were only significantly reduced after the age of 40 years. Other studies concur with this finding (Hull et al., 1992; Frederick et al., 1994). Our own data support the view that fecundity declines steadily with age and leads us to conclude that more aggressive protocols of IUI should be offered to women over 30 years of age, especially where a male factor is involved.

When the data were stratified according to female diagnosis, it was seen that most of the diagnoses did not differ significantly in outcome. The exceptions were endometriosis and partial tubal factor where each gave significantly poorer results in the IUI programme compared to the other female diagnoses. These two diagnostic groups were subsequently combined in the multiple regression analysis model and the chance of pregnancy was less with either of these diagnoses than for ovulatory dysfunction, cervical factor or when no female problem was identified. In our unit we will favour assisted reproduction as a treatment option in patients with severe endometriosis and where previous surgery was offered on Fallopian tubes (microsurgery or endoscopic surgery), especially where poor tubal prognostic factors were identified during surgery (Hull et al., 1992).

It can therefore be concluded that of the semen parameters, motility and morphology were found to be predictive of IUI outcome in the statistical model created in this study. Of the female factors, endometriosis and tubal factor were predictive of a poor outcome in this study. Increasing female age was negatively related to outcome in a linear fashion, whereas an increasing number of follicles was positively related to outcome in a linear fashion. These factors must be taken into consideration when treating patients, especially in cases with male factor infertility. Taking the detail of the male factor into consideration, one can thus always offer controlled hyperstimulation and IUI as second therapy options with hMG or clomiphene citrate (Ecochard et al., 2000) to achieve more than one follicle before reverting to assisted reproduction.

References


ANDROLOGIA 33, 135–141 (2001)
PREGNANCY RATE IN IUI


ANDROLOGIA 33, 135–141 (2001)
The following article reflects the scientific basis that supports the above argument:

Intrauterine insemination: a systematic review on determinants of success

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Intrauterine insemination (IUI) is a frequently indicated therapeutic modality in infertility. Here, a systematic review of the literature was performed to examine the current status of clinical and laboratory methodologies used in IUI and the impact of female and male factors on pregnancy success. Emphasis was centred in questioning the following: (i) the value of IUI against timed intercourse; (ii) IUI application with or without controlled ovarian hypostimulation; (iii) timing and frequency of IUI; and (iv) impact of various parameters (male/female) on the prediction of pregnancy outcome. The odds of multiple pregnancy occurrence and its risk factors, as well as the cost-effectiveness of IUI treatment compared with more complex assisted reproductive technologies are discussed. A computerized literature search was performed including Medline and the Cochrane library, as well as a crossover search from retrieved papers. It is concluded that although IUI is a successful contemporary treatment for appropriately selected cases of female and/or male infertility, further research is needed through well-designed studies to improve the methodologies currently utilized. Importantly, the clinical management of the infertile couple should be performed in an expedited manner taking into consideration the age of the woman, the presence of multifactorial infertility and cost-effectiveness of the available treatment alternatives.

Key words: cost-effectiveness/double insemination/female or male factors/intrauterine insemination/timed intercourse

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Introduction

Intrauterine insemination (IUI) is frequently used in the treatment of infertile couples with various causes of infertility, including cervical factor, ovulatory dysfunction, endometriosis, immunological causes, male factor and unexplained infertility. It is also the mode of treatment for various ejaculatory and coital problems. IUI is generally considered to be an intermediate step of low to moderate complexity before the application of more sophisticated assisted reproductive technologies (ART) such as IVF with or without ICSI (Oehninger, 2001).

The overall success rate of IUI remains controversial and depends on several factors, with published pregnancy rates ranging from as low as 5% to as high as 70% per patient; however, a 10-20% clinical pregnancy per cycle is an acceptable range for all aetiologies (Allen et al., 1985; Onime et al., 1995). IUI may be performed in natural cycles, as well as in conjunction with controlled ovarian hyperstimulation (COH). When combined with COH in unexplained infertility, cumulative pregnancy rates may approach those of ART (Hammond et al., 1998; Abougah et al., 1999; Goverde et al., 2000).

In this review, daily dilemmas that the physician confronts in the clinical setting when indicating IUI therapy were examined, with special emphasis on the analysis of success and cost-efficiency of IUI performed for male and unexplained infertility. The objectives were to: (i) compare the success rate of IUI with that of timed intercourse (TI) and intracervical insemination (ICI); (ii) compare the success rate of IUI cycles according to the management strategy (natural versus COH); (iii) review the methods and strategies used to time IUI with ovulation; (iv)
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identify the factors reported to determine IUI outcome; (v) review the risk factors reported for multiple pregnancy as a major drawback of IUI; and (vi) evaluate the cost-effectiveness of IUI as an option for infertility management.

Research methods

A computerized search of the published literature was carried out, including Medline and the Cochrane library. The search was not limited to English language literature. Key words used for the search included: intrauterine insemination, IUI, timed intercourse, human, randomized controlled trial, pregnancy, success, prog-nosis, sperm, intracervical insemination, natural, controlled ovarian hyperstimulation, ovulation induction, double, repeat, frequency, logistic regression, morphology, method, separation, preparation, selection, wash, density, gradient, swim-up, sperm stimulants, pentoxifylline, multiple pregnancy and cost-effective-ness. Retrieved articles were reviewed for content and their references were used to identify other articles of interest. For the selected objectives (i-vi), there were various randomized controlled trials as well as meta-analyses of them; therefore, studies with other types of design were not included. However, for the other objectives (iv, v) all published articles that could be retrieved were reviewed. The Breslow-Day model (Breslow and Day, 1980) was used to test statistical heterogeneity. If statistically homogeneous, data were pooled for each comparison and overall combined odds ratios (OR) with 95% confidence intervals (CI) were calculated using the Peto method (a fixed-effects model).

IUI versus TV/IUI

It has been documented that IUI is superior to TV in couples with male subfertility (Cohen et al., 2000). The results obtained from six randomized controlled trials indicated that IUI significantly improved the probability of conception compared with TV with an OR of 2.5 and 95% CI of 1.6-3.9 in natural cycles (Kerin et al., 1984; Kerin and Quinn, 1987; Ho et al., 1989; Veale et al., 1989; Martinez et al., 1990; Kirby et al., 1991). Seven randomized controlled trials showed a similar improvement in pregnancy with an OR of 2.2 (95% CI 1.4-3.6). In cycles of IUI combined with COH, against TV (Martinez et al., 1990, 1991; Evans et al., 1991; Croquigniot and Walters, 1994; Nan et al., 1994; Melis et al., 1995; Gregorius et al., 1996).

IUI is slightly more beneficial than TV or ICI in couples with unexplained infertility, in natural cycles. Based on two trials evaluating this issue in 1691 cycles, a border-line benefit was obtained by IUI over TV or ICI (OR = 2.7, 95% CI 1.0-4.4) (Kirby et al., 1991; Guzick et al., 1999). Intrauterine insemination combined with COH in unexplained infertility has also been proven to be superior to TV. A total of 980 cycles in seven prospective randomized studies (Croquigniot et al., 1991; Evans et al., 1991; Martinez et al., 1991; Karlston et al., 1993; Zikopoulos et al., 1993; Gregorius et al., 1995; Melis et al., 1995) yielded an improved probability of pregnancy (OR = 1.8, 95% CI 1.3-2.6) for couples with unexplained infertility treated with COH/IUI (Zeyneloglu et al., 1998). The studies included for this section and the level of evidence they provided are summarized in Table I.

Natural cycle versus ovarian stimulation in conjunction with IUI

In general, clomiphene citrate (CC) and/or gonadotrophins are used for COH in conjunction with IUI. For male subfertility, COH obtained by CC does not seem to increase the efficiency of IUI. Two randomized controlled trials (Martinez et al., 1990; Arici et al., 1994) were combined, which indicated the inefficiency of CC as a mode of COH for male subfertility (OR = 0.78, 95% CI 0.4-1.6). When gonadotrophins were used for COH/IUI on the other hand (Nilsen et al., 1993; Cohen et al., 1998), the probability of conception was increased (OR = 2.0, 95% CI 1.1-3.8) as compared with IUI only (Cohen et al., 2000). For unexplained infertility, COH by either CC (Arici et al., 1994) or gonado- trophins (Nilsen et al., 1993) improves the fecundity rate when compared with IUI alone. This is further supported by others (Guzick et al., 1999) who showed that the probability of pregnancy was 1.7 times more likely (95% CI 1.2-2.6) for COH/IUI when compared with IUI alone. Meta-analysis of IUI data was 3.2 times more likely to occur (95% CI 2.0-5.3) when COH/ IUI was compared with ICI (Guzick et al., 1999).

The studies included for this section and the level of evidence they provided are summarized in Table II, with the exception of two (Martinez et al., 1990; Guzick et al., 1999), which were already listed in Table I.

Timing/induction of ovulation, frequency of insemination

Timing of ovulation appears to be one of the crucial factors to determine the success of IUI therapy. It is the major goal of treatment to provide sperm that are capable of fertilizing the oocyte at the site of fertilization during a narrow time window, the so-called periovulatory period. Various strategies have been developed to achieve this goal. Urinary LH peak monitoring, hCG injection to stimulate ovulation and scheduling IUI with different frequencies at different time points are some of those strategies. hCG injection is a well-documented and accurate means of triggering ovulation by the time of optimal follicle maturation. However, it does not have superiority against spontaneous ovulation detected by urinary LH detection kits (Deaton et al., 1997; Zeik et al., 1999). Its main advantage is to give the physician a better control in the management of the cycle.

Several retrospective investigations in donor insemination programmes provided conflicting results on the impact of insemination frequency (single versus double) on pregnancy outcome (Matthews et al., 1979; Centola et al., 1990; Khalifa et al., 1995; Lincoln et al., 1995; Matilsky et al., 1998). In this review, three randomized controlled trials were found in the literature which aimed to investigate this topic on IUIs performed with husband’s sperm (Table III). The earliest of these was conducted on 49 cycles of COH/IUI (in cycles stimulated with hMG), and a higher cycle fecundity rate in favour of double insemination was reported (Silverberg et al., 1992). The next prospective randomized study on 169 cycles similarly managed, however, did not report a difference in outcome between these two options of management (Ranson et al., 1994).

The most recent prospective randomized study on 449 COH/ IUI cycles (with CC and gonadotrophins) indicated an increased cycle fecundity for double insemination performed 12 and 34 h
Table 1. Studies that compared the efficiency of intrauterine insemination (IUI) and timed intercourse (TI) for patients with male subfertility or unexplained infertility

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>n</th>
<th>Interventions</th>
<th>Outcomes</th>
<th>Comments</th>
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<tr>
<td>Crosignani et al.,</td>
<td>Random, cross-over, single centre</td>
<td>90 (130)</td>
<td>IUI vs TI, COH cycles (method not specified), unexplained infertility</td>
<td>PR per completed cycle, 10/15 for IUI, 5/96 for TI, NSD, NSD</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>1991</td>
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<tr>
<td>Crosignani and</td>
<td>Random, cross-over, single centre</td>
<td>NS (97)</td>
<td>IUI vs TI, COH cycles (method not specified), male subfertility</td>
<td>PR per completed cycle, 7/48 for IUI, 6/40 for TI, P = 0.006</td>
<td>Drawing black or white disk from a blinded bag, dropouts NS</td>
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<td>Walters, 1994</td>
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<td>Evans et al., 1991</td>
<td>Random, cross-over, single centre</td>
<td>22 (44)</td>
<td>IUI vs TI, COH cycles by CC+HMG, male subfertility (including men with anti-sperm Ab), unexplained infertility</td>
<td>PR per completed cycle, 0/22 for IUI, 1/22 for TI, NSD</td>
<td>Sealed opaque envelopes, dropouts &gt;10%</td>
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<td>Gregorini et al.,</td>
<td>Random, cross-over, single centre</td>
<td>67 (158)</td>
<td>IUI vs TI, COH cycles by HMG, male subfertility</td>
<td>PR per completed cycle, 15/130 for IUI, 5/128 for TI (P = 0.05)</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>1996</td>
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<td>Gatzick et al.,</td>
<td>Random, parallel, multicentre</td>
<td>932 (2678)</td>
<td>IUI vs ICI, natural and COH cycles by pure FSHI, male subfertility, unexplained infertility, unexplained infertility</td>
<td>PR per completed cycle, 35/717 for IUI, 14/706 for ICI, 54/418 for COH+IUI, 26/377 for COH+ICI (P = 0.01)</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>1999</td>
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<td>Ho et al., 1989</td>
<td>Random, cross-over, single centre</td>
<td>47 (238)</td>
<td>IUI vs TI, natural cycles, male subfertility</td>
<td>PR per completed cycle, 0/114 for IUI, 1/324 for TI, NSD PR per completed cycle, 4/22 for IUI, 7/47 for TI, NSD</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<tr>
<td>Karlton et al., 1993</td>
<td>Random, multicentre</td>
<td>148 (48)</td>
<td>IUI vs TI, COH cycles by CC+HMG</td>
<td>PR per completed cycle, 4/32 for IUI, 7/47 for TI, NSD</td>
<td>Randomization method, concealment of allocation, dropouts &lt;10%</td>
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<td>Kerin et al., 1984</td>
<td>Random, cross-over, single centre</td>
<td>35 (77)</td>
<td>IUI vs TI, natural cycles, male subfertility</td>
<td>PR per completed cycle, 8/39 for IUI, 0/36 for TI by LH (P = 0.05), 1/34 for TI by progesterone methods (P = 0.02)</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>Kerin and Quinn,</td>
<td>Random, cross-over, single centre</td>
<td>NS (509)</td>
<td>IUI vs TI, natural cycles, male subfertility</td>
<td>PR per completed cycle, 26/296 for IUI, 6/213 for TI (P = 0.01)</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>1987</td>
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<td>Kirby et al., 1991</td>
<td>Random, cross-over, single centre</td>
<td>261 (996)</td>
<td>IUI vs TI, natural cycles, male subfertility</td>
<td>PR per completed cycle, 30/342 for IUI, 15/453 for TI, NSD</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<tr>
<td>Martinez et al.,</td>
<td>Random, cross-over, single centre</td>
<td>38 (115)</td>
<td>IUI vs TI, natural and COH cycles by CC, male subfertility, unexplained infertility, unexplained infertility, cervical factor</td>
<td>PR per completed cycle, natural cycles: 3/22 for IUI, 20/40 for TI, NSD</td>
<td>Randomization method, concealment of allocation, dropouts &lt;10%</td>
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<td>1990</td>
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<tr>
<td>Martinez et al.,</td>
<td>Random, cross-over, single centre</td>
<td>16 (56)</td>
<td>IUI vs TI, COH cycles by HMG, male subfertility, unexplained infertility</td>
<td>PR per completed cycle, 3/40 for IUI, 2/37 for TI by HCG injection, 3/34 for TI by LH surge, NSD</td>
<td>Randomization method, concealment of allocation, dropouts &gt;10%</td>
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<td>1991</td>
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<tr>
<td>Melis et al., 1995</td>
<td>Random, parallel, single centre</td>
<td>184 (402)</td>
<td>IUI vs TI, COH cycles by pure FSHI, male subfertility, unexplained infertility</td>
<td>PR per completed cycle, 3/226 for IUI, 35/226 for TI, NSD</td>
<td>Numbered, sealed envelopes</td>
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<td>Net et al., 1994</td>
<td>Random, cross-over, single centre</td>
<td>76 (202)</td>
<td>IUI vs TI, COH cycles by HMG, male subfertility</td>
<td>PR per completed cycle, 11/107 for IUI, 4/95 for TI, NSD</td>
<td>Sealed opaque envelopes, dropouts NS</td>
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<td>te Velde et al.,</td>
<td>Random, cross-over, single centre</td>
<td>30 (202)</td>
<td>IUI vs TI, natural cycles, male subfertility, cervical factor</td>
<td>PR per completed cycle, 3/12 for IUI, 2/90 for TI, NSD</td>
<td>Sealed opaque envelopes, dropouts NS</td>
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<td>1989</td>
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<tr>
<td>Zlokopulos et al.,</td>
<td>Random, cross-over, single centre</td>
<td>48 (85)</td>
<td>IUI vs TI, COH cycles by GnRH (long luteal) + HMG</td>
<td>PR per completed cycle, 6/40 for IUI, 9/45 for TI, NSD</td>
<td>Randomization method, concealment of allocation, dropouts NS</td>
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<td>1993</td>
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*Values not in parentheses indicate number of complete cycles; values in parentheses indicate number of completed cycles.

COH: controlled ovarian hyperstimulation; ICI: intracervical insemination; NS = not stated; NSD = no significant difference; PR = pregnancy rate.
after hCG administration, as compared with both single and double inseminations performed 34 and 60 h after hCG injection (Ragini et al., 1999). Combining all the cycles with single IUI (n = 265) and those with double IUI that were performed 12–43 h after hCG injection (n = 241) from these three studies, a double IUI seems to increase the probability of pregnancy (OR = 2.3, 95% CI 1.4–3.9). However, further randomized controlled trials with better design are needed to confirm this finding.

### Prediction of pregnancy

**Factors related to the couple**

Despite the evident effectiveness of IUI for various causes of infertility—particularly cervical factor, ovulatory dysfunction and unexplained infertility—there is no consensus on the parameters that determine pregnancy success. Of several parameters that are claimed to have an important effect on IUI outcome, a parameter related to the couple is duration of infertility. In a retrospective analysis of 260 IUI cycles, logistic regression analysis revealed a 10% conception rate per cycle if the duration of fertility exceeded 72 months. For a shorter history of infertility, the conception rate was >20% (Tomlinson et al., 1996). However, another study, which was a randomized controlled trial based on a higher number of couples (and cycles), failed to demonstrate such an association (Goverde et al., 2000).

**Female parameters**

Another set of factors associated with IUI success is the aetiology of female infertility. It is a difficult task to isolate the influence of female factors on IUI outcome. One of the methods to achieve this goal is to select couples with no known cause of male infertility, prospectively. Another way to fulfill this task is by using logistic regression analysis, generally on retrospective data. This latter method was preferred in most of the studies mentioned in this section. In a retrospective review of 1728 cycles of IUI (Hendin et al., 2000), with data analysed by logistic regression, it was reported that the absence of history of any pelvic corrective surgery was one of the factors directly associated with a successful IUI outcome.

A recent retrospective report of 2473 cycles identified unexplained infertility and anovulation as favourable factors to predict the likelihood of pregnancy as compared with other aetiological factors, also by logistic regression analysis (Khalil et al., 2001). Another recent analysis of 495 cycles reviewed retrospectively by stepwise regression analysis revealed a negative impact of the diagnoses of endometriosis or tubal factor on IUI outcome (Montanaro et al., 2001). Based on these findings, a history of pelvic inflammation, regardless of the cause and whether its consequences are corrected or not, seems to decrease the likelihood of conception by IUI. On the contrary, unexplained anovulatory causes of infertility are aetiologies with relatively better prognostic value in terms of pregnancy. Further data, preferentially in prospective form, are needed to reveal the impact of other types of female aetiological problems on IUI success.

Other significant female factors that are associated with a positive IUI outcome are age, number of pre-ovulatory follicles and endometrial thickness by the time of ovulation, as well as indicators of vascular compliance in ovarian, uterine and spiral arteries (Campana et al., 1996; Tomlinson et al., 1996; Tohma et al., 1997; Stone et al., 1999; Hendin et al., 2000; Tsai et al., 2000; Khalil et al., 2001). The age of the female partner is a well known, indirect indicator of oocyte quality, a consensus that was reached as a result of several reports of ART. Evidence from several studies also indicates it as a determinant of IUI outcome (Campana et al., 1996; Kang and Wu, 1996; Stone et al., 1999; Hendin et al., 2000; Khalil et al., 2001; Montanaro et al., 2001). Other factors reported generally depend on the presence of COH in conjunction with IUI. In this respect, most of the female parameters claimed to affect IUI outcome are secondary indicators of the presence, as well as the impact, of COH.
The presence of severe male factor infertility is an indication to proceed to ART, rather than IUI (Oehninger, 2000). For male subfertility, however, IUI has a proven role as a clinical treatment modality, even though it has a lower success rate for this type of infertility (Oehninger et al., 1997; Khalil et al., 2001). Therefore, male parameters, especially those related to the ejaculate, may be more determinative for IUI outcome, especially for couples with known male subfertility. A combination of post-semen preparation sperm motility and concentration seems to be the major predictive factor, although other variables have also been proposed.

A total motile sperm count (TMSC) per insemination was reported to affect IUI outcome in 1115 cycles, with pregnancy rates of 2.1 and 6.7% for samples with TMSC per insemination <1×10^6 and ≥1×10^6, respectively (Campura et al., 1996). Another report on a retrospective analysis of 9963 IUI cycles also identified sperm motility in the inseminate as a major determinant of outcome, with <20% motility in inseminate significantly decreasing the possibility of pregnancy (pregnancy rates of 5.5 and 14.0% for motility in inseminate <20 and ≥20% respectively) (Stone et al., 1999). The total number of motile sperm inseminated was the only variable found by a group of European investigators to significantly affect the pregnancy rate; values <2×10^6 resulted in the poorest outcome (pregnancy rates of 4.6 and 9.2% for TMSC per insemination <2×10^6 and ≥2×10^6, respectively) (van der Westerlaken et al., 1998).

Logistic regression analysis of 1728 cycles also indicated post-wash sperm motility to be a determinant of IUI outcome, with a threshold of 40% (Hendin et al., 2000). The number of inseminated motile sperm was also reported as one of the six variables best predicting IUI outcome in a logistic regression analysis of 2473 cycles of the Scandinavian population (pregnancy rates 5.3 and 12.8% for TMSC per insemination <5×10^6 and ≥5×10^6, respectively) (Khalil et al., 2001).

It is very difficult to suggest a universal threshold for these parameters, since the inclusion criteria, methods of evaluation and even the pregnancy rate per cycle vary considerably among the studies mentioned. However, we believe that it would not be prejudiced to claim that the total motile sperm count and/or motility after semen processing are the parameters that have been cited most commonly as the predictors of IUI outcome. Instead of
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**Table IV.** Studies that described male-derived determinants of IUI outcome

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<tr>
<th>Reference</th>
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<th>Interventions</th>
<th>Statistics</th>
<th>Described determinants of IUI outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campana et al., 1996</td>
<td>Retrospective analysis of IUI cycles during a 5 year period</td>
<td>332 (1115)</td>
<td>Natural and COH cycles by CC or hMG</td>
<td>$\chi^2$, trend and life-table analyses</td>
<td>Cycle no. (first three cycles), age of woman (&lt;39 years), TMSC per insemination (1×10^7) Age of woman (&lt;38 years), history of corrective pelvic surgery, motility in the inseminate (≥40%)</td>
</tr>
<tr>
<td>Hendlin et al., 2000</td>
<td>Retrospective analysis of IUI cycles during a 3 year period</td>
<td>533 (1728)</td>
<td>Cycle management not stated</td>
<td>Logistic regression, life-table analyses with Kaplan-Meier methods</td>
<td>Age of woman (&lt;37 years), history of corrective pelvic surgery, motility in the inseminate (&lt;40%)</td>
</tr>
<tr>
<td>Karahesas and Gavle, 1997</td>
<td>Retrospective analysis of IUI cycles during a 2.5 year period</td>
<td>193 (538)</td>
<td>COH cycles by CC, hMG or CC+hMG; male subfertility, unexplained infertility, cervical factor, tubal factor, endometriosis, ovulation dysfunction</td>
<td>Least-squares methods using the general linear models</td>
<td>None, (No difference in PRs of groups with sperm morphology cut-off of 5, 10, 20 and ≥30%)</td>
</tr>
<tr>
<td>Khalil et al., 2001</td>
<td>Retrospective analysis of IUI cycles during a 9 year period</td>
<td>893 (2473)</td>
<td>COH cycles by CC, CC+FSH, CC+hMG, GrRHa+hMG Male subfertility, unexplained infertility, ovulatory dysfunction, one-sided tubal pathology</td>
<td>Logistic regression, $\chi^2$ analyses</td>
<td>Cycle no. (first cycle), number of follicles at the time of IUI (&gt;1), COH protocol (CC+FSH and CC+hMG better than CC), TMSC per insemination (&gt;5×10^7), time of insemination (day 13-16 of cycle), antithesis of infertility (ovulation dysfunction and unexplained infertility better than male subfertility)</td>
</tr>
<tr>
<td>Lindheim et al., 1996</td>
<td>Retrospective analysis of IUI cycles during a 4.5 year period</td>
<td>42 (176)</td>
<td>COH cycles by hMG, pure FSH, hMG+pure FSH</td>
<td>Student's t-test, $\chi^2$ analyses</td>
<td>Sperm morphology by strict criteria (≥4%)</td>
</tr>
<tr>
<td>Matzenkas et al., 1995</td>
<td>Prospective analysis of IUI cycles during a 2 year period. Male partners' sperm morphology evaluated 1 month before the first IUI cycle</td>
<td>74 (271)</td>
<td>COH cycles by hMG or FSH; male subfertility, cervical factor, tubal factor, endometriosis, ovulatory dysfunction</td>
<td>Kolmogorov-Smirnov, Student t-tests, $\chi^2$ analyses</td>
<td>None, (No difference in PRs of groups with sperm morphology cut-off of 4% or normal-slightly amorphous forms cut-off of 10%. This applies to both couples with male subfertility and whole population)</td>
</tr>
<tr>
<td>Montanaro et al., 2001</td>
<td>Retrospective analysis of IUI cycles during a 5.5 year period</td>
<td>373 (495)</td>
<td>Natural and COH cycles by CC, hMG or CC+hMG Male subfertility, unexplained infertility, ovulatory dysfunction, cervical factor, endometriosis, combined male and female factors</td>
<td>Student's t-test, logistic regression</td>
<td>Age of woman (&lt;35 years), number of follicles (≥2), motility before sperm preparation (&gt;80%), sperm morphology by strict criteria (≥4%), absence of endometriosis or tubal factor</td>
</tr>
<tr>
<td>Olive et al., 1997</td>
<td>Retrospective analysis of IUI cycles during a 7 year period</td>
<td>373 (792)</td>
<td>COH cycles by CC, male subfertility, unexplained infertility, cervical factor, endometriosis, ovulatory dysfunction, combined male and female factors</td>
<td>$\chi^2$, Student’s t-test, ROC curve analyses</td>
<td>Sperm morphology by strict criteria (≥4%)</td>
</tr>
<tr>
<td>Ston et al., 1999</td>
<td>Retrospective analysis of IUI cycles during a 6 year period</td>
<td>~3200 (9963)</td>
<td>Natural and COH cycles by CC, CC+hMG, CC+FSH+hMG, Extra-cc+FSH+hMG, FSH, FS+hMG, GsrRHa+FSH+hMG, hMG</td>
<td>ANOVA, $\chi^2$ analyses</td>
<td>Cycle no. (first three cycles), age of woman (&lt;32 years), number of follicles at the time of IUI (&gt;2), TMSC per insemination (&gt;2×10^7), motility in the inseminate (&gt;20%)</td>
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Table IV. Continued

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<tr>
<th>Reference</th>
<th>Study design</th>
<th>a²</th>
<th>Interventions</th>
<th>Statistics</th>
<th>Described determinants of IUI outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toner et al., 1995</td>
<td>Retrospective analysis of IUI cycles during a 1 year period</td>
<td>126 (395)</td>
<td>COH cycles by CC or IMG; male subfertility, unexplained infertility, ovulatory dysfunction, cervical factor, peritoneal factor, endometriosis</td>
<td>ANCOVA, χ² analysis, logistic regression, ROC analysis</td>
<td>Sperm morphology by strict criteria (≥14%), linearity of movement TMSC per insemination (≥2×10⁹)</td>
</tr>
<tr>
<td>van der Westelaan et al., 1998</td>
<td>Retrospective analysis of IUI cycles during a 8 year period</td>
<td>566 (1763)</td>
<td>COH cycles by CC; male subfertility, unexplained infertility, ovulatory dysfunction, one-sided tubal patholgy</td>
<td>χ² analysis</td>
<td>TMSC per insemination (≥2×10⁹)</td>
</tr>
</tbody>
</table>

*Values not in parentheses indicate number of couples; values in parentheses indicate number of completed cycles. CC = clomiphene citrate; COH = controlled ovarian hyperstimulation; FR = pregnancy rate; ROC = receiver-operating characteristic; TMSC = total motile sperm count.

Evidently, morphology of sperm assessed by strict criteria is one of the best predictors of IVF (Kruger et al., 1986; Oehninger et al., 1988; Engels et al., 1993). However, its predictive power for IUI outcome is not a matter of consensus. One of the reports claiming it as a predictive factor for IUI outcome relies on the data obtained from 176 cycles of 42 couples, indicating a 28.3-fold (95% CI 3.2–250.5) greater likelihood of achieving pregnancy with a favorable sperm morphology (Lindheim et al., 1996). Similarly, previous data from 395 IUI cycles accomplished at one of our centres also indicated a predictive capacity for sperm morphology (Toner et al., 1995). A recent logistic regression analysis of 495 cycles also demonstrated sperm morphology as one of the four variables to predict IUI outcome (Montanaro et al., 2001). Morphology has also been proposed to have a good predictivity for cases with <1×10⁹ sperm in the inseminate (Ombelet et al., 1997). However, similar pregnancy rates were also reported for samples with poor and normal morphology in both prospective (271 cycles) (Mastorides et al., 1995) and retrospective studies (538 cycles) (Karabulus and Gelety, 1997).

Based on existing data from the six studies mentioned above, a recent meta-analysis yielded a risk difference of -0.07 (95% CI -0.11 to -0.03) between pregnancy rates achieved in patients with poor (<4%) and normal (≥4%) sperm morphology (Van Waart et al., 2001). A risk difference of zero indicated the absence of any effect of poor sperm morphology on the outcome, whereas a negative risk difference—which was the case here—indicated a negative impact of poor sperm morphology on the outcome. The exclusion of zero from the 95% CI made this impact significant. The higher the absolute value of risk difference, the higher is the impact. Since both the impact calculated was relatively small and the majority of the studies evaluated were retrospective, we believe that prospective, more powerful, well-designed studies are needed to definitively establish the role of sperm morphology in predicting IUI outcome.

Several other parameters related to other features of sperm are currently under investigation. So far, motion characteristics evaluated by computer-assisted sperm analysis have not indicated a consistent prognostic value. With the advent of different methods of evaluation, prognostic values of several other sperm parameters, such as those related to energy metabolism, membrane characteristics and nuclear maturity/normality of sperm, will yet need to be determined. More data are also needed to examine the predictive value of the more validated available sperm functional assays, i.e. sperm-zona pellucida binding tests and induced-acrosome reaction testing, on IUI outcome (ESHRE Andrology Special Interest Group, 1996; Oehninger et al., 2000).

Sperm processing methods

There is no consensus on the use of sperm processing methodologies for IUI. Although most centres perform a simple wash in culture medium with or without protein supplementation, other programmes perform IUI following separation of purified sperm populations after swim-up, density gradient centrifugation (DGC) or other methods (Daya et al., 1987; Gonzales and Pella, 1993; Zimmerman et al., 1994; Centola et al., 1998). Only three published randomized controlled trials could be found which compared different methods of sperm preparation for IUI (Karlstrom et al., 1991; Carrell et al., 1998; Dodson et al., 1998). The first two studies were included to compare the efficiencies of two methods, wash and DGC (Table V), and the third study (Karlstrom et al., 1991) was excluded as it compared swim-up with self-migration in sodium hyaluronate. Combined data from the two studies (465 cycles in 443 couples) yielded a borderline benefit (OR = 1.7, 95% CI 1.0–2.9) in favour of DGC. Further randomized controlled comparisons are warranted to confirm these results. Until then, the selection of the sperm processing technique should be tailored to the individual case.

Unfortunately, the in-vitro use of substances to stimulate sperm functions and/or metabolic activities has not yielded expected results. Such stimulants have included, among others, xanthine derivatives (e.g. caffeine, pentoxyfylline and others), adenosine derivatives and analogues, kinin-enhancing drugs, follicular fluid and prostaglandins (Cummins and Yoshiv, 1993; Mizro et al., 1993; Nassar et al., 1998, 1999; Vandekerckhove et al., 2000; Brown et al., 2001; Toner et
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Table V. Studies that compared wash and density gradient centrifugation as sperm preparation methods for intrauterine insemination

<table>
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<th>Reference</th>
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<th>Interventions</th>
<th>Outcomes</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>Carrell et al., 1998</td>
<td>Random, cross-over, multicentre</td>
<td>363 (361)</td>
<td>Wash vs DCC (90 and 35% double-layer Percoll); natural and COH cycles (CC or gonadotrophin); male subfertility, unexplained infertility, wide range of male- and/or female-related disorders; samples with ≤0.1% progressive motile sperm not included</td>
<td>PR per completed cycle, 33/204 for DCC, 14/157 for wash. *P=0.04</td>
<td>Randomization method, concealment of allocation, dropout rate NS</td>
</tr>
<tr>
<td>Dodson et al., 1998</td>
<td>Random, neither cross-over nor parallel (cycle-specific randomization), single centre infertility, endometriosis, minor pelvic adhesions; patients with severe oligo-zoo-permia not included (threshold NS)</td>
<td>89 (153)</td>
<td>Wash vs DCC (90 and 45% double-layer Percoll); COH cycles only (gonadotrophin); male subfertility, unexplained</td>
<td>PR per completed cycle, 10/51 for DCC, 8/53 for wash. NSD</td>
<td>Computer-generated random numbers, concealment of allocation, dropout rate NS</td>
</tr>
</tbody>
</table>

*Values not in parentheses indicate number of couples; values in parentheses indicate number of completed cycles.
COH = controlled ovarian stimulation; DCC = density gradient centrifugation; NS = not stated; NSD = no significant difference; PR = pregnancy rate.

al., 2001). Although some of these substances clearly improve sperm functions under in-vitro conditions, their generalized use in the IUI setting has not been successful. More studies are needed to optimize such treatments.

Multiple pregnancy

Multiple pregnancy imposes a less favourable obstetric and perinatal outcome. There has been an increased prevalence of multiple births during the past two decades. A population survey from The Netherlands indicated that the delay in achieving pregnancy and the use of fertility-promoting therapies were responsible for this increase in prevalence in a given country (Steegers-Theunissen et al., 1998). The attitudes of couples undergoing IUI are especially more favourable towards multiple gestational pregnancies than those of IVF patients, although they have an accompanied increase in tendency for multifetal pregnancy reduction (Goldfarb et al., 1996). Thus, a multiple pregnancy rate of 14–39% has been reported in this high-risk group of couples (Valbuena et al., 1996; Goldfarb et al., 1997; Tur et al., 1997).

Major factors identified to predict multiple pregnancy outcome include peak estradiol level and number of pre-ovulatory follicles on the day of hCG, which are basically indirect indicators of COH (Pasqualotto et al., 1999; Dickey et al., 2001). Aspiration of supernumerary follicles before IUI has been associated with a multiple pregnancy rate of 10.4% without decreasing the overall pregnancy rate, though it has not been accepted as a routine practice (De Geyter et al., 1996).

Cost-effectiveness

One group (Peterson et al., 1994) compared COH/IUI (using hMG) to ART (IVF, gamete intra-Fallopian transfer (GIFT) and zygote intra-Fallopian transfer (ZIFT)) as a treatment modality in a prospective, non-randomized fashion. Using meta-analysis and theoretical assumptions, these authors found that one cycle of COH/IUI was inferior to that of ART, two cycles were comparable with IVF or ZIFT and inferior to GIFT, three cycles were superior to IVF or ZIFT and comparable with GIFT, and four cycles were superior to ART. They also reported that one cycle of IVF was more expensive than four cycles of COH/IUI (with hMG). Other investigators analysed the cost-effective treatment of the infertile couple. Of these, one group (Van Voorhis et al., 1998) concluded that IUI and COH/IUI (with CC or hMG) were similar procedures in terms of cost per delivery and all were more cost-effective than ART. Some factors, such as age of the female partner and number of inseminated motile sperm, were found to be determinants of cost for individual couples.

Two randomized controlled trials supported the cost-effectiveness of IUI and COH/IUI against IVF (Table VI). The first suggested IVF not to be a cost-effective first-line treatment in couples with unexplained infertility compared with a standard infertility treatment algorithm, with mean costs per pregnancy of US$38,021 and US$16,725 respectively (Karande et al., 1999). The second randomized controlled trial did not find any difference between cumulative pregnancy rates of IUI and COH/IUI, as well as those of both IVF and IVF for unexplained and non-severe male infertility (Goverde et al., 2000). The mean costs per pregnancy resulting in at least one live birth were 10,661 and 27,409 Dutch guilders (US$5,088 and US$13,132) for COH/IUI and IVF respectively. The impact of the female partner’s age on the cost of any treatment was also confirmed. The differences in costs between these two trials might result from the health policies administered in the countries where trials have been undertaken.

Unfortunately, neither of these studies evaluated the costs resulting from the prenatal care—an important issue that would contribute to the costs of the treatment options—and especially to
that of multiple pregnancies achieved. In addition to antenatal care, other costs such as those of neonatal intensive care should also be taken into account, since most multiple pregnancies end with premature delivery. We are not aware of any published study investigating such further outcomes of various treatment modalities for infertility. In order to establish more realistic numbers for cost-efficiency analysis, as well as to understand the consequences of different therapeutic options in a public health perspective, there is a current need for such studies with a comprehensive design. These results also need to be re-examined in the light of the continuing increase in pregnancy rates in IVF, especially in women aged <35 years (35% live birth/cycle) (Society for Assisted Reproductive Technology, American Society for Reproductive Medicine, 2002).

Conclusions

The treatment of infertility with IUI is a very frequently used approach. In our programmes, twice as many IUI cycles are performed on a yearly basis than ART procedures. There are, however, no national registries or reports that depict IUI numbers and success. This review demonstrates that there are consolidated facts about IUI therapy, but generally speaking more questions have been raised than questions answered. It can be concluded that IUI is a very useful and cost-effective treatment modality for some infertility aetiologies. Cumulative pregnancy rates by the fourth to sixth cycle are generally considered as optimal. IUI is superior to TI for non-severe male factor and unexplained infertility.

Several factors have been proposed to influence the likelihood of pregnancy after IUI. Of these, duration of infertility, age of the female partner, history of pelvic inflammation (such as pelvic inflammatory disease, surgery or endometriosis) and presence of a severe male factor have a negative impact on outcome, whereas cervical factor, unexplained and anovulatory causes of infertility are more favourable. The addition of COH to IUI, especially with gonadotrophins, increases its efficiency at the cost of increased expense and risk of multiple pregnancies, which is the major drawback of this mode of treatment. The use of GnRH agonists as adjuvants in gonadotrophin-treated cases, or GnRH antagonists in cycles treated with CC/gonadotrophins or gonadotrophins alone, may be indicated in selected cases to optimize ovarian response. The optimal timing of insemination(s) after hCG administration and the need of luteal phase support should be further investigated.

Of the parameters related to the inseminate, those related to motility—such as percentage or actual number of motile sperm—appear to have an important impact on outcome. The percentage of sperm with normal morphology according to strict criteria also seems to be correlated with a favourable IUI outcome, although this correlation needs further confirmation. Other semen parameters related to a successful IUI outcome need to be evaluated.
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pentosylfucose-induced hypertensive morbidity or acoronic reaction in human sperm. Fertil. Steril. 69, 748–754.


Tow, J.P., Mossad, H., Grow, D.R., Morshedi, M., Swanson, R.J. and
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Conclusion:

In this chapter it was shown that sperm morphology plays a role in fertilization and pregnancy rates in ART programs. It was also proved that the sperm morphology patterns affect pregnancy outcome not only in *In vitro* fertilization but also in IUI programs. Most important was the fact that evidence reflected in the international literature, supported the concept that sperm morphology patterns predict prognosis and can be used in clinical practice to make practical decisions about treatment options.
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Chapter 2
## Detailed Index – Chapter 2

### Summary - Chapter 2

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<td>Günalp S, Onculoglu C, Gürgan T, Kruger TF, Lombard CJ. A study of semen parameters with emphasis on sperm morphology in a fertile population: an attempt to develop clinical thresholds. Hum Reprod 2001;16:110-114</td>
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<td><strong>iii.</strong> A review of the literature on <em>In vivo</em> data comparing fertile and infertile men’s semen parameters.</td>
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<tr>
<td>Van der Merwe FH, Kruger TF, Oehninger SC, Lombard CJ. The use of semen parameters to identify the sub-fertile male in the general population. Gynecol Obstet Invest 2005;59:86-91</td>
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<tr>
<td>Lee Mee Ho, Alvin Soon Tiong Lim, Tse Hui Lim, Siew Chen Hum, Su Ling Yu, Kruger TF. Correlation between semen parameters and the hamster egg penetration test (HEPT) among fertile and sub-fertile men in Singapore. J Androl 2007;28:158-163</td>
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<tr>
<td>Human Semen and Sperm-Cervical Mucus Interaction, 4th Ed. Cambridge,</td>
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<td>Cambridge University Press, 1999</td>
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<td>Cambridge University Press, 2010</td>
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<td>reference values for human semen characteristics. Hum Reprod Update</td>
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<td>2010;16(5):559 (Abstract)</td>
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<td>c. To evaluate the application of the suggested thresholds in clinical</td>
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<td>practice.</td>
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<tr>
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<td>Botha DJ, Kruger TF, Van der Merwe JP, Nosarka, S. Semen profiles of male</td>
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<td>ii. An ovulation and semen thresholds</td>
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<tr>
<td>Siebert TI, Van der Merwe H, Kruger TF, et al. How do we define male sub-</td>
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<td>Infertility: Diagnosis and Treatment. SC Oehninger and TF Kruger (eds).</td>
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<td>iii. How to use these thresholds in clinical decision making</td>
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<td>Kruger TF. Should ICSI be done for all IVF patients? MEFSJ 2009;14(2):85-95.</td>
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SUMMARY

❖ Chapter 2

Based on a structured literature review new semen thresholds for the sub-fertile male were suggested (van der Merwe et al., 2005). One important inclusion criteria was the use of strict morphology criteria (Menkveld et al., 1990) in the studies on fertile and infertile couples in the in vivo situation. The following semen values were suggested for the sub-fertile male: Morphology < 5% normal forms, concentration below 10 million per ml. and motility below 30%. It was emphasized that by using the sperm morphology parameter in combination with the other semen values, the clinical value of the semen analysis should be improved.
Fertility/sub-fertility thresholds for sperm concentration, sperm motility/progressive motility and sperm morphology, using Tygerberg Strict Criteria

a. To investigate the rationale of the new sperm morphology threshold values and the suggested semen values to distinguish fertile from sub-fertile men.

i. **Semen thresholds to distinguish fertile and sub-fertile patients**

The use of thresholds to distinguish between fertile and sub-fertile patients has been under discussion for many years. There were suggestions about the sub-fertile/fertile thresholds by Freund in 1966 and MacLeod in 1952 (Freund et al., 1966; MacLeod et al., 1952). From the Tygerberg Hospital Unit, Van Zyl and Menkveld suggested lower thresholds for in vivo fertility in 1976 (Van Zyl and Menkveld, 1976). It was however the publication dealing with the P- and G-patterns that created renewed interest in this aspect of andrology (Kruger et al., 1988). It was clearly shown that there is a lower chance for fertilization and pregnancy rate if the sperm morphology is below 5% normal forms. This finding was also confirmed in an article on IUI (van Waart et al., 2002) as discussed in Chapter 1. Due to the importance of the article on the P- and G-patterns, a summary of the article will again be reflected, but a full text is available for the interested reader in Chapter 1.
The following article reflects the scientific basis that supports the above argument:

Predictive value of abnormal sperm morphology in in vitro fertilization

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Anibal A. Acosta, M.D.†† †† James F. Matta, Ph.D.§
Kathryn F. Simmons, M.S.§ Sergio Oehninger, M.D.†

Tygerberg Hospital, University of Stellenbosch, Parow, South Africa, and Eastern Virginia Medical School, Norfolk, Virginia

In patients with acceptable sperm count and motility, two patterns of abnormal morphology, judged with strict criteria, were identified and described. Patients with <4% normal forms and <30% morphology index (sumation of normal and slightly amorphous forms) had a fertilization rate of 7.6% of the oocytes (P pattern, poor prognosis). Patients with normal morphology between 4 and 14% had a significantly better fertilization rate of 63.9% of the oocytes (P < 0.0001). Cases with >14% normal forms fertilized within the normal range for the laboratory. By evaluating sperm morphology with the proposed strict criteria, its predictive value in in vitro fertilization is enhanced. Fertil Steril 49:112, 1988

Although there is still extensive debate about the role of sperm morphology in in vitro fertilization (IVF), the human model has greatly improved the understanding of the significance of this parameter for fertilization and pregnancy outcome. In previous publications it was noted that if evaluation of normal sperm morphology is done using strict criteria, this parameter has an excellent predictive value of fertilization. In patients with a sperm concentration > 20 × 10⁶/ml and a motility of >30% with a normal sperm morphology of <14%, the fertilization rate was markedly impaired (37% to 47% per oocyte), as opposed to a high fertilization rate (85% to 88%) when normal morphology was >14%.

Although there was severe impairment in the fertilization rate, some of these patients still fertilized the human egg; in these cases, a pregnancy was possible.

The purpose of this study was to evaluate patients with normal sperm morphology < 14% to try to establish a morphologic pattern which can differentiate the subgroup that fertilized from the subgroup that did not.

MATERIALS AND METHODS

Forty-five couples were allocated to the study group in a prospective way. All female partners in these couples had tubal infertility, and the males had either been considered normal or had some abnormal parameters by other laboratories evaluations. Twenty-eight patients were stimulated with a combination of hFSH/hMG/hCG (human follicle-stimulating hormone/human menopausal gonadotropin/human chorionic gonadotropin; 62.2%), 13 with hFSH/hCG (28.8%), and 4 with hMG/hCG (8.8%) following protocols previously published. In the Norfolk experience, all of these protocols have demonstrated provision of preovulatory oocytes with identical fertilization rates. All male patients had to have a sperm concentration > 20
ii. *In vivo fertility thresholds*

The question arose if the fertility thresholds, especially those used in *in vitro* fertilization, are the same in the *in vivo* situation. Due to the difficulty to obtain semen from fertile men this type of study was difficult to initiate. A collaborative study was planned with a colleague from Turkey (Gunalp *et al.*, 2001) who could obtain semen from fertile men. The semen parameters of the fertile group were compared with that of a sub-fertile population in an infertility clinic. The positive and negative predictive values were used to screen the general population to identify the sub-fertile group. A <5% normal morphology lower threshold was indicated with <30% motility and a concentration of 9 million per ml or lower as the sub-fertile *in vivo* values. It was also concluded that the <5% normal morphology value (*in vivo*) correlated with the *in vitro* reported values as well as the IUI report as summarized by Van Waart (Van Waart *et al.*, 2002).
The following article reflects the scientific basis that supports the above argument:

Günelp S, Onculoglu C, Gürgan T, Kruger TF, Lombard CJ. A study of semen parameters with emphasis on sperm morphology in a fertile population: an attempt to develop clinical thresholds. Hum Reprod 2001;16:110-114
A study of semen parameters with emphasis on sperm morphology in a fertile population: an attempt to develop clinical thresholds

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The aim of the study was to determine the semen parameters of a proven fertile population and to compare these parameters with that of a subfertile group in the same region. Sixty-nine fertile male patients were studied and compared with 93 patients recruited at an infertility clinic. A sub-sample of patients was matched according to age. Sixty-one were studied in the fertile group and 62 in the infertile group. Receiver operator characteristics analysis was done on the sub-sample. The threshold value of the progressive motility was 42% and it was the best parameter with sperm morphology to distinguish between the two groups. At 69% sensitivity and 67% specificity the sperm morphology threshold was 12% normal forms. If the positive and negative predictive value was used to screen the general population to identify the subfertile group, a 5% normal morphology threshold was indicated with 14% progressive motility, 30% motility and a concentration of \(9 \times 10^6/\text{ml}\) or lower. The negative predictive values of the parameters were good and achieved 90% in most cases. The sensitivity of the semen parameters at the reported thresholds was poor and indicated a large overlap in the distributions of these variables in the fertile and infertile groups. To distinguish between the fertile and subfertile population, the most significant finding of this study was the progressive motility with a threshold level of 14%. The cut-off value of the sperm morphology (5%) in vitro was consistent with the previous publications in assisted reproduction programmes for sperm morphology.

Key words: in-vivo fertilization/semen parameters/sperm morphology

Introduction

Over the last 10 years, attempts have been made to identify the male partners in couples at risk for a significantly lower chance of fertilization in vitro (Coetzee et al., 1998) or in intruterine insemination (IVI) programmes (Lindheim et al., 1996; Ombelet et al., 1997b; Montanar-Gauci and Kruger, 2000).

Although the 5% threshold (strict criteria) (Lindheim et al., 1996; Ombelet et al., 1997a; Coetzee et al., 1998; Montanaro-Gauci et al., 1999) has been shown to be of clinical significance in the situations mentioned above, it is still an unanswered question when considering the general population since thresholds are not well established for this group. In a recent publication, Ombelet et al. (1997a) pointed out that using the receiver operating characteristics (ROC) curve, thresholds have been established for a group of patients in Belgium. Regarding sperm morphology, a 10% threshold was used as an indicator of subfertility (when below this threshold) or fertile population when above this threshold.

The question arises of whether the threshold value might vary in populations in different parts of the world. Therefore, an attempt to study the threshold values in a subfertile population in Turkey was undertaken as a complementary study of the Ombelet study done in Belgium (Ombelet et al., 1997a). The differentiating aspect of the study is that the morphological evaluation of the spermatozoa was done by one of the original authors of strict criteria (T.K.). The aim of the study was to determine the semen parameters of a proven fertile population and to compare these parameters with that of a subfertile group in the same region.

Materials and methods

This is a prospective cross-sectional study comparing a fertile population with an infertile group.

During an 8 month period (from September 1996 to May 1997) and at a gestational age of 8–23 weeks, 69 pregnant women who attended the antenatal clinic at a university hospital were asked to
Clinical thresholds of semen parameters

participate in a study where the husbands’ semen was needed to define the fertile population in Turkey. The couples provided written consent after being given full background information. Inclusion criteria were as follows: pregnancy had to be achieved within 1 year of marriage and no habitual abortions (>3) were allowed. On the other hand, 93 consecutive infertile men were recruited into the study as they were consulted at the Andrology Division of the IVF Unit at Hacettepe University, Ankara, Turkey. There were no infertility factors in the female partners of the couples with no ovulatory dysfunction, with normal patency in hysterosalpingography and normal laparoscopic findings.

This study took place at the following two centres: (i) Andrology Division and Antenatal Polyclinic, Department of Obstetrics and Gynaecology, Hacettepe University, Medical Faculty, Ankara, Turkey; (ii) Reproductive Biology Unit, Department of Obstetrics and Gynaecology, Tygerberg Hospital, Tygerberg, Republic of South Africa.

Sperm parameters (except morphology) were collected and evaluated at the first centre. A single investigator (T.K.) at the second centre evaluated sperm morphology.

In short, the methods used for determination of the three variables in this study were as follows: the semen samples were obtained after 3–4 days of abstinence by masturbation at the laboratory. Immediately after liquefaction, a drop of the well-mixed specimen was placed on a clean and pre-warmed glass slide at 37°C covered with a coverslip, and left for a few minutes. The microscope was equipped with a hot stage to keep the slides at 37°C. The preparation was examined under a magnification of both ×10 and ×40 objectives. The motility assessment was done according to World Health Organization guidelines (WHO, 1992) in at least 10 separate randomly selected high-power fields, as described by Macleod (1965). The motility of each spermatozoon was graded a, b, c, d or of according to whether it showed (a) rapid progressive motility, (b) slow or sluggish progressive motility, (c) non-progressive motility or (d) immotility. The percentage of the progressive motility was done according to the formula a + b/a + b + c + d We did not use a computer assisted sperm analysis (CASA) system in our laboratory. At the same time presence of agglutination was observed and an estimation of the sperm concentration made. The volume of the semen samples was determined using a sterile disposable 5 ml pipette.

Depending on the estimated sperm concentration, a 1/10, 1/20, or 1/100 dilution of the semen sample was made with the use of a glass tuberculin syringe, instead of a white blood cell pipette (Menkveld et al., 1984), using HAM’S F10 + 10% BSA medium. An improved, double-rulled Neubauer haemocytometer (FPG) was used for counting the spermatozoa. Two dilutions were made for every sample. The difference between the two dilutions for each sample was not >20% for low concentrations and not >20% for concentrations of >60x10^6/ml (Eliaissi, 1971).

The following procedures were used for the assessment of the morphological characteristics of the spermatozoa. The slides were thoroughly cleaned, washed in alcohol and dried before use. For a reliable and reproducible assessment, a thin and well-spread smear was made so that each spermatozoon could be clearly and individually visualized. The smears were air-dried and the following day fixed and stained according to the Papanicoaulou method (Papanicoaulou, 1942). The morphological classification used at the Tygerberg Hospital unit was based on a modification of the methods described by Eliassii (1971) and Macleod (1962). This system takes the whole spermatozoon as well as the presence of germinal epithelial cells into consideration.

In this laboratory, a spermatozoon is considered normal when the head has a smooth, oval configuration with a well-defined acrosome comprising about 40–70% of the sperm head. Also there must be no neck, mid-piece, or tail defect and no cytoplasmic droplets of more than half the size of the sperm head. In contrast with other authors (Papanicoaulou, 1942; Macleod, 1962, 1965; Eliassii, 1971; WHO, 1980), borderline forms were considered abnormal (Kruger et al., 1986; Menkveld et al., 1990). At least 100, but preferably 200, spermatozoa with tails were classified into one of seven groups: normal (head and tail normal), normal head but with another abnormality present, large heads, small heads, tapering heads, duplicated heads or amorphous heads all with or without tail, neck or mid-piece defects. Tail, neck, and mid-piece defects, loose head, immature germinal cells and unknown cells were recorded separately and reported per 100 spermatozoa. The size of the spermatozoa was evaluated in five different areas on the slide to ensure a more randomized evaluation (WHO, 1999).

Statistical analysis

Basic descriptive statistics such as the mean, standard deviation and range were calculated for the two groups and compared using Fisher’s t-test. Linear regression analysis was used to investigate the effect of age on the mean difference between the semen parameters of the two groups. This analysis led to the restriction of the diagnostic analysis to subgroups with adequate overlap in the age distribution of the male and female partners in the study. The diagnostic ability of the individual semen parameters to differentiate between the fertile or infertile status of the male was analysed using ROC curve analysis (Hanley et al., 1983). The sensitivity, specificity, positive and negative predictive value, positive and negative likelihood ratio and area under the curve of each semen parameter was estimated assuming a 15% prevalence of infertility (Ombret et al., 1997a). Pairwise comparison of the area under the curve was done for the semen parameters of volume, concentration, motility, progressive motility and morphology.

Definitions used in this study

Sensitivity of a test is the percentage of individuals with infertility (disease) who are classified as having infertility. Specificity of a test is the percentage of individuals without the disease who are classified as not having the disease (infertility in this study). Positive predictive value is where the test is positive and the subject has the problem (subfertility). A negative predictive value is where the test is negative and the subject does not have the disease (subfertility), thus the subject is fertile. The last two definitions can also be applied to screen the general population with a given incidence of infertility (e.g., 1.5%) (Fisher et al., 1993).

Results

Sixty-nine fertile and 93 infertile patients were recruited. When the demographic characteristics of the groups were compared, the infertile group was found to be statistically older than the fertile group with respect to males (33 versus 29.7 years) and females (30 versus 26.6 years). The infertile group had few male outliers below 23 years and the fertile group had few outliers above 40 years. The fertile group had very few women above 32 years and the infertile group below 20 years. Therefore, a subset of patients from both groups was matched to make the groups comparable with respect to age, since it is very difficult to evaluate diagnostic criteria across the effect of a covariate. Limits were determined as 23–40 years for males and 20–32 years for females.

The statistics for the two subsets are given in Table I. The fertile group consisted of 61 subjects and the infertile group
of 62 subjects. After remodelling, the mean age for infertile males was 31.3 years, for fertile males 29.9 (not significant) and for females 27.3 versus 26.3 (not significant).

By comparison of two groups there was statistical difference regarding volume ($P < 0.02$), motility ($P < 0.03$), progressive motility ($P < 0.001$) and morphology ($P < 0.001$). However there was no difference in sperm concentrations of these groups (Table I).

The ROC analysis was done on the subset (Figure 1). The best performing semen parameters were progressive motility and morphology which had nearly identical predictive power as indicated by areas under the ROC curve (AUC), 0.707 and 0.697 respectively, and there was no significant difference between them, $P = 0.972$ (Table II). With respect to the AUC, progressive motility differed significantly from motility ($P = 0.002$) and concentration ($P = 0.011$) as did morphology for the latter ($P = 0.014$).

When using the sensitivity and specificity to analyse the data set, a cut-off value of 12% normal forms was detected for normal sperm morphology whereby the sensitivity and specificity were 69.1 and 66.7% respectively. Progressive motility threshold was calculated at 42% (specificity 65.6%, sensitivity 66.1%), motility at 52% (specificity 68.9%, sensitivity 44.6%), and concentration at $34 \times 10^6$/ml (specificity 43%, sensitivity 60.7%).

Assuming a prevalence of 15% infertility in the population the positive and negative predictive values for the semen parameters are reported in Table III. The corresponding sensitivity and specificity at the reported cut-off point are also given to describe the performance of the parameters in the study population. The positive predictive value of the semen parameters is low overall, using the mean values of the infertile group (Table I) as threshold values, i.e., progressive motility at 35 with a 33.6% chance of subfertility, morphology at 10% normal forms with a 29% chance and motility at 30 with a 28% chance. At 5% normal forms sperm morphology has a positive predictive value of 64.7% and this is similar to the 57.4% achieved by progressive motility at 14%.

**Discussion**

In a recent structured review (Coetzee et al., 1998) it was shown that the majority of authors used the strict criteria to judge sperm morphology. It was indicated that a threshold of 5% normal forms was of clinical relevance in IVF programmes as there was a significant difference in the total pregnancy
rate in the group <5% compared to the group >5% normal forms. It was also indirectly shown that the increase of pregnancy today could be at least doubled or tripled by instituting the ICSI procedure in the group of patients with sperm morphology below 5% normal forms (Coetzee et al., 1999).

The threshold of sperm morphology assisting the clinician was based on research done on the IVF model (Coetzee et al., 1998). There were also studies dealing with strict criteria and IUI where it was shown that the threshold of 5% was of value as indicated by Lindheim (1996). Ombleet et al. (1997b) added a dimension to the findings of Lindheim by bringing into play the concentration retrieved. No pregnancy was reported in patients where less than 1×10^6 spermatozoa were retrieved after swim-up and with a morphology <5%. Toner et al. (1994) used a threshold of 14% with a significant lower pregnancy rate below that threshold. The 5% threshold was also shown to be of value in the IUI programme at Tygerberg Hospital in a recent publication (Montanaro-Gauci and Kruger, 2000). In this study where all females were classified as normal, the pregnancy rate in the group 0-4% normal morphology was 2.63% per cycle compared to 24% in the group >14% normal morphology ($P = 0.003$).

It is clear that with the threshold indicated for strict criteria in IUI and IVF programmes, one of the problem areas is still the threshold for the in-vivo situation. The 14% threshold in the latest WHO manual was based on data from IVF studies (WHO, 1999). When using the principle of screening the population with the positive predictive value as indicator, it was clear from the data set that as far as sperm morphology is concerned the threshold is at the 5% level. With these data one can assume that 65% of patients below the threshold will be subfertile. On the other hand, the negative predictive value at this threshold was 89.4%, indicating that the probability of being fertile is above the 5% threshold. When using the same principle, the threshold for progressive motility was 14% or lower. For motility it was 30% or lower and for concentration 10×10^6/ml or lower (indicating the subfertile group below these thresholds) (Table III). The negative predictive values of the parameters are good and achieve 90% in most cases (Table III). The sensitivity of the semen parameters at the reported thresholds is poor and indicates a large overlap in the distributions of these variables in the fertile and infertile groups.

Based on the current data obtained by ROC analysis, the best predictors of subfertility were the normal sperm morphology and progressive motility. By statistical comparison of these two parameters there was no significant difference between them (Table II). By using the ROC curve and

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Value</th>
<th>+PV</th>
<th>-PV</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>3.2 ml</td>
<td>18.8</td>
<td>87.7</td>
<td>51.6</td>
<td>60.7</td>
</tr>
<tr>
<td></td>
<td>2.5 ml</td>
<td>20.1</td>
<td>87.3</td>
<td>41.9</td>
<td>60.7</td>
</tr>
<tr>
<td>Prog. Mot.</td>
<td>12%</td>
<td>26.8</td>
<td>92.4</td>
<td>69.1</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>29.0</td>
<td>91.6</td>
<td>61.8</td>
<td>73.3</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>38.3</td>
<td>91.1</td>
<td>52.7</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>64.7</td>
<td>89.4</td>
<td>34.5</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>25.3</td>
<td>91.6</td>
<td>66.1</td>
<td>65.6</td>
</tr>
<tr>
<td></td>
<td>35%</td>
<td>33.6</td>
<td>90.6</td>
<td>51.8</td>
<td>82.0</td>
</tr>
<tr>
<td></td>
<td>14%</td>
<td>57.4</td>
<td>86.4</td>
<td>12.5</td>
<td>98.4</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>20.2</td>
<td>87.4</td>
<td>44.6</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>27.8</td>
<td>86.1</td>
<td>14.3</td>
<td>93.4</td>
</tr>
<tr>
<td>Concentration</td>
<td>4×10^6/ml</td>
<td>15.2</td>
<td>85.3</td>
<td>51.9</td>
<td>49.2</td>
</tr>
<tr>
<td></td>
<td>2×10^6/ml</td>
<td>16.1</td>
<td>85.7</td>
<td>42.9</td>
<td>60.7</td>
</tr>
<tr>
<td></td>
<td>&lt;1×10^6/ml</td>
<td>22.4</td>
<td>85.6</td>
<td>10.7</td>
<td>93.4</td>
</tr>
</tbody>
</table>

*+PV = positive predictive value; -PV = negative predictive value; Prog. Mot. = progressive motility.
the population under discussion in this article (fertile and subfertile), the best threshold for sperm morphology indicating a possible problem in this group was at the 12% threshold (Figure 1) where the sensitivity was 69.1% and the specificity 66.7%. In the article by Ombelet et al. (1997a) they indicated a 10% threshold for sperm morphology using this method to evaluate their data. Barratt et al. (1995) also questioned the WHO (1992) 30% threshold and suggested a 10% threshold level for sperm morphology based on clinical considerations (Barratt et al., 1995). The question is, which population do we want to identify? With respect to the general population’s point of view (positive or negative predictive value), the 5% morphology threshold can be used with theoretically a 64.7% accuracy to identify a patient with subfertility (Table III). Above this threshold the negative predictive value was 89.4%. Progressive motility at the 14% level is also of clinical help, but concentration and motility are not (Table III).

Although there is scanty information concerning the geographical distribution of fertility and subfertility, it is interesting to note that with strict criteria applied in Turkey, Belgium and Singapore (Ombelet et al., 1997a; Chia et al., 1998) the trend is very much the same in these studies. We also tried to establish an index to predict the fertilization potential of the semen sample with three basic sperm parameters, namely sperm concentration, motility and morphology, but we were not able to achieve this.

To conclude, the available data on the fertile and subfertile groups in the in-vivo situation is of value. As far as sperm morphology is concerned it correlates with the findings of Ombelet et al. (1997a) and one can use these thresholds at least to identify a ‘high risk’ group with greater chance of having an infertility problem. One can also indirectly draw the line to IUI as well as IVF data, especially as sperm morphology is concerned, and use these thresholds at the 5% level practically to assist in clinical practice.

References


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iii. A review of the literature on In vivo data comparing fertile and infertile men’s semen parameters

As a research project, a review of the semen parameters comparing fertile and sub-fertile men was undertaken. The aim was to study the possibility to use the published literature to develop new semen thresholds for *in vivo* fertilization (Van der Merwe *et al*., 2005). One important inclusion criteria was the use of strict morphology criteria in the studies. The following threshold semen values were suggested: Morphology < 5% normal forms, concentration below 15 million per ml. and motility below 30%. It was emphasized that by using the sperm morphology parameter in combination with the other semen values, clinical value of the semen analysis should be improved. What made the review by Van der Merwe interesting were that one article came from Turkey, one from the USA, one from Belgium and one from the Netherlands (Van der Merwe *et al*., 2005).
The following article reflects the scientific basis that supports the above argument:

Van der Merwe FH, Kruger TF, Oehninger SC, Lombard CJ. The use of semen parameters to identify the sub-fertile male in the general population. Gynecol Obstet Invest 2005;59:86-91
The Use of Semen Parameters to Identify the Subfertile Male in the General Population

F.H. van der Merwe, T.F. Kruger, S.C. Oehninger, C.J. Lombard

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Key Words
In vivo fertilization · Semen analysis · Sperm morphology · Tygerberg strict criteria, sperm morphology · Structured literature review, semen parameters

Abstract
Aims: To present a structured review of the literature published on semen parameters and in vivo fertility potential and to establish fertility/subfertility thresholds for sperm morphology using Tygerberg strict criteria, sperm concentration, and sperm motility. Method: The published literature comparing fertile and subfertile populations between 1983 and 2002 was reviewed. Results: A total of 265 articles were identified by the sourcing methodology, but only four articles provided data that could be tabulated and analyzed. Using receiver-operating characteristics curves, morphology proved to be the best predictor of subfertility in 2 of the 4 articles, with concentration and motility also showing good predictive power. The thresholds calculated ranged between 4 and 10% for morphology, between $13.5 \times 10^6$/ml and $41 \times 10^6$/ml for concentration, and between 32 and 52% for motility. A second set of much lower thresholds was calculated in three of the articles using either a 15 or 50% prevalence of subfertility in the population or the tenth percentile of the fertile population. The adjusted thresholds were between 3 and 5% for morphology, between $9 \times 10^6$/ml and $20 \times 10^6$/ml for concentration, and between 20 and 30% for motility. Conclusions: Because these lower thresholds have a much higher positive predictive value, we suggest that thresholds of $<5\%$ normal sperm morphology, a concentration $<15 \times 10^6$/ml, and a motility <30% should be used to identify the subfertile male. The lower threshold for morphology also fits in vitro fertilization and intrauterine insemination data calculated previously. Using the parameters in combination increases the clinical value of semen analysis.

Introduction
Several semen parameters are used to discriminate the fertile male from the subfertile male. The most widely used parameters are sperm concentration, motility, progressive motility, and sperm morphology. Of these parameters, the sperm morphology is the single indicator most widely debated in the literature. A large number of classification systems have been used to describe which factors constitute a morphologically normal/abnormal
Table 1. Threshold values: fertile versus subfertile populations studied

<table>
<thead>
<tr>
<th>Authors</th>
<th>Morphology, %</th>
<th>Motility, %</th>
<th>Progressive motility, %</th>
<th>Concentration x10^9/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guzik et al. [17] (2001; CART)</td>
<td>9</td>
<td>32</td>
<td></td>
<td>13.5</td>
</tr>
<tr>
<td>Menkveld et al. [16] (2001; ROC)</td>
<td>4</td>
<td>45</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Gunalp et al. [15] (2001; ROC)</td>
<td>10</td>
<td>52</td>
<td>42</td>
<td>34</td>
</tr>
<tr>
<td>Ombelet et al. [14] (1997; ROC)</td>
<td>10</td>
<td>45</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

Methods

The articles were primarily sourced searching MEDLINE. The key words used in the search were semen analysis, semen parameters, sperm morphology, and fertility. Articles had to distinguish between fertile and subfertile populations by establishing thresholds for different semen parameters used in the basic semen analysis (i.e., raw, after liquefaction, including concentration, percentage motility, and percentage normal morphology). The search was limited to the English language, humans, and the time period between 1983 and 2002. The data bank of Tygerberg Hospital’s fertility unit was manually searched using the same criteria. Finally, the references of the articles obtained were cross-checked and also included if initially omitted. Two of the authors (F.H.v.d.M. and T.F.K.) searched independently and then compared the results.

The abstracts of 265 articles were evaluated. The articles were analyzed further, if the following criteria were met: (1) Tygerberg strict criteria were used in assessing sperm morphology, (2) statistical associations were investigated between sperm morphology, sperm concentration, sperm motility/progressive motility, and fertility/subfertility (motility and concentration measured manually), and (3) fertility/subfertility thresholds were calculated for the aforementioned parameters. Four articles [14–17] met the criteria as stated above and are listed in table 1.

Results

We do not contend that this review is complete, but only that the articles reviewed constitute a representative sample of studies published on the predictive value of sperm morphology, sperm concentration, and motility/progressive motility for in vivo fertility/subfertility.

Of the 265 articles evaluated, only 4 were found to fit the criteria as outlined. The four articles [14–17] compared the different semen parameters of a fertile and a subfertile group. The articles used either the classification and regression tree (CART) analysis or the receiver-operating characteristic (ROC) curve analysis to estimate thresholds for the different semen parameters. The ROC curve was also used to assess the diagnostic accuracy of the different parameters and their ability to classify subjects into fertile and subfertile groups.

Using ROC curve analysis, Ombelet et al. [14] calculated the following thresholds: proportion normal morphology 10%, proportion normal motility 45%, and normal sperm concentration 34 x 10^9/ml. The sperm morphology was shown to be the best parameter with the highest prediction power (area under curve or AUC 78%). Much lower thresholds were calculated using the 10th percentile of the fertile population, these thresholds being 5% for normal morphology, 28% for motility, and 14.3 x 10^9/ml for sperm concentration (tables 1, 2) [14].

Identification of Subfertile Males in the General Population

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Günelp et al. [15] also calculated thresholds using the ROC curve analysis. The thresholds were proportion normal morphology 10%, proportion normal motility 52%, proportion progressive motility 42%, and sperm concentration 34 × 10⁹/ml. The two parameters that performed best were progressive motility (AUC 70.7%) and morphology (AUC 69.7%). Assuming a 15% prevalence of subfertility in the population, Günelp et al. [15] used the positive predictive value as indicator to calculate a lower threshold for each parameter. Values of 5% for proportion normal morphology, 30% for proportion normal motility, 14% for proportion progressive motility, and 9 × 10⁹/ml for sperm concentration were calculated (tables 1, 2) [15].

Menkvedel et al. [16] found much lower thresholds than the others. Using the ROC curve, the following thresholds were calculated: 4% for normal morphology and 45% for normal motility. The morphology again showed a good predictive value with an AUC of 78.2%. Although a threshold for sperm concentration was not calculated (a sperm concentration <20 × 10⁹/ml was used as inclusion criterion), the authors proposed that a cutoff value of 20 × 10⁹/ml could be used with confidence, based on the resultant lower 10th percentile of the fertile population. Adjusted cutoff points calculated on the assumption of a 50% prevalence of male subfertility were as follows: 3% for proportion normal morphology and 20% for proportion normal motility (tables 1, 2) [16].

Guzick et al. [17], using the CART analysis, calculated two thresholds for each semen parameter which allowed for designation into three groups, namely normal (fertile), borderline, and abnormal (subfertile). The normal (fertile) group had values of >12% for morphology, >63% for motility, and >48 × 10⁹/ml for sperm concentration. The abnormal (subfertile) group had values of <9% for morphology, <32% for motility, and <13.5 × 10⁹/ml for sperm concentration. Borderline values were those between the normal and abnormal values. The ROC curve analysis showed that morphology had the best predictive power with an AUC of 66% [17].

**Discussion**

Semen analysis is used in clinical practice to assess the male fertility potential. To be of clinical value, the methods used for semen analysis should be standardized, and threshold values for fertility/subfertility should be calculated for the different parameters used in standard semen analysis.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Morphology, %</th>
<th>Motility, %</th>
<th>Progressive Motility, %</th>
<th>Concentration ×10⁹/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menkvedel et al. [16] (2001)</td>
<td>3</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Because there are so many different methods for semen evaluation, it would be difficult to standardize the methods used in semen analysis. This applies especially to the assessment of sperm morphology. The two classification systems most widely accepted are the WHO [1, 2] and the Tygerberg strict criteria [3–6]. Various methodological problems concerning sperm morphology have been identified. The variance among different methods of morphology assessment has been shown by Ombret et al. [18–20] and others [21, 22], and they recommended standardization of semen analysis methodologies. Some authors recommend that laboratories should adopt accepted standards such as those proposed by the WHO [21, 22]. Another problem identified is the variation in intra- and interindividual and interlaboratory sperm morphology assessment [18, 19]. This problem could be addressed by using the Tygerberg criteria [23] and by applying continuous quality control programs, as it was shown that consistent reading could be achieved [24, 25]. Cooper et al. [22] also urged for standardization of such quality control programs and that quality control centers should reach agreement with each other.

Previous WHO thresholds of 50 and 30% for sperm morphology were empiric values and not based on any clinical data. Several authors found these values to be of little or no clinical value [7, 9, 10]. These articles did, however, find a positive correlation between the high proportion of morphologically normal sperm and the increased likelihood of fertility and/or pregnancy. Other articles confirmed this correlation [26–29].

Van Zyl et al. [29] were the first to show the faster than linear decline in fertilization rate, when the proportion of normal forms dropped to <4%. Eggert-Kruse et al. [27] found a higher in vivo pregnancy rate for higher percentage normal forms at thresholds of 4, 7, and 14% using strict criteria for morphology assessment. Zinaman et al. [30] confirmed the value of sperm morphology (strict cri-
teria) by demonstrating a definite decline in pregnancy rate when normal morphology dropped below 8% and sperm concentration below $30 \times 10^5$ml$^{-1}$ [30]. In a study performed by Shima et al. [31], measuring the association between time to pregnancy and semen parameters, it was found that the proportion of morphologically normal sperm influenced the time to pregnancy up to a threshold value of 19%. This value is somewhat higher than that calculated in other studies.

A normal sperm morphology (strict criteria) has a positive predictive value in IVF and IUI programs. Normal sperm morphology thresholds produced positive predictive values for IVF success when using the 5% and the 14% thresholds, respectively, with the overall fertilization rates and overall pregnancy rates significantly higher in the group with normal morphology $\geq$5% as compared with the <5% group [12]. A meta-analysis of the data on IUI programs showed a higher pregnancy rate per cycle in the group with normal sperm morphology $\geq$5%. In the group with normal sperm morphology <5%, other semen parameters proved to be predicting IUI success [13]. In the IUI analysis, motility [32], total motile sperm count [33], and concentration [34] also played a role in some of the articles evaluated, whilst others [35] stated that sperm morphology alone was enough to predict the prognosis. Because of the high cost of assisted reproduction, males with good or reasonable fertility potential under in vivo conditions should be identified on the basis of semen quality. Conversely, males with a poor fertility potential should be identified and introduced to assisted reproduction programs.

The predictive power of any parameter was calculated as its AUC using the ROC curve. The sperm morphology had a high predictive power in the four articles studied [14–17], ranging from 66 to 78.2%, and in fact had the best performance of the different semen parameters in two articles [14, 17]. In these two articles, the threshold for sperm morphology was calculated at 10 and 9%, respectively. Günalp et al. [15] found morphology and progressive motility to have a nearly identical predictive power and calculated a threshold of 12% for normal sperm morphology using sensitivity and specificity to analyze their data set. The fourth article [16] calculated a 4% predictive cutoff point value for sperm morphology with the ROC curve analysis. Although sensitivity and specificity for these values are relatively high, the positive predictive values are not. This will result in classifying fertile males as subfertile, therefore, probably leading to a degree of anxiety and unnecessary and costly infertility treatment. Using the 10th percentile of the fertile population, Ombelet et al. [14] calculated a second and much lower cutoff value of 5%. Günalp et al. [15] calculated a lower threshold of 5% normal forms by screening the population with the positive predictive value as indicator. Assuming a 50% prevalence of subfertility in their study population, Menkveld et al. [16] calculated an adjusted cutoff point of 3%. The lower threshold thus ranging from 3 to 5% (table 2). These lower thresholds have a much higher positive predictive value than the higher thresholds, with the negative predictive value not much lower.

We suggest that the lower threshold should be used to identify males with the lowest potential for a pregnancy under in vivo conditions. Values above the lower threshold should be regarded as normal. These findings are in keeping with previous publications by Coetzee et al. [12] (IVF data) and Van Waart et al. [13] (IUI data) which showed a significantly lower chance of successful pregnancies in males with normal morphology below their calculated thresholds.

In all four articles [14–17], the thresholds for percentage motile sperm were also calculated. Higher threshold values (using ROC curve or CART analysis) ranged from 32 to 52%, while the lower threshold values ranged from 20 to 30%. Motility also had a high predictive power with an AUC of between 59 and 79.1%. Günalp et al. [15] calculated thresholds for progressive motility—a higher threshold of 42%, using the ROC curve, and a lower threshold of 14% with the positive predictive value as indicator. In the article by Günalp et al. [15], progressive motility proved to be a marginally better predictor of subfertility than sperm morphology with AUC values of 70.7 and 69.7%, respectively. Montanaro Gauci et al. [32] found percentage motility a significant predictor of IUI outcome. The pregnancy rate was almost three times higher in the group with motility $\geq$50% as compared with the group with motility $<$50%.

Ombelet et al. [14], Günalp et al. [15], and Guizick et al. [17] calculated cutoff values for sperm concentration: the higher threshold values ranging from $13.5 \times 10^6$ml$^{-1}$ to $34 \times 10^6$ml$^{-1}$ and the lower threshold values ranging from $9 \times 10^6$ml$^{-1}$ to $14.3 \times 10^6$ml$^{-1}$. An AUC value of between 55.5 and 69.4% served as confirmation of the predictive power of this parameter. Menkveld et al. [16] did not calculate a threshold value for sperm concentration, because values of $<20 \times 10^6$ml$^{-1}$ served as inclusion criteria in their study. However, they suggested a threshold value of $20 \times 10^6$ml$^{-1}$ to be used with confidence [16], because it did not influence the results from their fertile population. The clinical values of motility and sperm concentration serve as confirmation of findings reported in numerous other publications [7, 8, 11, 26–28].
Although the different parameters had a good predictive power, independent of each other, the clinical value of semen analysis increased, when the parameters were used in combination. Omebelet et al. [14] found that the differences between their fertile and subfertile populations only became significant, when two or all three semen parameters were combined. Bartoov et al. [36] concluded that the fertility potential is dependent on a combination of different semen characteristics. Eggert-Kruse et al. [27] found a significant correlation between the three parameters reviewed in their article. We, therefore, suggest that no single parameter be used isolated when assessing the male fertility potential.

Conclusions

Although each parameter showed a good predictive power, the clinical value of semen analysis increased, when the parameters are used in combination. The lower thresholds have a much higher positive predictive value and a high negative predictive value. We suggest that these lower thresholds should be used in identifying the subfertile male.

As suggested by the WHO in 1999, each group must develop their thresholds based on the population they are working in. Each laboratory should establish these thresholds, if possible. It seems as if the sperm morphology threshold of 0–4% normal forms indicates a higher-risk group for subfertility and fits the IVF and IUI data calculated previously [12, 13]. The four articles reviewed [14–17] showed the same trends and can serve as guidelines to distinguish fertile from subfertile males.

As far as concentration and motility are concerned, the thresholds are not clear, but a concentration <15 × 10^6/ml and a motility <30% seem to fit the general data [14–17]. However, more and possibly multicentered studies are needed to set definitive thresholds.

References


90 Gynecol Obstet Invest 2005;59:86–91

van der Merwe/Kruger/Oehninger/Lombard

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Identification of Subfertile Males in the General Population

iv. The quest for more data on In vivo semen thresholds

The quest to get more data continued. While giving lectures in Singapore it was learned that the group from the Singapore General Hospital was able to obtain semen from fertile men from that region (Lee Mee et al., 2007). By encouraging the scientists to continue with their research and in assisting them in analyzing the data and in writing the article a good end product developed from this region of the globe. The lower thresholds suggested by them, were 3% for morphology, and 28% for motility. They used a total normal count to report on concentration. Due to the fact that this method was not used in the previous publications, it could not be compared with concentration per ml. The other parameters, sperm morphology and motility, as reflected by Van der Merwe (Van der Merwe et al., 2005) correlated well with the published data.
The following article reflects the scientific basis that supports the above argument:

Lee Mee Ho, Alvin Soon Tiong Lim, Tse Hui Lim, Siew Chen Hum, Su Ling Yu, Kruger TF. Correlation between semen parameters and the hamster egg penetration test (HEPT) among fertile and sub-fertile men in Singapore. J Androl 2007;28:158-163
Correlation Between Semen Parameters and the Hamster Egg Penetration Test (HEPT) Among Fertile and Subfertile Men in Singapore

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ABSTRACT: The objective of this retrospective study was to distinguish between fertile and subfertile men based on their semen parameters and hamster egg penetration test (HEPT) outcome. This study involved 110 subfertile men recruited from an infertility clinic and 48 fertile men attending an antenatal clinic in Singapore. The men were required to donate a semen specimen for semen analysis and HEPT assay. The results indicated that the subfertile group had significantly lower normal sperm morphology according to the World Health Organization criteria, and lower progressive motility (P < .05). Semen volume, density, HEPT decondensation rate, and sperm penetration index were not significantly different between the two groups. Receiver operating characteristic curve analysis indicated that sperm morphology had the highest predictive power of 65.7% with a threshold value of 7%, and progressive motility had a predictive power of 61.8% with a threshold value of 50%. Using the tenth percentile of the fertile population as the cutoff, lower adjusted thresholds of 3% for sperm morphology and 28% for progressive motility were obtained, giving higher positive predictive values of 81.8% and 84.4%, respectively. This study shows that these new cutoff values can be used to screen the general population to identify subfertile men. In contrast, the HEPT proved to be an insensitive and unreliable assay in identifying subfertile males. To our knowledge the comparison of HEPT and semen parameters between subfertile and fertile men has not been previously reported in an Asian population.

Key words: Semen analysis, sperm, sperm function assay, sperm morphology, normal motile count.


The hamster egg penetration test (HEPT) is a complex sperm function assay that is based on the capacity of the human spermatozoa to fuse with zona pellucida-free golden hamster oocytes, leading to subsequent decondensation of the sperm nuclei. This screening test is thought to reduce wastage of precious oocytes and costly in vitro fertilization (IVF) cycles.

Several studies have reported good correlation between HEPT scores and IVF rates (Wolf et al, 1983; Ausman et al, 1985; Shibahara et al, 1998). On the other hand, there are reports that suggest that the HEPT assay has a poor predictive value of IVF fertilization and pregnancy rates (O’Shea et al, 1993; Rashid et al, 1998). Therefore, previous reports regarding the ability of the HEPT to predict natural human fertility have come to opposing findings. Part of this discrepancy may be due to the many different experimental protocols used in individual laboratories in inducing the acrosome reaction. For example, some protocols involve a short capacitation time with artificial induction using calcium ionophore (Saito et al, 1984; Aitken et al, 1987) or calcium ionophore with or without pentoxyfilline (Ford et al, 2001). Other protocols use TEST-yolk buffer (Romano et al, 1998) or exposure to progesterone during the spermatozoa/oocyte coincubation period (Francavila et al, 2002).

At the Department of Obstetrics and Gynaecology of the Singapore General Hospital, the zona-free HEPT was used as a screening test to simulate an IVF cycle using the patient’s husband’s spermatozoa. The HEPT protocol with the overnight capacitation system which has been previously described (Wolf et al, 1996) was used in the present study because it is similar to the actual capacitation protocol used in our actual IVF cycles. The calcium ionophore challenge or the test-yolk buffer systems were not employed, as these are not used in real IVF cycles. A poor HEPT result suggested that intracytoplasmic sperm injection (ICSI) was a better option than IVF. However, the threshold value for the...
Semen Analysis of Fertile/Subfertile Men

assay is difficult to determine, as is apparent from the reported false positive and negative results. In addition, the actual threshold value based on our local population had never truly been established and had been based largely on empirical data. Therefore it was essential to study the HEPT outcome of both the fertile and subfertile populations of local men in order to establish the cutoff predictive value.

In addition, while the laboratory had started using the Tygerberg strict criteria (SC) morphology scoring since early 1999, its predictive value for fertilization capability of spermatozoa has not been fully assessed. The objectives of our study were therefore to compare the semen parameters, including morphology, progressive motility and density, and the zona-free HEPT outcome between fertile and subfertile males, and to determine the parameters which provide good predictive value.

Materials and Methods

Study Population

This study involved 158 participants comprising 110 subfertile and 48 fertile men. Male partners of pregnant women attending the Antenatal Clinic at the O&G Centre, Department of Obstetrics & Gynaecology, Singapore General Hospital were approached to participate in the study. The fertile group consisted of men who had successfully impregnated their spouses by natural conception at the time of recruitment (Chia et al., 1998), either within 1 year of attempted conception or having more than 1 child with the youngest child less than 1 year old. In addition, these men had not undergone any surgical procedures related to male infertility, and the couples had never attended any assisted reproductive program.

The subfertile group was recruited from those undergoing IVF or ICSI procedures at the hospital's IVF unit, the Centre for Assisted Reproduction (CARE), Department of Obstetrics & Gynaecology, Singapore General Hospital. The subfertile group consisted of couples who were unable to conceive despite having unprotected coitus over a period of 1 year. Men who were azospermic and men whose wives were more than 40 years old were excluded from the study. Prior Institution Review Board approval was given to this study by the hospital's Ethics Committee. All participants had read, understood, and signed a consent form to allow their semen to be used solely for the purpose of this study.

Preparation of Zona-Free Hamster Oocytes

Sexually mature female Golden Syrian hamsters (Mesocricetus auratus) were superovulated with 40 IU pregnant mare serum gonadotrophin (Folligon; Intervet, Angers, France) on the morning of day 1 of their estrus cycle. Ovulation was induced with 40 IU human chorionic gonadotrophin (hCG) (Chorulon; Intervet) on the evening of day 3. The hamsters were sacrificed 16 to 18 hours post-hCG, their oviducts excised, and the cumulus masses released from the oocytes. The cumulus cells from all the hamsters were mixed and dispersed to free the oocytes by a brief exposure to 0.1% hyaluronidase (Sigma Chemical Co., St Louis, MO). The zona pellucida was digested using 0.1% trypsin (Sigma). The zona-free metaphase II oocytes were then quickly but thoroughly rinsed in Biggers, Whitten, and Whittingham medium (BWW; Biggers et al., 1971), supplemented with 8.0 mg/mL human serum albumin (HSA, Fraction V; Sigma) and with an osmolarity of 410 mOsm/kg.

Seminal Analysis and Sperm Preparation

On day 3 of the stimulation of the hamsters, fresh semen samples from both fertile and subfertile men were collected by masturbation into sterile containers after 2 to 5 days of abstinence and sent to the same laboratory for the semen analysis and HEPT assay. The semen samples were left to liquefy at room temperature for 30 minutes. Prior to HEPT assay, sperm density and progressive motility (rapid, slow, or sluggish) were determined according to the World Health Organization (1999) criteria, using phase-contrast microscopy at 400× magnification. Sperm morphology was performed on Papanicolaou-stained semen smears according to the Tygerberg SC (Kruger et al., 1986; 1988). The total normal motile count (sperm volume × % SC morphology × % progressive motility × sperm density) was also calculated and included in the evaluation.

Following initial assessments, the semen samples were washed once in BWW medium. For swim-up, sperm pellets were overlaid with 1.25 mL of BWW and incubated at 37°C in an atmosphere of 5% CO2 for 1 hour. After the incubation period, 1.0 mL of the topmost sperm suspension containing highly motile spermatozoa was aspirated and kept at room temperature to capacitate for 16 to 18 hours.

Insemination and Scoring of HEPT Decondensation and Sperm Penetration Index

In the following morning, the concentration of motile spermatozoa was adjusted to 5 million cells/mL and resuspended in 2.50-μL droplets in a 35-mm petri dish. Thirty zona pellucida-free hamster oocytes were distributed for insemination, with 15 oocytes per sperm suspension droplet. Incubation was carried out for the next 3 hours at 37°C in an atmosphere of 5% CO2. The oocytes were then washed by gentle pipetting to remove loosely bound sperm and placed onto the middle of a specially prepared microscope slide with 4 paraffin wax supports. A 22 × 22-mm glass coverslip was mounted over the wax supports, and then a gentle pressure was applied to compress the oocytes.

A successfully decondensed sperm head appeared as a large clear vacuole with a closely associated tail within an oocyte when observed under phase contrast microscopy (400× magnification). The percentage of HEPT decondensation was calculated as the number of oocytes with at least 1 decondensed sperm head, divided by 30 oocytes multiplied by
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Table 1. Semen parameters and HEPT outcome (subfertile men, N = 110; fertile men, N = 48)*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Subfertile Men</th>
<th>Fertile Men</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Semen volume, mL</td>
<td>2.8 ± 1.4</td>
<td>0.2–6.4</td>
<td>2.9 ± 2.0</td>
</tr>
<tr>
<td>Progressive motility, %</td>
<td>40.6 ± 16.5</td>
<td>2–69</td>
<td>47.0 ± 13.3</td>
</tr>
<tr>
<td>Sperm density, million/mL</td>
<td>57.1 ± 51.0</td>
<td>6.6–273</td>
<td>72.3 ± 58.7</td>
</tr>
<tr>
<td>SC morphology, %</td>
<td>5.6 ± 4.4</td>
<td>0–19</td>
<td>7.9 ± 4.8</td>
</tr>
<tr>
<td>Total normal motile count, million</td>
<td>5.83 ± 11.34</td>
<td>0–59.63</td>
<td>8.22 ± 10.15</td>
</tr>
<tr>
<td>HEPT decondensation, %</td>
<td>69.8 ± 30.7</td>
<td>0–100</td>
<td>79.2 ± 19.3</td>
</tr>
<tr>
<td>HEPT SPI</td>
<td>3.5 ± 3.1</td>
<td>0–15.0</td>
<td>3.1 ± 2.6</td>
</tr>
</tbody>
</table>

* SD indicates standard deviation; SC, Tygerberg strict criteria for normal sperm morphology; total normal motile count, semen volume × % SC morphology × % progressive motility × sperm density; HEPT, zona-free hamster eggs penetration test; and SPI, sperm penetration index.

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100. The sperm penetration index (SPI) was calculated as the mean number of denuded sperm heads per oocyte penetrated. Cryopreserved donor sperm was used as controls. The means for HEPT decondensation of the controls for intrasay were regularly more than 88%, and the coefficient of variation (CV) was less than 10%. The inter-assay means for HEPT decondensation were more than 86%, with CV of less than 17%.

Statistical Analysis

All statistical analyses were performed with the SPSS package version 10.1 (SPSS Inc, Chicago, Ill.). Basic descriptive statistics including means, standard deviation, medians, and ranges were calculated for the fertile and subfertile groups separately. Semen parameters and HEPT values of the 2 groups were compared for statistically significant differences at P < .05 (2-tailed) using the nonparametric Mann-Whitney U test.

The predictive value of the individual semen variables and HEPT outcome to differentiate between the fertile or subfertile status was analyzed using the receiver operating characteristics curve (ROC) analysis. The predicative power for fertility status is the area under the curve (AUC) and is expressed as a percentage. The ROC curves were plotted to compare the diagnostic performance of the semen parameters and HEPT outcome for the prediction of the fertility status of the males.

This study has defined sensitivity or true positive rate as the proportion of men with poor semen parameters (the subfertile group). Specificity or true negative rate is defined as the proportion of men with normal parameters (the fertile group) who are classified as fertile. The threshold values were calculated for optimal sensitivity and specificity. The positive predictive value (+PV) is the proportion or theoretical accuracy of identification of subfertile men with poor semen parameter with respect to the general population. The negative predictive value (−PV) is the proportion or theoretical accuracy of identification of fertile men with normal semen parameter with respect to the general population.

Results

The descriptive statistics for the semen parameters and HEPT outcomes are presented in Table 1. The mean progressive motility was significantly lower in the subfertile group compared to the fertile group (40.6% vs 47.0%, P = .041). The SC morphology and total normal motile count were also significantly lower in the subfertile group (5.6% versus 7.9%, P = .003; and 5.8 × 10⁶ versus 8.2 × 10⁴, P = .006, respectively). Semen volume, sperm density, and HEPT decondensation (%) were lower in the subfertile group but were not significantly different from the fertile group (P > .05). HEPT SPI was also not significantly different between the 2 groups of men (P > .05).

When the ROC curve analysis was used to predict fertility status, the SC morphology was found to be the best parameter, with a predictive power of 65.7%, P = .002 (95% CI: 56.6% to 74.7%) (Table 2). This was followed by progressive motility 61.8%, P = .02 (95% CI: 52.6% to 71.1%). On the other hand, HEPT decondensation (%) and HEPT SPI were not predictive, as the AUCs were only 54.0% and 51.1% (P > .05), respectively.

Table 2. Predictive test characteristics of semen parameters that significantly predict fertility status*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC</th>
<th>Threshold Values</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC morphology</td>
<td>65.7%</td>
<td>7%</td>
<td>67.3</td>
<td>60.4</td>
<td>.002</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>61.8%</td>
<td>50%</td>
<td>66.4</td>
<td>50.0</td>
<td>.02</td>
</tr>
<tr>
<td>Total normal motile count, million</td>
<td>64.1%</td>
<td>4.0</td>
<td>59.1</td>
<td>52.1</td>
<td>.006</td>
</tr>
</tbody>
</table>

* AUC indicates area under curve; SC, Tygerberg strict criteria for normal sperm morphology; total normal motile count, semen volume × % SC morphology × % progressive motility × sperm density.
Based on the results of this study, the optimal cutoff values that identify men as subfertile are 7% for SC morphology, with a sensitivity of 67.3% and a specificity of 60.4%, and 50% for progressive motility, with a sensitivity of 66.4% and a specificity of 50.0% (Table 2).

Although sperm morphology and progressive motility showed good predictive power, the clinical value of semen analysis increased when the parameters were used in combination, that is, the total normal motile count (AUC of 64.1%, \( P = 0.006 \), 95% CI: 55.0% to 73.2%). The optimal threshold value for total normal motile count was \( 4 \times 10^6 \), with a sensitivity of 59.1% and a specificity of 52.1%.

**Discussion**

Of all the parameters studied, the present study showed that the semen parameters that are of diagnostic value in identifying the subfertile male population are sperm morphology according to the Tygerberg SC and progressive sperm motility. In contrast, the high complexity HEPT assay and its SPI are found to be poor predictors, as are semen volume and sperm density.

SC sperm morphology at a threshold of 7% had the best sensitivity and specificity when calculated as its AUC using the ROC curve. This finding is in agreement with the few papers published on the comparison between fertile and subfertile men, in which the calculated threshold for sperm morphology ranged from 4% to 10% (Omebe et al., 1997; Günlap et al., 2001; Guzik et al., 2001; Menkveld et al., 2001). The predictive power of sperm morphology according to these 4 articles ranges from 66% to 78.2%, similar to our value of 65.7%. The tenth percentile of the fertile population was then used to derive a lower cutoff value of 3% sperm morphology. This resulted in a higher positive predictive value of 81.8%, the theoretical accuracy in identifying a man with subfertility with respect to the general population (Table 3). Using this lower threshold of 3% instead of 7% results in a more accurate classification of the subfertile population by excluding some fertile men who may have normal morphology at the upper limits of the abnormal range. Our finding was comparable with the earlier reports of 2% and 5% (Omebe et al., 1997; Menkveld et al., 2001).

Apart from sperm morphology, progressive motility was also found to be a fairly good predictor of fertility at the threshold of 30%, with an AUC of 61.8%. In the 4 articles that were mentioned earlier, motility also had a high predictive power, with AUC values ranging from 59% to 79.1% and calculated threshold values between 32% and 52%. When the threshold was set lower, to 28%, using the tenth percentile of the fertile population, the positive predictive value (or theoretical accuracy in identifying a man with subfertility) of 84.4% was obtained and was comparable to the adjusted lower thresholds in 3 of the articles, which were between 20% and 30% for motility.

It was found that the clinical value of semen analysis could actually be further enhanced when semen volume, sperm morphology, density, and progressive motility parameters were combined (Table 2). The total normal motile count gave an AUC value of 64.1% at the threshold of \( 4.0 \times 10^6 \), compared to 65.7% and 61.8% for sperm morphology and progressive motility, respectively. This threshold of \( 4.0 \times 10^6 \) was comparable to \( 3.24 \times 10^6 \), as reported earlier (Omebe et al., 1997). Using the tenth percentile of the fertile population, a lower threshold value of \( 0.35 \times 10^6 \) was derived for the total normal motile count, with a positive predictive value of 86.2% (Table 3), as compared to \( 0.52 \times 10^6 \) in the earlier report.

No other semen parameters were found to differ significantly between the subfertile and the fertile groups. In particular, neither the HEPT decondensation rate (69.8% vs 79.2%) nor the SPI (3.5 vs 3.1) were significantly different. This was a somewhat unexpected finding, as this assay had been offered to patients at our hospital for several years as a screening test for patients before their actual IVF cycle. This routine test was offered because of the good correlation found between the assay and in vitro outcome (Wolf et al., 1983; Ausman et al., 1985; Coetzee et al., 1989; Ibrahim et al., 1989; Johnson et al., 1991; Freeman et al., 2001), despite several later opposing findings (Lui and Baker et al., 1992; O'Shea et al., 1993; Rashid et al., 1998). These contrasting findings could have been in due part to the different protocols used in the assay and to the various sperm capacitation times used. Another reason could be that this screening test may not be representative of an actual IVF scenario because the sperm-zona pellucida interaction is totally precluded in the HEPT assay, leading to potentially artificially inflated HEPT decon-
denensation rates and SPI scores in the subfertile group. This suggestion is supported by a study by Lui and Baker (2000) which found that defective sperm-zona pellucida binding and penetration are the major factors of zero or low fertilization rates in IVF, since the zona pellucida selectively acts as a barrier to morphologically abnormal spermatozoa.

In conclusion, only sperm morphology according to the Tygerberg SC, progressive sperm motility, and total normal motile count were found to be the most useful parameters in discriminating the subfertile group from the fertile group of males. Using the tenth percentile values of the fertile population, the adjusted lower thresholds were 3% for sperm morphology, 28% for progressive motility, and 0.35 × 10⁶ for total normal motile count, with positive predictive values or theoretical accuracy in identifying a man with subfertility being 81.8%, 84.4%, and 86.2%, respectively. These findings are in concept with those reported from the United States (Guzick et al., 2001), Turkey (Günlap et al., 2001), and Europe (Omblet et al., 1997; Menkveld et al., 2001). This study sought to determine the reliability of the HEPT assay in comparison with semen analysis. In contrast, the HEPT was found to be an insensitive and unreliable assay in identifying subfertile men. To our knowledge, the comparison of HEPT and semen parameters between subfertile and fertile men has not been previously reported in an Asian population.

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The authors would like to thank Ms Swee Tec Lim for her tireless effort in recruiting the patients, Ms Stephanie Foo-Kong for her invaluable help with statistics, and Mr Yoke Chang Tan for his excellent bench work.

References

Aitken RJ, Thatcher S, Glasier AF, Clarkson JS, Wu FCW, Baird DT. Relative ability of modified versions of the hamster oocyte penetration test, incorporating hypotonic medium or the ionophore A23187, to predict IVF outcome. Hum Reprod. 1987;2:227-231.


b. **To explore the WHO acceptance of the new suggested thresholds for semen parameters**

i. **The WHO manual 1999**

The first change in the WHO approach on semen thresholds followed in 1999 with the publication of the semen manual (WHO, 1999). They accepted the Strict Criteria principles for sperm morphology but did not suggest any specific threshold value for normal sperm morphology. It was suggested that each clinic establish their own thresholds based on their own data. It was thought that this approach was not sound, but it also illustrates that new findings take time to be accepted.

ii. **The WHO manual 2010**

A task force convened in Geneva to discuss the new semen manual in 2007. As part of this prestigious manual the lower thresholds for semen analysis were debated by the appointed panel. The author formed part of this international panel. The Tygerberg Strict Criteria was accepted based on literature published as discussed in section 1 as well as on the semen values of a multicentre population study on fertile couples (Cooper *et al.*, 2010). In this population study the lower semen thresholds were established using the 5th centile of the semen parameters. It was suggested that lower thresholds were as follows: 4% normal forms or less, motility of 30% or less, concentration per ml. less than 15 million. These values correspond exactly with those established and suggested by Van der Merwe in 2005 (Van der Merwe *et al.*, 2005).

The author was responsible for the section on sperm morphology and did the classification of the cells for the 2010 manual page 66 to 102 (WHO, 2010).
Applicable chapters in the following text book will be reflected on:

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United States of America
Fig. 2.8. Schematic drawings of some abnormal forms of human spermatozoa. (Adapted from Kruger et al., 1993.)

(A) Head defects. (a) Tapered, (b) Pyriform, (c) Round, small and acrosome either absent or present.

(B) Amorphous, (c) Vacuolated, (d) Acrosomal area small, (e) Neck and midpiece defects, (f) Bent neck, (g) Asymmetrical insertion of midpiece, (h) Thick midpiece, (i) Thin midpiece.

(C) Tail defects, (a) Short tail, (b) Bent tail, (m) Coiled tail, (n) Cytoplasmic droplet defect, (m) Droplet greater than one third the area of the normal sperm head.
Applicable chapters in the following text book will be reflected on:

WHO laboratory manual for the
Examination and processing
of human semen
FIFTH EDITION
Acknowledgements

This publication was produced by the UNDP/UNFPA/WHO/World Bank Special Programme of Research, Development and Research Training in Human Reproduction (HRP), WHO Department of Reproductive Health and Research (RHR). The participation of the following individuals in the preparation and editing of this manual is gratefully acknowledged:

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The financial support of the International Society of Andrology is gratefully acknowledged.

This edition of the manual is dedicated to the memory of the late Geoffrey Waites (1928–2005), former manager of the WHO Task Force on Methods for the Regulation of Male Fertility and co-editor of the second, third and fourth editions of this laboratory manual. The editorial committee’s devotion to its task was driven by its appreciation of Geoff’s honesty, fairness and concern for the underprivileged.
Plate 1

Micrographs courtesy of C Brazil.
Morphology assessment of spermatozoa in Plate 1

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Plate 2

Micrographs courtesy of C Brazil.
Morphology assessment of spermatozoa in Plate 2

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The following article reflects the scientific basis that supports the above argument:

World Health Organization reference values for human semen characteristics†

Trevor G. Cooper1,10, Elizabeth Noonan2, Sigrid von Eckardstein3, Jacques Auger4, H.W. Gordon Baker5, Hermann M. Behre6, Trine B. Haugen7, Thinus Kruger8, Christina Wang9, Michael T. Mbizvo3,†, and Kirsten M. Vogelsong3,‡

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- Discussion
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BACKGROUND: Semen quality is taken as a surrogate measure of male fecundity in clinical andrology, male fertility, reproductive toxicology, epidemiology and pregnancy risk assessments. Reference intervals for values of semen parameters from a fertile population could provide data from which prognosis of fertility or diagnosis of infertility can be extrapolated.

* Dedicated to the memory of Professor GMH Walms (1938–2005).
† These authors (M.T.M., K.M.V.) are staff members of the World Health Organization. The authors alone are responsible for the views expressed in this publication; these views do not necessarily represent the decisions or policies of the World Health Organization.
‡ The list of authors who contributed data to this study is given in the Appendix.

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METHODS: Semen samples from over 4500 men in 14 countries on four continents were obtained from retrospective and prospective analyses on fertile men, men of unknown fertility status and men selected as normozoospermic. Men whose partners had a time-to-pregnancy (TTP) of ≤12 months were chosen as individuals to provide reference distributions for semen parameters. Distributions were also generated for a population assumed to represent the general population.

RESULTS: The following one-sided lower reference limits, the fifth centiles (with 95th percent confidence intervals), were generated from men whose partners had TTP ≤12 months: semen volume, 1.5 ml (1.4–1.7); total sperm number, 39 million per ejaculate (33–46); sperm concentration, 15 million per ml (12–16); vitality, 58% live (55–63); progressive motility, 32% (31–34); total (progressive + non-progressive) motility, 40% (38–42); morphologically normal forms, 4.0% (3.0–4.0). Semen quality of the reference population was superior to that of the men from the general population and normozoospermic men.

CONCLUSIONS: The data represent sound reference distributions of semen characteristics of fertile men in a number of countries. They provide an appropriate tool in conjunction with clinical data to evaluate a patient’s semen quality and prospects for fertility.

Key words: human semen / reference values / infertility diagnosis / fertile men

Introduction

The “WHO manual for the examination of human semen and sperm–cervical mucus interaction” (WHO, 1987, 1992, 1999) is widely used as a source of standard methodology for laboratories engaged in semen analyses. However, the interpretation and application of previous WHO ‘normal’ or ‘reference’ values for semen parameters used thus far have limitations, since the data were derived from imprecisely defined reference populations and obtained from laboratories with unknown comparability with respect to analytical methodologies. These values were limited by the lack of available data on semen variables in recent fathers, and did not define true reference ranges or limits. There has been no consensus around the suitability of these values, as some centres consider the cited values for characteristics of sperm concentration, morphology and motility too high, whereas others consider them too low.

If too high, a high percentage of fertile men would be classified as subnormal, especially when morphology, sperm concentration or motility is considered (Barratt et al., 1988; Chia et al., 1998; Nallela et al., 2006; Pasqualotto et al., 2006; Gao et al., 2007, 2008). Healthy men may also be investigated for infertility, or inappropriately treated by Assisted Reproduction Technologies, as a result of their lower semen quality if reference limits are too high (Bostofte et al., 1983; Lemcke et al., 1997).

On the other hand, a sperm concentration of $20 \times 10^6$/ml, the ‘normal’ or ‘reference’ value cited by WHO (1987, 1992, 1999), has been considered too low for a lower reference limit because the probability of pregnancy is essentially linear with sperm concentrations up to $40–50 \times 10^6$/ml (Bonde et al., 1998; Slama et al., 2002). Conversely, sperm concentrations above this value are repeatedly observed in infertile patients (Nallela et al., 2006). There may be no upper limit of any semen characteristics since pregnancy rates increase with superior sperm morphology and motility (Garrett et al., 2003). The then-current normal morphology value of WHO (1987) was considered inadequate by Check et al. (1992) as it did not distinguish between fertile and infertile men whose partners were healthy. With uncertain reference values, over- or under-diagnosis may result. Although much of the investigation conducted to date has considered the WHO ‘normal’ or ‘reference’ values as cut-off limits separating fertile from infertile populations, doubts have been raised about the validity of this approach (Bartoo et al., 1993; Barratt et al., 1995).

This article considers which men are most suitable for providing a reference population, presents data from such a population, mentions the possible limitations of the results obtained and discusses how the reference intervals could be interpreted as useful reference limits. The present analysis benefits from the availability and incorporation of multi-country data from recent fathers with known time-to-pregnancy (TTP). The development and application of clear reference ranges should help reduce the incidence of misdiagnosis of fertility problems and improve clinical care.

Individuals considered suitable for providing reference semen values have included unselected populations, that is, men of unproven fertility (Irvin et al., 1996; Paulsen et al., 1996; Lemcke et al., 1997; Junq et al., 2002); men from couples presenting with infertility (MacLeod and Wang, 1979; Bostofte et al., 1983; Berling and Wolker-Hanssen, 1997; Andol et al., 1999); candidates for semen donation, some proven fertile (Leo and Frenzilli, 1981; Auger et al., 1995; Bujan et al., 1996; Van Waeseghem et al., 1996) and men presenting for vasectomy (Sultan Sherman, 1983; Fisch et al., 1996). Whereas the first group may be considered drawn from the general population, semen donors may be, and vasectomy candidates most probably are, of proven fertility, although paternity may not have been recent relative to provision of the semen sample analysed. The majority of men have indefinable fertility status at any one moment: therefore a reference range comparing recently fertile men is defined by men whose semen variables may not reflect those of the general population. This is unusual among clinical laboratory tests and clearly presents a major challenge in defining a valid population reference range for human semen.

The present study examined semen quality in groups of men from the general population (having unknown fertility status) as well as fathers. For the investigation of male factor infertility, the most relevant reference group is that of proven fertile men, since for valid comparisons of patient data with the reference values, the patient should sufficiently resemble the reference individuals in all respects other than those under investigation (Petit Clerc and Solberg, 1987; Solberg, 1987), in this case fertility. The selection criteria determining
c. To evaluate the application of the suggested thresholds in clinical practice

   i. Endometriosis research and semen thresholds

The thresholds for concentration [<10 million/ml, motility (<30%) and morphology (<5%)] were used in a population of female patients suffering from endometriosis. In this publication it was indicated that the most common defect is sperm morphology abnormalities (teratozoospermia) which occurred in 25% of subjects. Of these patients, 11.96% had a serious male factor and it was concluded that in research studying for example the impact of surgery on e.g. endometriosis with pregnancy outcome as an endpoint, the male factor must be clearly defined and taken into consideration when analyzing the data over time. This was to our knowledge the first publication to assist researchers in the practical application of semen thresholds using Strict Criteria and to use the new semen thresholds in female research. (Dr DJ Botha is a post graduate student in our unit. He did his fellowship in Reproductive Medicine with the aim to continue with a PhD in future).
The following article reflects the scientific basis that supports the above argument:

Semen profiles of male partners in females presenting with endometriosis-associated subfertility

Danie J. Botha, M.Med.(O&G), FCOG, Thinus F. Kruger, M.D., FCOG, Jakohus P. Van Der Merwe, M.Med.(O&G), and Saleema Nosarked, M.Med.(O&G)

Department of Obstetrics and Gynecology, Stellenbosch University and Tygerberg Academic Hospital, Tygerberg, South Africa

Objective: To establish fertile and subfertile groups of males in couples presenting with endometriosis-related infertility in the female.

Design: Retrospective study of semen profiles on partners of women undergoing surgery for endometriosis-related infertility.

Setting: University-affiliated assisted reproduction center.

Patient(s): One hundred seventeen partners of females undergoing surgery for endometriosis related infertility.

Main Outcome Measured(s): Fertility potential according to Tygerberg strict criteria for sperm evaluation.

Result(s): A total of 65.8% of the semen analysis were reported normal according to the Tygerberg strict criteria and 34.2% were reported subfertile. Of the total, 11.96% of the patients studied had a severe defect (azoospermia, double, and triple defects).

Conclusion(s): Labeling of the male in couples presenting with infertility as fertile, subfertile, or infertile plays an important role in decision making regarding management of the female presenting with endometriosis-related infertility. It is important that in future studies on the effect of surgery on improving pregnancy outcome in patients with endometriosis the semen profile be reported and thresholds used should be mentioned. This will contribute significantly to the future comparison of data on the impact of the male factors in couples presenting with endometriosis-related infertility. (Fertil Steril 2009;91:2477–80. ©2009 by American Society for Reproductive Medicine.)

Key Words: Semen profiles, male factor infertility, Tygerberg strict criteria, endometriosis, surgery

Infertility is estimated to occur in 10% to 15% of the general population, and 30% of infertility is caused solely by a male factor (1). Semen parameters most widely used are sperm concentration, motility, progressive motility, and sperm morphology (2). Various classification systems have been used to describe the factors that constitute morphologic normal spermatozoa (World Health Organization [WHO] criteria of 1987, 1992, and Tygerberg Strict Criteria, now also used by the WHO since 1999) (3–5).

Males with good or reasonable fertility potential under in vivo conditions should be identified on the basis of semen quality (2). Conversely, males with a poor fertility potential should be identified and introduced to assisted reproduction programs. The percentage of normal sperm morphology using strict criteria has a positive predictive value in IVF/ intracytoplasmic sperm injection (ICSI) (6) and intrauterine insemination (IUI) programs (7).

Endometriosis is common in women of reproductive age, and affects 20% to 40% of women presenting with infertility (8). Although the precise pathophysiologic pathways of endometriosis are not fully understood, it is accepted that it is a benign disease that is multifactorial in origin, with hormonal, genetic, and immunologic/inflammatory components contributing (9, 10). For the patient, quality of life may be significantly impaired because of chronic pelvic pain, dysmenorrhea, and dyspareunia with or without accompanying infertility (11).

Surgery is generally accepted to improve endometriosis-related pain, and, together with medical treatment, should be offered to these patients. The optimal approach to manage the female patient with endometriosis-related infertility, however, is still not clear. Data on pregnancy outcome after surgery (12) is still conflicting, and there is possibly an indirect effect of endometriosis on oocyte and embryo quality (13).

Currently, the age of the patient, history of previous surgery, presence or absence of pain, duration of infertility, as well as failed conservative measures to improve fecundity are guiding the clinicians decision on whether or not to offer surgery.

Despite complete and often repeated surgical procedures for endometriosis in the female to improve fertility, many couples still end up in assisted reproduction programs.

The aim of this study is to establish fertile and subfertile groups of males in couples presenting with endometriosis-related infertility in the female partner.
MATERIALS AND METHODS
We conducted a retrospective analysis of semen profiles in patients presenting at the Tygerberg Fertility Clinic from January 2002 to May 2006 with female infertility who underwent surgery for endometriosis. Because of the retrospective nature of this study, institutional review board approval was not requested, with patient confidentiality and treatment outcome not influenced by publishing the data in this study.

Patients undergoing surgery for endometriosis-related pain where pregnancy was not planned in near future were excluded from this study. The records of 125 women who underwent surgery for endometriosis and for whom pregnancy outcome was available were searched. Staging of the endometriosis was according to the r ASRM classification 1996 of endometriosis (14).

Using this cohort of 125 patients, 117 patients’ partner semen profiles were available for inclusion in this study. Fresh semen samples were obtained by masturbation after 2 to 5 days of abstinence according to the clinic’s protocol. The methods used to examine the semen were according to the WHO guidelines (5), and for sperm morphology, Tygerberg Strict Criteria was used (5, 15, 16). The thresholds used for subfertility were those suggested by Van der Merwe et al. (2): 0% to 4% normal forms; ≤30% motility and <10 mil/mL concentration.

RESULTS
Results are shown in Table 1.

A total of 65.8% of semen analyses were reported normal according to Tygerberg Strict Criteria (normozoospermia) and 34.2% were reported subfertile.

A total of 27.4% had a single parameter defect, with teratozoospermia (18.8%) being the most common abnormality.

Double parameter defects were present in a total of 5.1% of patients.

Triple parameter defects were present in a total of 1.7% of patients.

Azoospermia was present in a total of 5.1% of patients, and immunologic factors (MAR > 60%) were present in a total of 1.7% of patients.

Severe defects (azoospermia, double, and triple defects) were present in a total of 11.96% of patients studied.

DISCUSSION
This is the first study to our knowledge to report on the semen profiles of couples presenting with endometriosis-related infertility where the strict criteria has been applied for sperm morphology. The aim of this study is to look at the profile of males presenting with infertility because of a known factor of endometriosis in the female partner. The question arises as to why some women will respond different to surgery regarding fertility with the same classification of endometriosis according to the r AFS classification. This study is a descriptive attempt to look at the male as the possible reason for the altered outcome. We also want to underline the fact that in clinical practice some couples are advised to undergo assisted reproductive technology (ART) without surgery in the female because of a severe defect in the male. The purpose of this study is not to report on pregnancy outcome, but merely to bring the male to the forefront when assessing these couples. This article aims to encourage debate on the role of the male partner in couples presenting with endometriosis-related infertility. Very little information is available in the literature on the male factor in patients with endometriosis-related infertility. Dahlberg (17) reported on the presence of asymptomatic bacteriospermia in male partners of patients with endometriosis, and Mahmood et al. (18) and Waller et al. (19) speculated on the possible association between male infertility and the development of endometriosis in the female. Arumugam (20) reported on sperm dysfunction in partners of infertile patients with minimal or mild endometriosis, using sperm penetration testing with bovine cervical mucus.

In our study, a total of 34.2% of patients presenting with endometriosis had a contributing male factor, with a total of 11.96% presenting with severe defects. The thresholds

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<td>Motility = &lt;30% Morphology = &lt;5%</td>
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used were concentration <10 ml/mL, motility <30%, and morphology <5%. Coetzee et al. (6), by using the 5% threshold for morphology, demonstrated significantly better overall fertilization and overall pregnancy rates in vitro in the group with a normal morphology of 5% or more, compared with <5% group. They demonstrated that patients with semen profiles of sperm concentration below 10 mil/mL have a higher potential to be subfertile in vivo, resulting in a higher chance of ending up in ART programs.

Two clinical questions should be addressed. First, when will a male factor be regarded to be so significant as to consider withholding surgery in the female partner with endometriosis and rather opt for ART earlier in the treatment planning? Second, with normal semen parameters, will surgery for endometriosis improve the couples’ chance of spontaneous pregnancy and successful ART outcome? The advantage of ART is that it addresses multiple problems including male factor infertility and tubal damage because of endometriosis, and it is the reason why in many units ART is routinely performed in patients with endometriosis.

A meta-analysis of data from IUI programs showed a higher pregnancy rate per cycle in the group with normal sperm morphology ≥5% (7). It is important to note that in the studies that were reviewed by Van Waarden, the group with <5% normal forms still had an accepted (7% per cycle) pregnancy rate, although significantly lower than the 11% success rate in the group with >5% normal forms. This finding highlights the principle in handling these patients. Thus, a patient with only one normal semen parameter abnormality must still receive IUI if swim-up before this suggested treatment yielded sperm in excess of 500,000 sperm/mL.

It is clear from the aforementioned that the labeling of the male in couples presenting with infertility as fertile, subfertile, or infertile plays an important role in decision making regarding management of the female presenting with endometriosis-related infertility.

Although the answer to the second question is a separate debate, it does appear that the correction of normal anatomy, especially the tubal-ovarian interaction, is probably partly responsible for the improvement in spontaneous pregnancy rates post-surgery.

It has been shown by prospective, randomized trials that surgery for minimal to mild endometriosis does improve fertility outcome (21, 22). A meta-analysis of these studies by Jacobson et al. (23) demonstrated that for minimal and mild stages of endometriosis, surgery does seem to improve pregnancy outcome in vivo. There are no randomized controlled trials confirming the generally accepted benefit of surgery for more advanced stages of endometriosis, but based on the above observations in milder stages of endometriosis, one can reason that there may be benefit for offering surgery to patients with severe endometriosis. This is especially true for couples in whom the semen profile is normal, suggesting a good chance of pregnancy in vivo according to the criteria mentioned, as well as those men with a single parameter defect, as spontaneous pregnancies are possible in this group.

From a research perspective we suggest that data on the semen profile be routinely included in all studies where patients are undergoing surgery for endometriosis to improve fertility. Currently, published studies often exclude couples with the presence of a male factor in evaluating the outcome of endometriosis-related infertility post-surgery. The excluded couples are what we perceive to be the clinical scenario in question. By including these infertile men into these studies and then performing subgroup analysis according to “fertile and subfertile” male groups, would give much more insight into the question at hand.

We want to challenge the design of surgical studies where the male factor is mentioned as a reason for exclusion from the study. It would be interesting to know how those couples, presenting with endometriosis and infertility with a male factor that were excluded, were managed. Was surgery offered to them, or ART without surgery, or both? If our data do reflect the true incidence of severe male factor infertility in these couples with endometriosis, it may be that some of these “excluded” couples did undergo surgery possibly unnecessarily. It also may mean that a lot of patients are excluded unnecessarily from trials because of vague definitions of a male factor without mentioning criteria or cutoff values used.

If we were to exclude these couples on the grounds of a male factor being present, we would have to exclude 34% of our patients when using the strict criteria for semen evaluation. That means that a third of the population with female infertility and endometriosis is excluded from clinical trials. The male factor cannot be ignored in all studies and then the perceived treatment proclaimed to be effective/not effective if a third off the population will be excluded when this treatment modality is applied in clinical practice. If the actual incidence of male infertility in these populations is reported, it may help the clinician to evaluate the relevance of a treatment modality that is studied. It would be interesting to know at which level of male infertility (depending on criteria used) would we start to question the value of a treatment modality, in this case, surgery for endometriosis, that is, if 80% of men are also infertile/subfertile, would we still study surgical treatment of endometriosis if it reflects on only 20% of the population who can be treated?

When patients are randomized and male factor infertility is taken into consideration, a better interpretation of data can be achieved based on clear guidelines and thresholds used in these studies. It is essential, especially when dealing with the endpoint as pregnancy outcome in vivo and long-term fertility in patients who underwent surgery for endometriosis. A prospective clinical trial is currently underway, studying the outcome of surgery in patients with endometriosis-related infertility and the effect that the male plays in outcome.
This is the first report to our knowledge on the prevalence of semen abnormalities using strict criteria in patients with endometriosis. We suggest that in endometriosis research it is mandatory if pregnancy outcome in vivo is a primary endpoint, to clearly state semen parameters and thresholds used. The role of ART in the management of these couples needs to be defined. This is an interesting debate. It is important that clinics develop their own thresholds for semen profiles or use international suggested thresholds as discussed in this article. If a clinician is dealing with the 12% of patients in Table 1 with a significant male factor (azoospermia/double parameter and triple parameter defects), these patients probably will do better with ART (ICSI) early in their treatment plans.

REFERENCES
ii. Anovulation and semen thresholds

The same principals were used in patients suffering from anovulation (PCOS) and the findings were published in chapter 18 in *Male Infertility: Diagnosis and Treatment* 2007. *(Tables 18.3 and 18.4)* (Siebert et al., 2007).
Applicable chapters in the following text book will be reflected on:

Siebert TI, Van der Merwe H, Kruger TF, Ombelet W. How do we define male sub-fertility and what is the prevalence in the general population? In: Male Infertility: Diagnosis and Treatment. SC Oehninger and TF Kruger (eds). Informa Healthcare: London. 2007:269
Male Infertility
Diagnosis and Treatment

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informa
healthcare
How do we define male subfertility and what is the prevalence in the general population?

INTRODUCTION

Several semen parameters are used to discriminate the fertile male from the subfertile male. The most widely used parameters are sperm concentration, motility, progressive motility and sperm morphology. Of these parameters, sperm morphology is the single indicator most widely debated in the literature. A large number of classification systems have been used to describe the factors that constitute a morphologically normal/abnormal spermatozoon. The most widely accepted classification systems for sperm morphology are the World Health Organization (WHO) criteria of 1987 and 1992\(^1,2\) and the Tygerberg strict criteria, now also used by the WHO since 1999\(^3-6\).

Although there is a positive correlation between normal semen parameters and male fertility potential, the threshold values for fertility/subfertility according to WHO criteria\(^1,2\) are of little clinical value in discriminating between the fertile and the subfertile male\(^7-11\). If these criteria were to be applied, a great number of fertile males (partners having had pregnancies shortly before, after or at the time of a spermiogram) would be classified as subfertile. The predictive values of sperm morphology using strict criteria in *in vitro* fertilization (IVF) and intrauterine insemination (IUI) have been reviewed recently and proved to be useful\(^12,13\).

Much less has been published on the use of this criterion regarding *in vivo* fertility.

In this chapter, we evaluate the classification systems for semen parameters after review of the literature published in English on semen parameters and *in vivo* fertility potential. We also use data from the literature to establish fertility/subfertility thresholds for semen parameters according to the WHO 1999 guidelines\(^3-6\). These thresholds should be of clinical value and useful when assessing male fertility potential for *in vivo* conditions, in order to identify those males with a significantly reduced chance of achieving success under these conditions.

**WHO CRITERIA OF 1987 AND 1992 AND MALE FERTILITY POTENTIAL**

The semen analysis is used in clinical practice to assess male fertility potential. To be of clinical value, the methods used should be standardized, and threshold values for fertility/subfertility should be calculated for the different parameters used in the standard semen analysis.

Because there are so many different methods for semen evaluation, it would be difficult to standardize the methods used in its analysis. This applies especially to the assessment of sperm
morbidity. The two classification systems most widely accepted are the WHO\textsuperscript{1,2} and the Tygerberg strict criteria\textsuperscript{3-6}. Various methodological problems concerning sperm morphology have been identified. The variants among different methods of morphology assessment have been reported by Ombelet \textit{et al.}\textsuperscript{14-16} and others\textsuperscript{17,18}, and they recommend standardization of semen analysis methodologies. Some authors recommend that laboratories should adopt the accepted standards, such as those proposed by the WHO\textsuperscript{17,18}. Another problem identified is the variation in intra- and interindividual and interlaboratory sperm morphology assessment\textsuperscript{18,19}. This problem can be addressed by using the Tygerberg strict criteria, as Menkveld \textit{et al.} showed that comparable and reliable results between and within observers could be obtained when using this method\textsuperscript{19}. Franken \textit{et al.} delivered dedicated work on continuous quality-control programs for strict sperm morphology assessment, and demonstrated that consistent readings could be achieved; they hence stressed the need for global quality-control measurements in andrology laboratories\textsuperscript{20,21}. Cooper \textit{et al.}\textsuperscript{18} also urged the standardization of such quality-control programs and that quality control centers should reach agreement with each other.

Previous WHO thresholds of 50% and 30% for sperm morphology were empirical values and not based on any clinical data. Several authors found these values to be of little or no clinical value\textsuperscript{7,9,10,22}. These studies did, however, find a positive correlation between a high proportion of morphologically normal sperm and an increased likelihood of fertility and/or pregnancy. Other studies have confirmed this correlation\textsuperscript{23-25}.

Van Zyl \textit{et al.}\textsuperscript{23} were the first to show a faster than linear decline in fertilization rate when the proportion of normal forms dropped to less than 4%. Eggert-Kruse \textit{et al.}\textsuperscript{23} found a higher \textit{in vivo} pregnancy rate for higher percentage normal forms at thresholds of 4, 7 and 14% using strict criteria for morphology assessment. Zinaman \textit{et al.}\textsuperscript{26} confirmed the value of sperm morphology (strict criteria) by demonstrating a definite decline in pregnancy rate \textit{in vivo} when the normal morphology dropped below 8% and sperm concentration below $30 \times 10^6$/ml. In a study performed by Slama \textit{et al.}\textsuperscript{27}, measuring the association between time to pregnancy and semen parameters, it was found that the proportion of morphologically normal sperm influenced the time to pregnancy up to a threshold value of 19%. This value is somewhat higher than that calculated in other studies.

\section*{THE USE OF SEMEN PARAMETERS IN IVF AND IUI PROGRAMS}

The percentage of normal sperm morphology (strict criteria) has a positive predictive value in IVF and IUI programs. Normal sperm morphology thresholds produced positive predictive values for IVF success when using the 5% and 14% thresholds, respectively, with the overall fertilization rate and overall pregnancy rate significantly higher in the group with normal morphology \textgeq 5% as compared with the <5% group\textsuperscript{12}. A meta-analysis of data from IUI programs showed a higher pregnancy rate per cycle in the group with normal sperm morphology \textgeq 5%. In the group with normal sperm morphology <5%, other semen parameters predicted IUI success\textsuperscript{13}. In the IUI meta-analysis, motility\textsuperscript{28}, total motile sperm count\textsuperscript{29} and concentration\textsuperscript{30} also played a role in some of the studies evaluated, while others\textsuperscript{31} stated that sperm morphology alone was enough to predict the prognosis. Because of the high cost of assisted reproduction, males with good or reasonable fertility potential under \textit{in vivo} conditions should be identified on the basis of semen quality. Conversely, males with a poor fertility potential should be identified, and introduced to assisted reproduction programs.
FERTILITY/SUBFERTILITY THRESHOLDS FOR SPERM MORPHOLOGY USING TYGERBERG STRICT CRITERIA, SPERM CONCENTRATION AND SPERM MOTILITY/PROGRESSIVE MOTILITY

In an effort to establish fertility/subfertility thresholds for the aforementioned parameters, we identified four articles in the published literature. It is our opinion that these articles constitute a representative sample of published studies of the predictive value of sperm morphology, sperm concentration and motility/progressive motility for in vivo fertility/subfertility. These articles compared the different semen parameters of a fertile and a subfertile group. They used either classification and regression tree (CART) analysis or receiver operating characteristic (ROC) curve analysis to estimate thresholds for the various semen parameters. The ROC curve was also used to assess the diagnostic accuracy of the different parameters and their ability to classify subjects into fertile and subfertile groups.

Using ROC curve analysis, Ombelet et al.\textsuperscript{32} calculated the following thresholds: proportion normal morphology 10\%, proportion normal motility 45\% and normal sperm concentration $34 \times 10^6$/ml. Sperm morphology was shown to be the parameter with the highest prediction power (area under the curve (AUC) 78\%). Much lower thresholds were calculated using the 10th centile of the fertile population, these thresholds being 5\% for normal morphology, 28\% for motility and $14.3 \times 10^6$/ml for sperm concentration (Tables 18.1 and 18.2)\textsuperscript{32}.

Günsel et al.\textsuperscript{33} also calculated thresholds using ROC curve analysis. These thresholds were: proportion normal morphology 10\%, proportion normal motility 52\%, proportion progressive motility 42\% and sperm concentration $34 \times 10^6$/ml. The two parameters that performed best were progressive motility (AUC 70.7\%) and

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morphology (AUC 69.7%). Assuming 50% prevalence of subfertility in the population, the authors used the positive predictive value as an indicator to calculate a lower threshold for each parameter. Values of 5% for proportion normal morphology, 30% for proportion normal motility, 14% for proportion progressive motility and 9 x 10^6/ml for sperm concentration were calculated (Tables 18.1 and 18.2)

In the most recent article of the four, Menkveld et al. found much lower thresholds than the others. Using ROC curve analysis, the following thresholds were calculated: 4% for normal morphology and 45% for normal motility. Again, morphology showed good predictive value with an AUC of 78.2%. Although a threshold for sperm concentration was not calculated (a sperm concentration less than 20 x 10^6/ml was used as inclusion criterion), the authors proposed that the cut-off value of 20 x 10^6/ml could be used with confidence, based on the resultant lower 10th centile of the fertile population. Adjusted cut-off points calculated on the assumption of 50% prevalence of male subfertility were as follows: 3% for proportion normal morphology and 20% for proportion normal motility (Tables 18.1 and 18.2).

In the fourth article by Guzik et al., the authors used CART analysis and calculated two thresholds for each semen parameter which allowed designation into three groups, namely normal (fertile), borderline and abnormal (subfertile). The normal (fertile) group had values greater than 12% for morphology, greater than 63% for motility and higher than 48 x 10^6/ml for sperm concentration. The abnormal (subfertile) group had values lower than 9% for morphology, lower than 32% for motility and lower than 13.5 x 10^6/ml for sperm concentration.

In these four articles, the predictive power of the different parameters was calculated as the AUC, using the ROC curve. The AUC for sperm morphology ranged from 66 to 78.2%, confirming the high predictive power of this parameter. In fact, it had the best performance among the different semen parameters in two articles. The thresholds calculated in these two articles were 10% and 9%, respectively, while Günalp et al. calculated a threshold of 12% using sensitivity and specificity to analyze their data, and the fourth study calculated a 4% predictive cut-off value. Although sensitivity and specificity for the values are relatively high, the positive predictive values are not. This will therefore result in classifying fertile males as subfertile, probably leading to a degree of anxiety as well as unnecessary and costly infertility treatment. A second and much lower threshold was calculated in three of the four articles. Ombelet et al. calculated this much lower threshold by using the 10th centile of the fertile population, while Günalp et al. screened the population with the positive predictive value as indicator, and Menkveld et al. assumed a 50% prevalence of subfertility in their study population. The lower threshold ranged from 3 to 5% (Table 18.2). These lower thresholds have a much higher positive predictive value than the higher thresholds, with a negative predictive value not much lower.

We suggest that the lower threshold should be used to identify males with the lowest potential for a pregnancy under in vivo conditions. Values above the lower threshold should be regarded as normal. These findings are in keeping with previous publications by Coetzee et al. (IVF data) and Van Waart et al. (IUI data), which reported a significantly lower chance of successful pregnancy in males with normal morphology below their calculated thresholds.

The higher threshold values for percentage motile sperm as calculated in the four articles (using ROC curve or CART analysis) ranged from 32 to 52%, while the lower threshold values ranged from 20 to 30%. Motility also had a high predictive power, with an AUC of between 59 and 79.1%. Günalp et al. calculated thresholds for progressive motility: a higher threshold of 42%, using the ROC curve, and a lower threshold of 14%, with the positive predictive value as indicator. In this study, progressive motility
proved to be a marginally better predictor of subfertility than sperm morphology, with AUC values of 70.7 and 69.7%, respectively. Montanaro Gauci et al. found percentage motility to be a significant predictor of IUI outcome. The pregnancy rate was almost three times higher in the group with motility > 50% as compared with the group with motility < 50%.

The higher threshold values for sperm concentrations calculated by Oombelet et al. and Günalp et al. and Guzick et al. ranged from 13.5 to 34 x 10^6/ml, while the lower threshold values ranged from 9 to 14.3 x 10^6/ml. An AUC value of between 55.5 and 69.4% served as confirmation of the predictive power of this parameter. Although Menkveld et al. did not calculate a threshold value for sperm concentration (because values of less than 20 x 10^6/ml served as inclusion criteria in their study), they suggested a threshold value of 20 x 10^6/ml to be used with confidence, because it did not influence the results from their fertile population. The clinical value of motility and sperm concentration serves as confirmation of findings reported in numerous other publications.

Although the various parameters had good predictive power, independent of each other, the clinical value of semen analysis was increased when the parameters were used in combination. Oombelet et al. found that differences between the fertile and subfertile populations only became significant when two or all three semen parameters were combined. Bartoo et al. concluded that fertility potential is dependent on a combination of different semen characteristics. Eggert-Kruse et al. found a significant correlation between the three parameters reviewed in their study. Although the different semen parameters demonstrate good individual predictive power, the clinical value of the semen analysis increases when the parameters are used in combination. We therefore suggest that no parameter should be used in isolation when assessing male fertility potential. The lower thresholds as discussed in this chapter have a much higher positive predictive value and a high negative predictive value. Therefore, we suggest that these lower thresholds should be used in identifying the subfertile male.

As suggested by the WHO in 1999, each group should develop their own thresholds, based on the population they are working in. It seems as if the sperm morphology threshold of 0-4% normal forms indicates a higher risk group for subfertility, and fits the IVF and IUI data calculated previously. The four articles discussed above showed the same trends, and can serve as guidelines to distinguish fertile from subfertile males.

As far as concentration and motility are concerned, the thresholds are not clear, but a concentration lower than 10^6/ml and a motility lower than 30% seem to fit the general data. More, preferably multicenter, studies are needed to set definitive thresholds.

SEMEN PROFILE OF THE GENERAL POPULATION: PARTNERS OF WOMEN WITH CHRONIC ANOVULATION

In general, there is quite a poor level of understanding and evidence regarding the semen analysis profile of the general population. Many male populations have been proposed to mirror the general population in terms of semen analysis. Using donors in a semen-donation program for normality is certainly not the best option, since this population is positively biased for fertility. Army recruits are biased by age. Husbands of tubal-factor patients can be biased by a positive history of infection (tubal factor due to pelvic infection) or a good fertility history (women with tubal sterilization). Therefore, we believe that possibly the best reference group for studying the semen profile in a general population includes partners of women who have been diagnosed with chronic anovulation/PCOS (polycystic ovarian syndrome) (maximum of three menstrual periods per year). We would thus like to propose employing the lower thresholds to indicate patients with subfertility, and, by using the cohort of
anovulatory women, we obtain a reflection of the semen profile in a general population.

Two different studies, one retrospective and one prospective, evaluating the semen analysis of partners of women presenting with anovulation were selected.

**Retrospective study of partners of women presenting with chronic anovulation (> 35 days) at Tygerberg Fertility Clinic**

Included in this study were all male partners of patients diagnosed as anovulatory at the Tygerberg Fertility Clinic. Methods used to examine the semen were according to WHO guidelines, and for sperm morphology Tygerberg strict criteria were used. The laboratory personnel initially evaluated all slides, and each slide was then evaluated by one observer (TFK) according to strict criteria. Sixty-two samples were eventually selected and included in the study (Table 18.3).

**Table 18.3 Retrospective study of partners of women presenting with chronic anovulation (> 35 days) at Tygerberg Fertility Clinic (< 10⁹/ml cut-off)**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normozoospermia</strong></td>
<td>29</td>
</tr>
<tr>
<td><strong>Sperm abnormality</strong></td>
<td></td>
</tr>
<tr>
<td>Single-parameter defect</td>
<td></td>
</tr>
<tr>
<td>azoospermia</td>
<td>3</td>
</tr>
<tr>
<td>oligozoospermia (O)</td>
<td>3</td>
</tr>
<tr>
<td>asthenozoospermia (A)</td>
<td>0</td>
</tr>
<tr>
<td>teratozoospermia (T)</td>
<td>16</td>
</tr>
<tr>
<td>polyzoospermia (P)</td>
<td>2</td>
</tr>
<tr>
<td>immunological factor (I)</td>
<td>1</td>
</tr>
<tr>
<td>Double-parameter defect</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>0</td>
</tr>
<tr>
<td>OT</td>
<td>4</td>
</tr>
<tr>
<td>AT</td>
<td>0</td>
</tr>
<tr>
<td>TP</td>
<td>1</td>
</tr>
<tr>
<td>TI</td>
<td>1</td>
</tr>
<tr>
<td>Triple-parameter defect</td>
<td></td>
</tr>
<tr>
<td>OAT</td>
<td>2</td>
</tr>
</tbody>
</table>

Threshold values used: concentration < 10⁹/ml, motility < 30%, morphology < 4% normal forms

**Prospective study of partners of women presenting with PCOS at Tygerberg Fertility Clinic**

Tygerberg Fertility Clinic conducted a study in patients with PCOS. The patients were diagnosed with PCOS according to the recent Rotterdam consensus statement. The aim of this study was to establish factors influencing ovulation induction in this group.

The semen of the partners of all these women was examined. Methods used to examine the semen were according to WHO guidelines, and for sperm morphology Tygerberg strict criteria were used. The laboratory personnel initially evaluated all slides, and all P-pattern morphology slides were re-evaluated by one observer (TFK) (Table 18.4). The thresholds used for subfertility were those suggested by Van der Merwe et al. in their recent review: 0–4% normal forms, < 30% motility, < 10⁹/ml, outlined in the first section of this chapter.

**DISCUSSION**

In the two studies (Table 18.3, retrospective; Table 18.4, prospective) ± 50% of patients had a normal semen analysis. The most common single abnormality was that of teratozoospermia (25.8% retrospective, 27.8% prospective). Azoospermia occurred in 1.4–4.8% of patients, with triple-parameter defects found in only 1.4–3.2% of cases (Tables 18.3 and 18.4).

The thresholds as calculated above were used in a group of anovulatory women. These thresholds reflect the prevalence of male factor infertility in the general population. It is interesting to note that in both the retrospective and prospective studies, the prevalence of teratozoospermia (< 4%
normal morphology) was 25.8–27.8%, making it the most common defect in this group. About 50% of all male patients had normal semen parameters in these two studies using the suggested thresholds as calculated based on the four articles discussed.\textsuperscript{32–35,38}

It is important to note that in PCOS patients the clinician needs to take into consideration that not only anovulation, but also, in up to 50% of these patients, the male factor needs attention, to assist in achieving a successful outcome in these couples. These lower thresholds are not absolute, but provide a continuum guiding the clinician to respond to the semen analysis. The golden rule is to repeat a semen analysis 4 weeks after the first (abnormal) evaluation to ensure that the correct approach will be followed. If the result is again abnormal, a thorough physical examination should be performed and the necessary treatment offered. In the case of PCOS, the female factor (anovulation) should obviously be corrected, starting, as first-line approach, with weight loss in women with a body mass index > 25. Although 50% of these patients had a male factor according to the definition used, it is also important to note that only ±5% of these factors were serious (azoospermia and the triple-parameter defects), with 7–9.7% with a double defect.

To our knowledge, this is the first attempt to use the specific suggested lower thresholds to define prevalence of the subfertile male in the general population by using an anovulatory group of women. These thresholds will guide the clinician towards a more directive management where indicated.

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iii. How to use these thresholds in clinical decision making

The knowledge of threshold values is also of value in clinical IVF and helps in decision making on which patients are in need of the ICSI treatment and who can continue with simpler measures at home. This was discussed in a recent debate as reflected in the 3rd paper in this section (Kruger et al., 2009).
The following article reflects the scientific basis that supports the above argument:

Kruger TF. Should ICSI be done for all IVF patients? MEFSJ 2009;14(2):85-95
DEBATE

Should ICSI be done for all IVF patients?

Complete failure of fertilization occurs in a small but significant number of non-male factor IVF cases. In cases of unexplained infertility, sperm abnormalities can be the cause. There are no reliable and guaranteed tests that can predict, with accuracy, the fertilization rate in vitro, prospectively. In many countries, ICSI is being performed for all IVF cases and there is a significant trend in the United States to do ICSI on cases of unexplained infertility, advanced maternal age, and poor ovarian reserve. In this issue, some of the world’s experts on IVF/ICSI attempt to address this problem and present their point of view. I am sure that the reader will find their comments to be helpful and enlightening.

Suheil J. Muasher, MD, FACOG
Deputy Editor,
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Comment by: E. Hakan Duran, M.D.
Sergio Oehninger* M.D., Ph.D.
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It has been several years since our last opinion paper on application of ICSI for all cases of in vitro conception (1). During the years 1998-2007 the use of ICSI increased from 40% to 63% for all assisted reproduction technology (ART) cycles performed in the USA (2, 3). Considering that the number of total ART cycles surged from 53,154 to 132,745 during that period, the actual application of ICSI increased approximately 4-fold. Figure 1 shows that the use of ICSI at our institution has followed the national trends. Today, there are many fertility centers throughout the world, which abandoned conventional IVF altogether in favor of ICSI on a pragmatic basis.

Since the advent of ICSI (4), there has been general agreement on its use for male factor infertility. In our program, the indications for ICSI in couples with male factor infertility have traditionally been: men presenting with varying degrees of oligo-asthenozoospermia (OAT), alone or in combination, anti-sperm antibodies, and obstructive and non-obstructive azoospermia (where ICSI is combined with testicular sperm extraction) (5). In our opinion, a thorough semen evaluation is mandatory for proper clinical management and directing patients to ICSI. Our program recommends the use of extended semen analysis and sperm functional testing to properly identify cases at risk for poor or failed fertilization (6, 7). Based on initially accumulated experience, the use of ICSI quickly expanded to cases with previous failed fertilization in conventional IVF and to diagnosed cases of unexplained infertility. The aims of these broadened indications were to avoid the undesired outcome of complete failure of fertilization, and to enhance the number of available embryos to optimize the total reproductive potential of a given IVF cycle.

A thorough search of the current medical literature reveals 12 randomized controlled trials with a reasonable sample size that addressed the question of whether ICSI should be the treatment of choice for all cases on in vitro conception. When analyzed in detail, some of these reports did not seem to have appropriate randomization methodology flawing the study design. Aboulghar et al. studied 116 couples with tubal factor infertility that underwent ART by either IVF or ICSI (8).
Similarly, Bukulmez et al. included 76 couples with tubal factor only (9). With these two similar studies combined (n=192), fertilization rate per oocyte was significantly greater for IVF (64.8% vs. 53.5% for ICSI). No differences were detected between these two groups for clinical pregnancy, miscarriage or live birth rates.

Ruiz et al. reported 70 couples with unexplained infertility and mild endometriosis undergoing ICSI vs. IVF for the sibling oocytes according to the order of retrieval (10). Despite the lack of appropriate randomization technique, this study failed to show any differences between the two groups for both fertilization rate and embryo quality. The authors concluded that although ICSI might be preferred for some oocytes to avoid fertilization failure, it was not superior to IVF as an insemination technique. Poehl et al. reported 91 cases with tubal or cervical factor infertility randomized in ICSI and IVF groups, although the randomization technique was not clearly stated (11). The baseline characteristics of the two groups were similar and the main outcome measures, i.e., fertilization, ongoing pregnancy and implantation rates, and embryo scores were not different.

Among the randomized controlled trials with appropriate randomization technique, Bhattacharya et al. had the largest series from a multicenter study (12) by randomizing 415
couples with non-male factor infertility to either IVF or ICSI. The authors found no significant difference between the two study groups on implantation or pregnancy outcomes; laboratory time was significantly longer for ICSI than IVF. Fishel et al. randomized 221 couples to either ICSI vs. IVF or ICSI vs. high insemination concentration IVF groups depending on the history of previous IVF performance and presence of male factor infertility (13). They reported overall higher fertilization rate for ICSI groups but similar embryo quality. However, there was no pregnancy outcome measure addressed by this study. Staessen et al. evaluated 56 couples with tubal factor infertility, randomizing sibling oocytes to ICSI vs. IVF (14). They found no difference in fertilization rate, implantation and clinical pregnancy rates. More patients had fertilization failure in IVF group but the numbers were too few to compare statistically. Westerlaken et al. randomized sibling oocytes of 38 patients with low fertilization (<25%) or total fertilization failure during a prior IVF cycle to ICSI and IVF/ICSI (15). Not only the fertilization rate was significantly lower in the IVF group, but also there were recurrent fertilization failures. No difference in clinical and ongoing pregnancy as well as implantation rates was detected; however, the numbers were quite low to provide adequate power. Another study by the same group randomized sibling oocytes from 106 couples with borderline semen characteristics (16). There was no significant difference in clinical and ongoing pregnancy rates between the IVF and ICSI groups, although numbers were more favorable for ICSI but power seemed inadequate. Foong et al. randomized 60 couples with unexplained infertility to IVF vs. ICSI groups (17). They did not find any significant difference in any of their outcome measures. Finally, Hang et al. randomized 1089 sibling oocytes obtained from 60 patients with polycystic ovarian syndrome (PCOS) to IVF vs. ICSI (18). Nine patients from the IVF group had fertilization failure, in addition to the significantly lower fertilization rate in this group (44.8% vs. 72.0% in the ICSI group). Embryo quality on the other hand was comparable between the study groups.

The pragmatic approach to perform ICSI for all ART cycles is therefore not supported by evidence which demonstrates that better clinical outcomes are obtained with ICSI in the non-male factor population. In an attempt to avoid unexpected fertilization failure or low fertilization rate in a couple with unexplained infertility (here maybe an “occult” male factor is present), or in cases where the number and/or quality of oocytes are poor secondary to a decreased ovarian reserve, it is tempting to opt for ICSI, but the evidence to support its use is lacking. Similarly, in light of the reports (18-21) indicating a reduced fertilization rate in patients with PCOS, routine application of ICSI may have practical benefits in this group of patients. Whether to prefer short-term practical benefits to evidence based decisions is at the discretion of the fertility center managing the patient at the moment.

This very issue is quite important when the long term effects and risks of ART are considered. A recent report analyzed the data from the US National Birth Defects Prevention Study and concluded some birth defects to occur more often among infants conceived with ART (22). These included septal heart defects, cleft lip / palate, esophageal and anorectal atresia. This particular study did not analyze ICSI and IVF subgroups separately, but this comparison has been done by a number of studies previously. A meta-analysis combining such studies identified a non-significant risk ratio of 1.12 (95% confidence intervals [CI]: 0.97-1.28) for major birth defects from four peer-reviewed publications (23). However, when three more sources of large non-peer-reviewed reports (the Australian-New Zealand, British and French databases) were included, the recalculated and significant risk ratio was found to be 1.20 (95% CI: 1.09-1.31). Among all, the increase in major birth defects was more notable from the British data and there was significant heterogeneity in risk ratios between the studies. It is important to note that the indications for ICSI were not stated.

It is well established that there is increased incidence of Y chromosome micro-deletions in cases with severe OAT. Similarly, males with congenital bilateral agenesis of vas deferens should be screened for cystic fibrosis. Both of these conditions may be inherited to the offspring by ICSI. The incidences of both de novo structural chromosomal abnormalities and sex chromosomal
abnormalities have been reported to be increased in ICSI offspring, particularly in cases of severe male infertility (24). Similarly, cases with the imprinting disorders Angelman and Beckwith-Wiedemann syndromes, have been associated with ICSI (25,26). Certain childhood cancers have been known to be associated with these disorders. The potential impact of sperm DNA fragmentation and particularly, of DNA damage in morphologically normal sperm found in men with teratozoospermia, needs to be further addressed (27,28). More studies are required to understand the pathogenesis of these disorders and to find out if any precautionary step can be taken to prevent them. Few studies have addressed the developmental outcome of ICSI babies in comparison to IVF babies. There was no difference in developmental outcome, measured by Bayley Scale at the age of 24-28 months between ICSI and IVF children (29).

In conclusion, there is an increasing application of ICSI in the clinical setting in spite of known (male factor population) and potential (non-male factor population) risks. The spectrum of ICSI indications has broadened in parallel to its application. As always, the physician must weigh the benefits versus the risk of using assisted fertilization techniques on an individual basis. Further data are needed to assess the long-term physical and psychological development of ICSI children, when used for male infertility and other indications. ICSI has become the dominant fertilization technique in the ART lab, but the above-mentioned findings highlight the need for continuous monitoring of ART results.

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Comment by: TF Kruger, M.D.
Tygerberg, South Africa

In 1992 intracytoplasmic sperm injection (ICSI) was reported as a treatment method for male factor infertility (1).

To date the use of this method for severe male factor infertility is widely supported (2). As with any new treatment modality there is always the pendulum effect regarding its real place in the assisted reproduction arena. There are authors suggesting ICSI to be used for all cases and indications of assisted reproduction even when clinical trials indicate that ICSI is no more effective than in vitro fertilization (IVF) (3).

In our unit we use the following criteria for male subfertility. A semen concentration/ml of less than 10 million. Percent motility < 30%, forward progression < 2 with normal sperm morphology less than 4% (4). When using these thresholds, we observed that 56.9% of patients attending our clinic, are normozoospermic. The rest with present with male subfertility of which 11.2% - 17.8% will be severe male factor infertility (double defects / triple defects and/or azaospermia) (5).

Severe male factor infertility

Indications for ICSI are ejaculated sperm (oligozoospermia, asthenozoospermia, teratozoospermia [<4% normal], Antisperm antibodies [MAR >60%], frozen sperm from cancer patients, ejaculatory disorders, eg retrograde ejaculation), azaospermia (absence of the vas deference, failed vaso-vasostomy, obstruction of both ejaculatory ducts) and testicular spermatozoa (all indications for failed epididymal spermatozoa, azaospermia caused by testicular failure [maturation arrest, germ cell aplasia], necrozoospermia) (2).

ICSI is the first choice treatment for severe male factor infertility to achieve pregnancy rates similar to IVF rates for non-male factor infertility (6).

Although no randomized controlled trials exist, retrospective analysis indicated that live birth rates using ICSI for severe male factor infertility are far better than those for conventional IVF (when used
as treatment for severe male factor infertility). IUI and subzonal sperm injection for the same indication (7).

Non-male factor infertility

In a prospective randomized multicentre trial, IVF was compared with ICSI, 219 cycles in the IVF group and 206 in the ICSI group (3). Their results showed that the use of ICSI in couples suitable for IVF treatment does not offer any clinical benefits. They found a significant difference in implantation rates in favor of IVF, but the pregnancy rates did not differ significantly. They also reported that the proportion of multiple pregnancies were similar.

In a small (60 patients) prospective randomized trial of conventional in vitro fertilization (IVF) vs ICSI in unexplained infertility, Foong et al (8) reported no differences in clinical outcomes associated with IVF vs ICSI in a treatment of unexplained infertility.

In a recent Cochrane review (9), it was concluded that in the single identified study (3), no difference in pregnancy rates were reported (OR 1.4, 95% CI, 0.95-2.2).

One could thus argue in favor of IVF in the light of simplicity and time involved in the laboratory as well as more and longer term data available on children born from the IVF technique.

Male subfertility (mild male factor)

Tournaye et al reported on randomized controlled trials dealing with “borderline semen parameters” (10). In these studies (n=9), more oocytes were fertilized after ICSI (1264/2015 - 62.7%) than after conventional IVF (549/1688 - 32.5%). The RR in all randomized controlled trials were all in favour of ICSI. Complete fertilization failure occurred in 37.4% of the IVF cycles vs 2.5% of ICSI cycles. However, Tournaye et al advocated the use of high insemination concentration (HIC) to counteract fertilization failure and in a prospective randomized trial, they showed that there was no significant difference in fertilization between ICSI and HIC IVF (10). This concept must be taken into consideration in handling patients with borderline semen parameters but more randomized controlled trials are necessary to test this concept.

I am in agreement with Tournaye stating that each IVF lab should try to improve their IVF procedure to the greatest extent and optimize the insemination procedures so as to cope with the specific demands of the couples involved (10). Corrective measures to promote IVF may have an important role to play, even in the era of ICSI (10). More randomized controlled trials comparing ICSI with optimized IVF procedures are certainly needed. In the meantime, at least a split IVF/ICSI approach, may be chosen as a first choice in couples with moderate male subfertility (10).

Fertilization failure

Total fertilization failure occur in 1-2% of all IVF cycles (11). According to the Capri Workshop Group, no recent randomized studies considered ICSI for secondary prevention of failed fertilization in couples with a history of failed or poor fertilization (2). It is however, common practice that most units will offer ICSI as the preferred method in such cases. It was suggested by the Capri Group that a trial should be done with patients as the unit of randomization and live birth as the outcome (2).

To summarize it is my opinion based on the evidence available, that IVF is the treatment method of choice in cases with non-male factor infertility. On the other hand, ICSI is the preferred treatment modality in cases with severe male factor infertility and fertilization failure. In patients with male subfertility, HIC can be considered with at least a split IVF/ICSI approach.

REFERENCES


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The injection of a single spermatozoon into the cytoplasm of a mature oocyte, ICSI (intracytoplasmic sperm injection), has been one of the remarkable progress in ART to treat male factor infertility (1). Interestingly, in the earlier days the indication to perform ICSI was purely for severe male factor infertility, i.e. cases with various degrees of spermatogenic disorders which had either no chance or an extremely low probability of fertilization with conventional in vitro insemination. Today the range of indications have expanded to include unexplained fertilization failure after conventional IVF (2,3), ejaculatory dysfunction, immunological infertility, endometriosis (4) and poor ovarian response (3). Another indication for ICSI is for cases utilizing thawed or re-warmed oocyte after cryopreservation (5). In these instances, oocytes that survive the freezing/thawing or the re-warming process require insemination by ICSI due to the thickening and hardening of the zona pellucida (6) and in fact, the application of ICSI has consistently led to an increase in the fertilization rate of thawed oocytes (7).

In 1997, five years after the initial successful report of a pregnancy by ICSI (1), the number of ICSI cycles comprised 35% of the ART procedures in USA. In the year 2000 the number of ICSI cycles increased to 47% of the ART procedures, and in the recent 2005 CDC report, the number of ICSI cycles have reached 60% of the ART procedures performed in USA. By comparing the 1997 and the 2005 statistics, ICSI utilization has almost doubled and represents now about two third of the insemination methods used during IVF. However, this progressive increase in the utilization of ICSI has not been dictated by a parallel increase in the number of male infertility cases and more importantly, has not been associated with an overall increase in live birth rates.

Several authors have been suggesting that ICSI should be offered to all patients needing IVF because of the significantly higher fertilization rate (3). However, the lack of natural selection of sperm and the circumvention of most of the known fertilization stages have led to concerns regarding perinatal outcome; therefore, the issue of whether to use ICSI for all in vitro insemination needs to be critically reviewed and discussed.

What are the rationale and justifications to apply ICSI for each IVF case? The justifications for utilizing ICSI should be: a) higher fertilization rate; b) prevention of fertilization failure and c) higher live birth rate for each of the conditions where this technique is preferred to the conventional IVF insemination. In this paper we will therefore
examine the following outcome points after utilization of ICSI: a) fertilization rate; b) embryo quality and rate of blastocyst development; c) safety: perinatal risks and child development; d) congenital malformations and epigenetic (imprinting) disorders.

**Fertilization rate**

The first question to address is whether the fertilization rate with ICSI is increased when compared to conventional IVF for non-male factor cases. The use of fertilization rate instead of clinical pregnancy rate as an outcome has drawbacks since it is an interim outcome measure in an IVF program, which may have little effect on the final outcome of a fresh cycle or that of a subsequent frozen embryo transfer. Several reports have been published in which ICSI and conventional IVF were compared when sibling oocytes were split between the two insemination methodologies (8,9,10).

Aboulghar et al. (10) made the comparison between ICSI and IVF within three groups of patients who were undergoing their first IVF treatment cycle. In the group with tubal factor infertility and normal semen parameters they observed a significantly higher fertilization rate per retrieved oocyte with IVF, but no significant difference in pregnancy rate between ICSI and IVF. In the group of patients with unexplained infertility and normal semen parameters there was no significant difference in fertilization rate, whereas in the group of patients with borderline semen parameters there was a significantly higher fertilization rate with ICSI (59%) than IVF (27%). Other studies have also reported a lack of significant difference in fertilization rates of ICSI versus IVF in patients with non male factor infertility (61% versus 67 %) (11) and unexplained infertility (60% vs. 54%) (12).

The data from two systematic reviews also revealed that in couples with normal semen parameters there is insufficient evidence to claim a difference in fertilization rates per retrieved oocyte or pregnancy rates, between ICSI and conventional IVF (13). Again, only couples with borderline semen parameters had higher fertilization with ICSI than IVF, a result confirmed by a subsequent meta-analysis. However, it was also suggested that similar fertilization rates could be achieved with a modification of the conventional IVF process, for example by using high sperm insemination concentration (HIC) IVF (14).

According to these reviews, ICSI should be the insemination of choice for severe male factor infertility and can be justified for cases with previous unexplained fertilization failure after conventional IVF.

**Embryo quality and blastocyst formation**

The second question to address is whether the embryo quality is superior if oocytes are inseminated by ICSI other than conventional IVF and whether the rate of blastocyst development after ICSI (a marker of embryo quality) is higher.

It is well known that embryo quality is an important factor affecting pregnancy potential. Implantation rates for ICSI-derived embryos continue to be lower than embryos derived from conventional insemination suggesting a diminished implantation potential of ICSI-derived embryos (15, 16). These negative influences on lower implantation rates have been attributed to the poor quality of injected spermatozoa.

It is known that spermatozoa requiring ICSI (either due to low production or low motility or low normal forms or a combination thereof) have higher levels of aneuploidy, DNA damage, DNA breaks and other subcellular defects which may impair embryo development (17, 18). Martin et al., (19) studied men with severe oligozoospermia (<106 sperm/ml), men with moderate (1-9 x 10^6 sperm/ml) or mild (10-19 x 10^6 sperm/ml) oligozoospermia. The FISH analysis using DNA probes specific for chromosomes 13, 21, X, and Y detected a significant inverse correlation between the frequency of sperm chromosome abnormalities and the sperm concentration for XY, XX, and YY disomy and diplody. These results demonstrate that men with severe oligozoospermia participating in reproductive treatments have an elevated risk for chromosome abnormalities in their sperm, particularly sex chromosome abnormalities.

In addition male factor infertility has been
shown to significantly reduce blastocyst production; it is not clear whether this is caused by the paternal genome or the ICSI technique itself (20-23). Although, production of blastocysts appears to be negatively affected by paternal or ICSI contributions, most studies demonstrated equivalent pregnancy and implantation rates between the blastocysts derived from ICSI and from conventional insemination (21).

One randomized controlled study which compared ICSI with IVF in non male factor cases concluded that implantation and pregnancy rates were not different (10).

On the other hand, in a recent study using oocytes from 35 couples with non male factor infertility, Khamisi and colleagues demonstrated increased formation of good quality embryos per retrieved oocyte after ICSI than after conventional IVF (64% and 47%, respectively) (24). However, there was no significant difference in the formation of good quality embryos per fertilized oocyte.

These results indicate that ICSI does not improve or diminish the formation of good embryos. Ultimately, embryo quality is likely depending on intrinsic factors of both gametes rather than on the fertilization method per se (25).

Safety: Perinatal Risks and Child Development

Unlike most therapeutic procedures used in medicine, assisted reproductive techniques in general and ICSI in particular, never underwent rigorous safety testing before their clinical use. In recent years, a large body of evidence has been accumulating demonstrating that many forms of male infertility have a genetic cause (26, 27). Researchers believe that perhaps 75% or more cases of all infertility have a contributing genetic basis, however the ability to diagnose these defects remains limited (28) Put simply, large numbers of couples undergo fertility treatments without a complete understanding of the basis of their infertility or the potential long term risks for their offspring. As a consequence, many infertile men requiring ICSI because of severe oligo-ozoospermia are at high risk of producing sperm with chromosomal aneuploidy or structural aberrations. In addition, men with normal semen parameters by conventional means may have high levels of chromosomal aberrations, apoptosis, and fragmented sperm DNA which can go unrecognized until poor embryo development during ART is encountered.

ICSI outcome should be assessed also in terms of safety by examining the perinatal risks and long term physical and development issues of ICSI-children. It is well known that pregnancies resulting from the use of assisted reproduction techniques are associated with significantly increased risks to mother and offspring due to multiple gestation, preterm delivery and congenital abnormalities in the offspring. A small increase in late preterm birth and low birth weight has been confirmed in most studies of outcome of pregnancies conceived via IVF with or without ICSI. However ICSI pregnancies appear to be associated with an increased the risk of monochorionic placentation, particularly when combined with day 5 embryo transfer (29).

There is little information about the long term outcome of children born after ICSI. In a systematic review from Cochrane database (30) children conceived via ICSI have been followed up to age 8 years, and investigated in terms of pubertal staging, neurological development and rates of surgery/hospitalization. Pubertal staging, neurological examination and rates of surgery/hospitalization was found to be similar for ICSI children and those conceived spontaneously. However the mechanical nature of the fertilization, that is, bypass of the zona pellucida and oolemma membrane, coupled with the indications to its use, that is, mostly for cases where the reasons of poor spermatogenesis are unknown, have and continue to raise concerns about the possible long term health consequences on the offspring (31; Sutcliffe A, personal communication).

Congenital Malformations and Epigenetic (imprinting) Disorders

A higher rate of inherited chromosomal anomalies has been reported in ICSI children, mainly due to potential structural chromosomal anomalies, as well as higher rate of de novo chromosomal anomalies related to paternal sperm characteristics (32). Malformation rates were
comparable between ICSI and IVF in most studies, but were two-fold higher compared with the general population (32, 33, 34).

A recent study evaluated both obstetric outcomes (344 pregnancies generated from ART compared with 344 spontaneous pregnancies) and rates of congenital anomalies in children (n=432) born after in vitro maturation of oocytes (n=55), or IVF (n=217) or ICSI (n=160), compared with children (n=360) born after spontaneous conception. In conclusion all ART pregnancies are associated with an increased risk of congenital abnormality (35). Reports of imprinting-related diseases such as Angelman and Beckwith-Wiedemann syndromes in offspring conceived via IVF as well as ICSI suggest a possible risk of in vitro culture procedures and requires further investigation (36).

CONCLUSION

Using the currently available data, ICSI performed with normal sperm parameters still cannot overcome fertilization failures due to oocyte abnormalities and in all maternal age groups, the rate of live birth per egg retrieval appears to be slightly higher for conventional IVF. There is no clinical evidence to prefer ICSI versus conventional IVF in patients with non-male factor infertility.

Furthermore, since ICSI is applicable only to mature metaphase II oocytes, properly identified as such after being stripped of their cumulus cells, the widespread use of ICSI may ultimately reduce the number of usable oocytes because the latter once denuded and found to be still immature, rarely progress to metaphase II. If these oocytes would be used for conventional insemination (thus not subjected to hyaluronidase to remove their cumulus cells) they could achieve maturity and fertilization.

Finally, ICSI bypasses the known biological mechanisms of sperm selection operating at the level of the zona pellucida. Even with the latest introduction of devices to increase the possibility of “choosing” the best spermatozoa for ICSI, with the technique of sperm selection via binding to hyaluronic acid (37) or with the intracytoplasmic morphologically selected sperm injection (IMSI) method (38), the “human factor” in making the final decision on which sperm to select, is still relevant.

In conclusion conventional IVF should be the option of choice for every couple requiring ART treatments to avoid the disadvantages of ICSI. A more acceptable strategy might be to perform a diagnostic comparison between conventional IVF and ICSI in all first treatment cycle of couples diagnosed with unexplained infertility. This would be beneficial in preventing total fertilization failure and in providing a clear answer to the couple of whether ICSI is a necessary tool to achieve fertilization.

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Conclusion:

In chapter 2 we have suggested new semen threshold values for sub-fertile and infertile men, based on the international literature available. It was established that sperm morphology with poor prognosis (<5% normal forms), not only apply for IVF and IUI pregnancy outcome, but also applies to the *in vivo* situation. The WHO accepted the suggestions of semen threshold values, based on the research evidence available and recommended the Tygerberg Strict Criteria for sperm morphology evaluation as the international standard to be used worldwide. The Tygerberg team not only showed how the threshold values can be applied to perform better research in endometriosis patients and in anovulatory women but can also be used to make sound clinical decisions.
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Chapter 3
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SUMMARY

❖ Chapter 3

In the light of the findings discussed in Chapters 1 and 2, an interest developed to understand the pathophysiological mechanisms involved in the low fertilization rates in men with severe teratozoospermia (P-pattern sperm morphology). For that reason, a study was undertaken to correlate the different morphological patterns with the hamster penetration assay (SPA test) (Kruger et al., 1988a). A good correlation was observed between poor penetration of the hamster oocytes (Kruger et al., 1988a) as well as low binding in the hemi-zona assay (HZA) (Franken et al., 1990) and P-pattern sperm morphology. It was also reported that poor morphology correlates with abnormal calcium influx (Oehninger et al., 1994) and abnormal acrosome reaction (Bastiaan et al., 2003). The hemi-zona assay (HZA) was found to be a good predictor of fertilization in vitro (Oehninger et al., 2000). The enzyme acrosin was also studied in 1988 but it could not be shown that a correlation exist between sperm morphology and the enzyme acrosin (Kruger et al., 1988b) although other researchers did find a correlation (Menkveld et al., 1995).

The hemi-zona test (HZA) became an important clinical tool and functional assay. An example of laboratory research with clinical application is the article about preservation of oocytes to assist laboratories in using this test on a day to day basis as fresh oocytes were not always readily available (Kruger et al., 1991). This article assisted the laboratories interested in the HZA, to be able to provide the test as a routine test to the clinician or scientist.
Extended semen analysis/ sperm functional assays

a. To explore the value of certain sperm functional assays and it’s correlation with sperm morphology.

   i. The extended semen analysis

   It was suggested that the extended semen analysis should include the following:

   - Measurement of reactive oxygen species and enzyme activities e.g. creatine phosphokinase.
   - Bioassays of gamete interaction [(Sperm penetration assay (SPA) test and the Hemi-
     zona assay test (HZA)] and induced acrosome reaction (AR) scoring.
   - Computer aided sperm motion analysis (CASA).

   ii. The sperm penetration assay (SPA test)

   In the light of the findings discussed in Chapters 1 and 2, an interest developed to understand the pathophysiological mechanisms involved in the low fertilization rates in men with severe teratozoospermia (P-pattern sperm morphology). For that reason a study was undertaken to correlate the different morphological patterns with the hamster penetration assay (SPA test). A good correlation was observed between poor penetration of the hamster oocytes and P-pattern sperm morphology (Kruger et al., 1988a). It was also reported that poor morphology also correlates with abnormal calcium influx (Oehninger et al., 1994) and abnormal acrosome reaction (Bastiaan et al., 2003).
The following article reflects the scientific basis that supports the above argument:

Abnormal sperm morphology and other semen parameters related to the outcome of the hamster oocyte human sperm penetration assay

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Summary
A new method for evaluation of sperm morphology using strict criteria is currently used in the andrology laboratory at the Eastern Virginia Medical School. A prospective study was designed to evaluate the following semen parameters in samples of all patients over a set period of time: sperm concentration and motility, and normal sperm morphology. These factors were correlated with results of the hamster zona-free oocyte/human sperm penetration assay (SPA). One hundred patients with a sperm concentration ranging from 2 to 219 × 10⁶/ml, a motile sperm fraction ranging from 6.9 to 87%, and normal sperm morphology ranging from 1 to 39%, were evaluated. The statistical analysis system general linear model was used to judge the influence of the different variables. There was a statistically significant relationship between the per cent of sperm with normal morphology and penetration rate in the SPA (P = 0.001). Outcome of the SPA was also correlated with in-vitro fertilization, retrospectively, in 84 patients. Thirty-eight patients had an SPA < 10%, with no fertilization in vitro in 13 patients (33.3%) and fertilization in 25 (66.7%). Forty-five had an SPA > 10% with fertilization in 37 (82.2%) and no fertilization in eight (17.8%) patients.

Keywords: sperm morphology, in-vitro fertilization, hamster oocyte, sperm penetration assay.

Introduction
The ability of traditional semen parameters to predict an individual patient’s sperm fertilizing ability has been evaluated by several different workers (e.g. Van Zyl et al., 1976). With the advent of human in-vitro fertilization (IVF) and the hamster zona-free oocyte/human sperm penetration assay (SPA), a better model emerged with which to determine the factors that control fertilization and to what extent each one contributes to the process. In two human IVF programmes a good

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correlation was found between normal sperm morphology, and both the fertilization rate of preovulatory oocytes and the cleavage rates of embryos (Kruger et al., 1986, 1987a). In both of these studies sperm morphology was evaluated using criteria described already (Kruger et al., 1986) and a clear threshold was noticed, namely <14% of sperm with normal morphology. A significantly lower fertilization rate was observed in the group with <14% normal forms (37% fertilization rate per oocyte) when compared with the group with >14% normal forms (fertilization rate of 88%) (Kruger et al., 1986). Rogers et al. (1983) also observed a correlation between sperm morphology and fertilizing ability in the hamster, but others have reported different results (Aitken et al., 1982).

A prospective study was designed to evaluate normal sperm morphology using the strict criteria published previously (Kruger et al., 1986, 1987a) as well as sperm concentration and motility, and to correlate these data with the SPA results. The latter were also correlated retrospectively with the fertilization rate per patient in the IVF programme over a 3-year period.

Patients and methods
One hundred patients referred to the andrology laboratory over a 5-month period (May–September 1986) for SPA were included in this study. A basic semen analysis was performed after liquefaction by computer analysis (Cellsoft Semen Analysis System, Labsoft Division of Cryo Resources Ltd, New York, U.S.A.). Sperm concentration and motility were evaluated using computer analysis. For investigation of sperm morphology, slides were prepared from each patient’s semen sample on the day of the SPA, after liquefaction, and stained by the Diff Quik technique (Dade Diagnostics, Miami, FL, U.S.A.) (Kruger et al., 1987b). Special care was taken to clean the slides thoroughly with 70% ethanol before use, and no more than 5 μl of semen were used, to make the smears as thin as possible. Morphology was evaluated and recorded on the same day, as outlined in detail by Kruger et al. (1986, 1987b). Spermatozoa were considered as normal only when the head had a smooth oval configuration with a well-defined acrosome involving 40–70% of the sperm head, as well as absence of defects in the neck, midpiece and tail. Borderline forms were counted as abnormal. At least 200 sperm were examined per sample.

In our experience, patients with sperm motility >30% perform well in IVF, and for that reason a threshold of 30% was used in this study (Kruger et al., 1987a). Patients were divided into four groups based on sperm concentration and motility; each group was subdivided into two subgroups: abnormal (a) and normal (b) morphology.

**Group 1**
(a) Sperm concentration of >20 × 10⁶/ml, motility >30%, with <14% normal morphology (pure teratozoospermia). (b) As for 1a, but with >14% normal morphology (normal sperm).

**Group 2**
(a) Sperm concentration of >20 × 10⁶/ml, motility <30%, with <14% normal morphology (asthenoteratozoospermia). (b) As for 2a, but with >14% normal morphology (pure asthenozoospermia).
Semen parameters and SPA

Group 3
(a) Sperm concentration of $<20 \times 10^6$/ml, motility $>30\%$, with $<14\%$ normal morphology (oligoaeratozoospermia). (b) As for 3a but with $>14\%$ normal morphology (pure oligozoospermia).

Group 4
Sperm concentration of $<20 \times 10^6$/ml, motility $<30\%$, with $<14\%$ normal morphology (oligoasthenoteratozoospermia).

The SPA was performed according to a protocol outlined in a previous publication (Swanson et al., 1984) with a few minor changes. A short incubation of 6 h was used, and the test was interpreted using phase microscopy without staining. A penetration rate of 0–10\% was considered as poor, >10\% as normal. In all experiments the normal donor used as the control had to penetrate 20\% or more of the hamster eggs to validate the test.

The relationship between semen parameters and the egg-penetration rate was examined using multiple regression analysis and the SAS general linear model procedure. Multiple regression analysis examines the contribution of the independent variables collectively and individually to the variation of the dependent variable penetration. The Chi-square test was used to evaluate the specific role of morphology in the three different groups. Group 4 was not evaluated because all patients were in the same subgroup.

A second objective of the study was to correlate SPA outcome with the fertilization rate per patient in the Norfolk IVF programme. Patients were evaluated retrospectively, from 1983 to 1986, series 2–24. Sperm penetration assay results were divided into penetration $<10\%$ and $>10\%$. Only patients with a donor penetration $>20\%$ were accepted into the study, and all cases in the IVF programme had to have mature oocytes with extrusion of the polar body.

Results

Based on the results of sperm concentration and motility in the analysis, patients were divided into four groups and then subdivided according to sperm morphology.

Group 1a (pure teratozoospermia)
The mean $\pm$ SD concentration of sperm was $67 \pm 38 \times 10^6$/ml, motility was $46 \pm 12.7\%$ and the % of sperm with normal morphology was $7 \pm 4\%$. In this group with poor sperm morphology an extremely high proportion of the SPA results were in the abnormal range. Of the 41 patients in this group, 35 (85.4\%) had an SPA value $<10\%$ and six out of 41 (14.6\%) had penetration rates $>10\%$ (Table 1).

Group 1b (normozoospermia)
The mean sperm concentration was $124 \pm 75.2 \times 10^6$/ml in this group, motility was $64 \pm 13.8\%$, and the % of sperm with normal morphology was $22.5 \pm 6.7\%$. Of the 29 patients in this group, 25 (86.2\%) penetrated $>10\%$ eggs, while four (13.8\%) penetrated $<10\%$ (Table 1). There was a highly significant difference between groups 1a and 1b, $P < 0.0001$ for sperm penetration when sperm motility was $>30\%$ and the concentration $>20 \times 10^6$/ml (Table 1).
Table 1. Sperm morphology as a predictor of the sperm penetration assay (SPA)

<table>
<thead>
<tr>
<th>SPA &lt;10%</th>
<th>Group 1a (n=41)</th>
<th>Group 1b (n=29)</th>
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<tbody>
<tr>
<td>Normal forms &lt;14% (Teratozoospermia)</td>
<td>35 (85.4%)</td>
<td>4 (13.8%)*</td>
</tr>
<tr>
<td>SPA &gt;10%</td>
<td>6 (14.6%)</td>
<td>25 (86.2%)*</td>
</tr>
</tbody>
</table>

* *P < 0.0001, in comparison with group 1a.*

**Group 2a (asthenoteratozoospermia)**
The mean sperm concentration was $62 \pm 55 \times 10^6$ ml, motility was $18.7 \pm 4.8\%$, and the % of sperm with normal morphology was $3.4 \pm 4.8\%$. Of the 15 patients in this group, 13 (86.6%) penetrated the hamster oocytes at a level $<10\%$ while two patients (13.4%) penetrated $>10\%$.

**Group 2b (pure asthenozoospermia)**
The mean sperm concentration was $64 \pm 35.7 \times 10^6$ ml, motility was $19 \pm 9.7\%$, and the % of sperm with normal morphology was $18 \pm 1.5\%$. Of the five patients in this group, three had an SPA value $<10\%$ while two patients penetrated $>10\%$. In this category there was no significant difference between groups a and b. The small numbers in this group do not allow final conclusions.

**Group 3a (oligoteratozoospermia)**
The mean sperm concentration was $8 \pm 4.6 \times 10^6$ ml, motility was $37 \pm 9.4\%$, and the % of sperm with normal morphology was $6 \pm 3.6\%$. Of the six patients in this group all had an SPA value $<10\%$.

**Group 3b (pure oligozoospermia)**
The mean sperm concentration was $4 \pm 2 \times 10^6$ ml, motility was $42 \pm 8\%$, and the % of sperm with normal morphology was $24.5 \pm 3.1\%$. Of the two patients in this group, both penetrated hamster oocytes at a level $<10\%$. In this category there was no significant difference between groups a and b.

**Group 4 (oligoasthenoteratozoospermia)**
The mean sperm concentration was $11.4 \pm 3.1 \times 10^6$ ml, motility was $19 \pm 5\%$, and the % of sperm with normal morphology was $9.5 \pm 2.2\%$. Of the two patients in the group, both penetrated hamster oocytes at a level $<10\%$.

With multiple regression analysis, a significant relation ($P = 0.001$) was found between the % of sperm with normal morphology and SPA rate.

Four hundred SPA tests were performed over a 3-year period and, of these, 84 patients underwent both SPA and IVF. In the group with an SPA rate of $<10\%$ (39 patients), 13 (33.3%) did not fertilize in vitro, while 26 (66.7%) fertilized at least
Table 2. Correlation between results from the sperm penetration assay (SPA) and in-vitro fertilization (n=84)

<table>
<thead>
<tr>
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<th>No fertilization</th>
<th>Fertilization</th>
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<tr>
<td>SPA &lt;10%</td>
<td>13/39 (33.3%)</td>
<td>26/39 (66.7%)</td>
</tr>
<tr>
<td>SPA &gt;10%</td>
<td>8/45 (17.8%)</td>
<td>37/45 (82.2%)</td>
</tr>
</tbody>
</table>

one oocyte. In the group with an SPA rate of >10% (45 patients), eight (17.8%) did not fertilize, and 37 (82.2%) did fertilize in vitro (Table 2). In the group with an SPA rate of <10%, 14 patients had a value of 0%. Of these patients 5/14 (35.7%) did not fertilize in vitro and 9/14 (64.3%) fertilized at least one oocyte.

Discussion
A significant positive relationship was observed between the percentage of sperm with normal morphology and hamster oocyte penetration when the SAS general linear model was used to evaluate all of the data (P = 0.001). No correlation could be found between the other semen parameters, namely sperm concentration, motility, and the SPA rate. Rogers et al. (1983) pointed out that there is a correlation between sperm morphology and the SPA rate. Sperm morphology is an important semen parameter often overlooked in patient assessment because its evaluation is difficult and requires experience and dedication. If the technician adheres to the strict criteria that we have outlined (Kruger et al., 1986), the results are reproducible between technicians (Kruger et al., 1987b) but valuable information can be obtained, especially in patients with a sperm concentration >20 x 10⁶/ml and motility >30%.

With all parameters in the normal range, 86.2% of patients (group 1b) penetrated >10% of hamster oocytes. Where normal sperm morphology was <14% (pure teratozoospermia) with a concentration of >20 x 10⁶/ml and motility >30%, 35 out of 41 patients (85.4%) did not penetrate at the >10% level (Table 1).

Our results are in agreement with those of Rogers et al. (1983) that sperm morphology can help to predict what will happen, not only in the SPA but also in human IVF, especially in patients with normal sperm concentration and motility (Kruger et al., 1986, 1987a). The threshold of 14% normal forms has proved to be of value in the human (Kruger et al., 1986) as seems also to be the case in the hamster, especially in patients with all semen parameters normal, including a normal morphology > 14% and also in those <14%.

A total of 66.7% of patients with an SPA of <10% fertilized eggs in-vitro while no fertilization occurred in 33.3%. In the group with an SPA rate of >10%, 82.2% fertilized eggs in vitro. These data suggest that there is a threshold of 10%, with a much better chance of successful IVF if the SPA rate is >10%. In the group with SPA <10%, 14 had 0% SPA. In spite of this, 64.8% of these patients did fertilize eggs in vitro while 35.7% had no fertilization, indicating that in this study an SPA rate of 0% does not necessarily indicate no fertilization in vitro.

The low penetration rate in the SPA of patients with <14% morphologically normal sperm, but with a normal sperm count and motility, is an interesting
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phenomenon. In IVF patients with the same semen parameters as in group 1b, (sperm concentration >20 × 10⁶/ml, motility >30%, and <14% normal morphology), the fertilization rate per oocyte was 37% (Kruger et al., 1986) to 47% (Kruger et al., 1987a), compared with 88.3% in the group with >14% normal sperm morphology (P = 0.0001).

When the SPA rate is below the 10% threshold, the test is not a good predictor of IVF outcome; however, it does warn the clinician of a possible male factor (Kruger et al., 1987c) as does a value of <14% normal sperm morphology (Kruger et al., 1986, 1987a). The close correlation between the SPA rate and sperm morphology (Table 1) highlights this important point. In order to clarify this issue and to increase the reliability of the prediction tests in these patients, two possibilities are available. The SPA with egg yolk buffer can be tried to see whether it will predict patients who will not fertilize in vitro (Rogers, 1986), or a detailed study of morphology patterns in patients with <14% normal sperm morphology can be undertaken to predict accurately what will happen in IVF. Efforts in this second direction are being pursued in our laboratory (Kruger et al., 1987c).

It is concluded that sperm morphology, as studied in our institution, has helped in the assessment of fertilizing potential, especially in patients with normal sperm concentration and motility (Kruger et al., 1987b). In patients in which the SPA could not be performed, or in patients treated at institutions where the SPA is not available, valuable information may be obtained if the strict criteria for classification of sperm morphology described previously (Kruger et al., 1986) are followed. By assigning patients to groups based on semen analysis an accurate prediction of the outcome of short-incubation SPA can be made. A high number of sperm with abnormal morphology is a reflection of poor testicular function and must be carefully evaluated because it can have a significant effect on fertilization and pregnancy rates (Kruger et al., 1986, 1987a).

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We thank Rosita Acosta and Anne Bogeart from the Andrology Laboratory for their devoted work, Sharon Durio for secretarial assistance and Dr Charlotte Schader for editorial assistance. T.F.K. thanks the Medical Research Council and Tygerberg Hospital for providing financial assistance during his stay at the Jones Institute for Reproductive Medicine.

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iii. **The enzyme acrosin**

The enzyme acrosin was also studied in 1988, but it could not be shown that a correlation exist between sperm morphology and acrosin (Kruger et al., 1988b). There was however a correlation between the acrosome reaction and the different morphology groups with a clear defective ability in the severe teratozoospermic (P-pattern) group to acrosome react (Bastiaan et al., 2003). Low acrosin activity has been associated with low IVF rates (Liu et al., 1990). However, the role of acrosin activity in IVF is not clear, because contradictory results have been published. (Liu et al., 1990; Senn et al., 1992; Kennedy et al., 1986; Tummon et al., 1991; Kruger et al., 1988b; De Jonge et al., 1993; Kruger et al., 1996)

Menkveld (Menkveld et al., 1996; Menkveld et al., 1995) found statistically significant differences between acrosin activity in the groups with fertilization rates of less than 50% and 50% or higher, with acrosin activity of $8.45 \pm 5.2$ and $30.06 \pm 16.8 \, \muIU/10^6$ sperm, respectively, and a correlation between acrosin activity and fertilization rates of $r = 0.7085$ ($p < 0.005$). No clear cut-off point with regard to expected fertilization rates of 50% or higher for acrosin activity was found. With an acrosin activity $\geq 19.8 \, \muIU/10^6$ sperm, the fertilization rate was 83% or higher. However, for men with an acrosin activity between 6.4 and 10.5 $\muIU/10^6$ sperm, the fertilization rate was 50% or higher. If the acrosin activity was below 6.4 $\muIU/10^6$ the prognosis for fertilization was poor (Menkveld et al., 1996; Kruger et al., 1996).
The following article reflects the scientific basis that supports the above argument:

CORRELATION BETWEEN SPERM MORPHOLOGY, ACROSIN, AND FERTILIZATION IN AN IVF PROGRAM


Acrosin, a neutral proteinase, is located within the acrosome. The aim of this study was to evaluate acrosin concentrations in patients with severe damage of the sperm head and to determine whether acrosin concentration could predict the chances of fertilization in an IVF program. Sixty patients were accepted into this study, prospectively. The patients were divided into two groups, those with a normal morphology of < 14% (group I, n = 33) and those with normal morphology > 14% (group II, n = 27). All the patients had a sperm concentration of > 20 million sperm/ml and > 30% progressively motile sperm. The acrosin assays were performed on the semen sample obtained on the day of IVF. Routine IVF insemination procedures were used, and only mature oocytes were considered. The only factor that showed a significant correlation with fertilization was normal morphology (p < 0.01). The mean acrosin level was 73.4 ± 38.6 mIU/10 million sperm in group I and 70.9 ± 42.7 mIU/10 million sperm in group II (no significant difference). The fertilization rate in group I was 45.4% and in group II, 77.7% (p < 0.002). Acrosin levels were not significantly different in patients with and without fertilization (72.0 ± 42.1 and 73.6 ± 8.5 mIU/10 million sperm, respectively).

Key Words: Sperm morphology; In vitro fertilization; Acrosin; Fertilization.

INTRODUCTION

Acrosin, a neutral proteinase, is located in the anterior portion of the sperm head within the acrosome [11]. It has been postulated that acrosin is involved in ovm penetration at various stages of gamete fusion [3]. When the activity of this enzyme is inhibited, fertilization does not occur [13]. In patients with a morphological abnormality in the sperm head, the fertilization rate per oocyte in a human in vitro fertilization (IVF) program was lower than normal: 47% in a group with normal morphology < 14% and 88% in a group with > 14% normal forms [4, 5].

The aims of this study were to determine whether there is a relationship between the concentration of acrosin and the number of spermatozoa with severe damage of the sperm
head and to evaluate the value of acrosin level as a predictor of success in in vitro fertilization.

MATERIALS AND METHODS

Sixty patients were accepted into this study, prospectively. Patients were divided into two groups: those with a normal morphology of <14% (group I) and those with normal morphology >14% (group II). There were 33 patients in group I and 27 patients in group II. Group I was further divided into those with normal morphology <4% (poor prognosis pattern, group Ia, \( n = 13 \)) and those with a normal morphology between 4% and 14% (good prognosis pattern, group Ib, \( n = 20 \)) [7]. Female partners of the couples selected for this project had only a tubal factor as cause of their infertility. Female or male patients with antisperm antibodies were excluded from this study.

All male patients selected were required to have a sperm concentration of >20 million per ml and a normally motile sperm fraction of >30% [4] in the basic semen analysis to try to minimize the impact of these two variables on the fertilization rate. The basic semen evaluation was performed after liquefaction of the specimen delivered for IVF insemination using computer analysis (Cellsoft Semen Analysis System, Lab-Soft Division of Cryo Resources Ltd., New York). Sperm concentration and percentage of normal motility were evaluated.

Evaluation of morphology was made by two independent observers unaware of the results of IVF. Two morphology slides were prepared for each patient on the day of laparoscopy directly after liquefaction and were stained by the Quick-stain technique [6]. The morphology was evaluated as outlined in detail by Kruger et al. [4, 7]. Semen bacteriologic studies were negative in all patients.

Acrosin was measured as follows: The sperm pellet was separated from the semen sample obtained on the day of IVF and prepared as soon as possible after liquefaction of the semen. Specimen preparation was done within 3 h after collection without any sperm selection procedure (e.g., swim-up) being done. The specimen was centrifuged at 1000 g for 15 min and seminal plasma/supernatant was discarded immediately, leaving the sperm pellet intact. Sperm pellets were kept frozen at \(-20^\circ\text{C}\) to \(-80^\circ\text{C}\) and assayed within 48 h after collection. All subsequent steps were performed at 5°C. The pellet was resuspended in 0.5 ml of 0.1 M sodium phosphate buffer of pH 7.2–7.5, layered onto 1–2 ml of sucrose solution and centrifuged at 6000 g for 30 min. The sperm pellet was resuspended in 250 \(\mu\)l to 1 ml of extracting solution (prepared by mixing equal volumes of 10% glycerol and 2% acetic acid, pH 2.8) so that the final concentration was 10 to 40 million sperm/ml of extracting solution. Finally, a micro-magnetic stirring bar was placed in each tube and the mixture was stirred constantly at \(4^\circ\text{C}\) for 4 to 12 h.

The extracted suspension was centrifuged at 27,000 for 20 min and the total acrosin activity of the supernatant measured spectrophotometrically at 253 nm by following the rate of hydrolysis of benzoyl arginine ethyl ester (BAEE) in Tris-HCl buffer (pH 8). The total acrosin activity was reported as mIU/10 million sperm [1].

The techniques of sperm preparation, oocyte insemination, and culture in the Norfolk IVF program have been described previously [12]. Only mature oocytes were used in this study and 50,000 sperm/ml/egg were used for oocyte insemination in a total of 3 ml of medium.

Relationships between sperm motility, concentration, morphology, acrosin concentration, and fertilization rate were examined using multiple regression analysis and the SAS general linear model procedure. The multiple regression analysis allowed the examination of the contribution of each independent variable to the variation in the dependent variable fertilization. Fertilization was standardized for the number of preovulatory eggs by dividing the number of eggs exhibiting fertilization by the number of preovulatory eggs.

To determine whether acrosin is an indicator of low normal morphology and low fertilization rate, the acrosin levels in groups I, II, Ia, and b, as well as in the groups of patients with and without fertilization, were further tested using the Kruskal-Wallis test.
RESULTS

Multiple regression analysis was performed on all the variables. The only factor that showed a significant correlation with fertilization was normal morphology ($p = 0.01$). There was no correlation between acrosin concentration and fertilization. The mean sperm motility in group I (33 patients, normal morphology <14%) was 51.5 ± 16.9%, mean concentration was 78.3 ± 55.5 million/ml, and normal morphology was 6.2 ± 4.0% (sperm head defects being the major abnormality in all patients). In group II (27 patients, normal morphology >14%), mean motility was 63.6 ± 17.1%, concentration was 138.3 ± 71.4 million/ml, and normal morphology was 18.7 ± 3.3%.

For group Ia (13 patients, normal morphology <4%) mean concentration was 67.8 ± 52.1 million/ml, mean motility was 46.2 ± 16.9%, and normal morphology was 2.1 ± 1.32%. For group Ib (20 patients, normal morphology between 4% and 14%), mean motility was 54.9 ± 16.4%, mean concentration was 85.1 ± 57.2 million/ml, and normal morphology was 8.9 ± 2.6%. The mean acrosin level was 73.4 ± 38.6 mIU/10 million sperm (range 16 to 156) in group I and 70.9 ± 42.7 mIU/10 million sperm (range 15 to 190) in group II (no significant difference). Neither were acrosin levels significantly different between groups Ia and b and II, or between Ia and b.

The fertilization rate in group I was 45.4% and in group II 77.7%, and these values were significantly different ($p < 0.002$) (Table 1). The per patient pregnancy rates were comparable in both groups (24.2% and 25.2%, respectively). The mean acrosin level in those patients in whom fertilization of at least one preovulatory oocyte occurred ($n = 50$) was 72.0 ± 42.1 mIU/10 million sperm, compared with 73.6 ± 8.57 mIU/10 million sperm in the group of patients with failed fertilization ($n = 10$) (no significant difference) (Table 2). The percentages of normal sperm forms in these groups were 12.8 ± 6.9% and 6.8 ± 7.0%, respectively ($p = 0.02$).

The mean acrosin levels were significantly lower ($p < 0.03$) in the group of patients in whom a pregnancy was achieved ($n = 15$, acrosin: 50.0 ± 7.0 mIU/10 million sperm) compared with the group of nonpregnant patients ($n = 45$, acrosin: 77.0 ± 8.8 mIU/10 million sperm). Sperm counts were similar in these two groups of patients (104.4 ± 10.2 million/ml and 105.5 ± 10.2 million/ml, respectively).

DISCUSSION

Ability to predict the chances of fertilization is of great significance to both clinicians and patients involved in IVF. The chances of a term pregnancy are good, even where a male factor is involved, if fertilization occurs [1, 5]. In a study performed under the same conditions in patients with a concentration of >20 million sperm per ml and a motility >30%, an ongoing pregnancy rate of 18.3% was indicated in the group with >14% normal morphology and 5% in the group with <14% (no significant difference). Normal sperm morphology can be of great value in predicting fertilization in an IVF program [4, 5, 7] and, although the strict criteria previously described seem to improve the predictive ability of the test, it is still a subjective test.

Abnormal round-headed spermatozoa are devoid of an acrosomal cap and are not expected to have any enzymatic activity. A semen sample containing a high percentage of these abnormal cells would contain a low level of acrosomal proteinase with spermatozoa that are unable
TABLE 1 Relationship between Sperm Acrosin, Morphology, and Fertilization Rate in Patients Undergoing IVF

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (33 Patients)</th>
<th>Group II (27 Patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean acrosin ± SD (mIU/10 million sperm)</td>
<td>73.4 ± 38.6</td>
<td>70.9 ± 42.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>45%</td>
<td>77.7%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>No significant difference.
<sup>b</sup><i>p</i> < 0.002.
SD, Standard deviation.

The per patient pregnancy rates were similar in both groups, thus confirming that once fertilization occurs, the pregnancy outcome of male-factor patients is similar to other infertile groups [1]. Neither were acrosin levels different when we compared the patients in whom fertilization of at least one oocyte occurred with the patients with failed fertilization. On the other hand, acrosin levels were lower in those patients that achieved a pregnancy, as compared with the nonpregnant patients, a finding not related to an effect of sperm concentration. The significance of this finding cannot be interpreted in light of our present knowledge.

Mohsenian et al. [10] found a significant difference between acrosin levels in normal fertile donors and patients with unexplained infertility and between fertile donors and oligospermic patients. One of the possible explanations given by the authors for the difference in acrosin levels in oligospermic males was the high percentage of abnormal morphology among those

TABLE 2 Relationship between Sperm Acrosin, Morphology, and Fertilizing Ability in IVF

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No Fertilization (10 Patients)</th>
<th>Fertilization (50 Patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean acrosin ± SD (mIU/10 million sperm)</td>
<td>73.6 ± 8.5</td>
<td>72.04 ± 42.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Normal forms</td>
<td>6.8 ± 7.0</td>
<td>12.8 ± 6.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>No significant difference.
<sup>b</sup><i>p</i> < 0.02.
SD, Standard deviation.
Sperm Morphology, Acrosin, and IVF

subjects, although the specific abnormalities were not mentioned in the study. Although some researchers have claimed that acrosin plays a role in penetration of cervical mucus [11], this observation was not supported by Beyler and Zaneveld [2]. They concluded, after using acrosin inhibitors, that in man acrosin activity is neither necessary nor facilitatory to sperm penetration of cervical mucus.

In the current study we could not find a relationship between acrosin and morphology or fertilization rate in the IVF populations studied. A possible explanation for these results could be technical problems with the acrosin extraction; more likely, the acrosin concentration of sperm is indeed of no prognostic value in an IVF program.

Acknowledgments: We thank the scientists in the embryology laboratory, Ms. Simona Simonetti, Dr. Jake Meyer, and Ms. Mary Maloney, for freezing semen samples daily, and Ms. Debbie Jones for retrieval of data. We also thank the technicians in the andrology laboratory, Mrs. Rosita Acosta and Ms. Anne Bogaart, for their devoted work, and Ms. Sharon Durio for secretarial assistance, as well as Dr. Charlotte Schrader and Ms. Anne Jones, for editorial assistance. We acknowledge the Medical Research Council and Tygerberg Hospital, who financially assisted the first author during his stay at The Jones Institute for Reproductive Medicine.

REFERENCES

The following article reflects the scientific basis that supports the above argument:

Kruger TF, Menkveld R.  *Acrosome reaction, acrosin levels, and sperm morphology in assisted reproduction.*  Asst Reprod Reviews 1996;6(1):27-37
Acrosome Reaction, Acrosin Levels, and Sperm Morphology in Assisted Reproduction

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In the fertility pathway, the acrosome reaction with the release of (pro)acrosin is one of the most important steps. Much has been written recently about the physiology of the acrosome reaction with reference to its biochemical regulatory pathway. The role of calcium influx during the acrosome reaction and the role of acrosin activity in sperm binding to, and penetration of the zona pellucida have been studied in detail, but the morphology of the acrosome has received less emphasis. It is now becoming increasingly evident that for these functions to take place normal sperm morphology is essential, especially with regard to sperm binding to the zona pellucida and the relation between acrosome status and acrosin activity. Extensive reviews on these matters have been published recently by Brucker and Lipford and Fénelich and Partnair, to name only the most recent ones.

In this review, a more clinical approach is followed with special reference to the role of the acrosome reaction and acrosin activity and its relation to sperm morphology and assisted reproductive procedures.

HUMAN SPERM ACROSOME

The acrosome is formed by the Golgi apparatus during spermatogenesis. The acrosome can be described as a secretory granule situated at the apex of the sperm head consisting of an inner acrosomal membrane that is closely associated with the nucleus and continuous with the outer acrosomal membrane. The acrosomal matrix proper is located between the two membranes. The whole acrosome, and indeed the whole spermatozoon, is covered by the plasma membrane. The acrosome has several characteristics in common with the lysosome, as it contains a number of enzymes, and the internal milieu is normally acid.

Identification and Evaluation of the Acrosome

Methods to identify the presence, and to evaluate the status of the acrosome have received more attention in recent literature. It is believed that the fertilizing ability of spermatozoa depends largely on the morphology of spermatozoa and in particular on the acrosomal integrity or status as well as that of the postacrosomal region.

The term acrosome “status” is not always clearly defined, as some authors use this term to describe the acrosome-reacted state of the sperm, whereas others use this term with regard to the morphologic appearance of the acrosome. In this review, the term acrosome status refers to the acrosome-reacted state, and acrosome morphology to the morphologic appearance as seen with the light microscope.

Most of the studies on acrosome status have been performed by electron microscopy or fluorescent staining techniques to determine the presence, intactness, and/or size of the acrosomes, because the ability of the ordinary light, or phase-contrast microscope was regarded to be insufficient to study the acrosome status in detail.

Evaluation of acrosomal morphology with the light microscope, however, received little attention. With the strict Tygerberg criteria, the size and appearance of the acrosome play an important role in the definition and evaluation of an ideal normal spermatozoon.

With good optics, at \( \times 1000 \) or, preferably, \( \times 1250 \) magnification and good staining of the spermatozoon with, for example, Papanicolaou stain, valuable information on acrosomal morphology can be obtained. The ability to evaluate acrosome morphology by light microscopy is now acknowledged by other researchers. Special staining for acrosomal morphology also has been described by Chinoy et al., using an alcoholic acidic silver nitrate stain, which is particularly suitable for staining of the acrosome and postacrosomal sheath.

Acrosomal Morphology, Sperm Function, and In Vitro Fertilization Results

Acrosomal defects as seen with the light microscope can be classified as specific defects with, in extreme cases, complete absence of the acrosomes or nonspecific alterations showing a variety of acrosomal malformations that are often associated with other morphologic malformations of the spermatozoa. Examples of specific acrosomal defects, which are genetically caused, are globozoospermia, crater defects, and the microacrosome defects. These conditions are rare but, when occurring, easy to detect by light microscope.

Nonspecific acrosomal defects are of more clinical importance, in that they occur in a much greater frequency and are more difficult to detect by light microscopy. The abnormalities...
mostly occur during spermatid differentiation, usually due to abnormal Leydig cell function or to infections or organisms in the epididymis. These acrosomal abnormalities can present on their own, and include small acrosomes, staining defects, and large acrosomes. Staining defects include irregular acrosomes, multiple vacuoles, cysts, and “empty” acrosomes. These staining defects may indicate damage of the acrosome membranes with subsequent leaking of proacrosin from the acrosomes. Jeulin et al. found low fertilization rates of semen samples containing predominant sperm with staining defects. They postulated that the low in vitro fertilization (IVF) rates associated with increased abnormal acrosome morphology may not be attributable to the presence of the abnormal acrosomes per se but might be due to a relation between acrosomal abnormalities and nuclear maturity or immaturity of the spermatozoa.

Hofmann and Haider distinguished two types of acrosomal malformations according to the Düsseldorf classification: Type AI, described as a slight disorder in which the acrosomes are small or missing and the spermatozoa are normal or slightly narrowed, and Type AII, in which the spermatozoa show the same acrosomal defects, but the sperm heads are round with a compact condensation of the chromatid. Acrosomal malformations can also appear as a result of specific sperm abnormalities, such as cases of small or microcephalic spermatozoa, in association with moderate elongated spermatozoa (Düsseldorf Type HII) or the more severe cases of elongated spermatozoa (Düsseldorf Type III), in which the acrosomes are severely affected and present as small or absent and typed as AII and AIII, respectively. Papanicolaou-stained spermatozoa showing only small or undetectable acrosomes are unable to undergo the acrosome reaction and to penetrate zona pellucida-free hamster oocytes, whereas spermatozoa with smaller acrosomal disturbances are able to penetrate the zona pellucida.

For the most part, spermatozoa with the ideal morphologically normal forms are found tightly bound to the zona pellucida. These sperm are oval shaped with normally formed acrosomes. Slightly amorphous as well as slightly and moderately elongated spermatozoa also have been found tightly bound to the zona pellucida, but not severely abnormal or elongated spermatozoa. Round-headed spermatozoa without acrosomes (globozoospermia) also are not able to bind to the zona pellucida. Liu and Baker found that small oval- and pyriform-headed spermatozoa with acrosomal areas shaped like those of morphologically normal spermatozoa could bind to the zona pellucida at moderate rates. Spermatozoa with acrosomal abnormalities or with globozoospermia also are not able to fuse with the oolemma of hamster or human ova, because of associated absence or abnormalities of the postacrosomal sheath, which seems to be involved in the initial sperm-oolemma binding process. It therefore appears that the presence and morphology of the acrosome is of primary importance.
ACROSUME REACTION

ACROSOME REACTION AND FERTILIZATION

Capacitation, Initiation, and Importance of the Acrosome Reaction

To effect normal fertilization, spermatozoa must undergo the acrosome reaction. This is necessary for the penetration of the zona pellucida and fusion with the egg oolemma. The ability of spermatozoa to undergo the normal acrosome reaction and the rate of the acrosome reaction may thus be important indicators of fertility.1,4 However, before spermatozoa are capable of the acrosome reaction, capacitation must occur.

Capacitation is a postejaculatory modification of the sperm surface, a time-dependent process that varies markedly among men,2 and occurs physiologically in the female reproductive tract. Capacitation primarily involves changes in the sperm membrane system. The sperm membrane is a complex, mosaic structure of heterogeneous proteins and lipid domains. According to Zaneveld and Derajski2 capacitation involves the mobilization and/or removal of certain surface components from the sperm plasma membrane, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor. Subsequently, an increase in membrane fluidity and permeability occurs. These events are followed by or are simultaneous with changes in the lipid composition of the sperm membrane, resulting in (1) a decrease in net surface charges, (2) the formation of specialized areas that are devoted of intramembranous proteins and steroids, and (3) increased concentrations of anionic phospholipids. These areas are thought to be the sites where fusion and vesiculation occur during the acrosome reaction.

Capacitation is, up to a point, regarded as a reversible event in which the membrane-surface-associated molecules are replaced on the plasma membrane, returning the spermatozoa to their decapacitated state. The altered permeability leads to an increased uptake of calcium ions, glucose, and oxygen, resulting in an elevated energy metabolism inducing hyperactivity of sperm movement and leading to the ability of the spermatozoa to undergo the acrosome reaction if an endogenous calcium ion threshold is reached while the spermatozoa are binding to the zona pellucida.6,7

The acrosome reaction occurs once spermatozoa are tightly bound to the zona pellucida. This is an essential exocytotic event that further permits the spermatozoa to penetrate through the zona pellucida and then gain access to the egg's oolemma directly.6 The acrosome reaction seems to be introduced by appropriate stimuli, which are believed to be follicular fluid, progestin, progesterone, and 17α-hydroxyprogesterone. Follicular fluid and cumulus cells, especially, have been indicated to initiate the acrosome reaction, because both have sufficient protein-bound progesterone concentrations, which have been identified as the acrosome reaction-inducing agent in follicular fluid.3,4,5 Morphologically, the steps involved are: (1) fusion of the outer acrosomal membrane with the overlying (sperm) plasma membrane, (2) vesiculation and disappearance of the fused membranes anterior to the equatorial segment, and (3) release of enzymes and other components contained within the acrosomal matrix and exposure of the inner acrosomal membrane with the associated bound enzymes.6,7

More and stronger evidence is now becoming available that only normal, intact, (i.e., acrosome-reacted) spermatozoa are able to bind to the zona pellucida.5,6,7 Furthermore, spermatozoa with acrosome defects have a low rate of acrosome reaction or are not able to undergo the acrosome reaction, whereas spermatozoa of semen samples with a prevalence of hyperelevated spermatozoa (HII and HII according to the Düsseldorf classification) have the same rate of acrosomal reactions as normozoospermia samples.5 Liu and Baker1 have diagnosed a condition in which spermatozoa bind to the zona pellucida, but because the acrosome reaction does not occur, fertilization is stopped at this point.

Methods for Identification of the Acrosome Status

The best results for the observation of the acrosome status were initially obtained with the electron microscope, which unfortunately is time-consuming and complex to use and therefore is not recommended for routine use.1 Other methods used to identify the acrosome-reacted state of spermatozoa are also complex and require expensive equipment (e.g., the use of fluorescein-conjugated lectins). Lectin receptors are located within the acrosomal contents, therefore, changes are found in the lectin-binding patterns of acrosome-reacted and -unreacted sperm.2

Antiserum methods also can be used to determine the acrosome-reacted state of spermatozoa. Monoclonal antibodies or polyclonal antisera can be raised against specific epitopes on the sperm surface. Because acrosome-reacted sperm, as opposed to acrosome-intact sperm, lack certain antigens, antibodies raised against acrosome-intact sperm may not react with acrosome-reacted sperm.2 According to Brucker and Alexander,2 labeling with polyclonal antisera has an advantage over lectins because of the possibility of evaluating the acrosomal status in the presence of glycoconjugates and other cellular material. Although polyclonal antisera usually do not detect one defined epitope, they are suitable for demonstrating different staining patterns in acrosome-intact and acrosome-reacted sperm in conjunction with immunofluorescence. According to Brucker and Alexander,2 the well-defined specificity of a monoclonal antibody and the possibility of producing virtually unlimited quantities of these specific antisera without the source of errors found with conventional antisera make them valuable tools to detect the changes accomplished with the acrosome reaction.

However, because of the complexity of the methods noted above, the need arose for a relatively simple assay that could quantitate the acrosome reaction at the light microscope level. A method for the evaluation by light microscopy is the triple staining technique as described by Talbot and Chacon.5 This cytochemical method can discriminate between live and dead.
spermatozoa with acrosome-reacted or acrosome-intact acrosomes, but spermatozoa usually are classified only as alive and acrosome-intact (acrosome-unreacted) or alive and acrosome reacted. Unfortunately, this method is also time-consuming. A variation has been described in which trypan blue, the viability stain, is omitted. Another method for the evaluation of acrosomal status is the silver staining technique as described by Gosálvez et al. and Chinoy et al.

**Relationship between Acrosome Reaction, Acrosome Function, and Fertilization Rates**

Evaluation of the acrosomal status is a useful approach for investigating sperm fertilizing ability. Acrosomal dysfunction can be classified as morphologically abnormal acrosomes, a high spontaneous acrosome reaction, a low inducible acrosome reaction, and defective enzymatic activity. These dysfunctions can be associated with reduced fertility and are of clinical importance for the prediction of IVF results and to help in the decision-making of the patient’s treatment (e.g., induction of the acrosome reaction or the use of micromanipulation instead of IVF/gamete intrafallopian transfer [GIFT]).

Under normal conditions, most of the freshly ejaculated spermatozoa are acrosome-unreacted. Schill found 80 to 90% of intact acrosomes using the triple stain. The spontaneous acrosome reaction rate during incubation in a defined medium is physiologically low. Carrel et al. reported that sperm with head abnormalities have a lower spontaneous acrosome-reaction rate and lower acrosome-reaction response to A23187 than do normal spermatozoa. Under capacitation conditions, Schill found that spermatozoa of fertile men showed a significant increase in the percentage of acrosome-reacted spermatozoa, with a maximum peak after 3 hours on average, but this could vary significantly among men, with a period of 1 to 6 hours. An important observation made by Schill was that in a group of infertile men the initial percentage of acrosome-reacted spermatozoa was increased compared with that of normal men and that there was no further increase after several hours of incubation. This may lead to a state of sterility, because the spermatozoa of these men cannot bind to the zona pellucida in sufficient numbers and are unable to penetrate the zona pellucida. Identifying these men is important in the fertility investigation of male patients, an aspect that seems mostly to be ignored. This condition is a specific entity, because spermatozoa from other men with pathologic semen parameters such as oligo-, astheno-, and teratozoospermia showed patterns compatible with those of normal men.

The inducibility of the acrosome reaction of spermatozoa has been used extensively as a functional test with the hamster ovum-penetration test and for predicting expected IVF outcome. Ionophore and increased albumin concentrations have been used experimentally but cannot be used for actual IVF, because the ionophores are toxic. A new method for the induction of the acrosome reaction that can also be used in actual IVF, is to expose spermatozoa to low (4°C) temperatures for 24 hours, followed by incubation at 37°C for 3 hours. The percentage of the induced acrosome reaction can be calculated by subtracting the initial percentage of acrosome-reacted spermatozoa from the percentage of acrosome-reacted spermatozoa after treatment. High IVF rates were observed with induced acrosome-reacted rates of between 11% and 35%.

It can therefore be concluded that normally fertile men present with a lower proportion of acrosome-reacted spermatozoa after ejaculation. The percentage of acrosome reacted spermatozoa must remain low during incubation in culture media even after 24 hours. High premature acrosome reactions in these conditions are associated with infertility. When the acrosome reaction is induced, fertile men have a drastic increase in acrosome-reacted spermatozoa, whereas in infertile men this often is not the case.

**Role of Acrosome Reaction in Subzonal Insemination and Intracytoplasmic Sperm Injection**

As stated previously, to obtain fusion and penetration of the sperm head into the oocyte, the spermatozoon must be acrosome-reacted. By resorting to assisted fertilization (i.e., subzonal insemination [SUZI]) and partial zona dissection in cases of unsuccessful IVF or severe male factors, the zona pellucida barrier is sidestepped, but for fusion with the oolemma to occur, the spermatozoon still must be acrosome-reacted. To enhance the results of assisted fertilization, methods to obtain a higher population of acrosome-reacted spermatozoa were introduced.

The techniques used to introduce the acrosome reaction were either incubation with follicular fluid or electroporation. With these methods, between 30% and 53.9% of acrosome-reacted spermatozoa could be obtained. Spermatozoa were then randomly chosen for SUZI, and therefore between 3 and 6 sperm were injected. However, the fertilization and pregnancy rates were low and still excluded men with globozoospermia, because these spermatozoa are not able to bind and penetrate the oolemma. Now these methods are only of academic interest because of the introduction of the intracytoplasmic sperm injection (ICSI) procedure in 1992.

With ICSI, zona pellucida penetration and oolemma fusion are bypassed and the acrosome reaction is thus not essential for this part of the procedure. The question remained however, whether the acrosome reaction was necessary for spermatozoa to undergo decondensation. Recent investigations have demonstrated that it is not necessary to carry out specific treatment of spermatozoa before ICSI. Even patients with globozoospermia who previously have been regarded as sterile, now can be treated with ICSI, because these spermatozoa are capable of decondensation once they have been introduced into the ooplasm, and successful pregnancies have been reported by several centers.

Another important group of patients who now can be treated are those described by Liu et al. with persistent failure of fertilization in IVF associated with disordered zona pellucida-induced acrosome reaction. The acrosome-unreacted spermatozoa were removed from the oocytes by aspiration through
a fine glass pipette and used for ICSI, resulting in successful pregnancies.

ACROSOME REACTION

Another important characteristic of the acrosome is that it contains several enzymes. Enzymes associated with the human acrosome are acid phosphatase, hyaluronidase, acid proteinase, a neutral proteinase (proacrosin), β-aspartyl N-acetyl glucosamine amino hydrolase, adenosinetriphosphatase (ATPase), and an enzyme that disperses the corona radiata of the ovum, referred to as the corona-penetrating enzyme.12

Proacrosin, when released from the acrosome during the acrosome reaction (of spermatozoa tightly bound to the zona pellucida), is transformed to the active form, acrosin. Acrosin was shown to be present in the human sperm by several researchers in the early 1970s.71 Acrosin is a unique neutral proteinase enzyme that plays a role in the softening of the zona pellucida and subsequent penetration of the sperm through the zona pellucida.50 Acrosin has a molecular weight of approximately 30,000. It is similar to trypsin and plasmin, although it differs from these enzymes in its inhibition spectrum and degree of inhibition by certain proteinase inhibitors. Acrosin activity is optimal at a pH of 8.0 and is inactive at a pH lower than 5.0 or higher than 10.5.12 Part of the acrosin becomes activated during the acrosome reaction, with the resultant acrosin being involved in the dispersion of the outer sperm membranes. Subsequently, the proacrosin that remains bound to the inner acrosomal membrane may become activated during the penetration of the spermatozoon through the zona pellucida, the acrosin aiding in the formation of the penetration slit and the passage of the spermatozoon.74

Methods for Acrosin Measurements

Different methods for the measurement of acrosin have been described by a number of investigators and include assay of esterase or amidease activity, active enzyme staining in electrophoretic gels, measurement of proteolytic activity using a gelatin film substrate,23 and immunochromatographic assay.8 A simple method has been reported for measuring the total acrosin activity of human spermatozoa by Welker et al27 based on the lysis of albumin spread thinly on a glass slide, by the proteinase (acrosin) released from the acrosome. The diameter of the halo formed in this way and the percentage of spermatozoa showing this halo formation are indicators of the acrosin activity. Men with globozoospermia show no halo formation with this test.76 The disadvantage of this test is that it is only quantitative, and no qualitative values of acrosin activity can be obtained. Difficulty is also encountered in standardization of the slides, because the thickness of the gelatin film may vary over the slide, affecting the diameter of the halos. Henkel et al,78 using this gelatinolytic test, found that a halo formation rate of more than 60% and a halo diameter of more than 10 μm is indicative of fertility (> 50% fertilization rate with IVF). An acrosin activity index (acrosin activity × halo formation rate/100) of less than 6 was indicative of a poor (< 50%) fertilization rate, with a specificity of 97.3% but a sensitivity of only 25.7%.78

A commercial kit also is available for the measurement of acrosin activity, based on the method of Kennedy et al,79 that is a faster and simpler miniature assay,80 but that yields poor correlations with the Kennedy method.79 However, Tummon et al81 found that acrosin activity values obtained with this test have good predictive use for the expected IVF outcome.

Today, most assays for the determination of acrosin activity are based on spectrophotometric methods. For these methods, the spermatozoa are first washed free of seminal plasma by centrifugation over Ficoll to remove the soluble proteinase inhibitors of the semen, which may interfere with the detection of acrosin activity. The sperm pellet is then suspended in a buffer that contains a detergent to facilitate the disruption of the acrosomes with the release of the acrosomal enzymes, including proacrosin. The buffer has a basic pH that allows activation of proacrosin into enzymatically active acrosin. A synthetic arginine amide substrate also is included in the buffer, and when this is hydrolyzed by the acrosin activity, it releases a chromophoric product. This reaction is stopped after 3 hours by adding a benzamidine solution. The intensity of the reaction is measured spectrophotometrically at 410 nm.79

Correlation between Acrosin Activity, Semen Parameters, and Functional Tests

Earlier studies were focused on the relation between sperm concentration and acrosin activity73 as well as other semen parameters, including sperm morphology.10,82 A strong relation exists between sperm morphology and acrosin activity. No acrosin activity can be measured in men with globozoospermia.73 The gelatinolysis test also is negative in globozoospermia men, inasmuch as no halo formation is observed,7 whereas a significant decrease has been found in men with severe teratozoospermia.14 As acrosin is released from the acrosome, it can be expected that the acrosin activity may be dependent on the percentage of normal intact acrosomes in a specific semen sample. Low acrosin activity has been observed in the presence of small acrosomes or in the absence of acrosomes.3,8,14,20 The important relation between morphologically normal acrosomes, as evaluated by strict criteria, and normal acrosin activities has been demonstrated by Menkveld et al14,29 who found a strong positive correlation between the percentage of morphologically normal acrosomes and acrosin activity (r = 0.8945; p < 0.0001).

Low acrosin activity also has been associated with male infertility4,7,17,83 and differs between so-called fertile and infertile populations and patients with unexplained infertility, in whom low acrosin activity has been observed.43 Decreased acrosin activity is also found in patients with polyzoospermia (>250 × 106 spermatozoa/mL), in whom acrosin activity can be reduced by as much as 60% compared with that in a normal population,44 and is associated with an increased rate of miscarriages.59 Other semen variables, such as motility and spermatozoon concentration, do not seem to influence acrosin activity;
as Schill\textsuperscript{7} reported that results in asthenozoospermia, oligozoospermia, and mild teratozoospermia cases did not differ statistically significantly from those with normozoospermia. However, Schill et al.\textsuperscript{8} made an interesting observation that the period of sexual abstinence may influence the activity of acrosomal protease and can thus lead to false interpretations of acrosomal functions. Acrosin activity was significantly higher after 1 day of abstinence compared with a period of 6 to 9 days. Their observation emphasizes the need for a prescribed period of abstinence, as advocated by others.\textsuperscript{8,9}

Several reports have been published on the relation between acrosin activity and the results of functional tests, such as the hamster ovum-penetration test.\textsuperscript{9} Francavilla et al.\textsuperscript{10} found that a low acrosin activity in the otherwise normal ejaculates from infertile patients was associated with an impaired sperm penetration into zona-free hamster oocytes. In these patients, the normal percentage of spermatozoa positive with immunostaining for proacrosin-acrosin suggested a possible functional defect of proacrosin activation and not of acrosine-reaction inhibition.

**Acrosin Activity versus In Vitro Fertilization Rates**

Low acrosin activity has been associated with low IVF rates.\textsuperscript{10,9} However, the role of acrosin activity in IVF is not clear, because contradictory results have been published.\textsuperscript{9,10,23,40,50,90}

Menkveld et al.\textsuperscript{42} found statistically significant differences between acrosin activity in the groups with fertilization rates of less than 50% and 50% or higher, with acrosin activity of 8.45 ± 5.2 and 30.06 ± 16.8 μIU/10⁶ sperm, respectively, and a correlation between acrosin activity and fertilization rates of r = 0.7085 (p < 0.005). No clear cutoff point with regard to expected fertilization rates of 50% or higher for acrosin activity was found. With an acrosin activity ≥ 19.8 μIU/10⁶ sperm, the fertilization rate was 83% or higher. However, for men with an acrosin activity between 6.4 and 10.5 μIU/10⁶ sperm, the fertilization rate was 50% or higher.

Considering their results, Menkveld et al.\textsuperscript{42} could distinguish three possible IVF prognosis groups according to the acrosin activity: a normal group with a good prognosis and an acrosin activity of ≥ 20.0 μIU/10⁶ sperm; a gray area with acrosin activity values between 19.8 μIU/10⁶ sperm and 6.4 μIU/10⁶ sperm where low fertilization rates may be expected; and a third group in which a poor prognosis can be expected with an acrosin activity of < 6.4 μIU/10⁶ sperm. These results are in accordance with the results of Kennedy et al.\textsuperscript{29} although their values are somewhat higher (normal = 25.0, gray = 14 to 24 and low = ≤ 13 μIU/10⁶ sperm) than those found in the study by Menkveld et al.\textsuperscript{42} Similar results were found by Van der Ven et al.\textsuperscript{40} and Zaneveld et al.\textsuperscript{40} who explored the relation between acrosin activity and IVF. Acrosin activity was assessed by the method described by Kennedy et al.\textsuperscript{29} Acrosin activity in the fertile group varied between 14 and 60 μIU/10⁶ spermatozoa with a mean ± SEM of 34.4 ± 2.9 μIU/10⁶ spermatozoa. In the infertile group, the acrosin activity was between 7 and 35 μIU/10⁶ spermatozoa with a mean ± SEM of 20.3 ± 3.2 μIU/10⁶ spermatozoa. These means were statistically significantly different. No fertilization occurred when the acrosin activity was less than 14 μIU/10⁶ spermatozoa, and nearly all oocytes were fertilized with acrosin activity at least 25 μIU/10⁶ spermatozoa. Based on these observations, acrosin activity of at least 25 μIU/10⁶ spermatozoa was regarded as normal and acrosin activity less than 14 μIU/10⁶ spermatozoa as abnormal. Good correlation with IVF rates also was found by others.\textsuperscript{70,80,90}

However, the good predictive value of acrosin activity in these studies is in contradiction to the results of others.\textsuperscript{9,10,90} Kruger et al.\textsuperscript{80} looked at acrosin activity distributions in groups with 14% or less and 15% or more morphologically normal forms, and found nonsignificantly different acrosin activity values of 73.4 ± 38.6 and 70.9 ± 42.7 mIU/10⁶ million sperm, respectively. The reason for the contradictory results for the predictive value of acrosin activity for IVF between the two groups is difficult to explain, but it is interesting to note that several of the studies reporting positive correlation, such as those of Menkveld et al.,\textsuperscript{14} Kennedy et al.,\textsuperscript{29} and De Jonge et al.,\textsuperscript{10} used the method of Kennedy et al.\textsuperscript{29} whereas those reporting a negative relation\textsuperscript{50,80,90,91} used other methods for the detection of the acrosin activity. Concern has been expressed by Harris et al.\textsuperscript{90} about the many different protocols used for the detection of acrosin activity. They have demonstrated several confounding effects that impact on the results of clinical (biochemical) acrosin assays. They came to the conclusion that, if acrosin activity is to be used as a diagnostic test, standardization of the assay protocols must be reached. Based on the above available data, it seems that the Kennedy assay\textsuperscript{29} should be recommended for optimal acrosin activity results.

**Relation between Functional Tests and Sperm Morphology**

The evaluation of sperm morphology according to strict criteria,\textsuperscript{29} together with the addition of an acrosome index, is an uncomplicated way of gaining important data on the functional ability of a specific semen sample. Therefore, sperm morphology still remains an important prognosticator for expected IVF results and underlines the important relation between normal sperm morphology and normal sperm function, especially in a negative way. If sperm morphology is low, poor results for the hamster ovum-penetration test,\textsuperscript{48} the hemizona assay,\textsuperscript{49} zona pellicuda–sperm binding,\textsuperscript{3} acrosin activity,\textsuperscript{48} and DNA content\textsuperscript{48} may be expected. Therefore, normal sperm morphology is needed for normal sperm function; that is, zona pellicuda–sperm binding, acrosome reaction, release of acrosin, and zona pellicuda penetration and sperm decondensation. On the other hand, there may be certain men with what appear to be morphologically normal spermatozoa who have a problem that may or may not be related to sperm morphology,\textsuperscript{29} and these men must be carefully investigated with sequential analysis to try to establish where the functional abnormality is, if an oocyte factor has with certainty been eliminated. Such men are, for example, those with normal morphology but poor or negative hamster ovum-penetration test results as reported by Coetzee et al.\textsuperscript{48}
SPERM MORPHOLOGY IN ASSISTED REPRODUCTION

The importance of sperm morphology in the diagnosis of male infertility has been discussed and debated in a number of articles recently. Various articles reviewing the topic of sperm morphology in assisted reproduction appeared in the literature, and this development stimulated discussions as well as a reevaluation of the value and role of the basic semen analysis in assisted reproduction. In the authors’ opinion, the basic semen analysis is the cornerstone in the evaluation of male fertility as is the screening for antibodies with the mixed antiglobulin reaction (MAR) test and/or immunobead tests with the first semen analysis.

How Reproducible is Sperm Morphology Evaluation?

Based on the work of Menkveld et al, an excellent inter- and intratechnician correlation was observed for manual sperm morphology evaluation in the Tygerberg laboratory. This observation was confirmed recently by another group studying inter- and intratechnician variation using strict criteria. There is, however, still worldwide uncertainty about the repeatability among different laboratories, and concerns regarding these aspects were expressed by researchers in the field of andrology. To effect worldwide uniformity, Davis et al. stated that an alternative approach, which may prove to be much less expensive and easier to control, is the use of automated laboratory assays.

Is the Use of Automated Sperm Morphology Analyzers Valuable in Clinical Practice?

In recent publications on sperm morphology evaluated by automated sperm morphology analyzers (ASMA), most workers were positive about the use of these instruments to evaluate sperm morphology on a cell-by-cell basis. Wang et al. evaluated a specific system and did not find any difference compared to manual evaluation. Garrett et al. found their system accurate, but too slow (100 minutes for 100 cells). The systems studied by Davis and colleagues and the Tygerberg group were competitive, with an average time of 4 minutes per slide (100 cells). The accuracy and precision of ASMA depend on (1) the microscope optics magnification and focusing capabilities, (2) video camera quality, (3) ray size and frame grabber, (4) image processing techniques, and (5) definitions of metric measurements.

The IVOS dimensions system from Hamilton Thorne Research (Boston, Massachusetts) (Versions 2.1 and 3.1) was evaluated by the authors. An excellent repeatability (cell by cell, \( k > 0.8 \)) was obtained. It was also shown that sperm morphology as analyzed by the IVOS dimension system was a predictor of fertilization, in a prospective study using multiple-regression analysis. The other predictors of fertilization in this study were the number of oocytes available and the total motile fraction retrieved after wash and swim-up. It was concluded from the study that this system can be of help in clinical practice assisted reproduction.

Sperm Morphology Is Not the Only Criterion of Male Infertility

The fact that the authors have been involved in research in the field of sperm morphology does not mean that there is a bias in the understanding of the fertilization process. As background, the study groups were standardized in the initial stages of the investigation of impact of sperm morphology on fertilization rate. Only patients with sperm concentrations of more than 20 million/mL as well as a motility above a certain threshold level (for this laboratory, > 30%) were used. It was in these initial prospective studies that the 14% threshold for sperm morphology was established, indicating a significantly lower fertilization below 14% normal morphology. It is also important to note that in those initial studies only up to 100,000 spermatozoa were used for insemination of oocytes.

This led to the next study, in which, under the same conditions, the P-pattern, or poor prognosis group (0% to 4% normal sperm morphology) was established, in which only 7.6% of the oocytes were fertilized in patients using the same insemination concentration (100,000 spermatozoa/mL).

It is important to realize that the fertilization rate can be affected if higher insemination concentrations are used, and thus the clear difference seen in the above-mentioned studies is not so obvious in later studies.

Prognosis of Patients with Normal Sperm Morphology (0% or 1%)

There is a misconception about the prognosis of the patients with 0% or 1% normal sperm morphology. Pregnancy can be brought about with GIFT, IVF, and in vivo fertilization, although the pregnancy rate in the studies conducted by the authors was significantly lower than that of the controls. However, the prognosis for fertilization and pregnancy in these patients can be improved by increasing insemination concentration 2- to 10-fold, and by selecting Metaphase II oocytes for transfer in GIFT procedures.

In patients like these, an IVF cycle with ICSI control is attempted. If insemination results lead to fertilization with good embryo quality in vitro, either IVF or GIFT is carried out in future cycles. If no fertilization occurs with IVF but does occur with ICSI in the first treatment cycle, ICSI is used in subsequent cycles.

The authors do not make clinical decisions on semen analysis and strict sperm morphology alone, but consider the information obtained as a warning of a significantly lower fertilization chance in, for example, a P-pattern morphology and low acrosome index with low hemizona binding results. The authors are in full agreement with Fraser et al. that there is no single test available that provides an absolute answer. However, all tests considered, the basic semen analysis, if done correctly, is a simple method and gives invaluable help in clinical practice.
Is a Sequential Analysis Necessary in the Evaluation of the Male?

As stated in the introduction as well as in an article by Oehninger et al., from the viewpoint of the male gamete, the fertilization process is complex, and a number of events must happen to lead to success in fertilization and implantation. Such phenomena include the process of capacitation leading to hyperactivated motility and acrosome reaction, which in turn allow a successful sperm–zona pellucida interaction and pre-embryo formation. Thus, it was proposed that a sequential diagnostic scheme should be utilized to assess sperm fertilizing potential. This sequential analysis should include basic functional and more complex bioassays to test for different sperm functions, and ultimately the final test is IVF.

Such an approach can lead to a much better selection of patients with a severe male factor who qualify, for example, for ICSI. In patients diagnosed with a P-pattern morphology, after sperm separation with more than 1 million spermatozoa/mL available, it would be a sound approach to do IVF as a control and ICSI as a test on the same cohort of oocytes where fertilization was never proved before. Based on the outcome of fertilization, a final long-term decision for treatment can then be made, and patients can be informed accordingly.

Intracytoplasmic Sperm Injection and Severe Teratozoospermia

Intracytoplasmic sperm injection is a major breakthrough in male infertility treatment. This fact, however, must not distract efforts to refine diagnostic methods, and ICSI should be offered to those who really qualify for the procedure. Cost per cycle is a major factor, even in the United States and Western Europe, and correct treatment must be offered, as is common practice in all branches of medicine.

Patients with P-pattern morphology (severe teratozoospermia) do well with ICSI, and it seems that, once the barrier is bridged, fertilization potential is excellent. This was not the case with SUZI, in which Cohen observed a correlation with low morphology and a lower fertilization rate.

Artificial Insemination and In Vivo Pregnancies

Some reports state a correlation between normal sperm morphology and pregnancy rate after insemination, but others do not suggest any correlation at all. Total motile sperm count is used by some as a predictor of outcome, but thresholds differ from laboratory to laboratory.

In an interesting study on 137 spontaneous pregnancies compared with a subfertile control group, Omebe et al. reported a threshold of less than 5% for normal morphology (strict criteria). This is the first report to compare the in vivo pregnancy rate with a subfertile control group using strict criteria. The threshold correlates with the authors’ concept of the P pattern in assisted reproduction. Others did not find any correlation with in vivo pregnancy rate and semen parameters.

Why Do All Groups Not Agree on the Value of Sperm Morphology as a Predictor in Assisted Reproduction?

The criteria applied in a specific laboratory can be wrong. Often, technicians are too strict, leading to patients being classified into the P pattern if actually fertile (>14% normal forms) or into the G pattern with a good prognosis in assisted reproduction or spontaneous in vivo pregnancy.

Corrective measures also can lead to an increase in pregnancy and fertilization rates as observed in the present programs. If these measures are applied, this can lead to less obvious differences in the morphology group study (P, G, and normal groups).

It is also important to use multiple-regression analysis to establish thresholds for a given laboratory, and not to use established thresholds from other laboratories. There can be subtle differences between laboratories (e.g., 10% threshold in one laboratory can clinically be the same as 14% in the next laboratory). In theory, this problem will be overcome using automated sperm morphology analyzers (ASMA).

SUMMARY

Strict criteria are clinically helpful and can be used to counsel patients and to plan correct treatment in assisted reproduction to alleviate male-factor problems. It can be concluded that normal sperm morphology is needed for normal sperm function, especially sperm–zona pellucida binding, the acrosome reaction with release of (pro)acrosin, and sperm–oolemma fusion. The inclusion of an acrosome index, however, can be a more valuable addition to the evaluation of sperm morphology for the prediction of expected IVF rates, especially in patients with 4% or less morphologically normal spermatozoa.

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REFERENCES


ACROSOME REACTION 37


The hemi-zona assay (HZA) [Relationship between functional tests and sperm morphology]

The hemi-zona assay was developed at the Jones Institute in 1986 and the findings were published in 1988 (Burkman, 1988). Numerous clinical (Oehninger et al., 2000; Franken, 1989) and laboratory related articles followed (Hodgen et al., 1988; Franken et al., 1990; Oehninger et al., 1991). It was observed that if sperm morphology is low, poor results for the hemi-zona assay, and zona pelucida-sperm binding (Franken et al., 1990) was obtained. Therefore, normal sperm morphology is needed for normal sperm function; that is, zona pellucid-sperm binding, acrosome reaction, release of acrosin, and zona pellucid penetration and sperm decondensation.

An example of a laboratory orientated article with clinical application is the article about preservation of oocytes to assist laboratories in using this test on a day to day basis as fresh oocytes were not always readily available. This article assisted the laboratories interested in the HZA, to be able to offer the test at a regular basis as required by the clinician or scientist (Kruger et al., 1991).
The following article reflects the scientific basis that supports the above argument:

Hemizona Assay: Use of Fresh Versus Salt-Stored Human Oocytes to Evaluate Sperm Binding Potential to the Zona Pellucida

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Salt-stored human oocytes (pH 7.0) showed sperm binding ability equal to that of fresh, living oocytes under hemizona assay (HZA) conditions.

KEY WORDS: human gametes; zona pellucida; sperm binding.

INTRODUCTION

Tight binding of spermatozoa to the zona pellucida represents a crucial association of the two gametes, requisite for fertilization and subsequent embryonic development. The hemizona assay (HZA), is a new homologous internally controlled bioassay for the evaluation of sperm binding to the zona pellucida with high predictive value for in vitro fertilization (IVF) outcome (1).

Earlier, the sperm binding capacity of salt-stored versus dimethyl sulfoxide (DMSO)-treated oocytes was compared (2). In that study, there was equivalent binding using salt- or DMSO-treated hemizonae. In addition, the sperm binding kinetics were similar and unaffected by the duration of zona storage (6 to 30 days in 1.5 M magnesium chloride solution) (2).

It has recently been suggested that the functional properties of zonae pellucidae of salt-stored oocytes differ from those of living oocytes and that this difference should be borne in mind when interpreting any test of sperm function using salt-stored human zonae pellucidae (3).

The question arose whether the binding ability of salt-stored oocytes is comparable to that of fresh oocytes. Here, we performed a prospective study during which the sperm binding ability of metaphase I salt-stored oocytes (6 to 30 days in storage) was compared to that of freshly obtained metaphase I oocytes under the conditions of the HZA.

MATERIALS AND METHODS

Oocytes were donated by patients undergoing gamete intrafallopian transfer (GIFT) therapy after written permission was obtained. Ovarian stimulation was performed using a combination of clomiphene citrate and human menopausal gonadotropin following established guidelines. A total of 22 human oocytes was utilized in the study, 11 fresh, immediately upon collection, and 11 after salt storage. Eleven metaphase I oocytes donated by patients undergoing GIFT therapy were salt-stored in 1.5 M magnesium chloride (Mallinkrodt Chemical Works, St. Louis, MO) supplemented with 0.1% polyvinylpyrrolidone (PVP; Sigma Chemical Co., St. Louis, MO) and 40 mM sodium Hepes buffer (Boehringer, West Germany) for a period of 6 to 30 days (4). In addition, on the day of the GIFT procedure, 11 donated, freshly obtained metaphase I oocytes were used in the HZA within 4 hr of collection. Microinjection of oocytes and semen preparation procedures were performed according to published guidelines (1,4).
SPERM–ZONA BINDING, SALT-STORED EGGS

Each salt-stored hemizona was incubated with swim-up processed spermatozoa from a fertile man (control), while the matching hemizona was coincubated with sperm from an infertile patient with a diagnosis of teratozoospermia (semen samples from the same men were used in the 11 HZA experiments). Simultaneously, a fresh, donated metaphase I oocyte was bisected and the hemizonae were incubated in separate droplets with the sperm from the same fertile control man and the same infertile patient. Gametes coincubation was allowed to occur for 4 hr, followed by rinsing of the hemizonae by vigorous pipetting. The number of tightly bound spermatozoa on the outer surface of the hemizona was counted using phase-contrast microscopy (×200) (1,4). The HZA index was calculated as follows (1):

\[
\text{HZA index} = \frac{\text{number of spermatozoa bound}}{\text{number of spermatozoa bound}} \times 100
\]

for fertile sample

Eleven experiments were completed comparing the hemizona index (HZA) of the salt-stored versus fresh oocytes.

RESULTS

For fresh oocytes, the mean numbers of tightly bound sperm for the hemizonae exposed to fertile and infertile semen were 118.6 ± 12.9 (mean ± SE) and 37.1 ± 7.6, respectively (\(P < 0.0006, \text{paired} t\text{ test}\)).

For salt-stored oocytes, the mean numbers of tightly bound sperm for the hemizonae exposed to fertile and infertile semen were 122.7 ± 14.0 and 33.4 ± 7.3, respectively (\(p < 0.0008, \text{paired} t\text{ test}\)).

The hemizona index using fresh oocytes was 35.2 ± 8.0, while that using salt-stored oocytes was 33.4 ± 7.9 (not significant, Student’s \(t\) test).

DISCUSSION

The sperm binding capacity of zonae pellucidae of metaphase I, fresh oocytes is similar to that of metaphase I salt-stored oocytes (6 to 30 days in storage) under the conditions of the HZA. This information is of crucial importance to validate the HZA as a predictor of human in vitro fertilization (1,2,4). We want to emphasize that with the HZA, specific and irreversible tight binding of the spermatozoa to the outer surface of the zona pellucida is evaluated, and based on this concept, the HZA was developed (4).

Others have reported that the zona pellucida of metaphase I and II oocytes aged in insemination medium and treated with a concentrated salt solution showed high penetrability, in contrast with oocytes not treated with salt solution (3). We have also observed loss of binding ability of human oocytes if left in salt solution for periods longer than 30 days (2). The use of nonbuffered solutions will cause pH changes over a 30-day period, which is an important factor to be considered (5). In those cases, the zona softens and virtually disintegrates. It is also important to adjust the pH to 7.0 by the addition of an adequate amount of 1 M NaOH before using the salt solution as a storage medium (5).

This study demonstrates that the zona pellucida of metaphase I human oocytes (fresh and salt-stored from 6 to 30 days under controlled-pH conditions) has equal ability to bind spermatozoa of both fertile and infertile men. Therefore, our studies extend the initial observations of Yanagimachi et al. (6) and of Yoshimatsu et al. (7) and demonstrate that salt-stored oocytes can be used with confidence when employing the HZA both as a predictor of in vitro fertilization and as a tool to investigate the cellular aspects of human fertilization (8,9).

SUMMARY

The hemizona assay (HZA) has a high predictive value for in vitro fertilization (IVF) results. Oocyte quality plays a significant role in the validation of this test. The question was asked whether human salt-stored oocytes (up to 30 days) are damaged and subsequently lose their sperm binding capacity when compared to fresh human oocytes. Equivalent binding in both the salt-stored and the fresh group of oocytes was observed in the hemizonae incubated with normal semen as well as in their matching halves incubated with semen from an infertile man. Based on the results, we conclude that salt-stored oocytes (pH 7.0) give reliable information regarding sperm binding potential under HZA conditions.

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b. Evaluation of the value of sperm functional assays (Hemi-zona assay and acrosome reaction) based on the international literature

i. The HZA, Acrosome reaction (AR) and SPA tests in perspective

In a literature review by Oehninger on 2906 subjects it was shown that there is a high predictive power in the HZA and induced acrosome reaction (AR) assays for fertilization in vitro but poor clinical value for the SPA test (Oehninger et al., 2000). The value of the Computer Assisted Semen Analysis (CASA) systems is still debated with a real need for standardization of this technology (Oehninger et al., 2000). As far as acrosome reaction is concerned there was a correlation between acrosome reaction and the different morphology groups with a clear defective ability in the severe teratozoospermic (P-pattern) group to acrosome react (Bastiaan et al., 2003).

The pathophysiology of severe teratozoospermia and other sperm abnormalities and the interpretation of sperm functional assays were discussed in detail by Kruger (Kruger et al., 1996; DSc Chapter 3,a,iii, 2012; Kruger et al., 1999; DSc Chapter 1,b,i 2012) as well as in a chapter written in Male infertility: Diagnosis and treatment (Arslan et al., 2007) and is reflected in the following section.
Applicable chapters in the following text book will be reflected on:

Clinical management of male infertility

Murat Arslan, Sergio Oehninger, Thinus F Kruger

INTRODUCTION

It is estimated that male subfertility is present in up to 40–50% of infertile couples, alone or in combination with female factors. There has been extensive progress in the diagnosis and treatment of male factor infertility since the inception of assisted reproductive technologies (ART). Moreover, the advent of intracytoplasmic sperm injection (ICSI) has resulted in a dramatically increased likelihood of pregnancy in couples suffering from most causes of male infertility. Fundamental advances have been made in the genetics of male disorders. Nevertheless, and at the same time, we are now witnessing a steady state in the development of assays that can be predictive of sperm functional capacities, both under in vivo and in vitro conditions.

Therefore, it is evident now, as it was a few years ago, that more research is needed to establish the causes and pathogenic mechanisms involved in male disorders leading to abnormal sperm function. The correct approach for male infertility evaluation should include a rational program composed of careful evaluation of the patient’s history, a complete physical examination, laboratory tests of basic/extended semen analysis and a urological, endocrinological and genetic work-up, as appropriate.

A comprehensive semen analysis following the World Health Organization (WHO) guidelines is fundamental at the primary-care level to make a rational initial diagnosis and to select the appropriate clinical management. Collection and analysis of the semen must be undertaken by properly standardized procedures in appropriately qualified and accredited laboratories. The ‘basic’ semen evaluation should include: (1) assessment of physical semen characteristics (volume, liquefaction, appearance, consistency, pH and agglutination); (2) evaluation of sperm concentration, grading of motility and analysis of morphological characteristics (using strict criteria); (3) determination of sperm vitality (viability), testing for sperm auto-antibodies (using the mixed antiglobulin test and/or the direct immunobead test), presence of leukosperma and immature sperm cells; and (4) bacteriological studies. The identification and separation of the motile sperm fraction is also an integral part of the initial semen evaluation.

Clinicians and scientists are still searching for semen parameter thresholds in the so-called ‘normal fertile populations’ in order to be able to define fertility, subfertility and infertility more accurately. Recent publications have appropriately readdressed these issues as part of both European and American studies. In a recent publication, van der Merwe et al., reassessed
fertility/subfertility thresholds for normal basic sperm parameters by a thorough, structured review of the current literature. Results demonstrated new and lower threshold levels for fertility/subfertility. These cut-off values included a sperm concentration < 15 million/ml, progressive motility < 30% and < 5% normal morphology. These thresholds also fit data from the in vitro fertilization (IVF) and intrauterine insemination (IUI) settings.

There are multiple structural and biochemical sperm alterations that are present in subfertile men. Anatomically, they can be divided into: membrane alterations (that can be assessed by tests of resistance to osmotic changes, translocation of phosphatidylserine and others), nuclear aberrations (abnormal chromatin condensation, retention of histones and presence of DNA fragmentation), cytoplasmic lesions (excessive generation of reactive oxygen species, loss of mitochondrial membrane potential and retention of cytoplasm – with excessive creatine kinase content or the presence of active caspases) and flagellar disturbances (disturbances of the microtubules and fibrous sheath). Some of these alterations are indicative of immaturity, the presence of an apoptosis phenotype, infection-necrosis or other unknown causes.

Attention has shifted to the examination of sperm nuclear abnormalities. Currently, various tests are available for the detection of chromatin/DNA defects, including aniline blue staining, acridine orange, the sperm chromatin structure assay (SCSA), the assessment of DNA fragmentation and fluorescence in situ hybridization (FISH) for aneuploidy.

Notwithstanding their occurrence and correlation with clinical outcomes, it is not clear how these abnormalities directly influence sperm function, particularly gamete transportation, fertilization and contribution to embryogenesis. Furthermore, most such assays are still experimental, and more research is needed to validate their results in the clinical setting and to determine their true capacity to predict male fertility potential.

On the other hand, there are other specific and critical sperm functional capacities that can be more reliably examined in vitro. These functions include: motility, competence to achieve capacitation, zona pellucida binding and the acrosome reaction. The assessment of these and what is typically considered as sperm functional testing.

The extended semen analysis should include the preferential examination of these essential sperm functional attributes. These assays have been categorized into: (1) tests that examine defective sperm function indirectly through the use of biochemical means (i.e. measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others); (2) bioassays of gamete interaction (i.e. the heterologous zona-free hamster-oocyte test and homologous sperm–zona pellucida binding assays) and induced acrosome-reaction scoring; and (3) computer-aided sperm motion analysis (CASA) for the evaluation of sperm motion characteristics.

We reported an objective, outcome-based examination of the validity of the currently available assays based upon the results obtained from 2906 subjects evaluated in 34 published and prospectively designed, controlled studies. The aim was carried out through a meta-analytical approach that examined the predictive value of four categories of sperm functional assays (computer-aided sperm motion analysis or CASA, induced acrosome-reaction testing, sperm penetration assay or SPA and sperm–zona pellucida binding assays) for IVF outcome.

Results of this meta-analysis demonstrated a high predictive power of the sperm–zona pellucida binding and induced acrosome-reaction assays for fertilization outcome under in vitro conditions. On the other hand, the findings indicated a poor clinical value of the SPA as predictor of fertilization, and a real need for standardization and further investigation of the potential clinical utility of CASA systems. Although this study provided objective evidence based on which clinical
management and future research may be directed, the analysis also pointed out limitations of the current tests and a need for the standardization of present methodologies and the development of novel technologies.

Typically, male infertility presents clinically as an abnormal basic or extended semen analysis. Abnormalities in sperm indices may occur as an isolated parameter or as a combination of various parameters. Oligozoospermia and teratozoospermia are the most frequently observed isolated defects in our clinical practices, but more frequently, various degrees of oligoasthenoteratozoospermia (OAT) are present. Here, it is our aim to examine the causes and clinical management of the various single and multiple sperm defects.

**ISOLATED SPERM ABNORMALITIES**

**Decreased sperm concentration (azo-/oligozoospermia)**

Pathologies classified as ‘decreased sperm concentration’ range from mild oligospermia (<15 million sperm/ml) to azoospermia (no sperm in the ejaculate). On a simplistic basis, the clinically known causative entities can be subdivided into those of pretesticular, testicular and post-testicular origin.

A variety of endocrinopathies that disrupt the hypothalamic–pituitary–testicular axis constitute pretesticular etiologies of oligozoospermia. These endocrinopathies might be congenital (Kallmann’s syndrome) or acquired (prolactinoma, other hypothalamic–pituitary tumors and pathologies), and require the measurement of serum prolactin levels together with follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone for differential diagnosis in a patient with decreased sperm concentration. Further evaluation with assessment of other pituitary hormones (thyroid stimulating hormone (TSH), growth hormone, cortisol) and intracranial imaging systems (computed tomography (CT), magnetic resonance imaging (MRI)) is crucial in cases of hypogonadotropic hypogonadism.

Six to 24 months of treatment in patients with idiopathic hypogonadotropic hypogonadism, either with gonadotropins or pulsatile gonadotropin-releasing hormone (GnRH), frequently results in sperm indices sufficient for fertility in these patients. Patients with a diagnosis of prolactinoma respond rapidly to anti-dopaminergic agents. Because of their impressive therapeutic effects in patients with prolactinoma, these agents have also been tried in idiopathic oligoasthenozoospermia to improve sperm parameters. However, it has recently been shown in a meta-analysis that although they decrease serum prolactin levels further within the normal range, they are not helpful in improving sperm indices or fertility.

Post-testicular etiologies resulting in reduced or absent sperm output include a variety of obstructive lesions of the genital tract (inflammatory-infectious, congenital or iatrogenic, such as vasectomy) and ejaculatory disorders, particularly retrograde ejaculation. Retrograde ejaculation should be suspected in any case of azoospermia with low seminal volume, and might be congenital, acquired (prostatic and bladder-neck surgery, diabetes mellitus, inguinal lymph node excision) or idiopathic in origin.

Testicular causes include hypospermatogenesis due to a reduction in the number of germ cells, incomplete/complete maturation arrest of germinal cell differentiation and/or germinal cell aplasia. These entities are characterized by disturbances of spermatogenesis and/or an aberrant apoptotic process occurring during mitosis, meiosis and/or spermiogenesis/spermiation. Some of these pathologies are end results or the sequelae of viral infections, iatrogenic agents (chemo- and radiotherapy) and varicocele, as well as disturbances secondary to genetic/chemical/environmental aberrations. Nonetheless, it is our experience that in almost all such cases oligozoospermia is associated with moderate to severe
degrees of astheno- and teratozoospermia (see below).

**Decreased sperm motility (asthenozoospermia)**

Asthenozoospermia is defined as the presence of progressive motility < 30%\(^1\). Its origin can be iatrogenic, structural, functional, genetic or environmental. Possible causes of isolated asthenozoospermia include: iatrogenic reasons (improper handling of the semen sample), anti-sperm antibodies, infections, partial axonemal defects, sperm-tail fibrous sheath defects and poor development of the outer dense fibers, the presence of fewer mitochondria in the midpiece or even aplasia, sperm centriole dysfunction, carboxymethyl transferase enzyme deficiency and epididymal pathologies (typically associated with inflammation-infection)\(^{56-62}\).

The autosomal recessive-inherited immotile cilia syndrome\(^{63}\) and sperm mitochondrial DNA mutations\(^{64-67}\) have been identified as two gene-related causes of isolated sperm motility disorders. Recently, Bacci et al.\(^{68}\), reported a patient with severe isolated asthenozoospermia characterized by an absence of the fibrous sheath in the principal-piece region of the tail in the whole sperm population, which strongly suggests a genetic origin.

In patients with documented asthenozoospermia, the diagnosis work-up should emphasize repeated semen analyses in order to exclude inappropriate handling of the specimen as the cause. Repeated semen and urine cultures together with immunological tests should also be performed. Structural analysis of the sperm tail (flagellum) under transmission electron microscopy is the method of choice for diagnosis of immotile cilia syndrome in suspected patients with isolated severe asthenozoospermia.

It is worth mentioning that for isolated asthenozoospermia, many different sperm preparation techniques, with or without *in vitro* motility enhancers, have been tried. These agents have included pentoxifylline, 2-deoxyadenosine, kalileikrin, platelet-activating factor and some antioxidants\(^{69,70}\). Although different levels of improvement have been reported with these agents, none of them has truly gained acceptance for routine use in clinical practice.

**Decreased normal morphology (teratozoospermia)**

The importance of sperm morphology in male factor infertility has been demonstrated in multiple reports\(^{5,12,71-76}\) even though there is no complete uniformity in the definition of normal sperm morphology and teratozoospermia\(^{3,71,77,78}\). After the introduction and validation of strict criteria by Kruger et al.\(^{5}\), sperm morphology gained acceptance as the most important sperm parameter in the prediction of IVF outcome\(^{72,79}\). Later on, many studies demonstrated good correlation between sperm morphology and sperm functional tests such as zona pellucida binding assays\(^{84,85}\) and the zona-free hamster-oocyte penetration assay\(^{84,85}\). Poor morphology also correlates with abnormal sperm calcium influx\(^{86}\) and an abnormal acrosome reaction\(^{87}\). Its prognostic value has also been validated in IUI cycles\(^{88-90}\).

On the other hand, the pathophysiology of teratozoospermia is not completely understood. Numerical and structural chromosomal defects have been claimed in its pathogenesis. Investigations of spermatozoa from somatically normal men during meiosis using the FISH technique resulted in findings of a higher percentage of disomy, trisomy or tetrasomy for chromosome 1\(^91\), chromosome 7\(^92\), chromosome 8\(^93\), chromosome 13\(^94,95\), chromosome 18\(^92,93,96\), chromosome 21\(^94\) and the sex chromosomes\(^{91-93,95,96}\). Importantly, these abnormalities occurred mostly in populations with combined defects of sperm parameters (OAT) and infertility. The authors of these studies proposed that the effects of factors that impair sperm indices during gametogenesis extend to the cytogenetic constitution of spermatozoa. Conversely, some other studies could not find any
correlation between sperm chromosomal abnormality and fertility. \(^{77-99}\)

Harkonen et al.\(^{92}\) focused on isolated teratozoospermia and demonstrated higher frequencies of disomies 7, 18, YY and XY and diploidy in patients having <10% normal morphology. Calogero et al.\(^{93}\) found higher incidences of disomies 8, 18, X and Y in patients with isolated teratozoospermia and OAT, compared with men with normozoospermia. These authors suggested that teratozoospermia might be the critical sperm parameter associated with aneuploidy. The same group also showed an increase in sperm aneuploidy rate in patients with OAT, particularly in the presence of an elevated percentage of spermatozoa with enlarged heads.\(^{100}\)

On the other hand, Gole et al.\(^{101}\) found a higher incidence of sex chromosomal disomy in patients with OAT compared with teratozoospermic patients. Recently, Burrello et al.\(^{102}\) reported a higher aneuploidy rate for spermatozoa with abnormal head shapes from OAT patients, compared with normally shaped spermatozoa from normal men. Their results showed that normal morphology in patients with OAT does not rule out the presence of aneuploidy in selected sperm for ICSI. These results weaken the possibility of a direct causal relationship between isolated teratozoospermia and sperm chromosomal abnormalities. However, there is consensus in the literature that infertile men and/or men with poor sperm indices carry a higher frequency of aneuploidy in their spermatozoa. More studies are needed to identify the effects of different chromosomal aberrations on different sperm parameters/functions.

There is also substantial evidence in the literature supporting that deregulation of specific genes might play a role in the appearance of morphological abnormalities in ejaculated spermatozoa. It has been shown in a mouse model that \(azh\) mutations (abnormal spermatozoan head shape) on chromosome 4 might cause specific structural changes in the sperm head.\(^{103,104}\) Adham et al.\(^{105}\) showed the development of sperm head abnormalities in mice containing \(Tnp2\) (transition protein

2) gene disruption, which takes part in the nuclear organization of spermatozoa. Xu et al.\(^{106}\) also demonstrated that male mice lacking a regulatory protein in the process of spermatogenesis (protein caspin kinase 2 \(\alpha,\) Csnk2a) due to Csnk2a gene disruption performed by transgenesis were infertile, with globozoospermia (acrosomeless sperm). In addition, the altered expression and arrangement of some cytoskeletal proteins (calcin, protein 4.1) has been associated with aberrant morphological changes during spermiogenesis.\(^{107,108}\) Recently, Milatiner et al.\(^{109}\) demonstrated a correlation between the severity of teratozoospermia in infertile men and changes in the nucleotide structure of the androgen receptor gene.

**COMBINED SPERM ABNORMALITIES: OLIGOASTHENOTERATOZOOSPERMIA**

As mentioned above, OAT is the most common clinical presentation of male infertility. It is typically the reflection of abnormal (testicular) spermatogenesis but it can also be due to post-testicular etiologies. Approximately half of clinical cases, however, still remain idiopathic.

There are numerous known spermatogenesis defects leading to OAT.\(^{76,74,110-114}\) They include: germ cell anomalies (depletion, aberrant apoptosis, defective differentiation), mitotic and meiotic defects and alterations of spermiogenesis/spERMiation. Aberrant apoptosis has been observed at the primary spermatocyte and spermatid levels and also in Sertoli cells. Arrest or quantitatively abnormal spermatogenesis at any stage may result in oligozoospermia. Meiotic alterations and spermiogenesis defects are probably associated with teratozoospermia.

The concept of sperm immaturity has gained acceptance. Retention of cytoplasm (including retention of organelles and enzymes participating in metabolism, apoptosis and other functions that become exaggerated) is probably the result of an abnormal Sertoli cell–late spermatid interaction, leading to the release of dysmorphic, dyskinetic
and dysfunctional spermatozoa\(^{15,19-21,23,118,119}\), (Figure 21.1). Abnormalities of sperm release from the seminiferous tubules (or spermatiation) are also probably present in some cases. Epididymal dysfunctions or pathologies can also influence sperm membrane domain constitution and may induce morphogenic/dysfunctional changes\(^{120}\).

**CLINICAL MANAGEMENT**

The treatment plan should be constructed based upon complete identification of both male and female factors (Figure 21.2). In the presence of pure male infertility (no identifiable female factors), therapy may be: (1) medical (endocrine such as in hypogonadism or hyperprolactinemia, antibiotics in case of infection); (2) urological (surgical or non-surgical treatments, such as conventional, microsurgical or laparoscopic surgery, including correction of varicocele, epididymo-vasovasostomy and modern approaches for ejaculatory disorders); and/or (3) low- or high-complexity assisted reproductive technologies (ART). The severity of male subfertility and some important prognostic risk factors in the female (e.g. age, duration of infertility, presence of endometriosis and other pathologies) may accelerate the indication for ART.

It is our opinion that, at the present time, there is no clinical role for the ‘empirical’ use of medical treatments of normogonadotropic subfertile men with idiopathic OAT. In the absence of a defined medical indication, there are no evidence-based data to support the use of gonadotropins, anti-estrogens, antioxidants, multivitamins or other unproven therapies.

Currently recommended ART options include: ‘low-complexity’ IUI therapy, ‘standard’ IVF and embryo transfer, and IVF augmented with ICSI. If the female partner is aged <35 years, typically 4–6 cycles of IUI using the husband’s sperm in combination with controlled ovarian hyperstimulation are recommended as a simple (low-complexity) ART approach, particularly if >1 million motile sperm can be recovered\(^{80,121}\).

Preliminary data suggest that in order to increase cost-efficiency and loss of valuable time, IUI should not be performed if the total mobile recoverable fraction is 42\(^{122}\), if the calcium ionophore-induced acrosome reaction is ≤22\(^{123}\), if the zona pellucida-induced acrosome reaction is <16%\(^{87}\) and/or if the proportion of sperm depicting DNA fragmentation is >12\(^{124}\).

Patients with a motile sperm fraction of <5 million motile spermatozoa following swim-up or gradient centrifugation, but with mild to moderate teratozoospermia (in the range 4–14% normal forms by strict criteria), may be offered ‘standard’ IVF therapy. In those cases, good fertilization and pregnancy rates are achieved with an increase in the sperm insemination concentration\(^{125,126}\). However, nowadays, these patients are offered ICSI in an effort to eliminate any risk of low or failed fertilization, or a combination of IVF and ICSI (in sibling oocytes) in the group with sperm morphology >14% normal forms, dependent on the individual IVF unit.
Figure 21.2 Algorithm for clinical management of the subfertile man. COH, controlled ovarian hyperstimulation; IUI, intrauterine insemination; ART, assisted reproductive technologies; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; TESE, testicular sperm extraction; MESA, microsurgical epididymal sperm aspiration.

In our programs, patients are selected for ICSI according to the following indications\textsuperscript{7,127}:

- Poor sperm parameters (i.e., <5 × 10\textsuperscript{6} total spermatozoa with adequate progressive motility after separation and/or severe teratozoospermia with <4% normal forms in the presence of a borderline to low total motile fraction);
- Poor functional abilities, including a defective sperm–zona pellucida binding capacity with a hemizona assay index < 30%\textsuperscript{82,128} and/or a low (<16%) zona pellucida-induced acrosome reaction or ZIAR\textsuperscript{87,129,130};
- Previous failed fertilization in IVF;
- Failure of IUI therapy in cases presenting with moderately abnormal sperm parameters (5–10 × 10\textsuperscript{6} total spermatozoa with adequate progressive motility after separation or morphology in the range of 5–14%), and also
including the presence of antisperm antibodies;

- Presence of obstructive or non-obstructive azoospermia, where ICSI is combined with sperm extraction from the testes or the epididymis\(^7,127,131-134\).

- In the presence of severe oligoasthenoteratozoospermia or if the outcome of sperm function testing indicates a significant impairment of fertilizing capacity, couples should be immediately directed to ICSI. This approach is probably more cost-effective and will avoid loss of valuable time, particularly in women > 35 years.

Based on currently available data, we estimate that ICSI should be indicated when male infertility is properly diagnosed based upon a state-of-the-art extended evaluation of the male partner, and also in cases with previous failed fertilization. Published prospective, randomized studies have demonstrated that it is not beneficial to perform ICSI in non-male infertility or unexplained infertility cases. Altogether, there are no data to suggest that ICSI should be performed in all cases of in vitro conception (reviewed in references 135 and 136). Consequently, to perform ICSI in all cases on a purely pragmatic basis appears to be a significant departure from principles of evidence-based medicine.

Greco et al.\(^137\) recently reported that ICSI with testicular spermatozoa provides the first-line ART option for men with high levels of DNA damage in ejaculated sperm. Nonetheless, more studies are needed clinically to validate methods of assessing DNA damage and the impact of DNA abnormalities on clinical outcomes.

Sperm cryopreservation represents a valuable therapeutic option in the management of male infertility. Current indications include: (1) mandatory use in artificial insemination programs with donor semen; (2) patient’s convenience (i.e. partner’s absence where IUI is performed in the presence of normal sperm parameters); (3) preservation of reproductive capacity in men with various types of neoplasias before undergoing radical surgery and/or radio-chemotherapy\(^138\); (4) aiding in the management of infertile men undergoing vasectomy reversal (vasovasostomy) or epididymovasostomy, when ‘banking’ may provide a future sperm source for possible use in IUI or ICSI therapies; and (5) because of the outstanding success with ICSI, even infertile men with different degrees of oligoasthenoteratozoospermia can now be offered the use of cryopreserved–thawed spermatozoa for assisted fertilization. Today, this applies not only to ejaculated but also to testicular and epididymal spermatozoa recovered for the purpose of ICSI\(^139,140\).

Interesting and challenging concepts to be applied to future treatment modalities of male infertility are germ cell transplantation and in vitro spermatogenesis\(^141,143\). Further progress in the identification of spermatogonial stem cells and techniques of germ cell transplantation\(^144\), in addition to the optimization of culture systems for in vitro spermatogenesis\(^145\), may give new options to patients with azoospermia.

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Conclusion:

In this chapter the pathophysiology of sperm morphology was highlighted. It was shown that normal sperm morphology is needed for normal sperm function; that is, zona pellucid-sperm binding, acrosome reaction, release of acrosin, and zona pellucid penetration and sperm decondensation. The extended semen analysis was discussed with the clinical role of the HZA as a sperm functional assay, highlighted.
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Chapter 4
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SUMMARY

Chapter 4

The publications on computerised strict sperm morphology were novel in this field. In 1993, the first publication in the international literature followed after research at Stellenbosch University and Tygerberg Hospital on the correlation between the computer and the manual method (Kruger et al., 1993).

The aim of the first research was to correlate Strict Criteria (manual method) with Strict Criteria evaluated by computer using image analysis (computer method). What was interesting was the finding of an excellent level of agreement between the human and the computer. The correlation was the same as if two experienced human readers were evaluating the sperm morphology slides. Using various statistical methods it was reported that an excellent measure of agreement was reached. These observations set the stage for studies between the computer method and the manual method.

The first publication on sperm morphology not only dealt with technical aspects of sperm morphology evaluation but also with clinical outcome (Kruger et al., 1993). Studying fertilization rates in patients with sperm morphology <14% and >14% normal forms in two centers, a significantly lower fertilization rates in the group below 14% normal forms highlighted the fact that a new diagnostic tool to predict fertilization in vitro was now available.
Automated Strict Criteria for sperm morphology

a. Correlation of manual evaluation of sperm morphology with the automated electronic computerized method (computer method) and the potential pitfalls.

i. Novel publications on computerized strict morphology

The publications on computerized strict sperm morphology were novel in this field. In 1993 the first publication in the international literature appeared following research at Tygerberg Hospital using the automated electronic computerized method (computer method) and determining the level of agreement with manual evaluation of sperm morphology (manual method).

The aim of the first research was to correlate Strict Criteria (manual method) with Strict Criteria evaluated by computer using image analysis (computer method). What was interesting was the finding of an excellent level of agreement between the human and computer. The correlation was the same as if two experienced human readers were evaluating the sperm morphology slides. Using various statistical methods it was reported that an excellent measure of agreement was reached. These observations set the stage for studies between computer and human.

It was highlighted that:

- Careful slide preparation and perfect microscopic focusing are of utmost importance to get excellent results.
- Slide preparation with no background clutter is also mandatory to obtain good results.
- On repeatability of computer; three repeat readings also reached the excellent category making this development clinically acceptable for day to day use as well as for research purposes.
- The time involved per slide with the system tested was also comparable with the manual method (3min 53 sec. vs. 5 min 39 sec.). Although statistically significantly faster manually, the computer method on average took 1 min 46 sec longer. The advantages of continuous throughput and ruling out the human factor offered huge advantages. The stage was set for
a new era in morphology reading. The above mentioned findings were published in the following publications.
The following article reflects the scientific basis that supports the above argument:

Urology-andrology

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A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading

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Objectives: To compare the ability of a computerized method of sperm morphology with the manually recorded method in predicting in vitro fertilization (IVF) results, to compare results obtained by both methods, and to determine the intraobserver variability.

Design, Setting, Patients: Forty-three semen slide preparations from two large level-three academic institutions’ reproductive endocrinology units (IVF programs) were blindly evaluated, and the sperm were classified into normal and amorphous forms.

Results: Experiment 1: Twenty-one slide preparations from the Tygerberg gamete intrafallopian transfer program were manually evaluated; the fertilization rates for the groups with <14% and >14% normal sperm forms were 53.3% (15/45 oocytes) and 78.6% (46/60 oocytes), respectively. Corresponding fertilization rates with FERTECH were 46.8% (30/64) and 75.8% (31/41). Experiment 2: Twenty-two slide preparations from the Norfolk IVF program were evaluated. The manual method reported a fertilization rate in the group with <14% normal forms of 27.4% (14/51 oocytes) compared with 90.0% (127/141 oocytes) in the group with >14% normal forms. Corresponding figures for the FERTECH method were 33.9% (18/53) and 88.4% (123/139), respectively. Experiment 3: When the 43 slide preparations were blindly evaluated using both methods, 84% of the FERTECH evaluations correlated well with the manual method and FERTECH ability to diagnose the subfertile male (<14% normal forms) was 95% (sensitivity). Experiment 4: A total of 16 different slides (8 per group) were randomly selected and analyzed five times (100 cells per reading) by a computerized method. The slides were obtained from men with normal sperm morphology of <14% and >14% as classified by the manual method. In the first group (<14%) 97.5% (30/31) of the readings classified the sperm in the proper category, whereas in the second group (>14%) 95% (38/40) of the cases were correctly identified.

Conclusion: Using strict criteria for morphology evaluation, there is a positive and significant correlation between FERTECH evaluation and manual assessment. The reproducibility of the computerized method and the ability to distinguish between fertile and subfertile groups using those criteria are good. Fertil Steril 1993;59:202-9

Key Words: Sperm morphology, strict criteria, computerized method, in vitro fertilization

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In the current literature, evidence supporting the crucial role of sperm morphology evaluation in the prediction of human fertilization in vitro is well documented (1-5). A clinically significant threshold

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of 14% normal forms when strict criteria are used has been described and validated (1, 2, 6). In vitro fertilization (IVF) rates were found to be significantly impaired in cases with normal sperm morphology below threshold (<14%). The impairment was even more significant when the number of normal sperm forms was below a second lower threshold of 5% (poor prognosis pattern) (2). In a prospectively designed blind study, multiple regression analysis demonstrated normal sperm morphology (percentage of normal sperm forms) to be the only semen parameter accurately predicting fertilization in vitro in the human (2). These studies demonstrated also that other sperm characteristics are not as valuable as morphology in predicting fertilization (1, 2).

The availability of an objective, reproducible method to evaluate human sperm morphology using strict criteria is of utmost importance to assist clinicians and scientists working in assisted reproduction programs. Over the last 2 years, these strict criteria were integrated into the software program of a computerized system (FERTECH, Norfolk, VA).

In this study, we evaluated sperm morphology using strict criteria comparing a new computerized sperm morphologist (FERTECH) with the manually recorded method. The specific objectives were as follows: [1] to determine the ability of both methods to predict IVF results; [2] to compare results obtained by both methods; and [3] to determine the intraobserver variability.

MATERIALS AND METHODS

Manual Assessment of Sperm Morphology

Semen specimens were obtained from 21 patients from the Tygerberg Clinic and 22 patients from the Norfolk program attempting IVP-gamete intrafallopian transfer (GIFT). Samples were obtained by masturbation after 2 to 4 days of sexual abstinence and were prepared for morphology evaluation after liquefaction was complete (<30 minutes) and within 1 hour of collection. Morphology slides were prepared as follows: each slide was cleaned thoroughly with 70% ethyl alcohol before use; no more than 5 μL of semen was used so the smear was as thin as possible. The 5-μL drop was pulled rather than pushed across the slide to avoid artificially detached heads. The slides were air dried at 37°C in a warm tray and stained using the Papanicolaou method (6). All slides were read at a magnification of ×1,000 by a trained observer (T.F.K.) using the strict criteria as previously published (1, 2, 6). In our laboratories, this method of evaluation has intratechnician and intertechnician coefficients of variation (CVs) of <10%, with Spearman’s correlation coefficients of 0.965 and 0.867 (P < 0.0001) for results of multiple observations of the same sample by one observer and different observers, respectively (6, 7).

Sperm Morphology Analyzer (FERTECH)

FERTECH is a computer-based imaging system programmed to implement a morphological sperm cell analysis partly based on strict criteria. FERTECH differs from the manual method in that it does not identify tail defects. It takes midpiece and neck defects indirectly into consideration.

The computer-assisted image analysis system included a light microscopy set with 100X objective (Nikon 100/1.25; Research Instrumentation, Johannesburg, South Africa) fitted to a Nikon Optiphot X-2 brightfield microscope (Research Instrumentation). The total magnification used was ×1,000 with a video camera attachment (National Panasonic WV-CD 130; Research Instrumentation). The video camera was linked to an AT (or 386) personal microcomputer, together with a video monitor (National Panasonic BT M1400 PSN, Research Instrumentation).

The computer was first programmed by one of us (T.F.K.) to classify the cell characteristics (shape and acrosome). This was first done for individual cells, and thereafter the program was evaluated and adjusted on a cell-by-cell basis (batch processing many cells without individual supervision). In developing the prototype of FERTECH, correlation of normal and abnormal forms was compared between FERTECH and the manual method. Morphometric adjustments were made to the image processing program to achieve a correlation on a cell-by-cell basis between FERTECH and the manual technique (T.F.K.).

Once the program was satisfactorily trained, it was not changed during the course of the experiments. We have considered the image processing program acceptable after it correlated with the manual method’s ability to classify sperm in the <14% and >14% normal morphology groups. Sperm characteristics and classification guidelines were fed to the FERTECH program as described.

Sperm cells on prestained slides were randomly chosen in a given microscopic field by the trained observer (T.F.K.). These cells were then analyzed by the FERTECH. The same spermatozoa were analyzed separately by FERTECH and technician during the two clinical studies. FERTECH is given a 512 × 512 pixel image to analyze (256 gray levels).
In this image, there may or may not be sperm cells. The first step is to locate all the objects (detection) in the image. Thereafter, each object is carefully separated from its background (segmentation). Objects that are too close to the image's borders, too large, or too small are ignored. This can lead to a biased interpretation of the morphometric analysis of those few sperm falling outside the criteria outlined by Kruger et al. (1, 2) for normal sperm. Each of the remaining cells is then analyzed in two ways: the shape is extracted and evaluated according to the strict morphology guidelines, and the presence and extent of the acrosome is evaluated. Depending on these characteristics, a cell is classified into one of two groups: normal forms and amorphous forms. Although cytoplasmic droplets and midpiece/tail attachments were not analyzed individually, they have a definite influence on the cell image. Therefore, their contributions were indirectly evaluated.

Two electronic cards were slotted into the extended bus of the AT computer. The first one is a frame grabber card that captures an image from the video camera, presents it to the computer, and displays it on the video monitor. A Data Translation® DT1851 product was used for this purpose. The second computer card is a transputer high speed processor card with one T800 processor and 2 MB memory.

**Performance**

The transputer processor card enables relatively fast processing of the images. The FERTECH morphologizer processes a patient (100 cells) in approximately 20 minutes. The computer program is designed to reject cells that are not in focus, objects or white blood cells that are much larger or smaller than the typical sperm cell, and artifacts. When a cell is not in focus, it lacks sharp edges in the image. By evaluating the sharpness of the edges, the FERTECH discriminates between objects in and out of focus. The morphometric information of an ideal sperm cell adheres to the previously published studies on strict criteria. Acrosomes are evaluated according to their relationship (proportion) to the head's size (1, 2).

**Experimental Design**

Experiments 1 and 2 used comparative analysis of the manual method and the FERTECH morphologizer as predictors of fertilization rates. In this prospectively designed blind study, semen samples of 21 patients from the Tygerberg GIFT/IVF program were analyzed. One hundred sperm cells on the slide preparation from each semen specimen were evaluated both manually and by the FERTECH morphologizer. Only those couples whose excess oocytes from the GIFT program were available were allocated into the study group, so that the fertilization rates of those oocytes could be used as an end point. For the cooperative Norfolk-Tygerberg study, 22 semen samples from the IVF program were blindly evaluated.

**Ovarian Stimulation**

In the Tygerberg program, a combination of clomiphene citrate and human menopausal gonadotropin (hMG) were used for ovarian stimulation (1). In the Norfolk program, a combination of follicle-stimulating hormone, hMG (Pergonal; Serono Laboratories, Randolph, MA), and a gonadotropin-releasing hormone agonist leuprolide acetate; Lupron, TAP Pharmaceuticals, North Chicago, IL) was used (8). These patients were monitored by serum estradiol and luteinizing hormone determinations, as needed, as well as by serial ultrasonographic measurements of the developing ovarian follicles. Administration of human chorionic gonadotropin, 10,000 IU, was performed at Tygerberg when the leading follicle had reached 18 mm in diameter and in Norfolk when it was 16 mm in size. Follicle aspiration was carried out 36 hours thereafter.

**Gamete Intrafallopian Transfer Procedure**

Translaparoscopic follicle aspiration was performed in Tygerberg under general anesthesia. Pneumoperitoneum was achieved by insufflation of CO₂. The recovered oocytes were evaluated for maturation stage (metaphase I or II) according to the criteria published by Veeck (9). Four metaphase II oocytes were randomly selected at the end of follicular aspiration. Two oocytes were then loaded into the transfer catheter together with 100,000 motile sperm (sperm concentration was increased to 500,000 motile/mL in the male factor patients when the morphology was <14% normal forms) (10). The loaded catheter was passed through the second puncture cannula used for aspiration and was inserted through the fimbriated end of the fallopian tube to a depth of 2 cm. Gametes were deposited into the tube, and the procedure was repeated on the other side.

All excess metaphase II oocytes were inseminated 5 hours after retrieval using the same criteria in terms of sperm density and placed in Ham's F-10 media.
culture medium (GIBCO, Grand Island, NY) supplemented with 10% maternal serum. In the Tygerberg program, 100,000 spermatozoa were used to inseminate all excess eggs if the normal morphology was >14% normal forms and 500,000 spermatozoa were used per oocyte if <14% normal forms were observed in the laboratory. Incubation was performed in 5% CO₂ in air with 95% humidity at 37°C. Laboratory procedures used in the Norfolk IVF program have already been described in detail (2, 10). The same insemination procedure was used as in the Tygerberg program.

Preparation and Morphological Evaluation of Spermatozoa Collected on the Day of GIFT and IVF Procedures

The patient was required to produce a semen sample 1½ hours before laparoscopy was performed. Semen was then prepared according to a double-wash, double-centrifugation, and swim-up technique (1).

The slides for morphology evaluation were prepared on the first semen sample according to techniques already mentioned (1, 2). According to the strict criteria, the spermatozoon is considered normal when the head has a smooth, oval configuration with a well-defined acrosome comprising about 40% to 70% of the sperm head. In addition, there must be no neck, midpiece, or tail defects, and no cytoplasmic droplets of more than one half the size of the sperm head (1, 2, 6). Borderline morphological aberrations are classified as abnormal forms. Infertility etiology was considered to involve a male factor when the percentage of normal sperm forms was <14% (1, 2).

Papanicolaou-stained slides of each sample were morphologically evaluated blindly using two methods: [1] manual reading by one investigator (T.P.K.) and [2] FERTECH sperm morphologist analysis. Observations were recorded under brightfield illumination and a 100X oil immersion objective, with a total magnification of 1,000X (Nikon Optiphot 2-X). Spermatozoa were classified by the two methods in two different categories: normal and amorphous forms (2). Patients were then divided according to the morphological categories in two different groups based on previous investigations (1, 2, 11), namely: <14% normal forms and >14% normal forms. The data were also reclassified according to a different threshold that has been used in other investigations (2), namely <10% and >10% normal forms. After the grouping of all semen samples according to the above mentioned criteria, the fertilization rates of the excess mature metaphase II oocytes (GIFT cases) and all IVF metaphase II oocytes were recorded and analyzed.

Experiment 3

Comparative analysis of sperm morphology as observed by the manually recorded method and FERTECH was determined. The forty-three fresh semen samples were evaluated by the manual method (gold standard) and the computerized method, and the outcome was recorded. The aim was to correlate FERTECH’s ability to classify a patient into the two groups of <14% and >14% normal sperm forms.

Experiment 4

Intra-assay variation of FERTECH was established. Eight slides from the groups of <14% and >14% normal morphology were randomly selected, and the reading was repeated five times per slide, using 100 cells per reading.

Statistical Analysis

In experiments 1 and 2, χ² analyses were used to evaluate the ability of both methods to predict IVF results. In experiment 3, a 2 × 2 contingency table was used to establish the sensitivity, specificity, and predictive value of the FERTECH analysis. The Bland and Altman procedure was used to compare the methods of determining the percentage normal sperm morphology by the two techniques.

RESULTS

Experiment 1 (Tygerberg Study)

When manual evaluation was used to enable normal morphology to classify the semen samples, the fertilization rate of excess oocytes in the group with <14% normal forms by strict criteria was 33.3% (15/45 oocytes); a significantly higher (P = 0.001) fertilization rate of 76.6% (46/60 oocytes) was found in the group with >14% normal forms. Corresponding figures for FERTECH were 46.8% (30/64 oocytes) and 75.6% (31/41 oocytes) (P = 0.001) (Fig. 1A).

There was no significant difference in the fertilization rates, neither in the group with <14% normal forms (33.3% and 46.8%, respectively) nor in the group with >14% normal forms (76.6% and 75.6%) when the manual and FERTECH methods were used.

When a cutoff figure of 10% normal forms was used in the manual method, the group with <10%
forms showed a significantly lower fertilization rate of 12.9% (4/31 oocytes) as compared with the group with >10% normal forms, which had a fertilization rate of 77.0% (57/74 oocytes) \( (P = 0.001) \) (Fig. 1B). Similar groups identified by FERTECH showed a fertilization rate of 20.6% (6/29) and 72.3% (55/76), respectively, \( (P = 0.001) \). Again, no significant differences in the fertilization rate were found in the groups identified by manual examination and by FERTECH.

**Experiment 2**

Twenty-two microscopic slide preparations from the Norfolk IVF program were evaluated. When the manual method was used, fertilization rate in the group with <14% normal forms was 27.4% (14/51 oocytes) as opposed to 96.0% (127/141 oocytes) when the normal forms were >14% (Fig. 2A). If the 10% cutoff figure was used, fertilization rates were 20.5% (7/34) and 84.8% (134/158) (Fig. 2B). There was a highly significant difference between the fertilization rate of the groups \( (P = 0.001) \).

When FERTECH was used for evaluation, the group with lower than 14% normal forms showed 33.9% fertilization rate (18/53 oocytes), significantly lower than the rate of 88.5% \( (123/139) \) \( (P = 0.001) \) in the group with >14% normal forms (Fig. 2A). Corresponding figures for the two methods when the threshold value of 10% was used were 18.1% (6/33 oocytes) and 84.9% (135/159 oocytes), also significantly different \( (P = 0.005) \) (Fig. 2B). No significant differences were found in the fertilization rates of the groups identified by both methods using either one of the thresholds.

**Experiments 1 and 2**

Of the 43 patients, only 7 patients did not correlate between the manual method and FERTECH (Table 1). Six of the 7 patients (patients 1 to 6, Table 2) were classified by FERTECH into the group <14% in contrast with the normal evaluation that was given by the manual method, and 1 patient (no. 7) was classified in the group >14% normal by FERTECH compared with the <14% classification by the manual method (Table 2). On the other hand, patients 1, 2, 4, and 6 were incorrectly classified (because of fertilization rates), whereas patients 3 and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparative Analysis of Results Obtained by the Manual Method and Computerized Morphologyzer (Strict Criteria)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;14%</td>
</tr>
<tr>
<td>Manual method</td>
<td></td>
</tr>
<tr>
<td>&lt;14%</td>
<td>18</td>
</tr>
<tr>
<td>&gt;14%</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>

* Sensitivity, 0.95; specificity, 0.75; overall correct, 0.84.
Table 2  Comparison Between Sperm Morphology Groups and Fertilization Rate per Oocytes as Recorded by FERTECH and the Manual Method of Seven Misclassified Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>FERTECH Morphology group</th>
<th>Manual Morphology group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;14%</td>
<td>&gt;14%</td>
</tr>
<tr>
<td>1</td>
<td>3/4 (75)*</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>5/6 (83)</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>0/6 (0)</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>7/8 (87)</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>0/6 (0)</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>4/4 (100)</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>0/2 (0)</td>
</tr>
</tbody>
</table>

* Values in parentheses are percents.

5 were theoretically correctly classified because of the poor fertilization rates recorded. Thus, 5 of 7 were correctly classified by the manual method compared with the 2 of 7 by FERTECH. Patients 3 and 5 did not fertilize and were thus theoretically correctly classified by the FERTECH method. Patient 7 also did not fertilize but was classified by FERTECH in the category > 14% normal forms.

Experiment 3

Using the manual method as the gold standard for the evaluation of sperm morphology with strict criteria, a sensitivity of 95% was found for the FERTECH method when the ability to identify patients with low fertilization potential was used as end point (i.e., <14% normal sperm forms). The percentage of normal morphology recorded by FERTECH and the manual method illustrated that the mean difference between the two procedures (manual versus FERTECH) was 1.84% (SE = 1, 26). This difference is not significantly different from 0 (P = 0.1538). The mean difference is a difference of the bias between the two methods, and there is no significant bias between them. Thus, when comparing the mean morphology between two groups, both methods will give the same estimates of the mean morphology levels of the groups. The limits of agreement are the confidence intervals for individual differences. In this comparison, the limits are wide (Fig. 3, Bland and Altman limits of agreement). Of the 43 cases, 2 exceeded the Bland and Altman limits of agreement. Using the manual method as the gold standard for the evaluation of sperm morphology with strict criteria, 18 of 19 patients were correctly classified by FERTECH in the group < 14% normal morphology; thus a sensitivity of 95% was found for the FERTECH method. The predictive capacity of the computerized method for patients with normal fertilizing potential, those with normal morphology > 14% normal forms, was as follows: 18 of 24 were in agreement with the manual methods (specificity of 75%), and 6 of 24 were not in agreement. The overall accuracy of the method was 84% (Table 1).

Experiment 4

Slide preparations from eight patients in the group with normal morphology > 14% and from eight patients in the group with normal morphology < 14% were randomly chosen, and five consecutive evaluations of sperm morphological characteristics were performed by FERTECH. One hundred cells from each slide preparation were read for a total of 4,000 cells per group. The intra-assay variation in the groups with normal morphology > 14% and <14% was evaluated showing reproducibility rates of 95% and 97.5%, respectively (Table 3). The overall mean was 18.1% among the slides. The variance within slides was 21.4. An intraclass correlation coefficient (P1) was 84%. The 95% confidence interval (CI) for P1 was 0.66 <P1 <0.97. The measurement of percent

Table 3  Intra-Assay Variation of FERTECH

<table>
<thead>
<tr>
<th>Normal morphology</th>
<th>&gt;14%</th>
<th>&lt;14%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient readings</td>
<td>8 x 5*</td>
<td>8 x 5*</td>
</tr>
<tr>
<td>(4,000 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>38/40 (95)†</td>
<td>39/40 (97.5)</td>
</tr>
</tbody>
</table>

* 8 different patients (slides), 5 times repeated; 100 cells/slide.
† Values in parentheses are percents.
of normal sperm morphology can be interpreted as reliable because the variance within slides is relatively homogeneous.

DISCUSSION

The identification of sperm with low fertilization potential, and for that matter, even with no fertilization potential is of utmost importance in programs of assisted reproduction (GIFT-IVF). It is not only helpful to the clinician to establish the correct prognosis and give proper advice to the patient but also to the scientist responsible for the IVF-embryology laboratory that uses the baseline andrologic evaluation to take appropriate corrective measures to enhance fertilization in vitro (10). Previous publications from our groups demonstrated that evaluation of sperm morphology by strict criteria is the best parameter to predict the sperm fertilizing ability in the in vitro system (1, 2, 10). These observations have now been confirmed by other investigators from different centers (3, 4, 5). Because of our previous research, it was shown that an increase in sperm concentration will enhance the fertilization rate. It is now a policy at both Tygerberg Hospital and the Norfolk program to increase the sperm concentration in cases of sperm morphology <14% normal forms. The patients are aware of these corrective measures, and it is our intention to try to improve the fertilization rate in these cases. For that reason, we could not standardize the insemination concentration for the purpose of this study. Despite this fact, there was still a difference in the fertilization rate when we looked at the two morphological groups <14% and >14% normal forms.

Wang et al. (12, 13), in recent publications, raised the question whether computer-assisted morphological evaluation of the sperm could be used to predict the results of IVF of human oocytes. In this study, we have tested for the first time the ability of a new computerized morphometric sperm analyzer to correctly evaluate sperm morphology according to strict criteria and to classify samples within previously established categories that have shown significantly different fertilizing capacity in the human IVF system. The predictive ability of FERTech was even enhanced when a threshold of 10% was used instead of the 14% previously established.

In a previous publication by Kruger et al. (2) it was clearly shown that if normal morphology was <5%, fertilization was markedly impaired (only 7.6% of all oocytes fertilized when inseminated using the regular protocol of 50,000 motile sperm/mL per egg).

No attempt was made here to subdivide the group with <14% normal sperm forms into the poor prognosis and good prognosis patterns as we did in the study mentioned above because very few of the slides fell into the poor prognosis pattern category.

Of the 43 patients investigated (43 slides evaluated), 84% (36/43) of the computer's ability to classify a sperm sample as <14% or >14% correlated well with the principal investigator's manual classification. This seems to be an acceptable variability when compared with the observed intertechnician variation in the laboratory (7). One of the main problems in morphology classification of a given semen sample is the large interobserver and intraobserver CVs between different laboratories. In our own laboratory in which we adhere to specific and stringent principles to distinguish between normal and abnormal forms, small CVs have been obtained (6, 7).

The ability to identify patients with poor fertilization potential (95% sensitivity) illustrates the power of this system. This new development in the evaluation of morphology will be extremely helpful in solving the differences between laboratories and even between technicians and will allow to properly classify specimens that, because of normal sperm concentration and motility and an inaccurate evaluation of morphology, could have been considered normal. When the female partner in these cases shows no oocyte factors, the couple may be classified as unexplained lack of fertilization, when in truth, it is an occult male factor (14). The diagnosis in these patients can be substantially improved if the laboratory has the ability to adequately diagnose the subfertile male because of morphology problems only.

The manual method of evaluating sperm morphology by strict criteria in experienced hands has a very low intraobserver and interobserver variability (6, 7). It is important in the clinical arena to get reliable reports, and in this context, the availability of a computerized system such as FERTech with very high reproducibility (97.5% in the <14% normal forms group, and 98% in the >14% normal forms group) is extremely useful.

In contrast to other reports (12, 13), FERTech shows an advantage over current morphologists because the time needed to analyze 100 cells (20 minutes) compares very well with that used by trained technicians in our laboratories to accomplish a similar task. This computerized objective method saves a considerable amount of technician time and gives reliable and reproducible reports that are clinically useful. The ability of the method to diagnose
the true normal spermatozoon and to differentiate it from sperm with minor and major abnormalities are the basis of its ability to classify the sample properly and to predict adequately fertilization in the in vitro system.

The method designed in our laboratory and described in the present work is very promising, and this development may allow most andrology laboratories working in the field of assisted reproduction and male infertility to use the strict morphology criteria in a similar manner and in a more accurate way. Furthermore, it has a good predictive ability for fertilization in vitro and allows a more objective evaluation of the male gamete fertilizing potential. To our knowledge, this is the first study in the literature incorporating the use of the strict criteria into a computerized system.

Acknowledgment. The authors are grateful to Ms. Pauline M. Clynes for her editorial assistance in the preparation of this manuscript.

REFERENCES


The following article reflects the scientific basis that supports the above argument:

Sperm morphology: assessing the agreement between the manual method (strict criteria) and the sperm morphology analyzer IVOS*†

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Thomas C. du Toit, M. Eng. † Carl J. Lombard, Ph.D. *
Daniel R. Franken, Ph.D. †

University of Stellenbosch, and Institute for Biostatistics, Medical Research Council, Tygerberg, Republic of South Africa


Design, Setting, Patients: Slides from 30 different patients from the Tygerberg IVF program were selected randomly. Microscopic fields and sperm cells were chosen randomly and percent normal morphology was recorded (objectives 1 and 2). The same slides were used and a cell-by-cell repeatability was done as outlined (objective 3).

Results: Experiment 1 (objective 1): there was no significant bias between T.F.K. and R.M. The limits of agreement were 8.6% and −7.3%. The SDs were not significantly different (P = 0.1283). The Spearman correlation coefficient between readers was 0.83. Experiment 2 (objective 2): the same findings were reported but the limits of agreement were 12.1% and −15.5%. The Spearman correlation coefficient was 0.83. The limits of agreement was tighter between 20% normal forms (+8.4 and −6.6). Experiment 3 (objective 3) (repeatability): 255 cells were analyzed three times in succession. Estimating pairwise agreement, the x statistic for the pairs are 0.85, 0.80, and 0.85, respectively, which compares favorably with the second canonical moment of 0.8329 (x = 0.83).

Discussion: The computer’s ability to classify normal morphology per slide is promising. Below 20% normal forms, the limit of agreement is tight. Because of the 6% higher reading compared with the manual method, different thresholds possibly will be developed to identify subfertile from fertile patients. The computer gives excellent repeatability of normal and abnormal cells. Based on results obtained, this system can be of clinical value both in IVF units and andrology laboratories but more clinical data is required in this field. Fertil Steril 1995;63:134–41

Key Words: Sperm morphology, strict criteria, computerized method

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† IVOS, Hamilton-Thorne Research, Beverly, Massachusetts.
‡ University of Stellenbosch and Tygerberg Hospital, Infertility Clinic.
§§ Reprint requests: Thinus F. Kruger, M.D., Reproductive Biology Unit, Department of Obstetrics and Gynecology, Tygerberg Hospital, Tygerberg 7505, Republic of South Africa (FAX: 27-21-933-3684).
† Fertech, Somerset, Republic of South Africa.
* Institute for Biostatistics, Medical Research Council, Tygerberg.

The use of strict morphology as a predictor for fertilization in vitro is well documented (1–11). Fertilization rates in IVF programs were found to be lower to a significant degree in cases with normal sperm morphology < 14% normal forms (1). Fertilization rates of 7.8% were observed in cases where normal sperm morphology was <5% (P pattern or poor prognosis pattern). Recently, Van der Merwe et al. (12) also reported a significantly lower pregnancy rate in a GIFT program for patients with a normal sperm morphology ≤ 14% versus those with normal morphology > 14%.

Menkveld et al. (13) observed an excellent inter-
observer and intraobserver correlation coefficient when using the strict method to evaluate sperm morphology. Subjective methods generally need a great amount of communication to ensure successful information transfer, especially between different laboratories. Once standardized, strict criteria can be used as a valuable tool. In theory, computerized programs for sperm morphology evaluation can achieve the above mentioned goals, e.g., more objective and reproducible than the human’s ability to identify normal and abnormal spermatozoa.

In this report the agreement on percent normal morphology was studied between different observers and a computerized method on a slide-by-slide basis. We thus have [1] compared an experienced observer (T.F.K.) with a second experienced observer (R.M.) on percent normal morphology per slide, [2] compared manual evaluation (T.F.K.) with sperm morphology analyzer (IVOS; Hamilton-Thorne Research, Beverly, MA) on percent normal morphology per slide, and [3] the repeatability of IVOS on normal and abnormal cells also was evaluated on a cell-by-cell basis.

MATERIALS AND METHODS

Manual Assessment of Sperm Morphology

Semen specimens were obtained from 30 patients from the assisted reproduction program at Tygerberg Hospital. Samples were obtained by masturbation after 2 to 4 days of sexual abstinence and were prepared for morphology evaluation after liquefaction was complete (<30 minutes) and within 1 hour of collection. Morphology slides were prepared as follows: each slide was cleaned thoroughly with 70% ethyl alcohol before use; no more than 5 μL of semen was used so the smear was as thin as possible. The 5-μL drop was pulled rather than pushed across the slide to avoid artificially detached heads. The slides were air dried at 37°C in a warm tray and stained using the Papanicolaou method (12,13). All slides were read at a magnification of ×1,000 by a trained observer (T.F.K.) using the strict criteria as published previously (2,3,12,13). In our laboratory this method of evaluation has intratechnician and intertechnician coefficients of variation of <10%, with Spearman’s correlation coefficients of 0.965 and 0.867 (P < 0.0001) for results of multiple observations of the same sample by one observer and different observers, respectively (13).

Thirty different patients’ slides from the Tygerberg IVF program were selected randomly. Fields and cells were chosen randomly and the percent normal morphology was recorded for two experienced observers. A Nikon microscope was used to evaluate morphology using ×100 oil immersion to give a total magnification of ×1,000. All the slides were stained using the Papanicolaou staining method.

Sperm Morphology Analyzer (IVOS)

The IVOS cell analyzer combines an internal optical system with an internal computer and image-digitizing and analyzing systems. A computer-controlled stage moves the specimen slide between fields. The system may be used for movement analysis of sperm as well as static cell morphology determination. In the sperm morphology application, the optical system uses a narrow-band 662-nm wavelength illumination in conjunction with a ×100 oil-immersion objective. Images are produced on a charge-coupled detector and transferred to the digitizer. Clear high-contrast images of Papanicolaou-stained cells are produced.

General Operation

On inserting a slide into the microscope stage, the computer verifies that the illumination is correct and allows the user to electronically maneuver the slide to select an image containing sperm cells and to adjust the focus. The computer then captures the image, processes it, and allows the user to select the next image. Processing can be either in interactive mode or in batch mode.

In interactive mode, an image is captured and processed immediately. The user can select to display the processing of every cell on the screen. The outline of the cell is superimposed with a color line on the microscope image, and the classification result is shown in a color-coded table, also superimposed on the image.

Every object located in the image is accepted as a valid sperm cell for further evaluation or rejected if it is not in focus or if it is considered as clutter. A cell is analyzed and classified into one of three categories: normal, subnormal, or abnormal (3), with a breakdown of the classification: size: normal, small, or large; and shape: normal, slightly amorphous, thin, tapered, elongated, round, midpiece, or severely amorphous (13). If the acrosome is <40% of the sperm head, the cell is abnormal (2,13) (Fig. 1).
Figure 1  Examples of some normal and abnormal forms.

In addition, numerical values are given for the focal quality of the cell, a shape acceptance factor, and the area of the cell in pixels. For a cell to be classified as normal, all three parameters of size, shape, and acrosome must pass as normal. If both the size and acrosome is normal, but the shape is slightly amorphous, then the cell is classified into the subnormal category. In any other case, the cell is classified as abnormal.

In batch mode, the captured images are first stored to disk and processed afterwards to minimize user operation time. At any stage, the resulting statistics per slide can be viewed by the user, such as the percentage of normal cells.

Hardware

The FERTech SMA software code is implemented in the IVOS instrument (1). The IVOS (Hamilton-Thorne Research, Boston, MA) is a PC-based imaging computer consisting of the processing unit, a high quality Super-VGA color monitor, standard PC keyboard, and mouse.

The processing unit contains a 50-MHz 486DX motherboard with 8MB RAM, a Data Translation DT3851 frame grabber with Super-VGA output, an optical unit, a 80-MB hard disk, and a 3.5" floppy drive. The optical unit consists of a Nikon-based microscope developed by Hamilton-Thorne Research with an effective magnification of ×1,000, a red illumination lamp, a Sony XC-75 CCD video camera, and an electronically controlled mechanical stage to hold a slide. Three push buttons are available to maneuver the stage: the Load button ejects the stage to enable the user to insert a slide into the stage and then retracts the stage to position the slide under the built-in microscope and the Jog In and Jog Out buttons move the slide under the microscope to allow panning. A Focus knob enables the user to adjust the focusing.

An image is digitized into 256 grey levels and a spatial resolution of 640 columns and 480 rows with aspect ratio of 1.0. The single Super-VGA monitor is used to screen both the PC functions and the real-time video images. For more technical details on the IVOS system, see Appendix.

Study Design

Experiment 1 (Human Versus Human)

In experiment 1 the same slides were evaluated by two experienced observers and the percent normal morphology was recorded for each slide. One hundred cells were evaluated per slide.

Experiment 2 (Human Versus Computer)

This experiment compared the results of the experienced worker (T.F.K.) with the sperm morphology analyzer using the IVOS optical system with a red illumination source. The percent normal morphology per slide was reported. Thirty patients’ slides were compared on the basis of percent normal morphology reported both by T.F.K. as well as the sperm morphology analyzer, IVOS. At least 100 cells were evaluated per slide.

Experiment 3

The ability of IVOS to repeat the same reading for normal and abnormal cells was studied. Two hundred fifty-five cells were selected randomly from 10 different patients’ slides and repeated three times.

Statistical Analysis

The Bland and Altman plot (14) and Spearman correlation coefficient were used to compare manual morphology assessment and IVOS morphology assessment per slide (percent normal sperm morphology/100 cells) (experiments 1 and 2). The κ statistic (15) is the usual measure of agreement reported when a categorical measurement is assessed by two repeat readings. A κ value < 0.4 represents poor agreement, between 0.4 and 0.75 represents good agreement, and >0.75 represents excellent agreement. In this study the methodology also was
extended to three repeat readings of the same cell by IVOS (experiment 3).

For the analysis of m multiple binary readings of sperm cell morphology, Lau (16) developed a measure for agreement. Agreement is assessed by the predictive probability of a single measurement from the other measurements. Exchangeability (order of reading is not important) is assumed between readings, and parameters called canonical moments are used to estimate the predictive probability of the k-th reading given the consensus (the extent of agreement) of the other k−1 readings, where k = 2, 3, . . . , m. The statistic used for assessing twoway agreement is related to the second canonical moment.

The binary classification used for sperm morphology is normal (n or 0) and abnormal (a or 1). In this study the main statistical evaluation of experiments 1 and 2 will be with the aid of the Bland and Altman plot and that of repeatability with the κ statistical index and predictive probabilities.

RESULTS

Experiment 1: Agreement per Slide (Percent Normal Morphology) Between Two Experienced Observers (T.F.K. versus R.M.)

Thirty slides were evaluated by both observers. From the Bland and Altman plot there is no significant bias between the two readers (mean difference = 0.63, P = 0.3984) (Fig. 2) and the limits of agreement are 8.6% and −7.3%. The interslide SD for T.F.K. was 5.5 and for R.M. was 7.0; the SDs were not significantly different (P = 0.1283). The Spearman correlation coefficient between the readers was 0.83.

Experiment 2: Agreement per Slide Between IVOS and Manual (T.F.K.)

Thirty slides were evaluated. From the Bland and Altman plot there is no significant bias between the two methods (Fig. 3) (P = 0.4333). The limits of agreement are 12.1% to −15.5% normal sperm morphology. In the range 0% to 20% normal sperm morphology, the agreement is tighter. Here the limits of agreement were 8.4% and −6.6% (Fig. 3).

In Table 1 the true readings (percent normal) between manual (T.F.K.) and IVOS can be viewed. The Spearman correlation coefficient between the readers was 0.85.

Experiment 3: The Agreement Among Three Readings by IVOS

A total of 255 individual sperm cells were assessed in triplicate by the machine and the results are given in Table 2. Estimating pairwise agreement, the κ statistics for the pairs are 0.86, 0.80, and 0.85, respectively (SE = 0.03) and this compares favorably with the second canonical moment of 0.8329, which is an overall estimate and falls in the excellent category > 0.75. The predictive probabilities over all pairwise readings of the data are as follows:

\[
P(X_2 = 1 : X_1 = 1) = 0.9371,
\]
\[
P(X_2 = 0 : X_1 = 0) = 0.8958,
\]

where \(X_j\) indicates the jth reading on a cell. Therefore, given that a cell has been read as abnormal \(X_j\),
The probability the next reading of the same cell will be abnormal ($X_2 = 1$) is 94%.

For the agreement between a third reading with the consensus of the other two readings, the predictive probabilities are as follows:

$$P(X_3 = 1 : X_2 + X_1 = 2) = 0.9687,$$

$$P(X_3 = 1 : X_2 + X_1 = 1) = 0.4667,$$

$$P(X_3 = 1 : X_2 + X_1 = 0) = 0.0620.$$

Therefore, given that there is a difference between the first two readings ($X_2 + X_1 = 1$) the probability the third reading of the same cell will be abnormal ($X_2 = 1$) is 47%.

**DISCUSSION**

Based on the analysis of the percent normal morphology between two human observers there was no significant bias using the Bland and Altman procedure. The same observation was made between IVOS and the manual (T.F.K.) method. The limits

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Percent Normal Morphology on a Slide-by-Slide Basis by Manual (T.F.K.) and Computer (IVOS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent normal morphology</td>
</tr>
<tr>
<td>Slide no.</td>
<td>Manual (T.F.K.)</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
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<td>9</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Triplicate Sperm Cell Morphology Readings by IVOS</th>
</tr>
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<tbody>
<tr>
<td>Outcome of three readings</td>
<td>Frequency</td>
</tr>
<tr>
<td>a*</td>
<td>a</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>a</td>
<td>n</td>
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<td>n</td>
<td>a</td>
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<td>n</td>
<td>a</td>
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<tr>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>

* a: abnormally cells.
† n: normal cells.

Of agreement, however, were wider in the last experiment (IVOS versus T.F.K.). This was probably because of a 6% higher reading in the patients with morphology > 20% normal forms. The agreement is tighter below the 20% threshold. This last observation has important clinical application because agreement below 20%, and more so below 14%, is often the level at which a reduced fertilization rate has been noted in IVF (1–3). Sperm morphology as evaluated by strict criteria has diagnostic and prognostic clinical implications in assisted reproduction because prior knowledge of teratozoospermic cases can lead ultimately to improved fertilization rate (6). Cases with >14% normal forms have similar fertilization rates as patients with normal morphology 15% to 30% and >30% (2). The bias in the data observed for the manual versus IVOS comparison for normal morphology > 20% can be because of several factors, one of which is too many cells per microscopic field, thus causing a discrepancy between manual and IVOS readings. Because the computerized system uses image analysis in these cases with a high concentration of spermatozoa per milliliter, a small droplet must be made (≤5 μL) to create the ideal of 5 to 10 sperm per high power field. This will reduce the chance of a computer observer fault, as cells overlapping or too many cells cannot be judged accurately, not only by IVOS, but also manually (13). It also is possible that, because of the 6% higher reading, different clinical thresholds will be identified for the computer to identify fertile from subfertile patients in clinical practice.

The IVOS system was standardized on Papanicolaou staining of spermatozoa. It is well known that staining techniques can influence the evaluation and thus the final interpretation of the normal or abnormal cell (e.g., with Quick Stain the measurements are different from Papanicolaou) (3). It is thus important to adhere to the Papanicolaou
method to prepare slides for the IVOS system until more data are available on other staining methods and its agreement with manual assessment and IVOS.

A number of different computerized systems have been introduced recently (17–19). An accurate length-width ratio is one of the principles to identify normal cells. This approach is, however, not accurate enough because often a totally amorphous cell can have a normal length-width ratio. Literature has shown that the shape of normal cells is very important in clinical practice (2, 3, 13). The evident advantage of the signature method used by the IVOS system is to approximate the cell head with an ellipse. The signature will show abnormally shaped cells other than purely the ratio of the major axis to the minor axis. If the cell head has the correct aspect ratio but is distorted, skewed, or a pertubated outline, the cell will be classified as abnormal with this approach. On the other hand, a small acrosome also will be taken into consideration and a cell will be classified as abnormal even if the shape is normal (13).

Repeatability of results is of utmost importance and forms the cornerstone of laboratory quality controls. The ability to repeat the same reading within normal and abnormal sperm cells will give clinically reliable and relevant results in andrology laboratories. The excellent results obtained in this section (κ > 0.83) as well as the above mentioned good agreement with manual strict evaluation per slide highlight the automated system as a valuable diagnostic modality in sperm morphological evaluations.

From both the two- and three-way predictive probabilities we see that the measurement procedure is very reliable. With conflicting results on any two readings the probability of the machine reading the third reading as abnormal is close to 50%, which is the ideal to have for a random choice between two categories. This situation occurred in only 5.8% (15/255) of the cells read. Given normal consensus between two readings the probability for an abnormal reading is 0.0620 compared with 0.0313 for a normal reading given abnormal consensus. Two of the three and as well as the second canonical moment fall in the range of almost perfect agreement.

Of the slides that differed between manual and IVOS evaluations on the percentage normal forms, three had severe background, causing false low readings. This observation emphasizes the need to be very meticulous with slide and smear preparation. Before smears are made, slides must be cleaned with alcohol and rinsed afterwards with deionized water. In cases with background problems a 1:1 rinse with Ham's F-10 and semen mixed and centrifuged at 200 x g for 5 minutes can improve the quality of the slide. A double wash was done on one of the false low readings (with severe background problems giving a clean smear afterwards), resulting in a good agreement between manual and IVOS (five of five readings correct on the "clean" smear). Another practical point regarding accuracy and repeatability was meticulous focusing on the cell head. The repeatability on a given cell is not good if careful attention is not given to this aspect.

In this study it was shown that there is good agreement between manual and computerized (IVOS) assessment of normal and abnormal cells. The repeatability and agreement per slide of IVOS is excellent in the clinically relevant range (<20% normal sperm morphology). This latter method offers great potential on a day-to-day basis in the clinical field (1) as well as in the research arena (1), but more clinical data is needed in this area.

APPENDIX

IVOS Technical Details
Software

The image processing sperm morphology analyzer software is written in Microsoft C++ using the version 7 compiler. The feature extraction and classification are described in more detail below.

Detection

First the intensity range of the input image is normalized. If the lighting is unacceptably low, the image is rejected. Object detection is done by adaptive thresholding of the spatial subsampled input image. Template matching is performed on the resulting binary low resolution image to isolate objects.

Segmentation

Once an object is located, high pass filtering on the original resolution subimage reveals the amount of high frequency information in the subimage, indicating the focal quality. If this is too low, the object is not properly in focus. In addition, if the size of the object is too large to be a cell, it is regarded as clutter. Objects not in focus or cluttered
are ignored completely and not taken into statistical consideration.

To separate the cell from its background, a Bayesian-based segmentation technique is implemented operating on both spatial and statistical probabilities. The spatial probability distribution function (pdf) is obtained by fitting a smooth "bubble" surface over the cell region, with maximum amplitude at the center and decreasing to zero away from the center. The statistical pdf is obtained by dividing the subimage into three regions (entirely cell, entirely background, and unknown) and generating histograms for two of the regions (entirely cell and entirely background). The classical Bayesian classifier separates the two classes, cell and background. A median filter removes spot noise.

A template-based adaptive filter is used to determine the location of the junction of the cell’s tail to its head. The head thus is separated from the tail and the orientation of the cell can be normalized.

**Classification**

The size of the cell head is calculated. If it is too small or too large, the cell is considered as abnormal (2, 3, 13). The acrosome is evaluated by measuring the shift of the true grey level centroid of the cell head from its binary centroid. In contrast to the binary centroid, which is at the geometrical center of the head, the true centroid is influenced by the size of the acrosome. Thus, if the acrosome is too small or absent, the true centroid tends to coincide with the geometrical centroid, indicating an abnormal acrosome.

The shape is extracted with a polar transformation using the cell head’s centroid as the distance origin and the tail junction as the angular origin. As the cell size already has been extracted and classified at this stage, the polar transformation is normalized with respect to its integral to reveal the "shape signature" (Fig. 4). The principle behind the shape classification is the isolation of the normal shape (2, 3, 13).

For this reason the shape is classified by comparing the cell’s signature with the normal signature using a least mean squared-based classifier. Figure 5 shows the principle, with the area between the actual and the normal curve indicating the fit error.

If the error is acceptably small, then the shape is considered as normal, otherwise not. If the signature only slightly deviates from the normal form (and the size and acrosome are correct), then the cell is classified into the subnormal (intermediate) class.

The evident advantage of the signature method over the traditional approach to approximate the cell head with an ellipse is that the signature will show out shape errors other than purely the ratio of the major axis to the minor axis. If a cell head has the correct aspect ratio but is distorted, skewed, or a pertubated outline, then the signature method still will throw it out into the abnormal class.

As the above method is very selective of the normal shape and there are about three variations on the normal or ideal shape, each of three normal signatures are fitted to the cell’s signature and the best fit is classified. Similarly, an abnormal shape is subclassified into several other categories, being thin, tapered, elongated, round, and midpiece (Fig. 1). If an abnormal shape does not fall into any of these subclasses, then it is categorized as severely amorphous (3, 13).

**Processing**

In interactive mode, the sperm morphology analyzer (program V1.04) captures and processes a single image with one cell in 5.6 seconds, an image with three cells in 8.9 seconds, and an image with nine cells in 19.2 seconds. In batch mode, the system captures and stores a single image in 1.7 seconds.

**Acknowledgments.** The authors thank Hamilton-Thorne Research, Beverly, Massachusetts, for supplying the IVOS system for laboratory evaluation and Mrs. Helena Kruger for preparing the manuscript.

**REFERENCES**


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**Figure 4** Normal shape signature. $\theta$, angle; $R$, distance from origin; $\pi$, pi.

**Figure 5** Abnormal shape signature. $\theta$, angle; $R$, distance from origin: $\pi$, pi.
of reading sperm morphology (strict criteria) is as efficient as technician reading. Fertil Steril 1995;63:202-9.


The following article reflects the scientific basis that supports the above argument:

Computer-assisted sperm analysis systems: morphometric aspects

Thinus Kruger

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Introduction
Sperm morphological assessment has been plagued by subjectivity and a lack of agreement among investigators (Freund, 1966). However, its reliability and reproducibility have been demonstrated in studies involving experienced observers who have used standardized criteria (Menkveld et al., 1990; Ombelet et al., 1994a,b). It was shown by Davis (1995) that there is a wide variation in repeatability between different human observers using both World Health Organization (WHO) and the strict criteria approach. However, this evaluation was carried out using slide projection, and the participants at the symposium had different levels of experience in judging sperm morphology. Based on this study, it was stated that the employment of laboratory technicians for subjective visual assays will require the development of appropriate training, testing and control procedures. An alternative approach which may prove to be much less expensive and easier to control is the use of automated laboratory assays (Davis, 1995).

Several attempts have been made to use morphometry to quantify sperm morphology (Schmassmann et al., 1979, 1982; Jagoe et al., 1986, 1987; Katz et al., 1986; Moruzzi et al., 1988). Schrader et al. (1990) reported that each morphometric parameter may provide important information about different aspects of spermatogenesis. However, this must still be evaluated in clinical practice (assisted reproduction programmes).

Clinical research in assisted reproduction relating sperm morphology to fertilization and pregnancy outcome (Kruger et al., 1988; Enginsu et al., 1991, 1992, 1993; Grow et al., 1994; Ombelet et al., 1994a,b) has led to a renewed interest in this field of diagnostic andrology. These results also stimulated the development of computerized equipment reading sperm morphology. This development could theoretically bring more objectivity and precision into the evaluation of sperm morphology.

Here we will discuss the latest developments in this field by looking at how computer morphology analysers work, reporting on the importance of slide preparation and the correlation between the manual and computerized evaluation of sperm morphology, and discussing repeatability studies as well as the first clinical studies in assisted reproduction using fertilization as an endpoint. We will also postulate how these systems can assist the clinician/scientist in the intracytoplasmic sperm injection procedure.

How computer morphology analysers work

Conventional pattern recognition image analysis systems
To obtain more detailed information regarding the differences between the CellForm-Human instrument (Hamilton-Thorn Research, Beverly, USA) and the IVOS (dimension) system (Hamilton-Thorn), we would like to refer the reader to the following two articles: Davis et al. (1992) and Kruger et al. (1995). A clear difference between the two systems is the evaluation of the acrosomal size and shape of spermatozoa by the IVOS (dimension) system, which was shown in previous studies to be of importance in clinical practice (Kruger et al., 1988; Grow et al., 1994). However, both systems take the lengths and widths of the spermatozoa into consideration.

Automated sperm morphology analysis instruments work much like current versions of computer-aided sperm analysis instruments for motion, except that no movement information is required
(Wang et al., 1991a,b; Davis, 1993; Kruger et al., 1993, 1995; Garrett and Baker, 1995). The system consists of a microscope, a video camera, a computer frame grabber and morphology software. The video camera delivers the image to the computer's frame grabber which stores it for analysis (Wang et al., 1991a; Davis et al., 1992; Kruger et al., 1993). The image is evaluated by the morphology software to determine whether spermatozoa are present. Sperm recognition is based on software specifications for size, shape, colour intensity and other characteristics. Once spermatozoa have been recognized and segregated from debris and other objects, metric measurements are performed on the head, midpiece, acrosome and other cytological features. These measurements are the basis for a sperm morphological classification. The accuracy and precision of automated sperm morphology analysis instruments depend on (i) the microscope optics, magnification and focusing capabilities; (ii) video camera quality; (iii) array size of the frame grabber; (iv) image processing techniques; (v) definitions of metric measurements (Wang et al., 1991a; Kruger et al., 1993, 1995); and (vi) staining methods (Kruger et al., 1993; Lacquet et al., 1995; R.Menkveld et al., unpublished data).

**Neuronal network image analysis system**

Artificial neural network models have been studied for many years in the hope of achieving a human-like performance in the fields of speech and image recognition. These models are composed of many non-linear computational elements operating in parallel and arranged in patients which are reminiscent of biological neural nets.

Analysis can be divided into feature extraction and classification.

**Feature extraction**

Feature extraction should be capable of finding objects in the image which look like 'spermatozoa', and calculated contours should faithfully reproduce real contours as accurately as possible. To check calculated contours, they are graphically superimposed on the original image in colour. For feature extraction, previous knowledge about the appearance of spermatozoa is used.

**Classification**

Each object is subdivided into the classes head, midpiece and tail. With the help of these three subdivisions, the user can allocate freely defined classes (e.g. for the headpiece: 'normal', 'amorph', etc.). For teaching the neuronal net, the user feeds the computer interactively with some samples that are set in advance. After unique teaching, the classificator is built and is able to classify what was taught to the computer (Mica Medical, promotion pamphlet).

This method described by Mika Medical (promotion pamphlet) seems promising, but more research is necessary before its value can be judged.

**Slide preparation and staining methods**

It was realized from the very beginning of our involvement in the field of computerized sperm morphology research that slide preparation and staining methods are of the utmost importance for reliable results (Kruger et al., 1993, 1995). Two aims must be achieved for accurate identification — namely good contrast and minimum background. Sperm shape, size and acrosome recognition are more reliable if images are sharp.

Two studies were performed in this respect. Background problems were solved with washing of the sample to remove seminal plasma. The Papanicolaou and Diff-Quik methods were compared between the manual and the computer system (IVOS) evaluation in a blind fashion using unwashed and washed samples (one or two washes; R.Menkveld et al., unpublished data). The one-wash Diff-Quik (computer, IVOS) method gave no significantly different results from the manual, Papanicolaou or Diff-Quik (washed or unwashed) methods evaluated in a blind fashion by R.Menkveld et al. (unpublished data). The Diff-Quik stain is the method of choice for computer use in our department because of the quick and effective staining results obtained.

We then aimed at standardizing and optimizing the Diff-Quik slide preparation. In a study by Lacquet et al. (1995), 22 semen samples were analysed by the IVOS system after different staining procedures, to assess whether changes in the staining methodology affected the normal morphology outcome. Each sample was divided into five aliquots: one aliquot was processed according to the standard procedure (procedure A) and the other four according to alternative procedures (Table I). For example, samples were processed fresh (<1 h) or after 5 h at room temperature and fixed immediately after air drying or after 24 h (Lacquet et al., 1995).

The non-parametric Friedman test was used to analyse the randomized block experimental design.
T. Kruger

Table I. Different staining procedures using Diff-Quik stains

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Slide preparation*</th>
<th>Fixation** post-preparation (10 s)</th>
<th>Staining*** post-fixing time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (standard)</td>
<td>&lt;1 h</td>
<td>Immediate</td>
<td>Immediate 7-7 s</td>
</tr>
<tr>
<td>B</td>
<td>&gt;5 h</td>
<td>Immediate</td>
<td>Immediate 7-7 s</td>
</tr>
<tr>
<td>C</td>
<td>&gt;5 h</td>
<td>Immediate</td>
<td>Immediate 20-20 s</td>
</tr>
<tr>
<td>D</td>
<td>&lt;1 h</td>
<td>Immediate</td>
<td>&gt;24 h 7-7 s</td>
</tr>
<tr>
<td>E</td>
<td>&lt;1 h</td>
<td>&gt;24 h</td>
<td>Immediate 7-7 s</td>
</tr>
</tbody>
</table>

*Slides were prepared at the stated time after collection.
**Sample slides were fixed at the stated time after slide preparation for 10 s.
***Slides were stained at the stated time after fixation. Staining time: for example, 7 s solution 1 and 7 s solution 2.

for the different staining procedures with the Diff-Quik stain.

The median percentages of normal forms for the five staining procedures (A–E) were 6.0, 6.5, 9.5, 8.5 and 5.5% respectively. No significant difference (P = 0.60) was found between the different staining procedures used. However, slides which had been prepared and stained according to procedure E did cause artifactual damage (swollen and ruptured sperm heads) to the spermatozoa in two cases (9.1%), resulting in no normal forms being read.

Therefore we recommend that slides are prepared immediately after liquefaction and fixed after air drying. Ultimately, these improvements will lead to the availability of a system that will objectively and accurately evaluate normal sperm morphology according to the strict criteria.

Correlation of normal morphology between manual and computerized evaluation (conventional pattern recognition)

In recent studies, sperm morphology was evaluated using computerized sperm analysers. These studies looked at the value of the computerized system (Wang et al., 1991a, b; Davis, 1993). Wang et al. (1991a, b) concluded that no benefit could be found in the system they tested above the manual method. One obvious disadvantage was the fact that this system was slow (45–60 min/slide) and thus not clinically competitive. However, Davis (1993) found the evaluation of the motion analysis system valuable, with a reported speed of 4 min/slide (Davis et al., 1992). Studies by Wang et al. (1991a, b) and Davis (1993) looked at comparative analyses between manual and computerized systems on a cell by cell basis.

The latest report regarding a new image analysis system is from Australia and seems promising, but as stated in the discussion, the 100 min/100 cells rate is still inferior to some of the other systems available (Garrett and Baker, 1995). The authors stated that the relatively slow assessment times would be improved substantially with upgraded hardware. According to them, the precision and sensitivity offered by the detailed image analysis of this system provide a powerful tool for morphometric semen analysis.

An acceptable time per slide is 4–6 min which was reported for IVOS dimensions (Lacquet et al., 1995) as well as by the CellForm instrument (Davis et al., 1992).

In recent studies by my group, using a slide by slide evaluation we considered the correlation of sperm morphology (normal forms, strict criteria) between the manual method and the computerized system.

In the first study of my group (Kruger et al., 1993), the intra-assay variation was established for the computerized system. Eight slides from the group <14% normal morphology and eight slides from the group >14% normal morphology were selected randomly. The reading was repeated five times using 100 cells per reading. In the group <14% normal forms, 97.5% (39/40) of the readings classified the spermatozoa into the proper category, whereas in the second group (≥14% normal forms), 95% (38/40) of the cases were identified correctly (Kruger et al., 1993).

The question asked in other experiments (Kruger et al., 1995) was what is the correlation between experienced human observers? Once the golden standard was known, the relationship between human and computer was studied. Thus we correlated the percentage normal morphology reported by different observers and a computerized method (IVOS dimensions) on a slide by slide basis using strict criteria: (i) experienced
observer (T.F.K.) versus experienced observer (R. Menkveld), and (ii) experienced observer (T.F.K.) versus sperm morphology analyser (IVOS; Kruger et al., 1995). Thirty patients' slides were chosen at random and evaluated, at least 100 cells per slide.

There was no significant bias between the measurements made by T.F.K. and R. Menkveld. The limits of agreement were 8.6 and −7.3%. The SD values were not significantly different ($P = 0.1283$; Figure 1). The Spearman correlation coefficient between readers was 0.83. In a subsequent experiment the same findings were reported but the limits of agreement were 12.1 and −15.5% (Figure 2). The Spearman correlation coefficient was 0.85. The limits of agreement were tighter below 20% normal forms (+8.4 and −6.6%). It was concluded that the computer's ability to classify normal morphology per slide is promising. Below 20% normal forms, the limit of agreement is tight. However, different thresholds for clinical use will be established for the computer based on the above-mentioned results.
Repeatability of manual observers and IVOS (dimensions) on a cell by cell basis

Repeatability per slide has been reported previously, and in our own unit an excellent correlation was found (Menkveld, 1992). However, repeatability is a constant point of criticism. Therefore we decided to study the repeatability per cell, which is a much more stringent test than a reading per slide (100 cells). A total of 300 cells were randomly chosen, stored on disc and read three times manually, 1 week apart (150 normal and 150 abnormal cells). An observer with >10 years of experience and one with only 2 years of experience were used.

For the IVOS system (dimensions), three readings were performed. For each reading, careful focusing was performed and a total of 300 cells were used. These cells were selected on a day to day basis (the Kappa statistical analysis was used to analyse the data).

For the experienced worker (>10 years), the overall Kappa (κ) value for repeatability was 0.80 (excellent category); for the worker with 2 years of experience the Kappa value was >0.72 (good) and for the computer it was 0.71 (good) (unpublished data).

From these experiments, it has been discovered that as far as the computer is concerned, the software is not the only factor which affects the results. Staining resulting in the good contrast of cells is also of utmost importance, as is focusing on a given cell. These factors can affect repeatability significantly.

In another study (Kruger et al., 1995), a total of 255 sperm cells were assessed in triplicate using the computerized system (Table II). By estimating pairwise agreement, the κ statistics in this study for the pairs were 0.85, 0.80 and 0.85 respectively (SE = 0.03), which compares favourably with the second canonical moment of 0.8329 (κ = 0.83; Kruger et al., 1995). It was concluded that the computer gives very good repeatability and can be of clinical use on a day to day basis.

Clinical studies in assisted reproduction

In our initial work, the computer evaluation was also compared with the manual evaluation in a clinical study using fertilization rate as an endpoint. The computerized system identified the <14% of normal forms very well, and showed a significant difference in fertilization rate in the groups with ≤14 and >14% normal forms, as well as at the 10% level (Figure 3). It was concluded that this new development holds promise for clinical practice (Kruger et al., 1993).

Recently, the IVOS system’s ability was evaluated for the prediction of fertilization in vitro in a prospective study (T.F. Kruger et al., unpublished data). In all, 80 patients from the Tygerberg gamete intra-Fallopian transfer (GIFT) programme were evaluated in a prospective manner. The same semen sample was analysed on a day to day basis by both the laboratory (manual method) and the computerized system for the percentage normal morphology, concentration, motility and forward progression. Only patients with more than two oocytes available after GIFT was performed were allowed into the study. In all cases, an insemination concentration of 500 000 spermatozoa per oocyte was used.
where normal morphology was \( \leq 14\% \). A logistical regression analysis was used to study the predictors of fertilization in vitro on excess oocytes. A total of 338 oocytes were obtained from the 80 patients, of which 239 fertilized.

The logistical regression analysis of the manual method (percentage normal morphology) and IVOS (dimensions) indicated that both were predictors of fertilization. The logistical regression analysis also showed that sperm morphology, as evaluated by IVOS in patients with \( < 10 \times 10^6 \) spermatozoa/ml after swim-up, was a significant predictor of fertilization. The influence of number of oocytes was also pointed out as significant by this model. Thus, the more oocytes obtained in the lower morphological groups, the better the chance of fertilization (T.F. Kruger et al., unpublished data). It was shown that in patients where \( < 10 \times 10^6 \) spermatozoa/ml were obtained, the role of morphology (evaluated by IVOS) as well as the number of oocytes were significant (for example, Figure 4 displays the data in the group where \( \leq 5 \times 10^6 \) spermatozoa were obtained; \( P = 0.0001 \)). In contrast, when \( > 10 \times 10^6 \) spermatozoa/ml were obtained, the percentage normal forms did not play a predictive role.

These factors (percentage normal morphology, number of spermatozoa retrieved and number of oocytes obtained) can be considered predictors of fertilization in patients with \( < 10 \times 10^6 \) spermatozoa/ml retrieved and \( < 10\% \) normal forms. The computer can be of great help in identifying the poor prognosis group as far as fertilization is concerned (T.F. Kruger et al., unpublished data).

Using a more simplistic approach with the same data, it was noted that the overall fertilization rate for IVOS in the 0–4% normal morphology group (p pattern) was 45.7% (37/81), in the 5–9% normal morphology group was 72.5% (87/120), in the 10–14% normal morphology group was 82.1% (46/56) and in the >14% normal morphology group was 85.2% (69/81) (\( P < 0.0001 \) for p pattern versus other groups).

One can identify the poor prognosis group in advance. By adapting a more aggressive ovulation induction programme, we theoretically obtain more oocytes with greater chance of fertilization success and possibly pregnancy success based on the model explained. This information can also help to identify patients for microinjection (intracytoplasmic sperm injection).

It is thus obvious from the above-mentioned data from different studies that the computer can become a helpful clinical tool in andrology laboratories and in-vitro fertilization centres. If careful slide preparation is adhered to, then computerized morphological evaluation can bring more objectivity in the evaluation of sperm morphology. However, more research in this field over the next few years is essential to find the answers.

Acknowledgements

The author wishes to thank Ms. Helena Kruger for preparing the manuscript and Dr. Axel Vandendael for reviewing the document.
References


The following article reflects the scientific basis that supports the above argument:

SLIDE PREPARATION AND STAINING PROCEDURES FOR RELIABLE RESULTS USING COMPUTERIZED MORPHOLOGY

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The purpose of this study was to standardize slide preparation and staining procedures to improve the efficiency and effectiveness of the IVOS system on normal sperm morphology readings with regard to the strict criteria. Semen samples from patients attending the Reproductive Biology Unit, Tygerberg Hospital, were used. In experiment 1, five different Diff-Quik staining procedures, including the standard procedure, were evaluated on each of 22 patients and the effect of slide preparation within 1 h or more than 5 h after collection and the effect of immediate fixation versus fixation after 24 h were observed. In experiment 2, the manual evaluation time per slide (n = 20) by two technicians was compared with the time taken by computer. In experiment 1 the median % normal for the 5 different staining procedures was 6, 6.5, 9.5, 8.5, and 5.5%. No significant difference was found.

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between the different staining procedures ($p < .00$, nonparametric Friedman test). In experiment 2 the mean time for manual assessment by two technicians was 3 min:6 s and 3 min.53 s per slide as compared to 4 min.39 s by computer. For experiment 1, slides can be prepared immediately or after 5 h. Fixation time also does not interfere with the computer’s ability to identify normal forms. For experiment 2, the IVOS system is competitive according to assessment time. Standardization of optimum staining procedures is important to ensure repeatability and comparability. Therefore, slides should be prepared immediately after liquefaction and fixed immediately after air drying.

**Keywords** computerized evaluations, normal sperm morphology, slide preparation, staining procedure, strict criteria

Sperm morphology determination has an important clinical value and can be used as a predictor for fertilization in vitro, a statement that has found support in a number of published articles [1, 3–6, 8, 11, 14, 15]. Manual reading, by light microscopy, has been the routine means of sperm morphology evaluation. The increased demand for sperm morphology readings have, however, increased the workload of andrology laboratories. In addition, the necessity of worldwide standardization of the strict criteria has made the development of computerized systems more and more important. A number of computerized systems have been developed and are operational [2, 9, 16]. The theoretical advantages of computerized readings are a high repeatability and objectivity compared to human readings.

By using the strict criteria for morphology evaluation, it was shown in recent publications [9, 10] that there is good agreement between the computerized reading and the manual assessment. The IVOS system (Integrated Visual Optical Systems, Hamilton–Thorne Research, Beverly, MA, USA) has provided excellent results with regards to repeatability [9, 10]. A recent study on the predictability of in vitro fertilization has also produced very promising results when using the IVOS to determine the percentage normal sperm morphology [9, 10].

The primary aim of this study was to standardize an optimum slide preparation and staining procedure as well as to evaluate whether the time taken by the IVOS to evaluate a slide is comparable to that of a manual assessment.

**MATERIALS AND METHODS**

Semen samples used in this study were produced for assisted reproductive procedures (GIFT, IVF) and routine semen analyses. Sperm concentration was determined using a Neubauer chamber as previously described [13].

**Manual Assessment of Sperm Morphology.** After liquefaction was complete (<30 min) and within 1 h of collection, morphology slides were prepared as previously reported [6, 12] using the Papanicolaou method. All slides were evaluated using strict criteria by one technologist. One hundred cells were evaluated from randomly chosen fields.

**Computerized Method for Sperm Morphology Reading.** Semen samples were obtained in the same way as for the manual method. Before making smears, semen was first washed once, as follows: an aliquot of semen ($=X \mu L$) was diluted in HAM’s F10 + 10% BSA medium according to its initial concentration. Diluted samples were then mixed by gentle shaking followed by centrifugation for 10 min at 1500 rpm. The supernatant was aspirated carefully using a Pasteur pipet, with the tube held at an angle. The pellet was resuspended in $Y \mu L$ medium to obtain a concentration of $100 \times 10^6$/mL.
Computerized Sperm Morphology Evaluation

\[ y = \frac{\text{initial concentration} \times \chi \mu L}{10^5} \mu L \]

Example: \[ y = \frac{50 \times 10^5/1000 \mu L \times 200 \mu L}{10^5} \mu L = 100 \mu L \]

Thin, evenly spread smears were made at an angle of 45° of these cell suspensions. For standardization, a drop of equal volume (25 μL) was used. A drop was pulled rather than pushed across the slide to avoid artificially detached heads. Slides were air-dried at room temperature and stained using the Diff-Quik [7] staining procedure. In the standard staining procedure (procedure A, Table 1), the slides were fixed within 1 h, after air drying for 10 s, in solution 1, and then immediately stained for 7 s in solution 2 and 7 s in solution 3. The slides were washed immediately after staining by gently being dipped 13 times into a container into which there was a continuous flow of tap water. The slides were placed in a vertical position to remove the excess water and allowed to air dry. The air-dried slides were then stored in the dark until evaluated.

A Hamilton–Thorn Version 10HTM-IVOS (Dimension V2.01) was used [10]. The IVOS cell analyzer combines an internal optical system with an internal computer image-digitizing and analyzing system. An automated stage moves the specimen slide between fields. In the sperm morphology application the optical system uses a narrow-band (662-nm) wavelength illumination in conjunction with a 100× oil-immersion objective. Images are produced on a charge-coupled detector and transferred to the digitizer.

The general operation of the IVOS and the measurement parameters used in the evaluation of sperm morphology were described in detail in a previous article [10]. The evaluations were performed as with the manual method by one technologist. One hundred cells were assessed on randomly (computer) selected fields.

**Experiment 1: Different Staining Procedures.** Twenty-two semen samples were analyzed by means of the IVOS using 5 different staining procedures, and their percentage normal sperm morphology outcomes were compared. Each sample was divided into 5 aliquots; one aliquot was processed according to the standard procedure (A) and the other 4 according to alternative procedures, as described in Table 1. For example, samples were processed fresh (<1 h) or after 5 h at room temperature and fixed immediately after air drying or after 24 h.

**Experiment 2: Time Study.** Twenty semen samples were analyzed by means of the manual method and 20 samples by means of the IVOS. This was done to compare the time taken to evaluate 100 sperm

<table>
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<tr>
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<td>A (standard)</td>
<td>&lt;1 h</td>
<td>Immediate</td>
<td>Immediate 7–7 s</td>
</tr>
<tr>
<td>B</td>
<td>&gt;5 h</td>
<td>Immediate</td>
<td>Immediate 7–7 s</td>
</tr>
<tr>
<td>C</td>
<td>&gt;5 h</td>
<td>Immediate</td>
<td>Immediate 20–20 s</td>
</tr>
<tr>
<td>D</td>
<td>&lt;1 h</td>
<td>Immediate</td>
<td>&gt;24 h 7–7 s</td>
</tr>
<tr>
<td>E</td>
<td>&lt;1 h</td>
<td>&gt;24 h</td>
<td>Immediate 7–7 s</td>
</tr>
</tbody>
</table>

*Note. Slides were prepared at the stated time after collection. Sample slides were fixed at the stated time after slide preparation, for 10 s. Slides were stained at the stated time after fixation, for example, 7 s solution 1 and 7 s solution 2.*
cells by the two methods. Timing of the manual assessment was started when the slide was placed on the microscope stage and ended when the evaluation of the 100 cells was complete. Times were recorded for two observers, one a routine observer and the other a nonroutine observer. Timing of the IVOS was started when the slide was introduced into the computer and ended when the final results appeared on the screen.

Statistics. In experiment 1, the nonparametric Friedman test was used to analyze the randomized block experimental design for the different staining procedures. In experiment 2, unpaired t tests were performed to examine the differences in reading time between the two manual observers and the computer.

RESULTS

Experiment 1

The median % normal for the 5 staining procedures (A–E) was 6.0, 6.5, 9.5, 8.5, and 5.5%, respectively (Figure 1). No significant difference ($p = .60$) was found between the different staining procedures used (Table 1). Slides prepared and stained according to procedure E, however, did cause artifactual damage (swollen and ruptured sperm heads) to the sperm in 2 cases (9.1%), resulting in no normal forms being read.

Experiment 2

The mean sperm concentration of the 20 samples prepared according to the manual method was $65.5 \times 10^6$/mL. The reading time per slide for the routine and nonroutine observers was 3 min and 6 s (SD = 54.87 s) and 3 min and 53 s (SD = 51.77 s), respectively. The mean sperm

![Percentage normal morphology distributions for the different staining procedures A–E from Table 1.](image_url)
concentration of the samples prepared for the computerized method was $60.1 \times 10^6$/mL. The mean reading time per slide for the computer was 5 min and 39 s (52.49 s). A significant difference was obtained between the reading time of both the routine ($p < .0001$) and the nonroutine ($p = .0082$) observers and the computer.

DISCUSSION

The production of reproducible and readable slides to ensure reliable results is a more critical factor for computerized evaluations than for manual evaluations of sperm morphology. Thin, evenly spread smears are required to ensure that all sperm are on the same focus plane, and uniform staining is needed for equal brightness over the entire slide. Contrast and background are also important factors that interfere with the computer's ability to select normal sperm. For instance, superposition of an object or unclear outlining of the sperm cell may result in an abnormal shape or size interpretation.

Washing the sperm sample once and then using the Diff-Quik staining procedure produced slides with a high-contrast resolution and with few background particles, enabling the computer to reach a sensitivity and specificity comparable with that of the manual method (unpublished, Menkveld), using the Papanicolaou method. A washing procedure prior to slide preparation has the added advantage of being able to concentrate the sample and to standardize the distribution of sperm cells (3–10 per frame) on screen. This modification also makes it possible to analyze oligozoospermic samples.

No statistical difference could be found between the 5 different Diff-Quik staining procedures compared in this study (experiment 1). Semen separated immediately from plasma or after >5 h did not interfere with the computer’s ability to distinguish normal forms. Samples can therefore be processed fresh or kept at room temperature until further analysis, depending on the workload and organization of the laboratory. No differences were found between the effect of different staining times and immediate versus delayed (>24 h) fixation. Unpredictable artificial damage does occur if fixation is not performed immediately after air drying.

In an IVE/GIFT program it is important to classify a man’s fertility potential prior to in vitro or in vivo insemination, because it will determine the number of sperm used per oocyte [14]. Using the wash–Diff-Quik staining procedure, good quality slides can be quickly obtained for morphology evaluation by the IVOS. Even though no washing procedure was used with the Papanicolaou method, the method is still more time-consuming by virtue of the numerous steps required. Although there was a significant difference in assessment time between the IVOS and the manual method, this is not of practical significance, since a 5-min reading time per slide is regarded as satisfactory. The IVOS is thus competitive as regards assessment time compared to the manual method, while remaining theoretically more objective.

The standardization of staining procedures is important to obtain reproducible and reliable results. We therefore recommend the wash–Diff-Quik procedure in which slides are prepared immediately after liquefaction and fixed after air drying. Ultimately, the improvement of slide quality in concert with the improvement of the IVOS will lead to shorter evaluation times.

REFERENCES


b. To investigate the role of automated semen analysis in *in vitro fertilization*.

i. *Prediction of fertilization rate in vitro using computer analysis of sperm morphology*

The first publication (Kruger *et al.*, 1993) on sperm morphology dealt with technical aspects of sperm morphology evaluation but also with clinical outcome, looking at fertilization rates in patients with sperm morphology < 14% and > 14% normal forms in two centers. The significantly lower fertilization rates in the group below 14% normal forms highlighted that a new diagnostic tool to predict fertilization *in vitro* was now available.

This novel work led to further studies in this field and also resulting in a PhD by Dr Coetzee in 1998. The research by Coetzee *et al.* that continued on the foundation set with the 1993 (Kruger *et al.*, 1993) and 1995 (Kruger *et al.*, 1995 a,b) publications by Kruger, as included in the previous section (a). These publications from the PhD thesis of Coetzee are listed under the reference section.
The following article reflects the scientific basis that supports the above argument:

A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading

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Daniel R. Franken, Ph.D.* Carl J. Lombard, Ph.D.¶
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Objectives: To compare the ability of a computerized method of sperm morphology with the manually recorded method in predicting in vitro fertilization (IVF) results, to compare results obtained by both methods, and to determine the intraobserver variability.

Design, Setting, Patients: Forty-three stained semen slide preparations from two large four academic institutions’ reproductive endocrinology units (IVF programs) were blindly evaluated, and the sperm were classified into normal and amorphous forms.

Results: Experiment 1: Twenty-one slide preparations from the Tygerberg gamete intrafallopian transfer program were manually evaluated; the fertilization rates for the groups with <14% and >14% normal sperm forms was 33.3% (15/45 oocytes) and 76.6% (46/60 oocytes), respectively. Corresponding fertilization rates with FERTECH were 46.8% (30/64) and 75.6% (31/41). Experiment 2: Twenty-two slide preparations from the Norfolk IVF program were evaluated. The manual method reported a fertilization rate in the group with <14% normal forms of 27.4% (14/51 oocytes) compared with 90.0% (127/141 oocytes) in the group with >14% normal forms. Corresponding figures for the FERTECH method were 33.9% (18/53) and 88.4% (123/139), respectively. Experiment 3: When the 43 slide preparations were blindly evaluated using both methods, 84% of the FERTECH evaluations correlated well with the manual method and FERTECH ability to diagnose the subfertile male (<14% normal forms) was 95% (sensitivity). Experiment 4: A total of 10 different slides (8 per group) were randomly selected and analyzed five times (100 cells per reading) by the computerized method. The slides were obtained from men with normal sperm morphology of <14% and >14% as classified by the manual method. In the first group (<14%) 97.5% (39/40) of the readings classified the sperm in the proper category, whereas in the second group (>14%) 95% (38/40) of the cases were correctly identified.

Conclusion: Using strict criteria for morphology evaluation, there is a positive and significant correlation between FERTECH evaluation and manual assessment. The reproducibility of the computerized method and the ability to distinguish between fertile and subfertile groups using those criteria are good. Fertil Steril 1993;69:202–9

Key Words: Sperm morphology, strict criteria, computerized method, in vitro fertilization

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¶ Institute for Biostatistics, Medical Research Council.

In the current literature, evidence supporting the crucial role of sperm morphology evaluation in the prediction of human fertilization in vitro is well documented (1–5). A clinically significant threshold
Conclusion:

The aim of the first publication in 1993 was to correlate sperm morphology, Strict Criteria (manual method), with Strict Criteria evaluated by computer using image analysis (computer method). An excellent level of agreement between the manual and computer methods was reported. The correlation was the same as if two experienced human readers were evaluating the sperm morphology slides. Using various statistical methods, it was reported that an excellent measure of agreement was reached. These observations set the stage for studies between computer and manual methods. A good clinical correlation was also reported with the computer and the computer method could thus be used to predict the chance of fertilisation *In vitro.*
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Chapter 5

The value of sperm selection has been studied extensively over the last 10 years in an attempt to improve fertilization and pregnancy rates in intra cytoplasmic sperm injection (ICSI) programs.

It was the work of Bartoov (Bartoov et al., 2001) that brought the concept of sperm selection to the forefront. The selection of spermatozoa with high magnification, selecting the ideal form if possible, solved poor pregnancy rates in couples with repeated failures. This method was called intracytoplasmic morphology selected sperm injection (IMSI). If no normal sperm could be found for sperm injection, Berkovitz observed a poorer embryo quality and lower pregnancy rates with higher abortion rates (Berkovitz et al., 2006).

Recently, in a randomized controlled trial Antinori reported a significant improved pregnancy rate in the IMSI group versus the ICSI group (Antinori et al., 2008). With annexin V binding it was observed that non-apoptotic sperm correlated with the percent normal forms. This observation confirmed the earlier finding that the random selection of the ideal form will assist in obtaining a ‘healthier’ sperm to be used for ICSI (Hoogendijk et al., 2009).

Huszar have shown that mature spermatozoa selectively bind to solid state Hyaluronic acid (HA) (Huszar et al., 2007). These spermatozoa are devoid of cytoplasmic retention, persistent histones, DNA fragmentation with lower frequency of chromosomal aneuploidy. The normal oval shape spermatozoa were also significantly richer in hyaluronic acid compared to the amorphous forms. A single blind collaborative study between Huszar at Yale and the Stellenbosch group followed. After hyaluronic acid selection took place of spermatozoa, with a non selected control group, the percentage normal forms were read. This led to the publication by Prinosilova (Prinosilova et al., 2009) confirming that a significant improvement in selection of the ideal forms took place in the test samples (hyaluronic acid) compared to control.

In this chapter, a new classification for spermatozoa to be used for IMSI is proposed based on physiological (Garolla et al., 2008; Huzar et al., 2007) and electron microscopical principles (Chemes et al., 2007).
To summarize: The original research reported on the normal form and its impact on fertilization and pregnancy rates in *In vitro* (Kruger *et al.*, 1986; Kruger *et al.*, 1988). In addition physiological defects were reported in the patients with severe abnormalities in sperm shape (P-pattern). Twenty years later the same observations were made for individual spermatozoa and the importance realized of selection of the ideal form for improved results in ICSI programs (Bartoov *et al.*, 2001; Berkovitz *et al.*, 2001; Huzar *et al.*, 2007; Prinosilova *et al.*, 2009).
The rationale of sperm selection

a. To assess the value of sperm selection for intracytoplasmic sperm injection (ICSI)

i. Should ICSI be done for all IVF patients?

It was written by Roldan that the widespread use of ICSI relies, in part, on the belief that it is not necessary to pay attention to aspects of sperm function because sperm selection becomes irrelevant if a spermatozoon is injected into the oocyte (Roldan et al., 2006). On the other hand, the understanding of the underlying mechanisms of sperm function during fertilization is important for successful results with ICSI. This understanding can lead to further improvement of the fertilization results leading to better embryo quality and thus better pregnancy rates. Roldan also suggested that acrosome reacted spermatozoa may give better pregnancy rates when used for the ICSI procedure. As was outlined in the first two chapters of this thesis that the understanding of semen thresholds will assist in selecting patients truly in need for the ICSI procedure. There is a trend towards offering ICSI to all patients. This approach is to the detriment of the patient and sometimes to the physician/scientist. It is important for the clinician to keep his/her skill to make good clinical diagnosis and to be able to offer simple solutions for infertility problems in many cases. Without insight in semen analysis for both the scientist and the clinician this skill will be lost with clear consequences to the patient and reproductive science as a whole. This was the background for the article written recently addressing the issue if ICSI must be offered to all patients (Kruger et al., 2009).
The following article reflects the scientific basis that supports the above argument:

Kruger TF. Should ICSI be done for all IVF patients? MEFSJ 2009;14(2):85-95
DEBATE

Should ICSI be done for all IVF patients?

Complete failure of fertilization occurs in a small but significant number of non-male factor IVF cases. In cases of unexplained infertility, sperm abnormalities can be the cause. There are no reliable and guaranteed tests that can predict, with accuracy, the fertilization rate in vitro, prospectively. In many countries, ICSI is being performed for all IVF cases and there is a significant trend in the United States to do ICSI on cases of unexplained infertility, advanced maternal age, and poor ovarian reserve. In this issue, some of the world’s experts on IVF/ICSI attempt to address this problem and present their point of view. I am sure that the reader will find their comments to be helpful and enlightening.

Subheil J. Muasher, MD, FACOG
Deputy Editor,
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Comment by: E. Hakan Duran, M.D.
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It has been several years since our last opinion paper on application of ICSI for all cases of in vitro conception (1). During the years 1998-2007 the use of ICSI increased from 40% to 63% for all assisted reproduction technology (ART) cycles performed in the USA (2, 3). Considering that the number of total ART cycles surged from 53,154 to 132,745 during that period, the actual application of ICSI increased approximately 4-fold. Figure 1 shows that the use of ICSI at our institution has followed the national trends. Today, there are many fertility centers throughout the world, which abandoned conventional IVF altogether in favor of ICSI on a pragmatic basis.

Since the advent of ICSI (4), there has been general agreement on its use for male factor infertility. In our program, the indications for ICSI in couples with male factor infertility have traditionally been: men presenting with varying degrees of oligo-astheno-teratozoospermia (OAT), alone or in combination, anti-sperm antibodies, and obstructive and non-obstructive azoospermia (where ICSI is combined with testicular sperm extraction) (5). In our opinion, a thorough semen evaluation is mandatory for proper clinical management and directing patients to ICSI. Our program recommends the use of extended semen analysis and sperm functional testing to properly identify cases at risk for poor or failed fertilization (6, 7). Based on initially accumulated experience, the use of ICSI quickly expanded to cases with previous failed fertilization in conventional IVF and to diagnosed cases of unexplained infertility. The aims of these broadened indications were to avoid the undesired outcome of complete failure of fertilization, and to enhance the number of available embryos to optimize the total reproductive potential of a given IVF cycle.

A thorough search of the current medical literature reveals 12 randomized controlled trials with a reasonable sample size that addressed the question of whether ICSI should be the treatment of choice for all cases on in vitro conception. When analyzed in detail, some of these reports did not seem to have appropriate randomization methodology flawing the study design. Abouhajar et al. studied 116 couples with tubal factor infertility that underwent ART by either IVF or ICSI (8).
Similarly, Bukulmez et al. included 76 couples with tubal factor only (9). With these two similar studies combined (n=192), fertilization rate per oocyte was significantly greater for IVF (64.8\% vs. 53.5\% for ICSI). No differences were detected between these two groups for clinical pregnancy, miscarriage or live birth rates.

Ruiz et al. reported 70 couples with unexplained infertility and mild endometriosis undergoing ICSI vs. IVF for the sibling oocytes according to the order of retrieval (10). Despite the lack of appropriate randomization technique, this study failed to show any differences between the two groups for both fertilization rate and embryo quality. The authors concluded that although ICSI might be preferred for some oocytes to avoid fertilization failure, it was not superior to IVF as an insemination technique.

Poehl et al. reported 91 cases with tubal or cervical factor infertility randomized in ICSI and IVF groups, although the randomization technique was not clearly stated (11). The baseline characteristics of the two groups were similar and the main outcome measures, i.e., fertilization, ongoing pregnancy and implantation rates, and embryo scores were not different.

Among the randomized controlled trials with appropriate randomization technique, Bhattacharya et al. had the largest series from a multicenter study (12) by randomizing 415
couples with non-male factor infertility to either IVF or ICSI. The authors found no significant difference between the two study groups on implantation or pregnancy outcomes; laboratory time was significantly longer for ICSI than IVF. Fishel et al. randomized 221 couples to either ICSI vs. IVF or ICSI vs. high insemination concentration IVF groups depending on the history of previous IVF performance and presence of male factor infertility (13). They reported overall higher fertilization rate for ICSI groups but similar embryo quality. However, there was no pregnancy outcome measure addressed by this study. Staessen et al. evaluated 56 couples with tubal factor infertility, randomizing sibling oocytes to ICSI vs. IVF (14). They found no difference in fertilization rate, implantation and clinical pregnancy rates. More patients had fertilization failure in IVF group but the numbers were too few to compare statistically. Westerlaken et al. randomized sibling oocytes of 38 patients with low fertilization (<25%) or total fertilization failure during a prior IVF cycle to ICSI and IVF/ICSI (15). Not only the fertilization rate was significantly lower in the IVF group, but also there were recurrent fertilization failures. No difference in clinical and ongoing pregnancy as well as implantation rates was detected; however, the numbers were quite low to provide adequate power. Another study by the same group randomized sibling oocytes from 106 couples with borderline semen characteristics (16). There was no significant difference in clinical and ongoing pregnancy rates between the IVF and ICSI groups, although numbers were more favorable for ICSI but power seemed inadequate. Foong et al. randomized 60 couples with unexplained infertility to IVF vs. ICSI groups (17). They did not find any significant difference in any of their outcome measures. Finally, Hang et al. randomized 1089 sibling oocytes obtained from 60 patients with polycystic ovarian syndrome (PCOS) to IVF vs. ICSI (18). Nine patients from the IVF group had fertilization failure, in addition to the significantly lower fertilization rate in this group (44.8% vs. 72.0% in the ICSI group). Embryo quality on the other hand was comparable between the study groups.

The pragmatic approach to perform ICSI for all ART cycles is therefore not supported by evidence which demonstrates that better clinical outcomes are obtained with ICSI in the non-male factor population. In an attempt to avoid unexpected fertilization failure or low fertilization rate in a couple with unexplained infertility (here maybe an “occult” male factor is present), or in cases where the number and/or quality of oocytes are poor secondary to a decreased ovarian reserve, it is tempting to opt for ICSI, but the evidence to support its use is lacking. Similarly, in light of the reports (18-21) indicating a reduced fertilization rate in patients with PCOS, routine application of ICSI may have practical benefits in this group of patients. Whether to prefer short-term practical benefits to evidence based decisions is at the discretion of the fertility center managing the patient at the moment.

This very issue is quite important when the long term effects and risks of ART are considered. A recent report analyzed the data from the US National Birth Defects Prevention Study and concluded some birth defects to occur more often among infants conceived with ART (22). These included septal heart defects, cleft lip / palate, esophageal and anorectal atresia. This particular study did not analyze ICSI and IVF subgroups separately, but this comparison has been done by a number of studies previously. A meta-analysis combining such studies identified a non-significant risk ratio of 1.12 (95% confidence intervals [CI]: 0.97-1.28) for major birth defects from four peer-reviewed publications (23). However, when three more sources of large non-peer-reviewed reports (the Australian-New Zealand, British and French databases) were included, the re-calculated and significant risk ratio was found to be 1.20 (95% CI: 1.09-1.31). Among all, the increase in major birth defects was more notable from the British data and there was significant heterogeneity in risk ratios between the studies. It is important to note that the indications for ICSI were not stated.

It is well established that there is increased incidence of Y chromosome micro-deletions in cases with severe OAT. Similarly, males with congenital bilateral agenesis of vas deferens should be screened for cystic fibrosis. Both of these conditions may be inherited to the offspring by ICSI. The incidences of both de novo structural chromosomal abnormalities and sex chromosomal
abnormalities have been reported to be increased in ICSI offspring, particularly in cases of severe male infertility (24). Similarly, cases with the imprinting disorders Angelman and Beckwith-Wiedemann syndromes, have been associated with ICSI (25,26). Certain childhood cancers have been known to be associated with these disorders. The potential impact of sperm DNA fragmentation and particularly, of DNA damage in morphologically normal sperm found in men with teratozoospermia, needs to be further addressed (27,28). More studies are required to understand the pathogenesis of these disorders and to find out if any precautionary step can be taken to prevent them. Few studies have addressed the developmental outcome of ICSI babies in comparison to IVF babies. There was no difference in developmental outcome, measured by Bayley Scale at the age of 24-28 months between ICSI and IVF children (29).

In conclusion, there is an increasing application of ICSI in the clinical setting in spite of known (male factor population) and potential (non-male factor population) risks. The spectrum of ICSI indications has broadened in parallel to its application. As always, the physician must weigh the benefits versus the risk of using assisted fertilization techniques on an individual basis. Further data are needed to assess the long-term physical and psychological development of ICSI children, when used for male infertility and other indications. ICSI has become the dominant fertilization technique in the ART lab, but the above-mentioned findings highlight the need for continuous monitoring of ART results.

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Debate Should ICSI be done for all IVF patients?

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In 1992 intracytoplasmic sperm injection (ICSI) was reported as a treatment method for male factor infertility (1).

To date the use of this method for severe male factor infertility is widely supported (2). As with any new treatment modality there is always the pendulum effect regarding its real place in the assisted reproduction arena. There are authors suggesting ICSI to be used for all cases and indications of assisted reproduction even when clinical trials indicate that ICSI is no more effective than in vitro fertilization (IVF) (3).

In our unit we use the following criteria for male subfertility. A semen concentration/ml of less than 10 million. Percent motility < 30%, forward progression < 2 with normal sperm morphology less than 4% (4). When using these thresholds, we observed that 56.9% of patients attending our clinic, are normozoospermic. The rest with present with male subfertility of 11.2% - 17.8% will be severe male factor infertility (double defects / triple defects and/or azoospermia) (5).

Severe male factor infertility

Indications for ICSI are ejaculated sperm (oligozoospermia, asthenozoospermia, teratozoospermia [<4% normal], Antisperm antibodies [MAR >60%], frozen sperm from cancer patients, ejaculatory disorders, eg retrograde ejaculation), azoospermia (absence of the vas deference, failed vaso-vasostomy, obstruction of both ejaculatory ducts) and testicular spermatozoa (all indications for failed epididymal spermatozoa, azoospermia caused by testicular failure [maturity arrest, germ cell aplasia], necrozoospermia). (2).

ICSI is the first choice treatment for severe male factor infertility to achieve pregnancy rates similar to IVF rates for non-male factor infertility (6).

Although no randomized controlled trials exist, retrospective analysis indicated that live birth rates using ICSI for severe male factor infertility are far better than those for conventional IVF (when used
as treatment for severe male factor infertility), IUI and subzonal sperm injection for the same indication (7).

**Non-male factor infertility**

In a prospective randomized multicentre trial, IVF was compared with ICSI, 219 cycles in the IVF group and 206 in the ICSI group (3). Their results showed that the use of ICSI in couples suitable for IVF treatment does not offer any clinical benefits. They found a significant difference in implantation rates in favor of IVF, but the pregnancy rates did not differ significantly. They also reported that the proportion of multiple pregnancies were similar.

In a small (60 patients) prospective randomized trial of conventional in vitro fertilization (IVF) vs ICSI in unexplained infertility, Foong et al (8) reported no differences in clinical outcomes associated with IVF vs ICSI in a treatment of unexplained infertility.

In a recent Cochrane review (9), it was concluded that in the single identified study (3), no difference in pregnancy rates were reported (OR 1.4, 95% CI, 0.95-2.2).

One could thus argue in favour of IVF in the light of simplicity and time involved in the laboratory as well as more and longer term data available on children born from the IVF technique.

**Male subfertility (mild male factor)**

Tournaye et al reported on randomized controlled trials dealing with “borderline semen parameters” (10). In these studies (n=9), more oocytes were fertilized after ICSI (1264/2015 - 62.7%) than after conventional IVF (549/1688 - 32.5%). The RR in all randomized controlled trials were all in favour of ICSI. Complete fertilization failure occurred in 37.4% of the IVF cycles vs 2.5% of ICSI cycles. However, Tournaye et al advocated the use of high insemination concentration (HIC) to counteract fertilization failure and in a prospective randomized trial, they showed that there was no significant difference in fertilization between ICSI and HIC IVF (10). This concept must be taken into consideration in handling patients with borderline semen parameters but more randomized controlled trials are necessary to test this concept.

I am in agreement with Tournaye stating that each IVF lab should try to improve their IVF procedure to the greatest extent and optimize the insemination procedures so as to cope with the specific demands of the couples involved (10). Corrective measures to promote IVF may have an important role to play, even in the era of ICSI (10). More randomized controlled trials comparing ICSI with optimized IVF procedures are certainly needed. In the meantime, at least a split IVF/ICSI approach, may be chosen as a first choice in couples with moderate male subfertility (10).

**Fertilization failure**

Total fertilization failure occur in 1-2 % of all IVF cycles (11). According to the Capri Workshop Group, no recent randomized studies considered ICSI for secondary prevention of failed fertilization in couples with a history of failed or poor fertilization (2). It is however, common practice that most units will offer ICSI as the preferred method in such cases. It was suggested by the Capri Group that a trial should be done with patients as the unit of randomization and live birth as the outcome (2).

To summarize it is my opinion based on the evidence available, that IVF is the treatment method of choice in cases with non-male factor infertility. On the other hand, ICSI is the preferred treatment modality in cases with severe male factor infertility and fertilization failure. In patients with male subfertility, HIC can be considered with at least a split IVF/ICSI approach.

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The injection of a single spermatozoon into the cytoplasm of a mature oocyte, ICSI (intracytoplasmic sperm injection), has been one of the remarkable progress in ART to treat male factor infertility (1). Interestingly, in the earlier days the indication to perform ICSI was purely for severe male factor infertility, i.e. cases with various degrees of spermatogenic disorders which had either no chance or an extremely low probability of fertilization with conventional in vitro insemination. Today the range of indications have expanded to include unexplained fertilization failure after conventional IVF (2,3), ejaculatory dysfunction, immunological infertility, endometriosis (4) and poor ovarian response (3). Another indication for ICSI is for cases utilizing thawed or re-warmed oocyte after cryopreservation (5). In these instances, oocytes that survive the freezing/thawing or the re-warming process require insemination by ICSI due to the thickening and hardening of the zona pellucida (6) and in fact, the application of ICSI has consistently led to an increase in the fertilization rate of thawed oocytes (7).

In 1997, five years after the initial successful report of a pregnancy by ICSI (1), the number of ICSI cycles comprised 35% of the ART procedures in USA. In the year 2000 the number of ICSI cycles increased to 47% of the ART procedures, and in the recent 2005 CDC report, the number of ICSI cycles have reached 60% of the ART procedures performed in USA. By comparing the 1997 and the 2005 statistics, ICSI utilization has almost doubled and represents now about two third of the insemination methods used during IVF. However, this progressive increase in the utilization of ICSI has not been dictated by a parallel increase in the number of male infertility cases and more importantly, has not been associated with an overall increase in live birth rates. Several authors have been suggesting that ICSI should be offered to all patients needing IVF because of the significantly higher fertilization rate (3). However, the lack of natural selection of sperm and the circumvention of most of the known fertilization stages have led to concerns regarding perinatal outcome; therefore, the issue of whether to use ICSI for all in vitro insemination needs to be critically reviewed and discussed.

What are the rationale and justifications to apply ICSI for each IVF case? The justifications for utilizing ICSI should be: a) higher fertilization rate; b) prevention of fertilization failure and c) higher live birth rate for each of the conditions where this technique is preferred to the conventional IVF insemination. In this paper we will therefore

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examine the following outcome points after utilization of ICSI: a) fertilization rate; b) embryo quality and rate of blastocyst development; c) safety: perinatal risks and child development; d) congenital malformations and epigenetic (imprinting) disorders.

Fertilization rate

The first question to address is whether the fertilization rate with ICSI is increased when compared to conventional IVF for non-male factor cases. The use of fertilization rate instead of clinical pregnancy rate as an outcome has drawbacks since it is an interim outcome measure in an IVF program, which may have little effect on the final outcome of a fresh cycle or that of a subsequent frozen embryo transfer. Several reports have been published in which ICSI and conventional IVF were compared when sibling oocytes were split between the two insemination methodologies (8,9,10).

Abouighar et al. (10) made the comparison between ICSI and IVF within three groups of patients who were beginning their first IVF treatment cycle. In the group with tubal factor infertility and normal semen parameters they observed a significantly higher fertilization rate per retrieved oocyte with IVF, but no significant difference in pregnancy rate between ICSI and IVF. In the group of patients with unexplained infertility and normal semen parameters there was no significant difference in fertilization rate, whereas in the group of patients with borderline semen parameters there was a significantly higher fertilization rate with ICSI (59%) than IVF (27%). Other studies have also reported a lack of significant difference in fertilization rates of ICSI versus IVF in patients with non male factor infertility (61% versus 67%) (11) and unexplained infertility (60% vs. 54%) (12).

The data from two systematic reviews also revealed that in couples with normal semen parameters there is insufficient evidence to claim a difference in fertilization rates per retrieved oocyte or pregnancy rates, between ICSI and conventional IVF (13). Again, only couples with borderline semen parameters had higher fertilization with ICSI than IVF, a result confirmed by a subsequent meta-analysis. However, it was also suggested that similar fertilization rates could be achieved with a modification of the conventional IVF process, for example by using high sperm insemination concentration (HIC) IVF (14).

According to these reviews, ICSI should be the insemination of choice for severe male factor infertility and can be justified for cases with previous unexplained fertilization failure after conventional IVF.

Embryo quality and blastocyst formation

The second question to address is whether the embryo quality is superior if oocytes are inseminated by ICSI other than conventional IVF and whether the rate of blastocyst development after ICSI (a marker of embryo quality) is higher.

It is well known that embryo quality is an important factor affecting pregnancy potential. Implantation rates for ICSI-derived embryos continue to be lower than embryos derived from conventional insemination suggesting a diminished implantation potential of ICSI-derived embryos (15, 16). These negative influences on lower implantation rates have been attributed to the poor quality of injected spermatozoa.

It is known that spermatozoa requiring ICSI (either due to low production or low motility or low normal forms or a combination thereof) have higher levels of aneuploidy, DNA damage, DNA breaks and other subcellular defects which may impair embryo development (17, 18). Martin et al., (19) studied men with severe oligozoospermia (<106 sperm/ml), men with moderate (1.9 x 10^6 sperm/ml) or mild (10-19 x 10^6 sperm/ml) oligozoospermia. The FISH analysis using DNA probes specific for chromosomes 13, 21, X, and Y detected a significant inverse correlation between the frequency of sperm chromosome abnormalities and the sperm concentration for XY, XX, and YY disomy and diplody. These results demonstrate that men with severe oligozoospermia participating in reproductive treatments have an elevated risk for chromosome abnormalities in their sperm, particularly sex chromosome abnormalities.

In addition male factor infertility has been
shown to significantly reduce blastocyst production; it is not clear whether this is caused by the paternal genome or the ICSI technique itself (20-23). Although, production of blastocysts appears to be negatively affected by paternal or ICSI contributions, most studies demonstrated equivalent pregnancy and implantation rates between the blastocysts derived from ICSI and from conventional insemination (21).

One randomized controlled study which compared ICSI with IVF in non male factor cases concluded that implantation and pregnancy rates were not different (10).

On the other hand, in a recent study using oocytes from 35 couples with non male factor infertility, Khamsi and colleagues demonstrated increased formation of good quality embryos per retrieved oocyte after ICSI than after conventional IVF (64% and 47%, respectively) (24). However, there was no significant difference in the formation of good quality embryos per fertilized oocyte.

These results indicate that ICSI does not improve or diminish the formation of good embryos. Ultimately, embryo quality is likely depending on intrinsic factors of both gametes rather than on the fertilization method per se (25).

Safety: Perinatal Risks and Child Development

Unlike most therapeutic procedures used in medicine, assisted reproductive techniques in general and ICSI in particular, never underwent rigorous safety testing before their clinical use. In recent years, a large body of evidence has been accumulating demonstrating that many forms of male infertility have a genetic cause (26, 27). Researchers believe that perhaps 75% or more cases of all infertility have a contributing genetic basis, however the ability to diagnose these defects remains limited (28). Put simply, large numbers of couples undergo fertility treatments without a complete understanding of the basis of their infertility or the potential long term risks for their offspring. As a consequence, many infertile men requiring ICSI because of severe oligozoospermia are at high risk of producing sperm with chromosomal aneuploidies or structural aberrations. In addition, men with normal semen parameters by conventional means may have high levels of chromosomal aberrations, apoptosis, and fragmented sperm DNA which can go unrecognized until poor embryo development during ART is encountered.

ICSI outcome should be assessed also in terms of safety by examining the perinatal risks and long term physical and development issues of ICSI children. It is well known that pregnancies resulting from the use of assisted reproduction techniques are associated with significantly increased risks to mother and offspring due to multiple gestation, preterm delivery and congenital abnormalities in the offspring. A small increase in late preterm birth and low birth weight has been confirmed in most studies of outcome of pregnancies conceived via IVF with or without ICSI. However ICSI pregnancies appear to be associated with an increased risk of monochorionic placentation, particularly when combined with day 5 embryo transfer (29).

There is little information about the long term outcome of children born after ICSI. In a systematic review from Cochrane database (30) children conceived via ICSI have been followed up to age 8 years, and investigated in terms of pubertal staging, neurological development and rates of surgery/hospitalization. Pubertal staging, neurological examination and rates of surgery/hospitalization was found to be similar for ICSI children and those conceived spontaneously. However the mechanical nature of the fertilization, that is, bypass of the zona pellucida and oolemma membrane, coupled with the indications to its use, that is, mostly for cases where the reasons of poor spermatogenesis are unknown, have and continue to raise concerns about the possible long term health consequences on the offspring (31; Sutcliffe A, personal communication).

Congenital Malformations and Epigenetic (imprinting) Disorders

A higher rate of inherited chromosomal anomalies has been reported in ICSI children, mainly due to potential structural chromosomal anomalies, as well as higher rate of de novo chromosomal anomalies related to paternal sperm characteristics (32). Malformation rates were
comparable between ICSI and IVF in most studies, but were two-fold higher compared with the general population (32, 33, 34).

A recent study evaluated both obstetric outcomes (344 pregnancies generated from ART compared with 344 spontaneous pregnancies) and rates of congenital anomalies in children (n=432) born after in vitro maturation of oocytes (n=55) or IVF (n=217) or ICSI (n=160), compared with children (n=360) born after spontaneous conception. In conclusion all ART pregnancies are associated with an increased risk of congenital abnormality (35). Reports of imprinting-related diseases such as Angelman and Beckwith-Wiedemann syndromes in offspring conceived via IVF as well as ICSI suggest a possible risk of in vitro culture procedures and requires further investigation (36).

CONCLUSION

Using the currently available data, ICSI performed with normal sperm parameters still cannot overcome fertilization failures due to oocyte abnormalities and in all maternal age groups, the rate of live birth per egg retrieval appears to be slightly higher for conventional IVF. There is no clinical evidence to prefer ICSI versus conventional IVF in patients with normal factor infertility.

Furthermore, since ICSI is applicable only to mature metaphase II oocytes, properly identified as such after being stripped of their cumulus cells, the widespread use of ICSI may ultimately reduce the number of usable oocytes because the latter once denuded and found to be still immature, rarely progress to metaphase II. If these oocytes would be used for conventional insemination (thus not subjected to hyaluronidase to remove their cumulus cells) they could achieve maturity and fertilization.

Finally, ICSI bypasses the known biological mechanisms of sperm selection operating at the level of the zona pellucida. Even with the latest introduction of devices to increase the possibility of “choosing” the best spermatozoa for ICSI, with the technique of sperm selection via binding to hyaluronan acid (37) or with the intracytoplasmic morphologically selected sperm injection (IMSI) method (38), the "human factor" in making the final decision on which sperm to select, is still relevant.

In conclusion conventional IVF should be the option of choice for every couple requiring ART treatments to avoid the disadvantages of ICSI. A more acceptable strategy might be to perform a diagnostic comparison between conventional IVF and ICSI in all first treatment cycle of couples diagnosed with unexplained infertility. This would be beneficial in preventing total fertilization failure and in providing a clear answer to the couple of whether ICSI is a necessary tool to achieve fertilization.

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ii. **Sperm selection using hyaluronic acid binding**

It was reported that men with low sperm morphology (P-pattern) had more DNA abnormalities and sperm aneuploidy than G-pattern and N-pattern groups (Lee *et al.*, 1996). These P-pattern groups also had poorer sperm binding to the zona pellucida and acrosome reaction ability compared to men with normal semen parameters (Oehninger *et al.*, 2000, Bastiaan *et al.*, 2003). It was this knowledge and the practical experience of poor fertilization rate in the P-pattern group that kindled the interest in the selection of sperm for ICSI. The publication of Menkveld (Menkveld *et al.*, 1999) on selection of the normal form by the zona pelucida, was often discussed. The question arose, why were all these forms bound to the zona pellucida almost perfect in shape? Also, the observation by Huzar (Huzar *et al.*, 2007) that the normal forms bound to the zona were rich in hyaluronic acid (HA) and that there were less aneuploidy reported in the shapes observed were of great interest to the scientific community. These authors (Huzar *et al.*, 2007) have also shown that mature spermatozoa selectively bind to solid state HA. These spermatozoa are devoid of cytoplasmic retention, persistent histones and DNA fragmentation with lower frequency of chromosomal aneuploidy. The normal oval shape spermatozoa were also significantly richer in hyaluronic acid compared to the amorphous forms. A collaborative study initiated by Huzar followed. In a blind study, after hyaluronic acid selection took place of spermatozoa, with a non selected control group, the percent normal forms were read. This led to the publication by Prinosilova (Prinosilova *et al.*, 2009). It was observed that a significant improvement in selection of the ideal forms took place in the hyaluronic acid group (test) compared to control.
The following article reflects the scientific basis that supports the above argument:

Prinosilova P, Kruger TF, Sati L, Ozkavukcu S, Vigue L, Kovanci E, Huszar G. Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology. RBM Online 2009;18(2)
Article

Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology

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Abstract

During spermiogenesis, a plasma membrane remodelling step facilitates formation of sperm zona pellucida and hyaluronic acid (HA) binding sites. Enrichment of Tygerberg normal spermatozoa in HA-bound versus semen sperm fractions was postulated. Semen was placed on the uncoated A side and HA-coated B side of a semen chamber. After 15 min, the HA binding score (proportion of HA-bound motile spermatozoa) was assessed on the B side, and unbound spermatozoa were removed by gentle rinsing. Following Diff-Quick staining, sperm morphology of A and B sides was evaluated by three blinded investigators at Yale and Tygerberg. The proportion of Tygerberg normal spermatozoa was higher in HA-bound versus semen spermatozoa (n = 63 subjects) with a 104-fold improvement (95% confidence limits: 1.9–4.7) in 37 teratozoospermic men, comparable with a 4.2-fold enrichment in zona pellucida-bound spermatozoa reported earlier by the Tygerberg group. The morphology scores of three investigators were different but related, indicating that the variations reflect individual to individual differences in the perception of shape normality. The selection power of HA and zona pellucida for normal spermatozoa are similar. The sperm biomarkers of creatine phosphokinase (reflecting retained cytoplasm in arrested maturity spermatozoa) and chaperone protein HspA2 (heat shock protein) were proportional with sperm HA binding. As HA binding reflects sperm maturity and function, the combination of Tygerberg morphology and HA binding is likely to improve male infertility management.

Keywords: hyaluronic acid binding, sperm biochemical maturity markers, sperm maturity, sperm selection, zona pellucida binding

Introduction

During late human spermiogenesis, simultaneously with the maturation step of cytoplasmic extrusion, a plasma membrane remodelling process occurs that facilitates the formation of the zona pellucida and hyaluronic acid (HA) binding sites in mature spermatozoa (Huszar et al., 1997; 2007). Previous studies have indicated a very close inverse correlation (r > 0.8) between the degree of cytoplasmic retention and the expression of the membrane maturity marker galactosyl transferase in human spermatozoa (Huszar et al., 1997).

Spermatozoa of arrested maturation, which also contain surplus cytoplasm, and show consequential abnormal morphology, have diminished ability to bind to the zona pellucida or to HA (Huszar et al., 1994; Cayli et al., 2003). Conversely, spermatozoa that are able to bind to solid state HA are mature and devoid of cytoplasmic retention, excess persistent histones, apoptotic processes, DNA chain fragmentation, and show a normal frequency of chromosomal aneuploidies (Clermont, 1963; Huszar et al., 1998a, 2003; Cayli et al., 2004; Jakub et al., 2005; Sathi et al., 2008).
Arrested maturity spermatozoa, due to the retained cytoplasm, may be characterized by larger and irregular heads, enlarged post-acrosomal and mid-piece region, and axial insertion of the tail. As a consequence of spermigenetic arrest, these cells also have shorter tails, and a lower tail length/head long axis ratio (Huszar and Vigue, 1993; Gergely et al., 1999; Chemés and Revet, 2003). Conversely, the shape attributes of mature spermatozoa conform with normal Tygerberg strict morphology, as these criteria were developed based on zona pellucida-bound spermatozoa (Kruger et al., 1986; Menkveld et al., 1990).

The relationship between sperm maturity and sperm shape has also been studied with computer-based objective morphometry assessment. In line with the attributes of mature spermatozoa, semen samples with low, intermediate and normal levels of sperm maturation, detected by the biochemical markers, were significantly different in head long axis, head area, sperm shape symmetry, tail length and the tail length/head long axis ratio (Gergely et al., 1999; Celikt-Özenci et al., 2003, 2004).

In earlier studies focusing upon sperm morphology in semen and in the respective zona pellucida-bound sperm fractions of the same ejaculate, the Tygerberg group has reported a 4.2 increase in spermatozoa with normal strict morphology in the zona pellucida-bound fraction (Menkveld et al., 1991). At that time, the investigators believed that selection of the normal spermatozoa was a consequence of rejection of the abnormal or amorphous sperm by the zona pellucida. It is now understood that due to sperm plasma membrane remodelling, the selection of mature spermatozoa with normal morphology is a sperm-initiated active process (Huszar et al., 1994, 1997, 2007). Because mature spermatozoa were shown selectively to bind either to the zona pellucida or HA, the present work has tested the hypothesis that, similarly to the sperm/zona pellucida studies, there will be an improvement in the proportion of spermatozoa with normal Tygerberg morphology in the HA-bound versus the respective semen sperm fractions.

**Materials and methods**

**Experimental design**

Sperm morphology was studied according to the Tygerberg criteria in 63 men. The proportion of Tygerberg normal spermatozoa in semen and in their respective HA-bound sperm fractions were evaluated by three independent investigators in a blinded manner. Two readers were from the USA (US1 and US2) and the third, TSA (TFK) was from Tygerberg, South Africa. All studies were approved by the Human Investigation Committee of the Yale School of Medicine.

**Preparation of spermatozoa for the studies**

For the sperm studies and sperm:HA binding assay, a modified Cell-View semen chamber was used (MidAtlantic Diagnostics Inc., USA). One side (A side) was composed of plain uncoated glass, whereas the second side (B side) was coated with hyaluronic acid. A 7 μl aliquot of semen (neat semen, without washing, centrifugation or any other intervention) were placed on the B side, and the spermatozoa were allowed to bind to the HA-coated surface in a wet chamber at room temperature. After 15 min, repeated gentle rinsing to the B side was applied in order to remove the unbound spermatozoa. Following that, a 7 μl aliquot of semen was smeared on the uncoated glass on the A side. Subsequently, spermatozoa on both sides of the chamber were fixed and stained with Diff-Quik (VWR, Bridgeton, NJ, USA). Following the staining step, cover slips were placed on both the A and B sides and were sealed with Permount (Fishier Scienti c, Fair Lawn, NJ, USA). For the assessment, 200 spermatozoa, from at least three or four areas of the A and B sides by each investigator. After evaluation by the two Yale investigators, the slides were sent to South Africa by overnight mail.

**Sperm morphology evaluation**

This assessment was carried out using the Tygerberg strict criteria (Kruger et al., 1986; Menkveld et al., 1990). A normal spermatozoon was classified as such if the head was oval shaped and had a well-defined acrosome covering 40–70% of the apical part of the head. The midpiece was smooth, and to the base of the head there was one tail symmetrically attached. The tail was not coiled or abnormally bent. All borderline forms were classified as abnormal. In addition, spermatozoa that were overlapping or not positioned fully at the glass surface were not considered.

**Determination of sperm HA binding score**

For the determination of the HA binding score in the semen samples, 68 μl aliquots of semen were applied to the HA-coated hyaluron binding assay glass chambers (sperm-HB test; MidAtlantic Diagnostics Inc.) according to the manufacturer’s recommendation. After application of the semen drop, sperm binding to HA was observed at room temperature under phase contrast microscopy. After 15 min, the proportions of spermatozoa that were HA-bound and exhibited vigorous tail beating versus the unbound spermatozoa that were freely swimming were assessed.

**Assessment of the biochemical markers of sperm maturity**

The determination of sperm creatine phosphokinase (CK) activity (i.e., cts surplus cytoplasm in arrested maturity spermatozoa) and the expression levels of the chaperone protein Hsp2A (heat shock protein) (measure of sperm maturity) are standard procedures in the laboratory. For these studies, aliquots of semen were washed with 10–15 volumes of ice-cold 0.15 mol/l NaCl and 30 mmol/l imidazole (pH 7.0) and centrifuged at 3,000 g, in order to remove seminal fluid. The sperm pellets were resuspended by vortexing in 0.1% Triton X-100, 30 mmol/l imidazole (pH 7.0), 10% glycerol, and 5 mmol/l dithiothreitol. The sperm extract was clarified by centrifugation at 5,000 g, and aliquots of the clear supernatant were subjected to CK activity determinations by spectrophotometric CK kit (Sigma Co., St Louis, MO, USA). For Hsp2A assessment, CK-B and Hsp2A were separated by electrophoresis on precast agarose gels (Helena Laboratories, USA). The separated bands were developed by overlaying the gel with a fluorescent ATP substrate, and the ATP-containing CK-B and Hsp2A bands were quantified under long-wave ultraviolet light with a scanning
torometer. The HspA2 levels were quantified as a ratio of percent [HspA2/(HspA2+CK-B)], as described previously (Huszar et al., 1990, 1992, 2000; Ehas et al., 2002).

Statistical analysis

Statistical analyses were carried out using one-way and two-way analyses of variance (ANOVA) with Tukey post-hoc analyses, chi-squared and Spearman tests as well as Pearson correlations with the SigmaStat version 2.0 program (Systat Software, San Jose, CA, USA). Likelihood ratio analysis was calculated by the Microsoft Excel spreadsheet program. The B side versus A side Tygerberg morphology improvement was calculated by taking the actual improvement value for each pair of data on each slide, and then calculating the mean improvement values for each investigator. All values are reported as mean ± SEM.

Results

Relationship between semen parameters, sperm morphology, biochemical markers, and HA binding scores

Semen samples from 63 men were studied. The sperm concentrations were $34.6 ± 3.4 \times 10^6$ sperm/ml semen (4.0–115) and sperm motility was $42.1 ± 2.2\%$ (10–70) (Table 1). Among the 63 men, 16 were oligozoospermic (group 1), 27 were in the 20–30 $10^6$ per ml low normal sperm concentration range (group 2), and 20 men were in the $30 > 10^6$ sperm/ml semen high normal sperm concentration range (group 3). The division of the >20 $10^6$ sperm/ml World Health Organization normal concentration samples into two groups was based on the consistent finding that within the 20–30 $10^6$ sperm/ml samples the biochemical markers showed a substantial proportion of samples with arrested maturity. Further, some of these men in subsequent semen analysis showed sperm concentrations in the >20 $10^6$ range (Huszar et al., 1988). Indeed, in the present study six of the 27 samples in group 2 (22%) showed arrested maturity. In comparison, the proportion of such men was 7 out of 16 in the oligozoospermic group 1 (44%).

There was a balanced representation of semen samples with various sperm concentrations, including oligozoospermic men (group 1, 20 \(10^6\)ml n = 16, 25.4% of the study population), men with low normal sperm concentration (group 2, 20–30 \(10^6\)ml, n = 27, 42.9% of the study population), and men with high normal concentration samples (group 3 with >30 \(10^6\) sperm/ml, n = 20, 31.7% of the study population, Table 1). Regarding Tygerberg strict morphology, 37 men were classified as teratozoospermic (<14% normal forms) by all three investigators. The other 26 men who were classified as ‘normal’ morphology, similarly to the Tygerberg design, were scored in the >14% normal range by at least one of the investigators (Menkveld et al., 1991).

Regarding groups 1, 2 and 3 (Table 1), in addition to the sperm concentration differences, there were also variations in the HA binding scores, and the sperm maturity markers of CK activity and HspA2 chaperone protein expression levels. The sperm CK activity (re-ecting surplus cytoplasm in arrested maturity spermatozoa) was higher in the oligozoospermic compared with the >30 $10^6$ sperm/ml normal sperm concentration population. The HspA2 chaperone levels and sperm/HA binding scores were higher in the >30 $10^6$ sperm/ml normal sperm concentration group (Table 1).

Tygerberg morphology results and B side versus A side score improvements

In Table 2, column pairs 1 and 2 (A side and B side, all 63 samples), the proportions of spermatozoa with strict normal morphology are presented in semen and in HA-selected spermatozoa on the HA-coated B side respectively. All comparisons, whether in the whole study group (n = 63), or in groups 1, 2 or 3, were significantly different (P < 0.001).

The column pairs of 3–4, 5–6 and 7–8 represent the respective A and B side scores by the three individual investigators US1, US2, and TSA in the 63 study samples, in groups 1, 2 and 3, as well as in the teratozoospermic and >14% normal morphology men. All 15 comparisons were different at the level of P < 0.001. The Tygerberg normal morphology scores reported by the three readers on the A sides (Table 2, columns 3, 5 and 7) were in a

<table>
<thead>
<tr>
<th>Table 1. Semen and biochemical attributes of the study population.</th>
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<tr>
<td></td>
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<tr>
<td>All samples</td>
</tr>
<tr>
<td>Group 1 (oligozoospermic men)</td>
</tr>
<tr>
<td>Group 2 (20–30 x10^6 sperm/ml)</td>
</tr>
<tr>
<td>Group 3 (men with &gt;30 x10^6 sperm/ml)</td>
</tr>
<tr>
<td>Teratozoospermic (by all three investigators)</td>
</tr>
<tr>
<td>Normal &gt;14% Tygerberg normal (by one or all readers)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*P <0.001; **P <0.01; ***P <0.05; ****P <0.01; *****P <0.05. **P <0.01.
comparable range: US1 (1–22%), US2 (1–26%) and TSA (1–
24%). The respective scores on the B sides (columns 4 and 6) were also comparable in case of US1 (4–37.2%) and US2 (3.5–
36.7%), but the wider ranges of column 8 (6–69%) indicated that US1 and US2 applied stricter criteria than TSA.

Further data in Table 3 show the improvements in the proportion of spermatozoa with normal strict morphology in the B versus A sides (‘times’ improvement, B versus A), whether considering the total 63 men in the study population or in the other ve subgroups. The B versus A improvements found by the three investigators were within the ranges of 1.6 to 4.7 (all comparisons are P < 0.001).

Reader-to-reader variations and consistency within the strict morphology scores

It is of particular interest in light of the well documented investigator-to-investigator differences in strict morphology evaluations, that despite the individual variations in the personal level of ‘strictness’ scores, and the fact that each investigator has likely selected different sperm elds for assessment, the scored were internally consistent, and weakly but signi cantly, correlated among the three investigators. Considering the A and B scores respectively: US1 versus US2: r = 0.67 and r = 0.57; US1 versus TSA: r = 0.53 and r = 0.51; US2 versus TSA: r = 0.61 and 0.60 (all six correlations were signi cant, P < 0.001, n = 63 men). Within the 37 teratozoospermic men, the respective correlations among the three investigators were also present: A sides: r = 0.65, r = 0.58; r = 0.57, and B sides: r = 0.44, r = 0.40 and r = 0.40 (P < 0.001 in all).

There were also variations (1.6 to 4.7) in reader-to-reader strictness within the A-side to B-side improvements within all 63 samples and in the ve concentration groups, although all comparisons were highly signi cant (P < 0.001, Table 3).

The relationship between the three investigators is further illustrated in Figure 1, in which the mean strict morphology scores on the A sides by the three investigators (turquoise line) is presented along with the B side improvements (i) there was a negative correlation between the initial strict morphology score and the degree of improvement (r values: US1: 0.59,

### Table 2. Tygerberg strict morphology score and improvement in the hyaluronic acid-bound sperm fractions.

<table>
<thead>
<tr>
<th>n</th>
<th>Column number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All investigators</td>
<td>A side</td>
<td>B side</td>
<td>US1</td>
<td>A side</td>
<td>B side</td>
<td>US2</td>
<td>A side</td>
<td>B side</td>
</tr>
<tr>
<td>All samples</td>
<td>63</td>
<td>9.8 ± 0.6</td>
<td>18.8 ± 0.9</td>
<td>8.6 ± 0.7</td>
<td>14.4 ± 0.9</td>
<td>10.5 ± 0.8</td>
<td>16.9 ± 1.0</td>
<td>10.3 ± 0.7</td>
<td>25.4 ± 1.4</td>
</tr>
<tr>
<td>Group 1 (oligozoospermic men)</td>
<td>16</td>
<td>7.9 ± 0.1</td>
<td>16.6 ± 1.1</td>
<td>7.9 ± 0.1</td>
<td>14.4 ± 2.2</td>
<td>8.4 ± 1.5</td>
<td>13.8 ± 2.2</td>
<td>7.4 ± 1.3</td>
<td>21.6 ± 1.8</td>
</tr>
<tr>
<td>Group 2 (20–30 × 10⁶ sperm/ml)</td>
<td>27</td>
<td>8.9 ± 0.8</td>
<td>17.7 ± 1.4</td>
<td>8.1 ± 1.0</td>
<td>13.5 ± 1.2</td>
<td>8.9 ± 1.1</td>
<td>15.0 ± 1.4</td>
<td>9.7 ± 0.8</td>
<td>24.5 ± 2.5</td>
</tr>
<tr>
<td>Group 3 (men with &gt;30 x 10⁶ sperm/ml)</td>
<td>20</td>
<td>12.5 ± 1.0</td>
<td>22.3 ± 1.3</td>
<td>9.9 ± 1.3</td>
<td>15.7 ± 1.5</td>
<td>14.2 ± 1.3</td>
<td>21.8 ± 1.5</td>
<td>13.5 ± 1.5</td>
<td>29.5 ± 2.3</td>
</tr>
<tr>
<td>Teratozoospermic by all three readers</td>
<td>37</td>
<td>6.5 ± 0.4</td>
<td>14.5 ± 0.7</td>
<td>5.8 ± 0.5</td>
<td>11.9 ± 0.9</td>
<td>6.6 ± 0.6</td>
<td>11.9 ± 0.9</td>
<td>7.2 ± 0.6</td>
<td>19.8 ± 0.9</td>
</tr>
<tr>
<td>&gt;14% normal morphology by one or all readers</td>
<td>26</td>
<td>14.4 ± 0.7</td>
<td>25.7 ± 1.1</td>
<td>2.6 ± 1.2</td>
<td>18.1 ± 1.4</td>
<td>16.0 ± 1.0</td>
<td>23.9 ± 1.3</td>
<td>14.7 ± 1.0</td>
<td>33.3 ± 2.5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
A side = plain uncoated glass; B side = coated with hyaluronic acid. US1, US2 and TSA were the three investigators.
All A vs B side comparisons (col 1 vs 2; 3 vs 4; 5 vs 6; 7 vs 8) are different at the level of P < 0.001.

### Table 3. Morphology score improvements in the hyaluronic acid-bound versus semen sperm fractions.

<table>
<thead>
<tr>
<th>n</th>
<th>Improvement (B side versus A side)</th>
<th>US1</th>
<th>US2</th>
<th>TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All samples</td>
<td>63</td>
<td>2.0 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Group 1 (oligozoospermic men)</td>
<td>16</td>
<td>2.3 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>Group 2 (20–30 × 10⁶ sperm/ml)</td>
<td>27</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Group 3 (men with &gt;30 x 10⁶ sperm/ml)</td>
<td>20</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Teratozoospermic by all three readers</td>
<td>37</td>
<td>2.3 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>&gt;14% normal morphology by one or all readers</td>
<td>26</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
A side = plain uncoated glass; B side = coated with hyaluronic acid. US1, US2 and TSA were the three investigators.
All B side versus A side improvements (by all three investigators) were statistically signi cant (P < 0.001).
Figure 1. B/A improvement in Tygerberg sperm morphology scores recorded by three individual investigators (US1, US2 and TSA) where A side = plain uncoated glass; B side = coated with hyaluronic acid.

Discussion

The present study explored the hypothesis that the binding sites for the zona pellucida and HA, which are under common spermiogenetic regulation, would provide a similar degree of power for selecting mature spermatozoa with normal morphology either by HA or by the zona pellucida (Menkveld et al., 1991). This hypothesis was developed based on an earlier demonstration of sperm plasma membrane remodelling in spermiogenesis, which facilitates the formation of both the zona pellucida and HA binding sites (Huszar et al., 1997; Oelh (niger, 2003).

In addition to strict morphology, various aspects of the research on sperm biochemical markers revealed that the HA-bound mature spermatozoa exhibit nuclear and cytoplasmic maturity attributes that are comparable to that of sperm bound to the zona pellucida of hemizygous, including lack of surplus cytoplasm, persistent histones, low levels of DNA chain degradation, chromosomal aneuploidy, and apoptotic processes (Huszar and Vigue, 1993; Huszar et al., 1994, 1998b, 2003; Cayli et al., 2004; Jakab et al., 2005).

The characteristics of spermatozoa with arrested maturation, particularly the incomplete extrusion of the surplus cytoplasm and diminished propagation of tail sprouting, decisively influence sperm shape with respect to the symmetry and size of the head, the shape and size of the midpiece area, including abaxial insertion of the tail, and tail length (Gergely et al., 1999; Celik-Ozenci et al., 2003, 2004). These factors also play a prominent role in the evaluation of sperm shape according to the Tygerberg criteria. An associated line of studies with objective morphometry measurements of sperm fractions further indicated that sperm shape closely reflects the degree of sperm maturity (Gergely et al., 1999; Celik-Ozenci et al., 2003, 2004; Zavaetzki et al., 2006).
The present results con rmed the hypotheses posed. The increase of the Tygerberg normal spermatozoa in the HA-bound fractions from the A to the B sides of HA-bound spermatozoa in the population of 63 samples, and in the ve subgroups covering oligozoospermic to high normozoospermic ranges, have all shown significant overall improvements. The increase in spermatozoa with normal morphology attributes were also associated with concomitant improvements in the sperm maturity biochemical markers, further indicating the relationship between sperm maturation and sperm shape.

The likelihood analysis based on data of the three investigators showed a 3.04 (95% confidence limit: 1.9 to 4.7) in HA-bound spermatozoa, which is in the range of the 4.2 improvement reported within the zona pellucida-bound sperm fraction (Menkvedhl et al., 1991). It is of interest that TSA, who has participated in both studies found a comparable improvement in the HA-bound and hemizona-bound sperm fractions.

With respect to the Tygerberg normal morphology scores of the three investigators, the observations are of interest from the point of view of this study, as well as to the strict morphology method itself. First, there was a substantial and significant increase in the proportion of normal spermatozoa from the A to the B sides with very few exceptions. Although both the A and B side scores varied among the three investigators, the scores were related in all sperm concentration groups. Thus, the variations in scores reflected a consistent individual-to-individual difference in the perception of normality.

Another related question is the reliability of Tygerberg morphology determination by single technicians. It is well established and reported that the ‘strict’ morphology evaluation, in spite of its utility, is an inexact science. Sample sets tested in various laboratories often resulted in inconsistent results. In further approaches investigators attempted to re-evaluate the clinical utility of sperm morphology, as shape properties or any other semen attributes alone, did not provide a sufficient measure of fertility or a solid ‘cut-off’ value, and there is a search for second lines of fertility testing (Ombelet et al., 1997, 1998; Gaszik et al., 2001; Menkvedhl et al., 2001; Nallella et al., 2006; Henkel et al., 2008).

Considering sperm function, sperm concentrations often fail to reflect sperm maturity and fertilizing potential. However, objective biochemical markers provide an objective assessment of sperm maturity and fertility. This has been demonstrated not only in laboratory experiments, but also in clinical studies of fertile oligozoospermic men, and of men with normal sperm concentrations with unexplained male infertility (sperm concentrations in the normal range coupled with diminished sperm maturity and fertility) (Huszar et al., 1992; Ergur et al., 2002).

Regarding the oligozoospermic group, in a study of couples treated with intratruite insemination, in 160 oligozoospermic samples the occurrence of pregnancies was significantly related to sperm Ck activity but was unrelated to sperm concentrations (Huszar et al., 1988). With respect to unexplained male infertility, in two blinded studies of IVF couples (82 and 119 couples), sperm Ck activity and sperm HspA2 (a chaperone protein regulating sperm maturity) were followed respectively. In these two studies, nine of 22 (41%) and 13 of 25 (52%) men with semen in the normal sperm concentration range and adequate sperm motility nevertheless had diminished sperm maturity and failed to achieve pregnancy in the IVF cycles (Huszar et al., 1992; Ergur et al., 2002).

It seems that the sperm–HA binding assay (an objective assessment performed with standardized HA platforms), which is based on basic principles of sperm biochemical measurements, may offer an advantageous test in addition to sperm morphology. HA binding re ects the zona pellucida binding ability of spermatozoa, and the presence of HA receptors that re ects normal spermigenesis and various upstream maturation events that affect DNA chain integrity, surplus cytoplasm and aneuploidy frequency (Huszar et al., 2007). The HA sperm binding assay is likely to be useful in evaluation of male infertility, particularly if the couple is treated with assisted reproduction.

In summary, the validity of the hypothesis tested in this study was confirmed due to the common regulation of the HA-binding and zona pellucida-binding sites in human spermatozoa, one may anticipate a similar improvement in the morphology of the HA-bound and zona pellucida-bound spermatozoa versus the semen spermatozoa fraction. In line with the variety of studies that demonstrated that normal sperm shape and sperm function are related, this study enhanced the scientific basis of the 20-year-old ‘strict morphology’ method. The findings also support the clinical utility of the sperm HA binding assay, as the HA-selected spermatozoa, in all attributes studied so far, seem to be comparable with those selected by the zona pellucida, including sperm shape, maturity properties, and genetic integrity. Thus, the sperm HA binding assay would improve the diagnosis and treatment of male factor infertility.

Acknowledgements

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Declaration: The HA-related diagnostic and sperm selection devices were invented by GH. The patent is owned by Yale University. Yale licensed the technology to Biocout Inc. GH is acting as scientific advisor.

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iii.  *Sperm selection using non apoptotic spermatozoa*

Hoogendijk, one of the PhD students at Stellenbosch University and Tygerberg Hospital was encouraged to look at other methods of sperm selection. The study looked at apoptosis in sperm populations. With annexin V binding it was observed that non apoptotic sperm selected for more normal forms compared to the apoptotic sperm. The observation that selection of spermatozoa using the ideal form will assist in obtaining a ‘healthier’ sperm to be used for ICSI, increasing the potential quality of embryos and pregnancy rates (Hoogendijk *et al.*, 2009; Antinori *et al.*, 2008).
The following article will be reflected on:

A novel approach for the selection of human sperm using annexin V-binding and flow cytometry

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Objective: To develop a method whereby sperm with phosphatidylserine externalization can be separated from those without this feature. Because annexin V binds phosphatidylserine, this study is using this feature to select functional spermatozoa. In addition, the relationship between annexin V binding in human spermatozoa and normal sperm morphology according to strict criteria was to be assessed.

Design: Prospective study.

Setting: Department of Obstetrics and Gynaecology of Stellenbosch University at Tygerberg Academic Hospital, Tygerberg, South Africa.

Patient(s): Semen from 14 healthy sperm donors. Exclusion criterion was the presence of less than 20 × 10^6/mL total motile spermatozoa in the original sample.

Main Outcome Measures: Annexin V-negative sperm, annexin V-positive sperm, normal sperm morphology.

Intervention(s): An aliquot of a semen sample after double density gradient centrifugation was incubated with annexin V fluorescein isothiocyanate conjugate (FITC). Cell fluorescence signals were determined using a FACSAria flow cytometer equipped with a FACSSort fluidics sorting module. The sorting procedure delivered two sperm subpopulations: annexin V-negative and annexin V-positive. Morphology slides were made and the sperm morphology was assessed according to strict criteria.

Result(s): There was a significant enrichment of annexin V-negative sperm as well as morphologically normal sperm in the annexin V-negative subgroup after separation with flow cytometry. The percentage of morphologically normal sperm increased from 8.3% in the control to 11.9% in the annexin V-negative fraction, whereas the percentage of annexin V-positive sperm decreased to 5.7%.

Conclusion(s): The annexin V-negative sperm subpopulation had morphologically superior quality sperm compared to annexin V-positive sperm. It is important to select morphologically normal sperm during intracytoplasmic sperm injection (ICSI) as it may contribute to increased implantation and pregnancy rates (PR). (Fertil Steril® 2009;91:1285-92. ©2009 by American Society for Reproductive Medicine.)

Key Words: Sperm, human, morphology, apoptosis, separation technique

A conventional semen analysis includes the assessment of sperm concentration, motility, and percentage of morphologically normal forms. In addition, it is and will remain, at least in the foreseeable future, the standard procedure for the evaluation of sperm fertilizing potential in given semen samples. Although it gives considerable information, such as the correlation of normal sperm morphology with fertilization potential (1), the results of a semen analysis are only moderately predictive of an individual’s fertility as these parameters show considerable high biological variability (2, 3).

Sperm DNA damage and sperm apoptosis have been considered as potentially useful indicators of male fertility. Cellular apoptosis, also called programmed cell death, is the

controlled disassembly of cells from within (4). Apoptosis is characterized by a cascade of events that include condensation and fragmentation of the chromatin, compaction of cytoplasmic organelles, reduced mitochondrial transmembrane potential (5), mitochondrial release of cytochrome c (6), production of reactive oxygen species (7), dilatation of the endoplasmic reticulum, and a decrease in cell volume (8).

Apoptosis is characterized by changes of the plasma membrane. An early sign of apoptosis is the translocation of phosphatidylserine (PS), which is a negatively charged phospholipid, from the normal location on the inner leaflet of the plasmamembrane to the outer side thereof. Phosphatidylserine is thus exposed on the external membrane surface (9, 10). It has a high affinity to annexin V, a phospholipid-binding protein of about 35 kDa (11); this binding is an early marker of apoptosis. Markers of terminal apoptosis include activated caspase-3, loss of the integrity of the mitochondrial membrane potential, and DNA fragmentation, which are expressed by varying proportions of ejaculated sperm (12, 13).
Apoptosis has been associated with male infertility (14–17). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (18, 19). It has been reported that the proportions of apoptotic sperm are higher in ejaculated semen samples from infertile men compared with fertile men (17). Furthermore, during cryopreservation, sperm caspases become more activated in patients with infertility than in healthy donors (20). Although apoptosis is considered a mechanism to ensure selection of sperm with undamaged DNA, sperm with DNA damage that are not eliminated by apoptosis may fertilize an ovum (13, 21, 22).

Several studies have explored the relationship between the parameters of a conventional semen analysis and apoptosis in ejaculated semen. These studies concur that there is a significant negative correlation between the proportion of apoptotic cells and sperm viability and motility in the ejaculate (17, 23–26). In contrast, there are only a few studies on the relationship between sperm morphology, applying Tygerberg’s strict criteria (1), and the proportion of apoptotic sperm (27, 28). Dissimilarly, when applying the World Health Organization (WHO) (29) criteria for sperm morphology, no correlations could be found between caspase-3 activation, intact mitochondrial membrane potential, and PS externalization (26).

There is the need to identify the normal sperm, especially for use in intracytoplasmic sperm injection (ICSI) in infertile men. Several methods to assess the status of the sperm DNA have been reported, but they require invasive steps such as fixation or sperm lysis. Other more reliable noninvasive methodologies are needed; these should enable the embryologist to select the healthiest sperm for fertilization during ICSI, which in turn will contribute positively toward better implantation and pregnancy rates (PR).

By conjugating fluorescein to annexin V it has been possible to use the marker to identify externalized PS, and thus apoptotic cells, by flow cytometry. This method does not involve enzyme activity and does not require cells to be previously fixed; accordingly this assay enables living sperm to be evaluated.

In this study the aim was to establish a noninvasive method whereby annexin V-positive and annexin V-negative sperm can be separated in such a way that the resultant annexin V-negative sperm subgroup can still be used for fertilization with ICSI. In addition, a relationship between the two subgroups with regard to normal sperm morphology according to Tygerberg’s strict criteria should be established.

MATERIALS AND METHODS
Experimental Design
These studies were performed upon approval of the Institutional Review Board (IRB) at the Faculty of Health Sciences, Stellenbosch University, Tygerberg Academic Hospital, Tygerberg, South Africa (project number 99/137). A total of 14 men, attending the Reproductive Biology Unit in the Department of Obstetrics and Gynaecology of Stellenbosch University at Tygerberg Academic Hospital for a semen analysis, were included in the study. Their age ranged from 28–54 years. The only exclusion criterion was the presence of less than 20 × 10^6/mL total motile spermatozoa in the original (after liquefaction) sample; this was determined to have sufficient numbers of sperm cells to perform the separation technique as well as subsequent motility and morphology analyses. Patients collected semen by masturbation into sterile cups after 2–4 days of sexual abstinence. The semen characteristics of volume, pH, and agglutination were normal; all samples had less than 0.1 × 10^6 round cells/mL.

Semen samples were allowed to liquefy for 30 minutes at room temperature followed by assessment of semen parameters using the criteria for normal concentration and motility according to the WHO (29) and the Tygerberg strict criteria (1) for normal sperm morphology, whereby normal sperm morphology is divided into three groups, p-pattern (0%–4% normal morphology), g-pattern (5%–14% normal morphology), and a-pattern (>14% normal morphology).

Semen samples were prepared by double density gradient (DDG) centrifugation, using a discontinuous (45%, 90%) Sil Select (FertiPro, Beernem, Belgium) gradient made with Quinn’s sperm wash medium (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT). The samples were loaded onto the discontinuous gradient and centrifuged at 400 × g for 12 minutes at room temperature. The resultant 90% pellet was washed by centrifugation (400 × g) for an additional 10 minutes and resuspended in Quinn’s sperm wash medium (SAGE In-Vitro Fertilization Inc.) to a final concentration of 10 × 10^6 sperm. One aliquot of this suspension served as control, and another aliquot was subjected to fluorescent cell sorting using fluorescein isothiocyanate conjugate (FITC)-labeled annexin V.

Annexin-V Staining
Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS. The translocation of PS residues to the outer layer of the plasma membrane was detected with annexin V-labeled with FITC. To label the sperm with annexin V the sperm suspension in Quinn’s sperm wash medium (SAGE In-Vitro Fertilization Inc.) with 10 × 10^6 sperm (total number) was diluted 1:10 with phosphate-buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO), washed, and centrifuged for 10 minutes at 500 × g at room temperature. The supernatant was discarded and the remaining pellet resuspended in 195 μL of binding buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 2.5 mmol/L CaCl_2, pH 7.4). Next, 5 μL of FITC-labeled annexin V (Annexin V FITC Kit; Bender MedSystem Diagnostics, Vienna, Austria; catalog no. BMS306FI) was added and the samples were incubated in the dark for 15 minutes at room temperature. Subsequently, 800 μL of binding buffer was added.
Fluorescent Cell Sorting

Cell fluorescence signals were determined immediately after staining using a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NY) equipped with a 15-mW argon laser with an excitation wavelength of 488 nm, as well as a FACSort fluid sorting module. The FITC was identified by using a 530-nm band pass filter. The analysis was performed using CellQuest software (version 3.3; BD Biosciences, San Jose, CA). A primary gate based on physical parameters (forward and side light scatter) was set to exclude dead cells or debris. The background level was estimated by omitting the annexin V FITC reagent. Green fluorescence (480–530 nm) was measured in the FL1 channel of the flow cytometer. Spermatozoa were examined at a flow rate of <100 cells/s. After determining the initial annexin V positivity of each of the semen samples, the annexin V-positive and annexin V-negative sperm fractions were separated. A sort region of annexin-positive events was set and the events were collected to represent those cells that had bound the fluorescent marker indicative of PS externalization. The sort region negative (annexin V-negative) was collected to represent those sperm cells that had not externalized the marker.

The fractions composed of annexin V-positive and annexin V-negative were collected separately in 50-mL polypropylene tubes (Becton Dickinson). Before collection, these tubes were flushed out with Quinn’s sperm wash medium (SAGE In-Vitro Fertilization Inc.) to prevent the sperm from attaching to the inside surface. After separation the 50-mL fractions were centrifuged at 500 × g for 20 minutes at room temperature. The resultant pellet was resuspended in 50 µL of binding buffer. An aliquot of 20 µL was used to make the morphology smear and the remaining sample was reanalyzed in the flow cytometer to determine the efficiency of the sorting process as well as the purity of the sorted fraction.

Sperm Morphology Assessment

A concentration-dependent droplet of semen was smeared thinly across cleaned slides and allowed to air dry at room temperature. Thin smears facilitated sperm morphology assessment by avoiding sperm cell overlap and ensuring that the sperm were scattered at the same focal depth. The air-dried slides were stained with Diff-Quik stain (Merck Diagnostics, Darmstadt, Germany) as previously described (30). Briefly, the slides were fixed for 20 seconds in solution 1, stained for 10 seconds in solution 2, and for 10 seconds in solution 3, and washed in water. All slides were assessed by one observer using a morphological classification based on the strict criteria for normal sperm morphology. Strict quality control was maintained as each slide was coded. The scorer was blinded to the category that each slide had been assigned. The code was only broken once the results were known.

Statistical Analysis

After testing for normal distribution by means of the Kolmogorov-Smirnov test, parametrical tests (paired samples t-test) for normally distributed data, as well as nonparametrical tests (Wilcoxon test for paired samples) for data not normally distributed were performed. Results are presented as mean ± SD or median and P<0.05 was considered significant. The software used was MedCalc version 9.3.2.0 obtained from MedCalc Software, Mariakerke, Belgium.

RESULTS

Fourteen men were included in this study. Summary statistics of basic semen parameters are presented in Table 1. The age of the men included in this study ranged between 28 and 45 years (mean 34.57 ± 5.26 years). All semen parameters measured were above the minimum thresholds for normality.

Table 2 presents the summary statistics for flow cytometry results of the double density gradient centrifugation (DDGC) (control), annexin V-positive and annexin V-negative sperm subgroups analyzed in this study. The high variation of values, reflected by the high SD is expected, because biological parameters were measured in this study. Results of the univariate comparisons, as performed with two-tailed t-tests, of the median normal sperm morphology in the sperm subgroups are presented in Table 3.

The annexin V-negative sperm subgroup had a significantly higher median (12.00) normal sperm morphology compared to the median normal sperm morphology of the control sperm.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
</table>

Summary statistics of basic semen parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>14</td>
<td>34.57 ± 5.26</td>
<td>28.00–45.00</td>
<td>33.50</td>
</tr>
<tr>
<td>Forward progression</td>
<td>14</td>
<td>2.63 ± 0.24</td>
<td>2.00–2.75</td>
<td>2.75</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>14</td>
<td>49.29 ± 13.28</td>
<td>20.00–70.00</td>
<td>50.00</td>
</tr>
<tr>
<td>pH</td>
<td>14</td>
<td>7.45 ± 0.26</td>
<td>7.20–6.00</td>
<td>7.50</td>
</tr>
<tr>
<td>Sperm concentration (x 10^9/mL)</td>
<td>14</td>
<td>92.57 ± 43.54</td>
<td>41.00–205.50</td>
<td>67.75</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>14</td>
<td>3.50 ± 1.58</td>
<td>1.50–7.00</td>
<td>3.75</td>
</tr>
</tbody>
</table>


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TABLE 2

Percentage of annexin V-positive and annexin V-negative spermatozoa as well as percentage of normal sperm morphology in the double density gradient centrifugation (control) and the flow cytometrically separated sperm subgroups annexin V-positive and negative.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal morphology (%)a</td>
<td>14</td>
<td>8.36 ± 4.14</td>
<td>2.00-16.00</td>
<td>8.50</td>
</tr>
<tr>
<td>Annexin V-negative (%)a</td>
<td>14</td>
<td>76.21 ± 9.47</td>
<td>62.05-88.44</td>
<td>79.75</td>
</tr>
<tr>
<td>Annexin V-positive (%)a</td>
<td>14</td>
<td>23.80 ± 9.47</td>
<td>11.56-37.95</td>
<td>20.26</td>
</tr>
<tr>
<td>Normal morphology (%)b</td>
<td>13</td>
<td>5.77 ± 3.49</td>
<td>1.00-13.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Annexin V-negative (%)b</td>
<td>14</td>
<td>14.62 ± 14.10</td>
<td>1.25-61.53</td>
<td>11.86</td>
</tr>
<tr>
<td>Annexin V-positive (%)b</td>
<td>14</td>
<td>85.38 ± 14.10</td>
<td>38.47-98.75</td>
<td>88.14</td>
</tr>
<tr>
<td>Normal morphology (%)c</td>
<td>13</td>
<td>11.92 ± 4.43</td>
<td>7.00-22.00</td>
<td>12.00</td>
</tr>
<tr>
<td>Annexin V-negative (%)c</td>
<td>14</td>
<td>95.59 ± 2.70</td>
<td>89.88-98.44</td>
<td>96.77</td>
</tr>
<tr>
<td>Annexin V-positive (%)c</td>
<td>14</td>
<td>4.41 ± 2.70</td>
<td>1.57-10.12</td>
<td>3.23</td>
</tr>
</tbody>
</table>

*a* Double density gradient sperm fraction (control).

*b* Annexin V-positive sperm fraction.

*c* Annexin V-negative sperm fraction.


subgroup (median = 8.50) (n = 13; P<.0001) (Table 2) as well as the annexin V-positive sperm subgroup (median = 5.00) (n = 12; P<.0001) (Table 2). Furthermore, the annexin V-positive sperm subgroup had a significantly lower median (5.00) of normal sperm morphology when compared with the control sperm subgroup (median = 8.50) (n = 13; P<.0001) (Table 2).

After fluorescent cell sorting, reanalysis of the resultant annexin V-positive and annexin V-negative subgroups were performed. The purpose for the reanalysis, performed with the exact same instrument settings as for the original sample, was to determine the efficiency of the separation assay. Univariate comparisons, with the Wilcoxon test, showed that the median normal sperm morphology was significantly higher in the annexin V-negative subgroup (n = 14; P=.0001), when compared with both the control and annexin V-positive subgroups (Fig. 1). In addition, these tests showed that the median normal sperm morphology in the annexin V-positive subgroup was significantly lower (n = 14; P=.0001) than in the control group.

**DISCUSSION**

The results of this study confirm the shift in the normal sperm morphological profile in sperm preparations, before and after

----

TABLE 3

Comparison of normal sperm morphology between the different groups isolated after the sperm separation (paired sample t-test).

<table>
<thead>
<tr>
<th>Sperm subgroup</th>
<th>No.</th>
<th>Median</th>
<th>95% CI</th>
<th>Mean</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controla</td>
<td>13</td>
<td>8.50</td>
<td>5.56-9.98</td>
<td>7.77</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Annexin V-positiveb</td>
<td>13</td>
<td>5.00</td>
<td>3.66-7.88</td>
<td>5.77</td>
<td></td>
</tr>
<tr>
<td>Controlb</td>
<td>13</td>
<td>8.50</td>
<td>5.70-10.91</td>
<td>8.31</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Annexin V-negativec</td>
<td>13</td>
<td>12.00</td>
<td>9.25-14.60</td>
<td>11.92</td>
<td></td>
</tr>
<tr>
<td>Annexin V-positivec</td>
<td>12</td>
<td>5.00</td>
<td>3.43-8.07</td>
<td>5.75</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Annexin V-negativec</td>
<td>12</td>
<td>12.00</td>
<td>8.94-13.23</td>
<td>11.08</td>
<td></td>
</tr>
</tbody>
</table>

Note: Sperm morphology of the double density gradient centrifugation (DDGO) (control) and the subsequent two sperm subgroups resulting from flow cytometric cell sorting were compared using paired sample t-tests. All hypotheses testing were two-tailed; P<.05 was considered statistically significant. The 95% confidence intervals (CI) are given for each case.

*a* DDGO sperm subgroup (control).

*b* Annexin V-positive subgroup.

*c* Annexin V-negative subgroup.

the isolation of the annexin V-positive and annexin V-negative sperm (Table 2). Isolation of these sperm was performed on the basis of PS externalization using annexin V labeling and flow cytometry. Normal sperm morphology was assessed according to strict criteria (1). Compared with the annexin V-positive sperm subgroup, the annexin V-negative subgroup revealed an improved normal sperm morphology profile, as demonstrated by significantly higher proportions of sperm with normal morphology (Table 3). In addition, the normal sperm morphology of the annexin V-negative sperm subgroup had a similar favorable shift when compared with the DDGC (control) sperm subgroup. Furthermore, the study results demonstrate that the flow cytometric cell separation technique proved to be an adequate method for the separation of annexin V-positive and annexin V-negative sperm, as the resultant subgroups were significantly enriched for the specific selection criterion.

Other studies have previously attempted to correlate apoptotic markers in the ejaculated sperm and sperm morphology of the neat semen (27, 31, 32). These studies, however, did not accurately distinguish between moribund or necrotic sperm and motile sperm expressing apoptotic markers. In addition, there are studies that correlated sperm morphology in neat semen with apoptosis in selected motile sperm subpopulations after swim-up or DDGC techniques (25, 33, 34). All of these studies applied different morphology assessment methodologies: WHO 1992 standards (34), WHO 1999 standards (31, 33), and strict criteria (25, 27, 32).

In this study a highly motile sperm population was selected with a DDGC technique followed by a flow cytometric cell separation technique to facilitate the direct correlation of normal sperm morphology, according to strict criteria, and the expression of PS in the same sperm subpopulation avoiding interference by round cells and immotile or necrotic sperm found in semen. The highly significant correlation between normal sperm morphology, according to strict criteria, and the apoptotic marker, PS externalization, observed in the current study is in agreement with one other study (28), which also selected a highly motile sperm subpopulation through DDGC, but in contrast they separated the resultant sperm subpopulation by means of a magnetic cell sorting technique. This study found that the nonapoptotic sperm subpopulation had morphologically superior quality sperm compared with apoptotic sperm, as reflected by significantly lower sperm deformity index scores. The sperm deformity index score is a novel expression of the quality of sperm morphology and it has previously been shown to be a more powerful predictor of male fertility and of IVF outcome compared with the assessment of the proportion of sperm with normal morphology (35). In addition, there are two more studies (31, 33), that found a correlation between normal morphology and the apoptotic marker, percentage of PS externalization (31). In these studies normal sperm morphology was assessed using WHO 1999 criteria.

In an attempt to elucidate the possible correlation between normal sperm morphology as assessed with strict criteria (1), and sperm physiological, chromosomal, and genetic properties, in relation to fertilization, embryo quality, and pregnancy, many investigators have studied the correlations between normal sperm morphology and zona binding tests (36), acrosome reaction (37), sperm nuclear DNA normality (38, 39), double-stranded DNA content of the sperm nucleus (40), sperm chromosome complement (41), and sperm nucleus DNA fragmentation. All of these studies have shown that normal sperm morphology correlates positively with the sperm cell’s functional competence. In addition, the present study confirms that normal sperm morphology correlates significantly negative with the externalization of PS (P<0.0001). Some studies attribute this to the process of apoptosis, as the externalization of PS is an early marker of the programmed cell death and assume that annexin V binding is a good marker of semen quality in andrological diagnosis (14, 24). On the other hand, Henkel et al. (13) showed that the early parameters of apoptosis annexin V binding and Fas expression are not predictive of fertilization or of pregnancy. Only the late parameter of apoptosis, DNA fragmentation, could significantly predict the onset of pregnancy by using ejaculated sperm.

Although there are indications that apoptosis in ejaculated sperm might have importance in identifying the sperm with
superior quality and the highest fertilizing potential (24, 42), there are also indications that apoptosis does not play a major role, that is, Fas expression has no relevance to pregnancy (13), caspase-independent pathway to induce apoptosis present in sperm (43, 44), sperm DNA fragmentation is induced by reactive oxygen species (45, 46), and the externalization of PS takes place during capacitation, acrosome reaction, and cryopreservation (47, 48). In line with these results, Moustafa et al. (49) revealed that apoptosis may not significantly contribute to DNA damage in ejaculated sperm.

The importance of normal sperm morphology, as assessed with strict criteria (1), is that it has previously been shown to be predictive of fertilization and PRs in vitro (50–53). In a structured literature review (meta-analysis) conducted to assess the importance of sperm morphology on fertilization and PRs in IVF, Coetzee et al. (54) confirmed the importance of sperm morphology in relation to the positive predictive value for fertilization and pregnancy.

From the literature it is evident that the normal form sperm per se is not physiologically, chromosomally, or genetically normal but, it is superior in many ways to its abnormal counterpart. During the ICSI technique, this observation becomes increasingly important as the natural barriers against abnormality are bypassed and selection of the sperm for fertilization is based on the visual assessment of the shape of the sperm, which rests with the embryologist. In a study where the effect of individual sperm morphology in relation to fertilization and pregnancy outcome was observed, significantly lower PR and implantation rate were obtained after transfer of embryos resulting from morphologically abnormal sperm cells, especially in those patients where there were spermatozoa with amorphous heads, elongated heads, the presence of cytoplasmic droplets (reflecting incomplete maturity), or broken necks (55).

Rather than rely only on the visual assessment for the normal form, Jakab et al. (56) developed a sperm selection method based on the binding of mature sperm to hyaluronic acid (57). Hyaluronic acid bound sperm have been shown to be devoid of persistent histones and apoptosis as evidenced by aniline blue staining and the absence of active caspase-3, respectively (58). In comparison, the current study has applied a novel method of sperm selection based on the fact that apoptotic sperm externalize PS, which binds fluorescence-labeled annexin V, and thus be isolated from the non-labeled subpopulation by means of flow cytometric cell sorting.

The importance of the significant positive correlation between annexin V-negative sperm and normal sperm morphology, according to strict criteria, lies in the fact that value is added to the significance of the initial sperm morphology assessment. Not many laboratories have the necessary infrastructure to provide specialized techniques such as flow cytometry. On the contrary, sperm morphology assessment according to strict criteria is an inexpensive and standardized method for the evaluation of male fertility potential and the embryologist should therefore be aware of these positive correlations and subsequently pay special attention during the selection of sperm at the time of ICSI.

Because annexin V-negative spermatozoa show a significantly superior morphology, the selection process of such sperm enriched the number of morphologically normal spermatozoa, which are beneficial for the use in assisted reproduction. This is also what could be observed using other selection methods based on the annexin V binding (42). However, in the light of the concerns about the importance of apoptosis in ejaculated sperm and the involvement of PS externalization in other processes, this methodology might not only select morphologically superior sperm but also capacitating and acrosome-reacted spermatozoa and more investigations will have to clarify this issue.

In conclusion, the results of this study suggested that the annexin V-negative sperm subpopulation has morphologically superior quality compared with the annexin V-positive sperm subpopulation, as reflected by the shift observed in normal sperm morphology between these subpopulations. We recommend meticulous morphology evaluation (according to strict criteria) on all patients attending an infertility clinic. The initial evaluation of the sperm morphology can assist in the clinical decision making regarding treatment options. If ICSI is the treatment of choice, emphasis should be placed on selecting morphologically the most normal-appearing spermatozoa to ultimately increase implantation rate and PR in these patients.

REFERENCES


b. To study sperm selection’s impact on clinical practice in ART based on the international literature

   i. Reasons for careful morphology evaluation prior to ICSI

   It was reported by De Vos (De Vos et al., 2003) that with careful selection of the ideal form, using the inverted microscope, pregnancy rates can be improved significantly. This clinical observation can possibly be explained by the fact that abnormal spermatozoa have four times higher structural chromosomal aberrations (Lee et al., 1996).

   ii. The selection of spermatozoa using high magnification IMSI (Intracytoplasmic Morphology Selected sperm Injection)

   The work of Bartoov (Bartoov et al., 2001) brought the concept of sperm selection to the forefront. The selection of spermatozoa with high magnification, selecting the ideal form if possible, solved poor pregnancy rates in couples with repeated failures. This method was called IMSI. Berkovitz (Berkovitz et al., 2001) observed a poorer embryo quality and lower pregnancy rates with higher abortion rates if no normal sperm could be found for sperm injection.

   Recently, in a randomized controlled trial Antinori reported a significant improved pregnancy rate in the IMSI group versus the ICSI group (Antinori et al., 2008). A number of scientists in recent years studied single sperm and its physiological characteristics. Garolla (Garolla et al., 2008) reported that the ideal spermatozoa have less aneuploidy, less mitochondrial damage and less DNA fragmentation than sperm with 1 or more vacuoles. The observations of Vanderzwalmen and Franco correlated with those of Garolla. (Garolla et al., 2008; Vanderzwalmen et al., 2008; Franco et al., 2008).

   To summarize, it was reported in 1986 that the percent normal forms have an impact on fertilization and pregnancy rates (Kruger et al., 1986). Initially it was observed that zona binding, acrosome reaction, calcium influx and the sperm penetration assay were all affected in the P-pattern groups (physiological defects) (Oehninger et al., 2000, Oehninger et al., 1994, Franken et al., 1990, Bastiaan et al., 2003). These physiological defects were reported in the patients with severe abnormalities in shape of sperm (P-pattern). Twenty years later
the same observations were made for individual spermatozoa and the importance realized of selection of the ideal form for improved results in ICSI programs.

c. **To suggest a new classification method based on Strict Criteria for treatment of severe male factor infertility using ICSI.** Attention will be given to electron microscopic evidence as well as taking the pathophysiology of sperm into consideration.

   i. **Classification of the normal forms for IMSI**

   In a lecture by Vanderzwalmen at the ESHRE 2010 meeting in Rome, he suggested the following classification system for spermatozoa to be used in the IMSI treatment arena.

   - **Grade 1** – no abnormalities with a perfect oval shape or 1 or 2 small vacuoles (Figure 1)
   - **Grade 2** – 1 big or 2 smaller vacuoles (Figure 2)
   - **Grade 3** – amorphous forms and numerous vacuoles (Figure 3)


   ![Spermatozoa grade 1 diagram](image)

   **Figure 1**
Normal Form, at least 1 large or >2 Small vacuole

Abnormal Form and Vacuole(s)

Figure 2

Figure 3

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ii. Pathology of the sperm head

When the process of chromatin maturation and compaction is altered, the heads of the spermatozoa display large lacunar defects (2-3 μ in diameter), where the compact arrangement of the chromatin is replaced by granulofibrillar or ‘empty’ areas that occupy as much as 20 to 50% of the nucleus. (Chemes and Rawe, 2007). These abnormalities originate in the testis as a consequence of abnormal spermiogenesis. Furthermore spermatozoa with chromatin abnormalities frequently demonstrate abnormal headshapes, have diminished fertility potential or are associated with 1st-trimester abortions (Chemes and Rawe, 2007). In addition DNA breaks, abnormal histone-protamine transition or apaptotic changes have been reported, as well as insufficient chromatin condensation, immaturity and intranuclear lacunae (vacuoles) that are their ultrastructural correlates (Chemes and Rawe, 2007). A positive correlation between aneuploidy and teratozoospermia has also been reported. (Lewis Jones et al., 2003; Vicari et al., 2003; Lee et al., 1996; Calogero et al., 2001; Kovanci et al., 2001; Perrin et al., 2008; Nagvenkar et al., 2005).

As mentioned in previous paragraphs, the patho-physiological findings in spermatozoa with vacuoles compared with those with normal oval shapes in IMSI programs (Garolla et al., 2008) and the improved pregnancy rates with IMSI as reported by (Antinori et al., 2008) which all tie the electron microscopic findings together.

iii. Pathology of the midpiece

During mammalian fertilization the centrosome deriving from the sperm is used in the first cell division to organize the radial array of microtubules comprising the sperm aster. Aberrant microtubule organization suggests centrosomal dysfunction and may be a cause of fertilization arrest (Ugajin et al., 2010).

The release of the sperm centriole after fertilization probably involves the action of sperm proteasomes localized in the neck region of human spermatozoa (Chemes et al., 2007; Wojcik C et al., 2000; Rawe VY, 2005). It was recently shown that spermatozoa with a straight midpiece has a normal electron microscopic configuration, but those with a tapered shape have misaligned mitochondria and vacuolar structures. Clear differences were not observed in centrosome structures in the 2 groups studied but in the straight group the total rate of sperm aster formation was 80.5% and in the tapered group 33.3% (P<0.05) (Ugajin et
al., 2010). Rawe suggested that the arrest of oocytes at the pronuclear stage is due to a lack of sperm aster formation (Rawe et al., 2000). In the article by Ugajin it is shown that proper centrosomal function (sperm aster formation) is essential for fertilization (Ugajin et al., 2010). For that reason, it is suggested in this thesis that a 4th category must be added to sperm classification for IMSI. This will lead to improved fertilization rates and pregnancy rates in clinics and will refine the selection of sperm even more. It is also important to note that with the Strict Criteria it has clearly been shown that the normal spermatozoon selection is based on no midpiece or neck defects (Kruger et al., 1986; Menkveld et al., 1990).

Based on the evidence above and our approach regarding Strict Criteria used in IVF, the following classification for IMSI is suggested that will better assist in research and possibly improve pregnancy outcome. Spending more time to find the ideal sperm will definitely result in improving clinical practice.

**Proposed classification system for IMSI**

Grade 1  –  no abnormalities with a perfect oval shape and with a straight-shaped midpiece (4a)
Grade 2a – 1 or 2 small vacuoles
Grade 2b – 1 big or 2 smaller vacuoles equal to the size of a big vacuole
Grade 3 – amorphous forms and numerous vacuoles
Grade 4 – midpiece defects
   a  –  straight midpiece
   b  –  subtle defects e.g. tapering midpiece
   c  –  severely abnormal midpiece

For research purposes it is important to report on head and midpiece abnormalities, for example, Grade 2b/4a. The ideal sperm must be reported as Gr1/4a. This approach will shed even more light in future on the impact of different semen abnormalities on fertilization rate, embryo quality and pregnancy rates.
Conclusion:

The percentage sperm morphological normal forms in IVF and IUI are playing a role in pregnancy outcome. It was also shown that careful sperm selection (selection of the perfect normal form) is also playing a role in an improvement in pregnancy outcome in the ICSI procedure. In this chapter different techniques of sperm selection were discussed in an attempt to improve pregnancy outcome. A new sperm classification method was suggested based on electron microscopic and sperm physiological evidence to be used in ICSI programs.
References


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Lee JD, Kamiguchi Y, Yanagimachi R. Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. Hum Reprod 1996;11(9):1942


Menkveld R, Coetzee K, Stander FSH, et al. The use of the acrosome index as an additional morphology parameter in the clinical selection of patients for ICSI. Hum Reprod 1999;14:156


Roldan ERS. Better intracytoplasmic sperm injection without sperm membranes and acrosome. Proceedings of the National Academy of Sciences USA 2006;103(47):17585
Ugajin T, Terada Y, Hasegawa H, et al. The shape of the sperm midpiece in intracytoplasmic morphologically selected sperm injection relates sperm controsomal function. JARG 2010;27:75

Vanderzwalmen P, Hiemer A, Rubner P, et al. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. RBM Online 2008;17:5617


Chapter 6
## Summary – Chapter 6

### 1. The impact of the research on pre- and post-graduate education

#### a. The pre-graduate textbooks that the author has been involved in will be discussed, the philosophy behind them and learning objectives to be achieved for medical students. The impact on the medical community and their handling of infertile patients will be mentioned.

<table>
<thead>
<tr>
<th>i. Pre-graduate level</th>
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#### b. To assess the effect of sperm morphology (Tygerberg Strict Criteria) on education both at post-graduate level nationally and internationally.

<table>
<thead>
<tr>
<th>i. Clinical impact at post-graduate level nationally and internationally:</th>
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<td>474</td>
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<tr>
<td>476</td>
</tr>
</tbody>
</table>

**Siebert TI, Van der Merwe H, Kruger TF.** *How do we define male subfertility and what is the prevalence in the general population.* In: Male infertility: Diagnosis and treatment. Kruger TF, Oehninger SC (Eds). Informa Health Care: London 2007;18:269-276


### c. The impact of training on the scientists’ knowledge and understanding of sperm morphology.

#### i. Scientists’ knowledge and understanding of sperm morphology

<table>
<thead>
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<th>Page</th>
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#### ii. A concluding remark on the educational aspects of sperm morphology

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<td>471</td>
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</table>

### 2. A chronologic summary of the impact of sperm morphology on clinical practice internationally and concluding remarks

**Conclusion**

<table>
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<th>Page</th>
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<td>474</td>
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**References**

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</table>
SUMMARY

❖ Chapter 6

The research on sperm morphology had an impact on the interpretation of the semen analysis by students in training and also general practitioners country wide. As the research progressed, the chapter on male infertility was updated. The medical community thus knew how to handle a male factor and when to refer to a tertiary centre for fertility treatment.

At postgraduate and international level, the textbooks and sperm morphology atlases updated knowledge and gave more practical information on how to handle a patient with an abnormal semen analysis. The basis of acceptance by the WHO in 1999 (WHO 1999) and more so in 2010 (WHO 2010) of Strict Criteria was also highlighted and discussed in this section.

❖ Conclusion

Since the first publication in 1986 (Kruger et al., 1986), the international scientific community’s understanding of sperm morphology has changed. Not only were new morphology patterns described with clinical application, but it was also shown that the P-pattern group had more DNA abnormalities (Garolla et al., 2008), sperm binding/function was defective (Franken et al., 1990) and the potential to fertilize in vitro or in vivo was reduced (Kruger et al., 1988; Van Waart et al., 2001). New semen fertility thresholds were suggested which was accepted in 2010 by the WHO (Van der Merwe et al., 2005; WHO 2010). The computerised method (Kruger et al., 1993) to evaluate sperm morphology, brought a new objective tool to evaluate the male factor.

Initially an attempt to overcome the low fertilization chance was to increase the sperm insemination concentration (Franken et al., 1990). With the development of the ICSI technique this problem was partially overcome but with better sperm selection (strict evaluation) the prognosis of severe male factor patients and those with P-pattern morphology was further improved (Antinori et al., 2008).
The finding that sperm morphology affects fertilization *in vivo* and *in vitro* stimulated research in this field tremendously which brought new insight in male factor infertility.
1. The impact of the research on pre- and post-graduate education

a. The pre-graduate textbooks that the author has been involved in will be discussed, the philosophy behind them and learning objectives to be achieved for medical students. The impact on the medical community and their handling of infertile patients will be mentioned.

i. Pre-graduate level

In 1993 a chapter was written on male infertility for pre-graduate students and general practitioners. Definitions on terminology, causes of male infertility, semen thresholds and treatment options were discussed in the chapter. A table was developed on semen thresholds to indicate fertile and sub fertile values. These values were there to assist the general practitioner to be able to distinguish between those patients to be referred and those not, based on the semen analysis and known threshold values (Kruger et al., 1993, Table 1). The suggested thresholds were based on the international research and the Strict Criteria morphology values as discussed in Chapters 1 and 2. It is interesting to note that very little changed regarding general facts on male factor infertility in the updated chapters between 1993 and 2011. However, there were constant changes over the years regarding semen threshold values reflecting the active research in this field of diagnostic andrology over the last 25 years. These findings are reflected in the tables in the pre-graduate chapters on male infertility. This information can be seen in the tables in the 1993, 2001, 2007 and 2010 textbooks Clinical Gynaecology as shown below. The most important sources for the changes in the values were based on the international research in the field (Kruger et al., 1986; Kruger et al., 1988; Coetsee et al., 1998; Van der Merwe et al., 2005; Cooper et al., 2010).

In the books that followed the 1993 version, threshold values were adapted to the current international research. Please note that the first acceptance of the concept of Strict Criteria by the WHO came in 1999 and was fully accepted in 2010 (WHO 1999, WHO 2010). The table used in the 2011 textbook Clinical Gynaecology reflects the suggested semen thresholds published in 2005 (Van der Merwe et al., 2005) and these thresholds were endorsed by the WHO in the latest semen manual (WHO 2010).
Applicable chapters in the following text book will be reflected on:

Clinical Gynaecology 1993 – 1st Edition - Chapter 11, table 1, page 130
(Criteria for normal semen analysis)
<table>
<thead>
<tr>
<th>Criteria for normal semen analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Count per millimeter</td>
</tr>
<tr>
<td>o Normal or fertile: above 20 million</td>
</tr>
<tr>
<td>o Subfertile: between five and 20 million</td>
</tr>
<tr>
<td>o Possible infertile: less than five million</td>
</tr>
<tr>
<td>• Volume 2,5 – 3,5 ml</td>
</tr>
<tr>
<td>• Motility:</td>
</tr>
<tr>
<td>o Fertile: &gt;30% motile sperms within half an hour of collection</td>
</tr>
<tr>
<td>• Normal morphology:</td>
</tr>
<tr>
<td>o Fertile (in vitro): more than 20% normal forms.</td>
</tr>
<tr>
<td>o Fertile (in vitro): more than 14% normal forms.</td>
</tr>
<tr>
<td>• Forward progression:</td>
</tr>
<tr>
<td>o Fertile: &gt;2 (scale 0-4)</td>
</tr>
</tbody>
</table>

*Clinical Gynaecology 1993 – First Edition - Chapter 11, table 1*
Applicable chapters in the following text book will be reflected on:

Clinical Gynaecology 2001 – 2\textsuperscript{nd} Edition - Chapter 26, table 26.1, page 269. (Classification of male fertility potential used at Tygerberg Hospital)
### Classification of male fertility potential used at Tygerberg Hospital

<table>
<thead>
<tr>
<th>Semen parameter</th>
<th>Infertile</th>
<th>Subfertile</th>
<th>Fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (x10⁶/ml)</td>
<td>&lt;2.0</td>
<td>2.0-9.9</td>
<td>&gt;10.0</td>
</tr>
<tr>
<td>Motility (% motile)</td>
<td>&lt;10</td>
<td>10-29</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Forward progression (0-4)</td>
<td>&lt;1.0</td>
<td>1.0-1.9</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Motility index</td>
<td>&lt;20.0</td>
<td>20.0-49.9</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Morphology (% normal)</td>
<td>&lt;5</td>
<td>5-14</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>&lt;1.0</td>
<td>&gt;6.0</td>
<td>1.0-6.0</td>
</tr>
</tbody>
</table>

Fertile: optimal change for conception  
Infertile: very small change for conception  
Subfertile: reduced chance for conception  
Sterile: azoospermia or globazoospermia

**Clinical Gynaecology 2001 – Second Edition - Chapter 26, table 26.1**
Applicable chapters in the following text book will be reflected on:

Clinical Gynaecology 2007 – 3rd Edition - Chapter 28, table 28.1, page 329. (Classification of male fertility potential used at Tygerberg Hospital)
### Classification of male fertility potential used at Tygerberg Hospital

<table>
<thead>
<tr>
<th>Semen parameter</th>
<th>Infertile</th>
<th>Subfertile</th>
<th>Fertile</th>
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<tbody>
<tr>
<td>Concentration ($x10^6$/ml)</td>
<td>&lt;2.0</td>
<td>2.0-9.9</td>
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</tr>
<tr>
<td>Motility (% motile)</td>
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</tr>
<tr>
<td>Morphology (% normal)</td>
<td>&lt;5</td>
<td>5-14</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>&lt;1.0</td>
<td>&gt;6.0</td>
<td>1.0-6.0</td>
</tr>
</tbody>
</table>

- **Fertile:** optimal change for conception
- **Infertile:** very small change for conception
- **Subfertile:** reduced chance for conception
- **Sterile:** azoospermia or globazoospermia

Applicable chapters in the following text book will be reflected on:

(Classification of male fertility potential used at Tygerberg Hospital)
### Classification of male fertility potential used at Tygerberg Hospital

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<tbody>
<tr>
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<td>&lt;2.0</td>
<td>2.0-9.9</td>
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<tr>
<td>Morphology (% normal)</td>
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<td>5-14</td>
<td>&gt;15</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>&lt;1.0</td>
<td>&gt;6.0</td>
<td>1.0-6.0</td>
</tr>
<tr>
<td>MAR test</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&lt;50%</td>
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Fertile: optimal change for conception  
Infertile: very small change for conception  
Subfertile: reduced chance for conception  
Sterile: azoospermia or globazoospermia

*Clinical Gynaecology 2011 – Forth Edition - Chapter 28, table 28.1*
Male infertility

It is estimated that in 40–50 percent of infertile couples, the male is infertile which, in the general population, equals about 5–10 percent of all married men. However, infertility should not be viewed as solely male-related or female-related, but as a question of varying degrees of fertility potential in both partners. Marginal male fertility can often be offset by excellent female fertility and vice versa. Therefore, it is strongly advised that both partners simultaneously undergo a fertility evaluation. In this chapter, definitions, causes of male infertility, evaluation of the male, special investigations and treatments available for the infertile male will be discussed.

DEFINITIONS

- Normospermia: normal semen parameters, ejaculate likely to be fertile (>5 percent normal morphology) (Table 28.1)
- Oligozoospermia: sperm count less than 10 million/ml
- Asthenozoospermia: less than 30 percent motile spermatozoa with forward progression of less than two (scale of 0 to 4)
- Teratozoospermia: less than 5 percent spermatozoa with normal morphology.

The Tygerberg strict criteria are now the accepted criteria by the World Health Organisation in Geneva, and the suggested international standard
- Oligoastheno-teratozoospermia: signifies disturbance of all three variables (amount, motility, morphology). Combinations of only two parameters may also be possible
- Azoospermia: no spermatozoa in ejaculate
- Globozoospermia: sperm with round head and no visible acrosome
- Aspermia: no ejaculate.

Table 28.1 Classification of male fertility potential used at Tygerberg Hospital

<table>
<thead>
<tr>
<th>Semen parameter</th>
<th>Infertile</th>
<th>Subfertile</th>
<th>Fertile</th>
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<td>&lt;20.0</td>
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<td>&gt;50</td>
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<tr>
<td>Morphology (% normal)</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>&lt;1.0</td>
<td>&lt;1.5</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td>MAR test</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&lt;50%</td>
</tr>
</tbody>
</table>

Fertile: optimal chance for conception
Subfertile: reduced chance for conception
Infertile: very small chance for conception
Sterile: azoospermia or globozoospermia
CAUSES OF MALE INFERTILITY

Male infertility can be categorised into five aetiological groups (Table 28.2):

- Pretesticular or pregerminal causes
- Testicular causes
- Post-testicular causes
- Genitourinary infections
- Immunological causes.

EVALUATION OF THE MALE

When evaluating the infertile couple, it is important to take a thorough history. It is essential to obtain information from both partners regarding their perception of the "fertile period" and to make sure that adequate intercourse is taking place during that time. In this chapter, the same approach is followed as described in the section on female infertility (Chapter 27).

The general practitioner should always use ovulation and anovulation in the female partner as a guideline (Fig 28.1). If the patient is anovulatory, serum prolactin and TSH should be determined and a semen analysis (SA) of her partner should be performed. If the SA as well as the other two tests are normal, ovulation induction is proceeded with as described in the chapter on female infertility. On the other hand, if the patient is ovulatory and infertile for more than two years with a history of regular intercourse around the time of ovulation, one must suspect a more serious problem. Here, the male factor will be responsible, causing infertility in about 40 percent of infertile couples.

The general practitioner can request an SA from a recognised andrology laboratory at this stage. If a subfertile SA is obtained, the patient can be referred to a clinic or colleague specialising in human reproduction. It is important to note the following guidelines:

- Interpret an SA with care
- Do not rely on one abnormal SA
- Choose wording carefully and inform the patient that the presence of abnormal semen requires further investigation. This does not necessarily mean that there is a

Table 28.2 Causes of male infertility

<table>
<thead>
<tr>
<th>I Pretesticular or pregerminal causes</th>
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</thead>
<tbody>
<tr>
<td>Central gonadotropin deficiency</td>
</tr>
<tr>
<td>Hypothalamic: congenital GnRH</td>
</tr>
<tr>
<td>deficiency; tumour, infection, head</td>
</tr>
<tr>
<td>trauma</td>
</tr>
<tr>
<td>Pituitary: congenital FSH/LH deficit</td>
</tr>
<tr>
<td>deficiency; tumour, infarction, infection, trauma</td>
</tr>
<tr>
<td>Other: sarcoidosis, haemochromatosis</td>
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<tr>
<td>Endocrine excess syndromes</td>
</tr>
<tr>
<td>Gestrogen: functional tumour of</td>
</tr>
<tr>
<td>adrenal gland; cirrhosis</td>
</tr>
<tr>
<td>Androgen: congenital adrenal hyperplasia; androgen-producing tumour</td>
</tr>
<tr>
<td>Glucocorticoid: Cushing's syndrome;</td>
</tr>
<tr>
<td>steroid treatment (ulcerative colitis, asthma)</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Hypothyroidism</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>II Testicular causes</td>
</tr>
<tr>
<td>Chromosomal abnormalities</td>
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<tr>
<td>Klinefelter's syndrome (47, XXY)</td>
</tr>
<tr>
<td>Cryptorchidism, unilateral or bilateral</td>
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<td>Radiation, chemotherapy</td>
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<tr>
<td>Mumps, viral orchitis</td>
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<tr>
<td>Trauma</td>
</tr>
<tr>
<td>Sertoli-cell-only syndrome</td>
</tr>
<tr>
<td>Idiopathic maturation arrest</td>
</tr>
<tr>
<td>Androgen receptor abnormality</td>
</tr>
<tr>
<td>Androgen insensitivity syndrome</td>
</tr>
<tr>
<td>III Post-testicular causes</td>
</tr>
<tr>
<td>Congenital ductal obstruction</td>
</tr>
<tr>
<td>Vas deferens, epididymis</td>
</tr>
<tr>
<td>Acquired ductal block</td>
</tr>
<tr>
<td>Infection: gonorrhoea, tuberculosis</td>
</tr>
<tr>
<td>Vas ligation</td>
</tr>
<tr>
<td>Impaired motility</td>
</tr>
<tr>
<td>Kartagener's syndrome</td>
</tr>
<tr>
<td>Immotile cilia syndrome</td>
</tr>
<tr>
<td>Enzyme deficiencies</td>
</tr>
<tr>
<td>Protein carboxymethylase</td>
</tr>
<tr>
<td>IV Genitourinary infections</td>
</tr>
<tr>
<td>V Immunological causes</td>
</tr>
<tr>
<td>(According to Wentz A C, 1988)</td>
</tr>
</tbody>
</table>
male factor, but an expert's opinion at this stage is necessary. In the past it was found that incorrect information was given to patients on the basis of only one SA.

- A second SA is thus necessary when there is any abnormality in the first analysis. The quality and characteristics of the ejaculate may be improved by:
  - restricting smoking and excessive alcohol intake
  - proper diet, adequate rest, relief of emotional tension, and treatment of any chronic illness or metabolic disease
  - avoiding underwear that keeps the testicles in contact with the body, and heat exposure during excessively prolonged hot tubs or steam baths because heat is deleterious to testicular function
  - specific hormone therapy. However, it is estimated that this will benefit only 10 percent of men with idiopathic oligospernia or hypogonadotropic hypogonadism.

**SPECIAL INVESTIGATIONS**

**Basic semen analysis**

The basic semen analysis is still the cornerstone of tests used to evaluate male fertility. The semen specimen is usually obtained by masturbation and must be collected after two to three days of abstinence to standardise the SA in all patients. The general practitioner should make use of a laboratory with the necessary expertise. The clinical technologist involved in the semen analysis should have been trained at a teaching hospital and should hold a qualification equivalent to a certificate from the Health Professions Council in Reproductive Biology.

Spermatozoal abnormality is often associated with disorders of the testes, such as a varicocele. Male infertility is likely to be present if more than 95 percent of abnormal forms (less than 5 percent normal morphology) are encountered.

The quality of semen is judged by the number of spermatozoa in millions per
Figure 28.2 Close-up photograph of spermiogram showing normal (having long tails, normal acrosomes and <3 vacuoles) and abnormal spermatozoa.

(a) spermatozoon with a bent neck; (b) spermatozoon with a thick and broken tail; (c) spermatozoon with atypical, curling tail causing a ring effect; (d) spermatozoon with big head and enlarged cytoplasmic body (arrow); (e) spermhead with elongated shape; (f) normal sperm; (g) normal sperm.

millilitre, the percentage of normal forms, the percentage and type of abnormal forms, the motility and the forward progression (Table 28.1) (Fig 28.2).

The morphology is assessed after using one of the staining procedures, and the morphologic rating should include the counting of apparently normal spermatozoa. At Tygerberg Academic Hospital this is considered the most significant aspect of the evaluation because it gives the best information regarding fertility. This parameter is expressed as the percentage of normal forms or normal morphology (Table 28.1).

The total volume, pH and viscosity are also of some importance. A large volume of ejaculate may produce a low sperm concentration. An increased presence of leukocytes (more than two to five leukocytes per highpower field) may suggest prostatitis or another infection. A white cell count is then performed which is considered abnormal if more than one million white cells per millilitre are obtained. Care must be taken not to confuse white cells with immature spermatozoa as the appearance is similar. Aerobic and anaerobic cultures of the ejaculate should always be requested.

The motility is assessed under constant temperature conditions immediately after liquefaction and again four hours later. The different criteria indicating a normal SA are shown in Table 28.1.

Several specialised tests are available for the assessment of semen. Specialists in the field of infertility use most of these and it is essential to know what these investigations measure and for what reason they are indicated. However, it is important to realise that most of the information regarding fertility can be obtained by a basic semen analysis done in a good laboratory.

**Mixed agglutination reaction test (MAR test)**

This is a simple screening method for sperm antibodies which may impair male reproduc-
tive ability. It detects IgG antibodies in the semen sample. After several modifications, the MAR test has become one of the two mixed agglutination assays routinely used to demonstrate membrane-bound antibodies on sperm. A MAR test is considered abnormal if the values are >50 percent.

**Immunobead test**

If the MAR test is positive, the immunobead test is performed next. This assay employs immunoglobulin-coated latex particles as an indicator source. It also uses antitoplin to produce a mixed agglutination between antibody-bound sperm and the indicator. The assay not only demonstrates the presence of antibodies but also indicates the region of binding on the sperm surface and determines the class and/or subclass of immunoglobulin involved. The types of antisperm antibodies detected with the immunobead test in the semen sample are IgG, IgA and IgM.

**Sperm penetration assay (SPA)**

This is a test for assessing the sperm penetration ability using hamster oocytes. The zona pellucidae of hamster eggs are removed and if the penetration rate is more than 10 percent, the patient is considered to be possibly fertile. If the rate is less than 10 percent, the male may be infertile.

**Hemizona assay (HZA)**

Here the scientist looks at tight binding of spermatozoa to the human zona pellucida. The oocytes from ovaries obtained from patients undergoing hysterectomy and oophorectomy are cut into two halves and one half (hemizona) is used to assess the patient’s spermatozoa binding ability. The other hemizona serves for comparison with a proven fertile male. This is a test with promising ability to predict the chances of fertilisation in vitro.

**TREATMENTS FOR DIFFERENT MALE FACTOR CONDITIONS**

**Azoospermia**

Azoospermia can be due to pretesticular, testicular or post-testicular causes. Among the last, retrograde ejaculation must always be excluded in these patients. The practitioner should ask the patient to pass urine after the semen sample is obtained to make sure that there are no spermatozoa in the bladder due to retrograde ejaculation.

Men with azoospermia should always be referred to a specialist to decide whether surgical or medical treatment can be offered to correct the condition. No decisions on these patients should be taken before thorough investigations have been performed. It is important to note that in the majority of cases intracytoplasmic sperm injection (ICSI) after testicular biopsy is the treatment of choice. Intrauterine insemination (IUI) can be offered in cases with retrograde ejaculation.

**Asthenozoospermia**

If the patient presents with asthenozoospermia, it is important to repeat the SA. If the abnormality is confirmed in at least three semen samples, referral for further treatment is indicated. Such a patient can benefit from sperm manipulation procedures, and the problem of infertility can be treated with intrauterine insemination (IUI), gamete intrafallopian transfer (GIFT), in vitro fertilisation (IVF) or, in severe cases, by using sperm microinjection techniques (ICSI).

**Teratozoospermia**

If the male has a low sperm morphology (less than 5 percent normal forms), it is well known that these couples take longer to achieve a pregnancy. If infertility is present for more than two years, IVF or GIFT can be offered. If the sperm morphology shows between 0 percent and 4 percent normal forms but has a normal count and motility, the pregnancy chance at present using GIFT is 9–11 percent.
per cycle. On the other hand, if the morphology is between 5 percent and 14 percent normal forms, the pregnancy chance is 40 percent per cycle, which compares favourably with patients having normal semen parameters. Currently we consider the microinjection technique (ICSI) for the poor prognosis group (0–4 percent) normal forms if no pregnancy has occurred after three to four IUI attempts.

Antisperm antibodies
In these cases, assisted reproductive methods can be employed, that is, artificial insemination (IUI). Sperm manipulation procedures are used to improve the sample and motility and, if more than 500,000 sperm can be obtained after preparation, the prognosis is good (3–4 cycles are allowed). Systemic administration of corticosteroids in patients with antisperm antibodies is not indicated. In the case of low sperm morphology (0–4 percent normal forms) with anti-sperm antibodies, ICSI is the treatment of choice.

Micromanipulation – ICSI (intracytoplasmic sperm injection)
Micromanipulation is a new development in the field of male infertility used for injecting selected spermatozoa into the oocyte. Indications for this treatment are: if the patient has a very low sperm count (oligozoospermia), severe asthenozoospermia, severe teratozoospermia or azoospermia. In the case of azoospermia, spermatozoa can be obtained and injected after testicular biopsy. A biopsy specimen can also be frozen in small quantities and used when required with excellent results. In these cases micromanipulation can be applied in conjunction with IVF to try to achieve a pregnancy.

The current pregnancy rate at our institution is 40 percent per cycle in the most severe cases, if embryo quality is good. The cumulative pregnancy rate is ± 60–70 percent in three treatment cycles in patients for whom no pregnancy chance or treatment could be offered a few years ago.

See DVD for more information on treatment modalities.

CONCLUSION
It is essential for the general practitioner to understand that assisted reproduction has opened new doors for patients suffering from infertility, and more so for the male partner with serious infertility conditions. It is important to refer the infertile couple with a possible male factor as soon as possible for specialised treatment.

Further reading
b. To assess the effect of sperm morphology (Tygerberg Strict Criteria) on education both at post-graduate level nationally and internationally.

i. Clinical impact at post-graduate level nationally and internationally

The findings on sperm morphology in 1986 (Kruger et al., 1986) and 1988 (Kruger et al., 1988) led to a renewed interest in male infertility problems. This stimulated Kruger and Acosta, after a post-doctoral visit to the Jones Institute in 1986/1987 to embark on a textbook on male infertility. The aim of this project was to inform Gynaecologists, Urologists and Andrologists of the latest developments in the field of diagnostic Andrology but also to cover the wider field of male infertility. The first book in 1990 was a combined effort between the Jones Institute and the Tygerberg Fertility Unit with a few invited authors from other institutions. In the 1996 version the approach changed with more invited authors but again with the aim to update the current knowledge in the field of male infertility. In 2005 Kruger and Oehninger embarked on a new edition after the retirement of Dr Anibal Acosta. The publication ‘Male Infertility: diagnosis and treatment’ followed with the contributors the leading international researchers in the field of Andrology.

In this section the reflection will be on the table of contents of the different books published in 1990, 1996, 2006 and the chapters to do with sperm morphology and its clinical impact on treatment and decision making will be displayed.
Applicable chapters in the following text book will be reflected on:


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Section A3:

BASIC SEMEN ANALYSIS:
CLINICAL IMPORTANCE OF MORPHOLOGY

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ROELOF MENKVLEID, PH.D., AND SERGIO OEHNINGER,
M.D.

Today in vitro fertilization (IVF) not only plays an important role in the management of infertile couples but also has led to a better understanding of the basic physiological principles of, for example, ovulation induction and male infertility (1). Several categories of male infertility can be encountered: severe oligozoospermia, oligoteratozoospermia, pure teratozoospermia, asthenoteratozoospermia, asthenozoospermia, and oligoasthenoteratozoospermia (2). It is very important to recognize that one of the problems in the evaluation of male infertility is that, when patients are not categorized as mentioned above, the investigative results are often confusing and difficult to interpret. One category of great interest to us is pure teratozoospermia: normal spermatozoa concentration and motility but a low percentage of normal sperm morphology.
Sperm Morphology

EVALUATION

Evaluation of the percentage of normal sperm morphological features is subjective and difficult to compare among the laboratories of the world, and different means of assessing these features have been described (3, 4). Furthermore, the role of sperm morphology as a predictor of penetration capacity in the hamster zona-free oocyte sperm penetration assay (SPA) (5) and of fertilization potential in human IVF programs (6) has been questioned by some researchers. Although it is difficult to compare morphological features, the critical issue is what these features tell us in an IVF program.

Spermatozoa are considered normal when the head has a smooth, oval configuration, with a well-defined acrosome involving about 40% to 70% of the sperm head; absence of defects in the neck, midpiece, and tail; and no cytoplasmic droplets of more than half the size of the sperm head. Kruger (4) counts borderline forms as abnormal, in contrast to Eliasson (3).

In a study of sperm morphology, at least 200 cells per slide were evaluated. All male patients selected for study were required to have a concentration of $>20 \times 10^6$ sperm per milliliter and a normally motile sperm fraction of $>30\%$ (7, 8) in the basic semen analysis to minimize the impact of these two variables on the fertilization rate (8).

PREDICTIVE ROLE OF BASIC SEMEN ANALYSIS

In a prospective study of 129 male patients in 190 cycles, Kruger (4) divided the patients into four groups based on their percentage of normal morphology. According to criteria set by van Zyl (7), all patients had a sperm concentration of $>20 \times 10^6$/ml, with a normal motility of $>30\%$. Group 1 had a normal morphology of 0 to 14%; group 2, 15% to 30%; group 3, 31% to 45%; and group 4, 46% to 60%. In group 1, 104 oocytes were obtained; of these, 37% fertilized. In group 2 there were 324 oocytes, with a fertilization rate of 81%. In group 3, 309 oocytes produced a fertilization rate of 82%, and in group 4, 69 oocytes produced a fertilization rate of 91%.

A clear threshold of 14% normal sperm morphology was observed. No pregnancy was achieved in the group with $<14\%$ normal morphology. A pregnancy rate per embryo transfer of 25.8% was seen in the group with $>14\%$ normal morphology if three or more oocytes were retrieved; the rate was 11.4% if one or two oocytes were retrieved.

In a similar study at the Jones Institute, Kruger (8) used the same threshold, with a fertilization rate of 49.4% in the group with $<14\%$ normal morphology and a rate of 88.3% in the group with $>14\%$ normal morphology ($P<0.0001$). To select an accurate threshold, stricter criteria of normal morphology than those of the World Health Organization were used. Borderline forms were classified as abnormal, contributing to the low percentage of normal morphology. With the possibilities offered by evaluating egg-sperm interaction in vitro, a much better understanding of the meaning of normal morphology can be obtained.

Patterns in Patients with $<14\%$ Normal Morphology

Although there is severe impairment in the fertilization rate of patients with $<14\%$ normal morphology, some can still fertilize the human egg. Researchers at the Jones
Institute (9) studied 45 patients with <14% normal morphology to seek a morphological pattern which would differentiate the subgroup who fertilize from the subgroup who do not. All male patients had a sperm concentration of $>20 \times 10^6$/ml and a motile sperm fraction of $\geq 30\%$ in the basic semen analysis to minimize the impact of these two variables on the fertilization rate. They were divided into two groups. Group 1 consisted of those who had fertilized no oocytes; group 2 consisted of those who had fertilized at least one oocyte. There was a significant difference between their percentages of normal morphology: 1.8% (standard error, 2.4) in group 1 and 7.7% (standard error, 3.3) in group 2 ($P<0.0001$) (9). The percentage of slightly amorphous head abnormalities was 14.8% (9.7) in group 1 and 28.4% (7.8) in group 2 ($P<0.0001$). The predictive value ($R^2=0.44$) of normal morphology was better than that of slightly amorphous forms ($R^2=0.36$). No other sperm abnormalities showed a significant difference between the two groups. When the percentage of normal morphology and the percentage of slightly amorphous abnormalities were added and analyzed by multiple regression, there was a highly significant regression relationship ($P<0.0001$), with an even better predictive value ($R^2=0.56$). The combined percentage of slightly amorphous and normal forms (morphology index) was 19.7% (standard error 11.7) in group 1 and 42% (standard error 7.8) in group 2.

The Statistical Analysis System (SAS) general linear model was used with the number of embryos as the dependent variable to determine a threshold at which the chances of fertilization were significantly impaired. A threshold of <4% was indicated for normal morphology; <30% was indicated for the morphology index (combined percentage of normal morphology and slightly amorphous forms) (9). The fertilization rate per oocyte in group 1 (morphology index <30%, normal morphology <4%) was 7.1%; in group 2 (>30%, >4%), 60.7%. The mean number of embryos in the 13 patients in group 1 was 0.4; for the 32 patients in group 2 it was 2.6. These means were significantly different ($P<0.0001$). The ongoing pregnancy rate in group 1 was 1 in 13, or 7.6%; in group 2 it was 6 in 32, or 18.75%, with 3 abortions and 1 ectopic pregnancy (9).

Our results indicate that severe impairment of fertilization takes place at a level of <4% normal morphology, based on the strict criteria explained above. Results also indicate that by adding the slightly amorphous and normal forms, a morphology index can be established with a cut-off figure of 30%. Patients with a value of <30% have a significantly smaller chance to fertilize than patients with a level $>30\%$ ($P<0.0001$). None of the other semen parameters evaluated were of any help in predicting a patient’s chance to fertilize.

The advantage of strict morphology evaluation is that it is reproducible between patients and between technicians (10). It also allows the clinician to classify the patient into one of two specific groups (<14% and $>14\%$ normal morphology), providing a reliable criterion for counseling the patient and planning the approach in future IVF cycles (4, 8).

Based on the significant differences between normal morphology and the slightly amorphous forms in groups 1 and 2, we propose that two patterns can be observed in the <14% group. The P pattern (poor prognosis) has a mean normal morphology of <4%, and the G pattern (good prognosis) has 5% to 14% normal morphology. The G pattern gives the patient a significantly better chance to fertilize ($P<0.0001$) than the P pattern.

The evaluation of sperm morphology is a controversial issue. Results in fertilization rates differ among IVF units (4, 8, 11, 12). Do we look at the same spectrum of
Table 5.8.  
Fertilization Rate of Patients with Teratozoospermia

<table>
<thead>
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<th>P Pattern (&lt;4% Normal Forms)</th>
<th>G Pattern (4% to 14% Normal Forms)</th>
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<tr>
<td>Metaphase I oocytes</td>
<td>7 of 29 (24%)(^a)</td>
<td>68 of 155 (43.8%)(^b)</td>
</tr>
<tr>
<td>Metaphase II oocytes</td>
<td>3 of 15 (20%)(^a)</td>
<td>65 of 112 (58.04%)(^b)</td>
</tr>
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\(^a\)Not significant.  
\(^b\)P<0.01.

Abnormalities, explaining the difference in results, or is our classification of abnormal and normal sperm morphology in need of revision? We believe that the latter is true and needs urgent attention (4, 8, 9).

*Manipulation of Gametes In Vitro to Improve the Fertilization Rate*

Once fertilization and cleavage have occurred in the group of patients with <14% normal morphology, their chance of a pregnancy is good. This is perhaps more applicable to the G pattern (10). Increasing the concentration of spermatozoa in vitro from 100,000/ml to 500,000/ml improves the fertilization rate significantly (13). However, the pregnancy outcome in the P-pattern group was 4.1%, compared to 17.8% in the G-pattern group (9).

Another factor of utmost importance in handling patients with teratozoospermia—and perhaps all male factor patients—is the maturity of the oocytes. Patients were divided into two groups: those with <4% normal forms (P pattern) and those with 5% to 14% normal forms (G pattern). Oocytes were classified into metaphase I and II, according to the criteria of Veeck (14), 6 hours after retrieval and were fertilized at that time with 100,000 sperm per milliliter. The fertilization rate in the P-pattern group was 7 of 29 metaphase I oocytes (24.1%) and 3 of 15 metaphase II oocytes (20%) (Table 5.8). In the G-pattern group, the fertilization rate was 68 of 155 metaphase I oocytes (43.8%) and 65 of 112 metaphase II oocytes (58.04%) (P<0.01). The conclusion was drawn that, in the G-pattern group, selection of oocytes according to the criteria of Veeck (14) can influence the prognosis of fertilization and pregnancy outcome. In the P-pattern group, the fertilization rate did not differ significantly; although the numbers were small, it is well known that the prognosis of these patients is very poor (9). The delayed insemination principle reported by Trounson (15) is questionable when we treat male infertility, and specifically those with teratozoospermia.

It is also important to note that the oocyte classification which we used in the past, derived from the work of Testart (16), did not distinguish between metaphase I and II, although a so-called mature oocyte was retrieved. In a study by Stander (unpublished data), this fact was pointed out. Kruger (17) used a grading system with an 8-point scale to indicate oocyte maturity, with >6 of 8 indicating a mature oocyte. This system was correlated with metaphase I and II oocytes in patients with all semen parameters normal and with a sperm morphology of >14% normal forms. In the metaphase I group, 66 oocytes had a score of ≥6; in the metaphase II group 71 had this score. No correlation was found between the scoring system and the metaphase stage of the oocyte. The
oocytes were evaluated 6 hours after retrieval; soon afterward, insemination was performed. The fertilization rate in both groups was good, 87.9% in the metaphase I group and 98.6% in the metaphase II group.

Although the fertilization rate was good in the fertile group, the same observation was not made in the group with <14% normal morphology. In that group, the quality of the oocytes, particularly the distinction between metaphase I and II oocytes, became extremely important.

Thus, a clear trend was observed. Not only the recognition of the specific male problem was important but also the ability to distinguish between metaphase I and II oocytes. By doing this and by increasing the concentration of the spermatozoa to 500,000/ml for in vitro insemination, the fertilization rate will be significantly improved.

**Summary**

It has been pointed out in this chapter that if sperm morphology is classified according to the strict criteria we have laid down, this parameter can be used as a predictor of fertilization. Swim-up morphology can also be used to distinguish between different categories of patients with poor prognosis in vitro.

We advocate that failure of fertilization in vitro can often be attributed to a a morphological problem of the spermatozoa. Patients with a sperm morphology <14% normal sperm fertilize fewer eggs in vitro when we use 100,000 sperm per milliliter and can be divided into two groups (P pattern and G pattern) according the fertilization rate and prognosis. By increasing the concentration of spermatozoa to 500,000/ml and taking into consideration the maturity of the oocyte, the prognosis of these patients can be significantly improved.

**References**

Section B:

SPERMATOZOA MOTILE FRACTION SEPARATION

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In our laboratory, washed semen is used for artificial insemination by husband (AIH), in vitro fertilization (IVF), and gamete iatralfalloplian transfer (GIFT). Trounson's method of preparation (1) is used.

Liquefied semen samples obtained by masturbation after 3 days of abstinence are mixed with 2 ml of Ham's F-10 medium plus 10% human serum (midcycle from wife). The mixture is centrifuged at 1500 rpm for 10 minutes. This is repeated once with 10 ml of Ham's F-10 plus 10% serum. The resultant pellet is overlaid with 0.5 to 1 ml of Ham's F-10 plus 15% serum and incubated at 37°C for 30 to 60 minutes. Motile sperm swim up into the layer of medium, which is then pipetted off for use in AIH, IVF, or GIFT.

In all cases, an estimated count, motility, and forward progression are noted in the samples before the wash procedure. A slide for morphology evaluation is also prepared. Slight modifications are made, depending on the procedure to be carried out.
Methods of Sperm Wash

FOR AIH

For intrauterine insemination, the whole semen sample is mixed with 2 ml of Ham’s F-10 plus 10% serum and centrifuged at 1500 rpm for 10 minutes; the supernatant is discarded. The pellet is washed once more with 2 ml of Ham’s F-10 plus 10% serum. The final pellet is overlaid with 0.8 ml of Ham’s F-10 plus 10% serum and incubated for 1 hour at 37°C to allow sperm swim-up. Then 0.5 to 0.7 ml of the top layer is aspirated; the count, motility, and forward progression are noted and used for insemination. A morphology slide of the swim-up sample is prepared for later evaluation.

FOR IVF AND GIFT

In the wash procedure for IVF and GIFT, 1 ml of semen is washed twice with 2 ml of Ham’s F-10 plus 10% serum and centrifuged at 1500 rpm for 10 minutes. The final sperm pellet is overlaid with 1 ml of Ham’s F-10 plus 10% serum. The sperm are allowed to swim up for 30 minutes at 37°C in an incubator with 5% CO₂ in air. The top layer containing the swim-up sperm is aspirated and investigated for concentration, motility, and forward progression; a slide is made for morphology. A count for the determination of the final swim-up sperm concentration is performed, and the appropriate volume is added to the ova to obtain the recommended number of sperm per ovum.

For GIFT, the resultant swim-up samples are diluted to a sperm concentration of $4 \times 10^7$/ml and $20 \times 10^6$/ml to get a final count of 100,000 and 500,000 sperm in normal males (>14% morphologically normal sperm) and in teratozoospermic males (<14% morphologically normal sperm), respectively.

We have found that when we use sperm preparations as described, we obtain improved semen samples. The resulting swim-up samples contain a high proportion of motile sperm (80% to 100%), with improved forward progression and free of any foreign cells or debris. Sperm survival is usually very good, up to 72 hours. We have also found an increased percentage of morphologically normal sperm in the swim-up samples.

FOR ANTISPERM ANTIBODIES

Our studies have shown that washed, swim-up sperm from men with antisperm antibodies do not differ from unwashed samples in terms of sperm-bound antibodies. Swim-up procedures are exactly the same as for AIH preparation, and antibodies are detected with the immunobead test (IBT) and sperm cervical mucus contact (SCMC) test before and after sperm separation. However, the semen parameters—morphology, forward progression, and motility—are all improved by the wash and swim-up procedures.

We also did a study on washed semen samples obtained by the method of Boettcher (3) and Harrison (4). Semen samples were ejaculated in 15 ml of Ham’s F-10 plus 10% serum, mixed thoroughly, and incubated until liquefaction was completed. The mixture was centrifuged at 1500 rpm for 10 minutes. Pooled pellets were washed once more, and the final pellet was covered with 1 ml of medium to allow swim-up. After 10 to 60 minutes at 37°C, the top layer was aspirated and analyzed. Results again showed no significant differences in sperm-bound antibodies between washed and unwashed samples, as detected by the IBT and SCMC tests. Resultant sperm parameters were again improved, as in the normal wash procedure (5).
Our results suggest that patients can achieve pregnancy despite antisperm antibodies (2, 5). Improvement in sperm parameters (2, 5), minimizing the distance between sperm and ova, and bypassing the cervical mucus may be reasons for the relatively high pregnancy rate.

**Comparison of Sperm Morphology before and after Swim-Up for IVF**

The correlation of sperm morphology using strict criteria and IVF fertilization rates is based on evaluations of morphology in the initial specimen. Since specimens undergo a double swim-up before insemination, the sperm used for insemination may not be accurately reflected by the sperm morphology of the initial specimen. Previous evaluations using the criteria of the World Health Organization have documented an improvement in sperm morphology following the double swim-up preparation (6). That study used less rigid criteria and did not correlate the magnitude of the improvements with the degree of abnormality in the initial specimen. Therefore, sperm morphology was analyzed before and after swim-up and was correlated with the percentage of improvement and morphology patterns.

The initial specimens of 73 consecutive IVF patients were evaluated for sperm morphology. The double swim-up was performed by standard techniques. The specimens were coded, randomized, and read in a double-blind manner. Statistical evaluations were performed using paired data analysis and contingency table analysis, as indicated.

Of the 73 patients, 62 showed an improvement in their normal forms ($P<0.001$). The mean percentage of normal forms in the pre- and post-swim-up specimens was 19.8% and 23.4%, respectively. This represents an 18% difference, which is statistically significant ($P<0.05$).

Of the 73 patients, 27 had abnormal morphology ($\leq14\%$ normal forms) upon evaluation of their initial specimens. Of these, 18 (67%) showed an improvement in normal forms, eight (30%) were unchanged, and only one (4%) showed a decline in the percentage of normal forms. The mean improvement in normal forms was from 8.2% to 20.0%, an increase of 243.9% ($P<0.005$). These data support our observation that the double swim-up preparation enhances sperm morphology. The most substantial benefit seems to be in patients with abnormal morphology in their initial specimens.

**Predictive Role of Swim-Up Morphology**

In a study at the Reproductive Biology Unit of Tygerberg Hospital (Grobler, unpublished data), the role of swim-up morphology as a predictor of fertilization was studied in 122 IVF cycles of patients with $<14\%$ normal morphology by basic semen analysis. A sperm morphology slide was prepared after swim-up. The fertilization rate was 5.1%, or 3 of 58 oocytes, in the group with $<14\%$ normal swim-up morphology, and 35.2%, or 179 of 508 oocytes, in the group with $>14\%$ normal swim-up morphology ($P<0.01$). It was concluded that if, after swim-up, the normal sperm morphology is $<10\%$, the chance of fertilization is small; this fact can be used as a predictor of IVF outcome.
Glass Wool Filtration

Many techniques have been developed to recover viable sperm from an ejaculate for artificial insemination, IVF, and GIFT. However, many of these techniques yield a poor recovery of sperm or do not produce consistent results, particularly when the ejaculate is oligozoospermic, asthenozoospermic, or viscous. A technique that yields a high recovery of viable sperm from these ejaculates is therefore desirable. Glass wool filtration, as described by Jeyendran (7–8), is such a method.

Using the filtration technique, it was shown that all or almost all viable sperm in samples following centrifugation (washed sperm) or 50% to 70% of the viable sperm present in the unwashed ejaculate were recovered (7–9). The repeatability of the filtration technique was good, as assessed by determining the recovery of viable sperm from different aliquots of the same ejaculate. The reliability of the technique was tested by mixing known numbers of frozen-thawed sperm with untreated sperm prior to filtration. The relationship between the expected and observed sperm recovery was high ($r = 0.86, n = 12$) (10). The recovery of viable sperm by this filtration technique is much better than that obtained by swim-up procedures, especially in cases of viscous and/or oligozoospermic ejaculates (8), or asthenozoospermic ejaculates (11). A higher recovery of motile and hypo-osmotic swelling (HOS)-positive sperm is obtained from asthenozoospermic ejaculates by glass wool filtration than by a two-layer Percoll density gradient centrifugation technique (9).

The main purpose of treating an ejaculate is to select viable and potentially fertile sperm so that the sperm population has a higher fertilizing potential than the original ejaculate. Glass wool filtration has been criticized for causing latent injury to the membrane and acrosome of sperm heads (12), which may be detrimental to the fertilizing capacity of the sperm. These observations were based on an electron microscopic study of five ejaculates, during which some evidence of membrane damage was found. However, the sperm obtained by filtration survived cryopreservation as well as, or better than, those obtained from the unfiltered ejaculates. Also, the glass wool-filtered sperm survived storage at 22°C for 12 hours, better than sperm in the original ejaculate. Therefore, the functional studies do not support the electron microscopic observations. Another study did not find ultrastructural changes after glass wool filtration (10). Other data also argue against the occurrence of significant damage to the sperm membranes.

The filtration technique results in a significantly increased percentage of sperm with intact membranes as determined by the dye exclusion (viability) test and the HOS test (7). Sperm obtained by glass wool filtration have already been shown to be fertile. Seventy-six percent of the sperm samples ($n = 64$) obtained by glass wool filtration fertilized intact human oocytes (8). Of these, 11 resulted in pregnancies after embryo transfer. Paulson and Comhaire (13) and Jeyendran and Perez-Pelaez (14) reported pregnancies resulting from artificial insemination with glass wool-filtered sperm. Therefore, no good evidence exists that glass wool filtration leads to significant functional changes in the sperm.

Sperm recovered after glass wool filtration penetrate denuded hamster oocytes more successfully than untreated sperm, even after the samples are adjusted so that they contain the same number of motile sperm as unfiltered samples (15). Sperm in the first fraction of the filtrate were of higher quality than those of subsequent fractions or the
original ejaculate, based on motility, velocity, hypo-osmotic swelling, acrosin activity, and the ability to penetrate denuded hamster oocytes (16).

In view of these data, filtering sperm through glass wool produces a much higher yield of viable sperm than the swim-up technique, possibly than other methods, particularly when the ejaculate is asthenozoospermic and/or oligozoospermic. Sperm recovered by glass wool filtration are fertile. Therefore, the technique is useful for the preparation of sperm for IVF, GIFT, or for intrauterine insemination. Some glass wool fibers may appear in the filtrate even after the column has been rinsed extensively. It is important to assure the absence of fibers by light microscopic observations before using glass wool-filtered sperm for artificial insemination.

References
Applicable chapters in the following text book will be reflected on:


Human Spermatozoa in Assisted Reproduction

2nd edition

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Preface to first edition

The field of andrology as a discipline has moved and developed slowly and laboriously since its inception in 1891, in spite of tremendous efforts made by many excellent basic and clinical investigators. During all these years, the final judgment on sperm quality and the determination of normality in sperm parameters in the various tests depended on the ability of the patient to establish a pregnancy in his partner. That was the only available yardstick. There are significant drawbacks to this approach. On one hand, female fertility is not always easy to determine. On the other hand, unless artificial insemination is used the quality of the sperm which established a pregnancy is, for the most part, unknown. Therefore, this endpoint by which sperm is judged — the ability to produce a gestation — is quite unreliable, particularly when conception is not achieved.

The situation changed dramatically in the late 1970s when *in vitro* fertilization (IVF) and embryo transfer came into being, followed by other methods of assisted reproduction. For the first time, investigators interested in male infertility were able to judge human oocyte-sperm interaction. Regardless of whether or not pregnancy was established, the capability of normal or abnormal sperm to fertilize the female gamete could be readily determined. A new and more reliable endpoint was now at our disposal. New approaches to diagnosis and treatment of male factors, and also new procedures for sperm evaluation, manipulation and preparation were designed. Interest began to drift away from pregnancy as a yardstick by which to measure sperm quality, and the evaluation of the many sperm tests was done by comparing their results with the results of IVF in the human system. The era of using animals for that purpose was ending. Clinicians began to look for new criteria to predict results in IVF and to develop guidelines for the acceptability of male factors into the rapidly moving field of assisted reproduction.

In spite of all efforts, a group of patients were still unable to achieve fertilization *in vitro*. The interest of scientists shifted once again to developing methods to allow abnormal sperm to fertilize, and a new field — assisted fertilization — emerged.

With these new developments in the area of andrology, because of assisted reproduction and assisted fertilization, it seems timely to compile, summarize, review critically and set forth this knowledge for gynecologists, urologists, andrologists, reproductive endocrinologists, basic scientists and laboratory technologists. Such a book may be crucial for consolidating information and establishing a platform for future explorations in the field.

The collective experience of the Andrology Laboratories of Eastern Virginia Medical School and Old Dominion University, the program of assisted reproduction at The Jones Institute for Reproductive Medicine, the Tygerberg Clinic at the University of Stellenbosch and many outstanding clinicians and researchers from other institutions who generously contributed to this book has been compiled to update the knowledge in the field and to describe the diagnostic and therapeutic tests now in use. This collaborative effort stresses the need for a multidisciplinary–multicenter approach to solve the intricacies of the male infertility problem.

We have come a long way since sperm was discovered as one of the main elements of human reproduction, but we still have a long way to go to unveil all the scientific facts of sperm function. Past and future, ignorance and knowledge, despair and hope, faith and skepticism, empiricism and science have been the extremes through which we have moved in this very delicate area of reproduction and perhaps represent the extremes through which we will continue to move, at least in the near future. To make these efforts worthwhile, this work was conceived and published.

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Preface to second edition

Four years have passed since the original edition of this book was published. The goals and ideas that triggered that effort are still alive and valid. The reception has been excellent and, for the most part, the objectives were fulfilled. Complacency, none the less, should not interfere with renewed challenges and needs; a careful new look at the field of andrology gives clear evidence of areas where knowledge should be updated critically, as the approach to male factor diagnosis and treatment enters the era of assisted reproduction and assisted fertilization.

Many important centers and distinguished researchers have made substantial contributions to our understanding of this problem, but the majority of those contributions have been in semenology. Knowledge of physiology and pathophysiology of the testis contains enormous gaps in understanding; we have not made substantial progress in those fields. The fact that we are finding pure technical solutions to complex male reproductive function problems has given technology an edge over basic sciences. Nevertheless, although we continue to look at the sperm in assisted reproduction in this edition of the book, it does not mean that we fail to recognize the need to upgrade our knowledge of testicular physiology and pathophysiology to understand the roots of these deficiencies. Technology, although extremely useful, should never be allowed to replace scientific knowledge; same-way empiricism should never be allowed to replace sound scientifically based diagnosis and treatment.

Molecular genetics has now invaded medical science and the possibilities of true preventive medicine seem to be around the corner. A whole new field in andrology is now widely open and should be looked upon in a careful and hopeful manner; scrutiny is certainly warranted.

Once again, the andrology programs of the Eastern Virginia Medical School and the Tygerberg Clinic at the University of Stellenbosch join this effort. A long history of collaborative work has made this endeavor easier.

We timidly asked the best centers of the world to cooperate with us in updating this long list of subjects; the response was unanimous, generous and unrestricted; we could not have put together a better group of invited scientists and clinicians. The discipline still requires a multidisciplinary approach; otherwise, this book would have been incomplete and, in some areas, not at the level of excellence required. We are very grateful to all our contributors.

The extremes through which the field is moving, mentioned in the preface to the first edition, are still present. As the past is used for launching new ideas, the future looks brighter. As ignorance decreases, knowledge opens new doors for research therapies, despair is yielding to hope, faith is overcoming skepticism and science is fortunately replacing empiricism in our discipline.

Let us review again what we have learned in the last four years. Put it into the hands of an avid younger generation of physicians and scientists, and let them carry the torch and make it light brighter. This is a challenge that will prevent complacency. The second edition of this book is aimed to accomplish all this; the editors were mere instruments.

A kind invitation from the Chairman of the Department of Obstetrics and Gynecology at the University of Stellenbosch, Professor Hein Odenaal, to one of us (AAA) to become a Visiting Professor at his department for one month allowed us to get together to complete the task. It was an excellent and rewarding opportunity for which we are most grateful.

T. F. Kruger
A. A. Acosta
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HUMAN SPERMATOZOA IN ASSISTED REPRODUCTION

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Anatomy of the mature spermatozoon

T. F. Kruger, R. Menkweld and S. Oehninger

INTRODUCTION

A summary of light microscopic and electron microscopic features is given in this chapter outlining the basic characteristics of the anatomy of the human spermatozoon.

LIGHT AND ELECTRON MICROSCOPIC MORPHOLOGICAL CHARACTERISTICS OF SPERMATOZOA

Spermatozoa are highly specialized and condensed cells which do not grow or divide. A spermatozoon consists of a head, containing the paternal heredity material (DNA), and a tail, which provides motility (Figures 1 and 2). The spermatozoon is endowed with a large nucleus but lacks the large cytoplasm which is characteristic of most body cells. Men are unique among mammals in the degree of morphological heterogeneity of the ejaculate.

SPERM HEAD

Light microscopy

Human spermatozoa are classified using bright field microscope optics on fixed, stained specimens.

The heads of stained human spermatozoa are slightly smaller than the heads of living spermatozoa in the original semen, although the shapes are not appreciably different. The normal head should be oval in shape. Allowing for the slight shrinkage that fixation and staining induce, the length of the head is about 4.0–5.5 μm, and the width 2.5–3.5 μm. The normal length-to-width ratio is about 1.50–1.75. These values span the 95% confidence limits of comparative data for both Papanicolaou stained and living sperm heads. Two slightly different types of normal spermatozoal head forms have been described based on spermatozoa found in endocervical canal mucus post-coitally. The first and most common form, as identified under the microscope with bright field illumination, is the perfectly smooth oval head; the second form is oval, still having a smooth or regular contour but slightly tapered at the postacrosomal end. Since diversity is a fact of all biological systems, trivial variations must be regarded as normal.

The following head aberrations can be observed: head shape/size defects, including large, small, tapering, pyriform, amorphous, vacuolated (>20% of the head surface occupied by unstained vacuolar areas), and double heads, or any combination of these.

Human spermatozoa have a well-defined acrosomal region comprising about two-thirds of the anterior head area. It does not exhibit an apical thickening like many other species, but shows a uniform thickness/thinning towards the end forming the equatorial segment. Because of this

Figure 1 Schematic drawing of light microscopic human spermatozoon
thinning, the area will appear more intensely stained when examined with the light microscope. Depending on this staining intensity, the acrosome will appear to cover 40–70% of the sperm head.

**Scanning electron microscopy**

Scanning electron microscopy (SEM) is useful for the demonstration of surface structures of spermatozoa in great detail. Due to its three-dimensional picture, furthermore, it is possible to observe and interpret the complex structure of a human spermatozoon more easily and completely than with either light or transmission electron microscopy.

The sperm head is divided into two unequal parts by a furrow that completely encircles the head, i.e. the acrosomal and postacrosomal regions. The acrosomal region can represent up to two-thirds of the head length and in some cases a depression is noted in this area which is regarded as morphologically normal. The equatorial segment is not always clearly visible with SEM. Just after the equatorial segment is the beginning of the postacrosomal region, which is marked by maximal thickness and width of the spermatozoon. The postacrosomal region is divided into two parts by the posterior ring forming two equal bands. The band closest to the acrosome often stands out.

The surface of human spermatozoa, washed free of seminal plasma, appears smooth without coarse particles. The only exception is the acrosome and especially the anterior part that may frequently appear rough.

**Electron transmission microscopy**

The electron microscopic morphological characteristics of human spermatozoa are presented in Figures 2–6. The sperm head is a flattened ovoid structure consisting primarily of the nucleus. The acrosome is a caplike structure covering the anterior two-thirds of the sperm head (Figures 2 and 3), which arises from the Golgi apparatus of the spermioid as it differentiates into a spermatozoon. Unlike that in many other mammalian species, the acrosome of human spermatozoa does not exhibit an apical thickening, but has an anterior segment of uniform thickness. The acrosome contains several hydrolytic enzymes, including hyaluronidase and proacrosin, which are necessary for fertilization.

During fertilization of the egg, the enzyme-rich contents of the acrosomes are released at the time of acrosome reaction. During fusion of the outer
Figure 3  Schematic drawing of longitudinal section of sperm head

Figure 4  Longitudinal section of the region between midpiece and principal piece of human spermatozoon

Figure 5  Longitudinal section through midpiece

Figure 6  Cross-section of human sperm tail
acrosomal membrane with the plasma membrane at multiple sites, the acrosomal enzymes are released. The anterior half of the head is then devoid of plasma and outer acrosomal membrane and is covered only by the inner acrosomal membrane. The equatorial segment of the acrosome persists more or less intact since it does not participate in acrosome reaction (Figure 3).

The posterior portion of the sperm head is covered by the postnuclear cap, which is a single membrane. The equatorial segment consists of an overlap of the acrosome and the postnuclear cap (Figure 3).

The nucleus (Figure 3), comprising 65% of the head, is composed of DNA conjugated with protein. The chromatin within the nucleus is very compact, and no distinct chromosomes are visible. Several nuclei have incomplete condensation with apparent vacuoles. The genetic information carried by the spermatozoon is 'coded' and stored in the DNA molecule, which is made up of many nucleotides. The hereditary characteristics transmitted by the sperm nucleus include sex determination.

**SPERM TAIL**

**Light microscopy**

The sperm tail formation arises at the spermatid stage. The centriole during spermatogenesis is differentiated into three parts: midpiece, principal piece, and endpoint (Figures 1, 2). The midpiece has a similar length to the head, and is separated from the tailpiece by a ring, the annulus (Figure 5). The following tail aberrations can be observed:

1. Neck and midpiece aberrations, including their absence (seen as ‘free’ or ‘loose’ heads), noninserted or ‘bent’ tail (the tail forms an angle of about 90° with the long axis of the head), distended/irregular/bent midpiece, abnormally thin midpiece (i.e. no mitochondrial sheath), or any combination of these;

2. Tail aberrations include short, multiple, hairpin, broken (angulation >90°) tails, irregular width, coiling tails with terminal droplets, or any combination of these;

3. Cytoplasmic droplets greater than one-third the area of a normal sperm head are considered abnormal. They are usually located in the neck/midpiece region of the tail, although some immature spermatozoa may have a cytoplasmic droplet at other locations along the tail. The endpoint is not distinctly visualized by light microscopy.

**Scanning electron microscopy**

With SEM the tail can be subdivided into three distinct parts, i.e. midpiece, principal piece and endpoint. In the midpiece the mitochondrial spirals can be clearly visualized. This ends abruptly at the beginning of the midpiece. The midpiece narrows towards the posterior end. A longitudinal column and transverse ribs are visible. The short endpoint has a small diameter due to the absence of outer fibers.

**Transmission electron microscopy**

The midpiece possesses a cytoplasmic portion and a lipid-rich mitochondrial sheath that consists of several spiral mitochondria, surrounding the axial filament in a helical fashion (Figures 2, 5, 6). The midpiece provides the sperm with the energy necessary for motility. The central axial core of 11 fibrils is surrounded by an additional outer ring of nine coarser fibrils (Figures 2, 6). Individual mitochondria are wrapped around these outer fibrils in a spiral manner to form the mitochondrial sheath, which contains the enzymes involved in the oxidative metabolism of the sperm (Figures 2, 4–6). The mitochondrial sheath of the midpiece is relatively short, slightly longer than the combined length of the head and neck.

The principal piece (mainpiece), the longest part of the tail, provides most of the propellant machinery. The coarse nine fibrils of the outer ring diminish in thickness and finally disappear, leaving only the inner fibrils in the axial core for much of the length of the principal piece (Figure 2). The fibrils of the principal piece are surrounded by a fibrous tail sheath, which consists of branching and anastomosing semicircular strands or 'ribs' held together by their attachment to two bands that run lengthwise along opposite sides of the tail. The tail
ANATOMY OF THE MATURE SPERMATOZOOON

interpretes the endpiece with a length of 4–10 µm and a diameter of <1 µm. The small diameter is due to the absence of the outer fibers and sheath and a distal fading of the microtubules.

CONCLUSION

Although many of the structures described here, especially the ultrastructure characteristics based on electron microscopy studies, are not visible by light microscopy a basic knowledge of these structures is very important for the correct evaluation and interpretation of sperm morphology under light microscopy; in turn this will help in the determination of male fertility potential, as will be discussed in chapters to follow. These fundamental anatomic characteristics are also useful in understanding certain functional aspects of the male gamete during the fertilization process.

Original light and electron microscopy photographs, as well as schematic morphology/anatomy drawings (light- and electron microscopy), are depicted in Figures 1–6. If followed carefully, a clear picture of the basic sperm anatomy will be obtained.

References

INTRODUCTION

The currently reported high incidence of male factor infertility mandates a complete andrological consultation and basic semen evaluation in all male partners of couples consulting for infertility. Clinicians and research scientists are still confronted with the question of whether a given laboratory test or a battery of tests can predict the outcome in assisted reproduction. No simple solution to these problems is available and there is no wide consensus on the value of the different tests available in clinical practice today.

BASIC SEMEN ANALYSIS

As far as routine evaluation of male infertility is concerned, the basic semen analysis is still the cornerstone of laboratory evaluation of the male.

Physical examination of semen, e.g. coagulation, liquefaction and viscosity, are important and gross abnormalities when present should be detected and carefully reported by the Andrology Laboratory regardless of the quality of other semen parameters. Semen processing using different separation techniques is often hampered by physical abnormalities.

Semen volume, unless extremely low, seldom represents a problem for assisted reproduction (normal >2.0 mL). pH should be checked routinely because its value may indicate the presence of chronic or acute infections that can impair fertilization in vitro or in vivo (normal pH = 7.2–8.0).

The basic semen analysis will be considered normal for in vitro fertilization according to World Health Organization’s (WHO) latest manual, when a concentration of >20 million/mL, percentage of motile sperm %50% (categories a and b) and normal sperm morphology >30% are found. The morphology threshold for assisted reproduction according to the Tygerberg and Norfolk groups is at the level of 14% normal forms if strict criteria are used.

We consider a bacteriological as well as immunological screening essential parts of the initial semen analysis evaluation.

Influence of motility on fertilization in vitro

In a report by Acosta et al. from the Jones Institute, it was noted that even a very low percentage of motile spermatozoa (10%) in a given sample did not have a significant negative influence on fertilization and pregnancy rates. They divided their patients into three groups: those with motility between 10% and 20% (group 1), 21% to 30% (group 2), and 31% to 40% (group 3). The fertilization rate was 68% for group 1, 72% for group 2 and 73% for group 3 with a pregnancy rate per embryo transfer of 42.9% (group 1), 92.5% (group 2), and 24.4% (group 3). There were no significant differences between the groups. However, it is possible that motility values <10% may represent a problem in in vitro fertilization (IVF).

Sperm concentration

The number of sperm is evaluated in terms of both sperm density and recoverable sperm. This is very important in handling patients for assisted reproduction. If the sperm density is <20 million/mL, the specimen is considered abnormal. However, in assisted reproduction patients with severe oligozoospermia can still do well in terms of fertilization and pregnancy outcome if enough sperm can be obtained with separation techniques. In a prospective study performed at our own institution, no impact could be found on pregnancy outcome using, for example, the concentration/ml in the initial sample as a yardstick (Table 1).

The number of recoverable sperm is determined by the swim-up procedure. A minimum figure
Table 1 The effect of different semen parameters on pregnancy rate. Reproduced from Kruger et al. 1993 with permission of the Hemisphere Publishing Corporation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ongoing pregnancies (%)</th>
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</thead>
<tbody>
<tr>
<td>Sperm morphology (%)</td>
<td></td>
</tr>
<tr>
<td>0.0–4.0</td>
<td>14*</td>
</tr>
<tr>
<td>3.0–14.0</td>
<td>21*</td>
</tr>
<tr>
<td>Insemination semen volume in oviduct</td>
<td></td>
</tr>
<tr>
<td>0.01–0.09</td>
<td>20*</td>
</tr>
<tr>
<td>0.1–0.19</td>
<td>14*</td>
</tr>
<tr>
<td>0.2–0.9</td>
<td>19*</td>
</tr>
<tr>
<td>Seminal sperm concentration/mL</td>
<td></td>
</tr>
<tr>
<td>3.0–10.0</td>
<td>17*</td>
</tr>
<tr>
<td>11.0–19.0</td>
<td>8*</td>
</tr>
<tr>
<td>&gt;20.0</td>
<td>22*</td>
</tr>
<tr>
<td>Swim-up concentration</td>
<td></td>
</tr>
<tr>
<td>0.1–4.9</td>
<td>18*</td>
</tr>
<tr>
<td>3.0–9.9</td>
<td>11*</td>
</tr>
<tr>
<td>&gt;10.0</td>
<td>30*</td>
</tr>
</tbody>
</table>

* No significant difference

compatible for fertilization seemed to be 1.5 million total motile sperm assuming that the other parameters are normal. In our own gamete intrafallopian transfer (GIFT) program, it is possible to achieve a good pregnancy rate if only 50 000 sperm/mL are recovered.

Spermatozoon morphology

In our experience, morphological characteristics appear to be the best predictors for fertilization. Strict criteria should be employed during the morphological examination of spermatozoa. Based on previous publications, this gives the best predictive ability.

The normal spermatozoon should have a single sperm head with a well-defined acrosome comprising between 40% and 70% of the sperm head area, and a perfectly smooth oval head, or one that tapers slightly at the level of the postacrosomal region. The head length should be 4–5 μm and the width 2.5–3 μm when Papanicolaou staining is utilized. When the DiffQuik stain is used, the length should be 5–6 μm and the width 2.5–3.5 μm. (WHO 1992 with a stricter approach towards morphology criteria gives measurements for Papanicolaou of 4–5.5 μm [length]×2.5–3.5 μm [width].) No abnormalities in the neck, midpiece or tail should be present. The midpiece should be slender, axially attached and <1 μm wide, and its length should be approximately 1.5 times the head length. No cytoplasmic droplet greater than half the area of the sperm head should be present. The tail should be uniform, slightly thinner than the midpiece, uncoiled and approximately 45 μm long. Trivial variations in head morphology should be considered normal, but borderline normal heads are classified in a subgroup called slightly abnormal.

The 14% threshold for normal forms

When these strict criteria for normality are utilized in patients undergoing IVF, patients with fewer than 14% normal morphological forms were found to have a decreased fertilization rate according to previous publications (Table 2). Furthermore, in this same group of patients, two subgroups could be identified. The first appeared to have a good prognosis in terms of fertilizing ability although the percentage of eggs fertilized was lower than normal. This group was said to demonstrate the good prognostic pattern, or G pattern (lower fertilization potential than the normal group if only 100 000 sperm/mL/egg are used to fertilize the oocytes) (Table 2).

The second group in which fertilization was observed, exhibited a poor prognostic pattern, or P pattern (Table 3).

These morphological patterns have been found to be consistent and interpretation of the slides reproducible among technicians. In patients with 0–4% normal sperm morphology, classified as the P pattern, fertilization rates per oocyte were found to be 7.6% compared to the fertilization rate of 63.9% in the G pattern group (P<0.0001).

Recently, independent researchers from various international centers looking at the role of sperm morphology in assisted reproduction have confirmed these results. It was also observed in studies by Enginsu, that the predictive ability of strict criteria was significantly better compared to the WHO 1987 approach.

In a prospective study of patients in our GIFT program, we have also observed a significant difference in pregnancy rate when utilizing the 14% threshold of normal sperm morphology. A significantly lower pregnancy rate was observed in the group of patients with <14% normal morphology compared to those individuals in whom normal morphological forms comprised >14% of the
PREDICTION OF SPERM FERTILIZING POTENTIAL

Table 2  Sperm morphology as a predictor of human IVF. Reproduced from Acosta (1988)6 with permission of B. C. Dekko Inc.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 25)</th>
<th>Group 2 (n = 71)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal forms &lt;14%</td>
<td>Normal forms &gt;14%</td>
</tr>
<tr>
<td>Fertilization rate (%) per oocyte</td>
<td>49.4</td>
<td>88.3*</td>
</tr>
<tr>
<td>MI–MII</td>
<td>0=28%</td>
<td>0=1.4%</td>
</tr>
<tr>
<td></td>
<td>&lt;50%=36%</td>
<td>&lt;50%=11.2%</td>
</tr>
<tr>
<td></td>
<td>&gt;50%=36%</td>
<td>&gt;50%=87.4%</td>
</tr>
<tr>
<td>Pregnancies</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>Pregnancy rate/laparoscopy</td>
<td>12%</td>
<td>34.8%</td>
</tr>
<tr>
<td>Ongoing</td>
<td>4%</td>
<td>18.3%*</td>
</tr>
<tr>
<td>Pregnancy rate/transfer</td>
<td>16%</td>
<td>34.3%</td>
</tr>
<tr>
<td>Ongoing</td>
<td>5%</td>
<td>18.5%*</td>
</tr>
</tbody>
</table>

*P<0.00001; * no significant difference

Table 3  Prognostic patterns of sperm morphology in human IVF. Reproduced from Kruger et al. (1988)4 with permission of the American Society for Reproductive Medicine

<table>
<thead>
<tr>
<th></th>
<th>Fertilization in P pattern group (n = 13)</th>
<th>Fertilization in G pattern group (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal forms (%)±SD</td>
<td>1.8±2.4</td>
<td>7.7±3.3</td>
</tr>
<tr>
<td>Concentration (million/mL)±SD</td>
<td>63.3±42.8</td>
<td>83.3±57.8 (NS)</td>
</tr>
<tr>
<td>Motility (%)±SD</td>
<td>45.6±13.2</td>
<td>55.3±18.6 (NS)</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>7.6</td>
<td>63.9*</td>
</tr>
<tr>
<td>per oocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryos (%)±SD per patient</td>
<td>0.4</td>
<td>2.6*</td>
</tr>
</tbody>
</table>

SD = standard deviation; NS = not significant; *P<0.0001

specimen. In this study 423 cycles were analyzed prospectively, and the ongoing pregnancy rate in the group with abnormal sperm morphology (<14% normal forms) was 12%; meanwhile, an ongoing pregnancy rate of 23% was recorded in those couples in whom the male was found to have adequate sperm morphology (*P<0.01)5.

Two factors can be taken into consideration when handling a patient with low morphology to improve prognosis in assisted reproduction: (1) fertilization rate can be improved by simply increasing the insemination concentration/mL/egg from 100 000 sperm to at least 500 000 sperm5; and (2) to carefully consider the maturation stage of the oocyte. Patients with metaphase II oocytes will do better with IVF14 and have an improved pregnancy rate in GIFT5.

NEW DEVELOPMENTS IN THE FIELD OF SPERM MORPHOLOGY

In recent studies, sperm morphology was evaluated using computerized sperm analyzers. Four recent studies examined the value of the computerized system15–18. Wang15,16 concluded that no benefit could be found in the system she had tested above the manual method. Davis17, however, found the evaluation of the Motion Analysis System valuable. The studies by Wang and Davis looked at comparative analysis between manual and computerized systems on a cell by cell basis. In a recent study by our own group, we looked at the correlation between sperm morphology (normal forms, strict criteria) using slide by slide evaluation between manual and computerized systems (FERTECH). A good correlation was observed between the two methods18. The computer evaluation was also compared with the manual evaluation in a clinical study using fertilization as an endpoint. The computerized system identified the <14% of normal forms very well and showed a significant difference in fertilization rate in the groups with <14% and >14% normal forms (Figures 1,2). This difference was even more pronounced when a group of patients with <10% normal forms was considered (Figures
It was concluded that this new development holds promise for clinical practice.

In a recent, still unpublished, study we evaluated the IVOS system readings against the human readings (experienced worker) in a slide by slide evaluation. Thirty slides were compared in each group. The Kappa statistical evaluation was used and man-versus-computer (IVOS) was in the same category as man-versus-man looking at percentage normal morphology per slide (Kappa, respectively, 0.73 and 0.58; both in the good agreement group).

When the Spearman correlation coefficient was applied to the same set of data, Kappa was 0.88 in the man-versus-man and 0.85 man-versus-IVOS experiments.

The repeatability per cell for the IVOS system showed a Kappa value of 0.93 (excellent category). This new development, fast enough for day to day use, holds promise in routine andrology laboratories as well as in clinical practice in assisted reproduction.

### Bacteriological screening

Routine bacteriological screening includes cultures for Neisseria gonorrhoea, Ureaplasma urealyticum and other mycoplasma strains, and Chlamydia trachomatis detected by the monoclonal antibody immunofluorescence technique in the urethral smear. In men with a history of chronic urogenital infectious disease or a white cell count in semen of >1 million leukocytes/mL, regular bacteriological investigation should also be performed. In general, a microorganism count of >3000 Gram-negative pathogens/mL of semen or >10 000 Gram-positive non-pathogenic bacteria/mL of 1:2 diluted seminal plasma should be considered significant.

### Immunological factors

In our institution, we use the mixed agglutination reaction test to screen for sperm antibodies in semen. If a value of more than 10% is obtained, we consider this test positive and confirm it with a
direct immunobead test on the semen. In a study performed at our institution\(^9\), we found a good correlation doing sperm antibodies in serum and in semen, thus supporting the use of the mixed agglutination reaction (MAR) test as a screening procedure to detect sperm autoantibodies.

When antibodies are detected, we tend to offer assisted reproduction techniques to these patients if there are no other abnormalities present, one reason being that we find the best pregnancy result with GIFT\(^9\). There was a significantly lower pregnancy rate within the same patients if only intrauterine insemination was performed compared to the GIFT outcome (3% pregnancy per cycle, compared to a 16% pregnancy rate per cycle, respectively).

In a recent study performed at the Jones Institute and Tygerberg Hospital, it was shown that low normal sperm morphology is playing a detrimental role in the fertilization process both in the control group and in the antibody positive group. One can clearly note the influence on fertilization and pregnancy outcome in the different morphology groups, as well as between the antibody positive and control groups (Tables 4, 5) (see Chapters 13, 27 and 28\(^2\)).

### Spermatozoa separation methods

Sperm separation is essential in assisted reproductive procedures to remove spermatozoa from the seminal plasma, thus eliminating anticalcification factors in the seminal plasma, and to obtain a highly motile sperm fraction with increased normal forms. In our program, we use Ham’s F-10 media plus maternal serum for preparation of semen in a double wash and swim-up procedure\(^3\). Other methods of separation include layering and swim-up, albumin gradient separation, glass-wool separation, glass bead column separation and Percoll separation\(^2\). Recently, a new method of semen preparation by MiniPercoll has been reported that was shown to be particularly beneficial in patients with severe oligozoospermia\(^2\). It was also used successfully in cases in which epididymal sperm was aspirated to be used in IVF. It is the authors’ opinion that each male patient must be approached as an individual. The assisted reproduction laboratory must have different separation techniques available for oligozoospermic and asthenozoospermic patients. Some patients will do better using the swim-up technique and deliver sufficient sperm for

---

**Table 4** Outcome of IVF in the Norfolk group. Reproduced from Acosta et al. (1994)\(^3\) with permission of the American Society for Reproductive Medicine

<table>
<thead>
<tr>
<th></th>
<th>Fert. rate (%)</th>
<th>Total preg rate (%)</th>
<th>Term preg rate (%)</th>
<th>Abort. rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle (%)</td>
<td>Transf. (%)</td>
<td>Cycle (%)</td>
<td>Transf. (%)</td>
</tr>
<tr>
<td>Study group</td>
<td>41.9</td>
<td>21.0</td>
<td>23.5</td>
<td>14.7</td>
</tr>
<tr>
<td>(29 patients, 38 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
</tr>
<tr>
<td>G pattern</td>
<td>52.2</td>
<td>24.1</td>
<td>25.0</td>
<td>14.3</td>
</tr>
<tr>
<td>(21 patients, 29 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>42.8</td>
</tr>
<tr>
<td>P pattern</td>
<td>18.5</td>
<td>11.1</td>
<td>16.7</td>
<td>16.7</td>
</tr>
<tr>
<td>(8 patients, 9 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Control group</td>
<td>77.9</td>
<td>65.2</td>
<td>60.0</td>
<td>48.0</td>
</tr>
<tr>
<td>(23 patients, 23 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>40.0</td>
</tr>
<tr>
<td>G pattern</td>
<td>82.8</td>
<td>81.2</td>
<td>72.2</td>
<td>61.1</td>
</tr>
<tr>
<td>(16 patients, 16 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>30.8</td>
</tr>
<tr>
<td>P pattern</td>
<td>67.8</td>
<td>28.6</td>
<td>28.6</td>
<td>14.3</td>
</tr>
<tr>
<td>(7 patients, 7 cycles)</td>
<td></td>
<td></td>
<td></td>
<td>100.0</td>
</tr>
</tbody>
</table>

Fert. = fertility; preg = pregnancy; Transf. = transfer; Abort. = abortion
assisted reproduction. On the other hand, other patients would need support to obtain a sufficient number of motile sperm, i.e. using the glass-wool technique or other separation techniques with or without pentoxifylline. In a patient with asthenozoospermia the days of abstinence can be important and often, one day or less of abstinence can improve the motility of sperm in the sample.

**Testing of sperm function**

Despite progress in IVF and other assisted reproduction techniques, the diagnosis and treatment of male factor infertility is still a major challenge for reproductive biologists as well as clinicians in reproductive medicine. Since evaluation of sperm function test results prior to any male factor treatment seems to be mandatory, the diagnostic work-up and the success in male infertility have recently incorporated more sophisticated diagnostic methods for evaluation of sperm quality. Some of these assays determine biochemical parameters which have been found to be important for sperm function. Most of them, however, determine the biological functions of spermatozoa and consequently the capability to fertilize an oocyte.

**Biochemical assays**

**Creatine kinase**

Recently, a cellular marker of sperm quality, creatine kinase, has been found to be a key enzyme in the synthesis of energy transport factors. Higher levels seem to indicate a defect in sperm cytoplasmic extrusions. Motile sperm fractions from oligozoospermic samples enriched by the swim-up method were found to have lower creatine kinase levels than the original samples. Furthermore, when IVF was performed in oligozoospermic men, the group that proved to be fertile could be predicted on the basis of their sperm creatine kinase activity. However, more work is necessary to make this observation valuable for routine clinical use.
Recently, we have found that mature sperm selectively bind to the zona pellucida using immunocytochemistry for creatine kinase\(^2\). Spermatozoa with immature creatine kinase-staining patterns seem to be deficient in oocyte recognition and binding. This corroborates the report of Menkveld et al.\(^2\), that morphologically superior sperm have a higher binding capacity than abnormally shaped sperm forms. Furthermore, a good relationship has been found between the sperm biochemical parameters of creatine kinase concentration, lipid peroxidation and abnormal sperm morphology\(^3\).

### Bioassays of sperm–oocyte interaction

**Acrosin activity**

Acrosin (EC 3.4.21.10), a trypsin-like serine proteinase which is exclusively found within the acrosome of mammalian spermatozoa\(^4\), is associated with zona pellucida binding and zona pellucida penetration of spermatozoa\(^5\). Human spermatozoa that are almost devoid of acrosin fail to fertilize an egg, even if they are motile and possess an intact plasma membrane\(^6\). Considering the important function of acrosin for the fertilization process, several techniques have been described in the past to assess human sperm acrosin activity\(^7\). However, there are still uncertainties regarding the value of acrosin as a sole predictor of fertilization\(^8\). More work is needed in this field.

Two different assays quoted from the literature are evaluated in IVF programs: one spectrophotometric assay, using BAPNA as a substrate\(^9\), and another based on lysis of a gelatine layer which is evaluated in our unit\(^10\). Using a cutoff point of 10 μm for the halo diameter and 60% for the halo formation rate in the gelatinolytic assay, our data indicated that men whose spermatozoa show these parameters in the low range are infertile with high probability (Table 6). Using that gelatinolytic assay, we found relatively low sensitivity besides high specificity (Table 6). This clearly supports the hypothesis that functional parameters of spermatozoa are more or less independent from each other. To reach the main goal of reproductive events, i.e. successful fertilization of oocytes by spermatozoa, each of these specific spermatozoal functions must be optimal. If one parameter is suboptimal, fertilization is affected.

<table>
<thead>
<tr>
<th>Halo diameter (10 μm)</th>
<th>Halo formation rate (60%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity (%)</td>
<td>98.7</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>25.7</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>90.0</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>74.0</td>
</tr>
</tbody>
</table>

### Hypo-osmotic swelling test

The hypo-osmotic swelling (HOS) test is a measure of sperm membrane integrity and has been proposed as a predictor of IVF success\(^11\). Some investigations, however, have not found significant correlations between the results of the HOS test and the fertilizing ability of sperm *in vitro*\(^12\).\(^13\).

**Acrosome reaction**

Apart from motility, membrane integrity, ability to bind to the zona pellucida, acrosin activity and membrane fusion abilities, the acrosome reaction is of essential importance for fertilization. The acrosome is a membrane-bound organelle which appears during spermatogenesis as a product of the Golgi complex. Acrosomal membranes underlying the plasma membrane are referred to as outer acrosomal membranes, those overlying the nuclear membrane as inner acrosomal membranes. The acrosome reaction is an exocytotic process involving fusion of sperm plasma membrane and outer acrosomal membrane. Only acrosome-reacted spermatozoa can penetrate the zona pellucida\(^14\). Patients showing aberrations of the acrosome are subfertile\(^15\).

Data recently obtained by us\(^16\) support the hypothesis by Tesarik\(^17\) that higher levels of acrosome-reacted spermatozoa are required for fertilization, which will occur under physiological induction of the acrosome reaction. This means that the spontaneous acrosome reaction of capacitated spermatozoa, induced by random formation of clusters of the ZP3 receptors in the sperm membrane\(^18\) and reported to be 4–10%\(^19\), is not sufficient for fertilization.
Table 7  Statistical calculations of predictive values for
cutoff values of the inducibility of the acrosome
reaction (AR) (difference between test value of AR after
low-temperature induction and the spontaneous AR)
(7.5%) and the percentage of acrosome-reacted
spermatzoa after induction of acrosome reaction by
means of low temperature to predict the fertilizing
capacity of spermatozoa from different patients.
Reproduced from Henkel et al. (1995) with permission
of IRL Press Ltd

<table>
<thead>
<tr>
<th>Inducibility (7.5%)</th>
<th>Percentage of acrosome-reacted sperm (13.0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity (%)</td>
<td>50.0</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>86.0</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>63.1</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>78.2</td>
</tr>
</tbody>
</table>

Table 8  Sperm morphology as a predictor of the
sperm penetration assay (SPA). Reproduced from Kruger
et al. (1988) with permission of Blackwell Science Ltd

<table>
<thead>
<tr>
<th>Group 1a (n=41)</th>
<th>Group 1b (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal forms &lt;14%</td>
<td>Normal forms &gt;14%</td>
</tr>
<tr>
<td>(azoospermia)</td>
<td>(normospermia)</td>
</tr>
<tr>
<td>SPA&lt;10%</td>
<td>SPA&gt;10%</td>
</tr>
<tr>
<td>33 (85.4%)</td>
<td>6 (14.6%)</td>
</tr>
<tr>
<td>4 (13.8%)*</td>
<td>25 (86.2%)*</td>
</tr>
</tbody>
</table>

*P<0.0001, in comparison with group 1a

The inducibility of oocytes. This is in accordance with a
recent report by Henkel et al., that the percentage
of acrosome-reacted spermatozoa should exceed
13% after induction of acrosome reaction in a diagnostic
system using low temperature to induce acrosome
reaction (Table 7). Consequently, an increased
number of acrosome-reacted spermatozoa (more
than those occurring during spontaneous acrosome
reaction) is a prerequisite for fertilization. Neverthe-
less, the percentage of acrosome-reacted spermatoz-
oa after induction of the acrosome reaction (AR) by
means of low temperature in patients showing good
fertilization is not significantly different from that in
patients showing poor fertilization. In contrast, the
inducibility of the acrosome reaction, i.e. the differ-
ence between spontaneous acrosome reaction and
the percentage of acrosome-reacted spermatozoa
after induction, shows highly significant correlation.
Inducibility of acrosome reaction should be at least
7.5% to be assessed as normal. Normal acrosome
reaction and/or normal inducibility of the acro-
some reaction indicate good fertilizing capacity of
spermatozoa (Table 7).

Sperm penetration assay

The zona-free hamster ovum–sperm penetration
assay (SPA) is often used as a prognostic test to assess
male fertility in vitro as well as to measure the
improvement of sperm function after therapy. This
heterologous bioassay evaluates the ability of the
proportion of acrosome-reacted sperm in a given
sperm population and its ability to fuse with the
oolemma and decondense within zona-free hamster
eggs. Originally proposed as a test for assessing the
fertilizing capacity of human spermatozoa, the SPA
reflects specifically the ability of sperm samples to
undergo the acrosome reaction.

The existence of a correlation between SPA and
conventional semen parameters has been examined
by numerous authors, yet no consensus has been
attained, mainly because of the varied experimental
conditions and assessment criteria used by different
laboratories. Most early investigators found no cor-
relation between SPA and the traditional semen
parameters, sperm density, motility or morphology.
In contrast, Rogers et al. reported a correlation with
motility and morphology, but found the latter to be
more important. A prospective study of the SPA
at Norfolk evaluated 70 patients with a normal
sperm concentration (exceeding 20×10^6/mL), a
normal motile sperm fraction (>30%) and varied
levels of sperm morphology, ranging from 1% to
39% normal forms. Biggers–Whitten–Whittingham
(BWW) medium was utilized for incubation, with 3%
bovine serum albumin (BSA) as the protein source
during a short (6-h) incubation protocol. A statistical
significant relationship was noted between the per-
centage of sperm with normal forms (>14%) and
the penetration rate in the SPA (P=0.001) (Table 8).

Furthermore, the outcome of the SPA was cor-
related with IVF retrospectively in 84 patients.
Thirty-eight patients had an SPA of <10%; no fer-
tilization in vitro was demonstrated in 13 patients
(34.2%), whereas successful fertilization was noted
in 25 (65.8%). Forty-six couples had an SPA exceed-
PREDICTION OF SPERM FERTILIZING POTENTIAL

Fertilization occurred in 38 (82.6%) and no fertilization in 8 (17.4%). Based on these results, it was concluded that SPA appeared to have a positive predictive value for IVF when it was normal, but was less reliable when egg penetration was <10%. This relatively poor predictability of fertilization by means of SPA might be attributed to the fact that this bioassay is still not really evaluated. According to all previous protocols, acrosome reaction has to be induced before co-incubation of human sperm with zona-free hamster oocytes. However, no attention is paid to the percentage of acrosome-reacted spermatozoa (high percentage or low percentage). Thus, one does not know whether low binding and/or penetration results from a poorly induced acrosome reaction or from an impaired binding of sperm to the oolemma. Moreover, there is no agreement about the cutoff value. Apart from the large-scaled procedure for performance of SPA, after comparing the new criteria of sperm morphology and our SPA standards, and taking the mentioned disadvantages into consideration, morphology seems to be a better predictor of IVF outcome and a more useful adjunct for counseling patients.

Hemizona assay

Tight binding of human spermatozoa to the human zona pellucida is a critical event in gamete interaction leading to fertilization. This binding step may provide unique information predictive of sperm's ultimate fertilizing potential. However, due to species specificity human spermatozoa will bind firmly only to human zona pellucida. In view of this, the feasibility of tight human sperm binding to the zona pellucida of non-living human oocytes was first examined by Overstreet and Hembree. Sperm binding and zona penetration were tested using oocytes recovered from post mortem ovarian tissue and, later, from ovaries removed from surgical indications. This study, however, did not investigate the kinetics of zona binding or the specific relationship between the binding event and male fertility.

Burkman and colleagues developed a new sperm function assay based on the relative binding of patient versus control spermatozoa to the matching halves of a bisected human oocyte. This hemizona assay (HZA) assesses tight binding of sperm to the outer surface of the zona hemisphere. A threshold of 30% for the hemizona assay index (HZA) was established with the better prognosis in IVF for those sperm samples with an index >30%. In a prospective, blinded study, we investigated the relationship between sperm binding to the hemizona and IVF success. The positive predictive value of this test was 85%, and negative predictive value was 70%.

This functional test holds promise as an adjustment to the basic semen analysis to counsel patients prior to an assisted reproduction procedure.

It is interesting to note that most of the spermatozoa bound to the hemizona were morphologically normal, and 80% acrosome-reacted. The zona glycoproteins from salt-stored human zona pellucida retained not only the capability for tight binding, but also the ability to induce acrosome reaction. The ZP3 complex thus serves both as a sperm receptor and a trigger for the acrosome reaction (see Chapter 20).

Additional investigations

Acridine orange test

The acridine orange (AO) test has recently been introduced as an indicator of the DNA status of human spermatozoa. Tejada et al. introduced a simplified method for fixation and denaturing sperm DNA. Specimens with high percentages of denatured sperm heads were associated with decreased fertility, whereas specimens with high numbers of sperm heads showing a distinct green fluorescence were indicative of normal semen samples containing a high percentage of sperm with normal DNA content.

The results of the AO test were compared with human sperm morphology and fertilization in vitro. Seventy-six patients from the IVF and GIFT programs were randomly selected for the study. All patients underwent routine semen analysis, sperm DNA evaluation and standard IVF procedures. The results indicated a moderate positive correlation (r=0.38, P=0.0006) between the AO test results and normal sperm morphology. Patients with an AO test value exceeding 24% had significantly higher oocyte fertilization rates than did patients with lower values: results for metaphase I were 74% versus 51% (P=0.0008) and, in metaphase II oocytes, 88% versus 60% (P=0.0001).
We concluded from this study that, although there is a good predictive value in the AO test, sperm morphology is still the best predictor and easier to do on a day to day basis.

Chromatin condensation

Another parameter of spermatozoal function which has been shown to be predictive of fertilization in vitro is the chromatin condensation\textsuperscript{62}. Normally, during spermatogenesis, the histones in late spermatids are replaced by protamines. The ratio of replacement can be determined by means of aniline blue staining\textsuperscript{63}, because acid aniline blue binds to the very basic residues of histones. If this replacement is not performed properly sperm will stain blue, indicating immaturity of nuclei and, consequently, probability of successful fertilization will decrease. According to Liu and Baker\textsuperscript{64}, poor fertilization in patients with normal sperm morphology might be caused by poor zona binding or poor nuclear maturity. Recently, it was demonstrated that the percentage of mature nuclei can be enriched by means of glass wool filtration\textsuperscript{65}.

Sequential analysis

It was proposed by Oehninger \textit{et al.}\textsuperscript{66} that, by combining the two bioassays, the HZA, a zona penetration assay, and the heterologous SPA, it might be possible to evaluate tight sperm–oocyte binding, zona penetration, sperm oocyte fusion and sperm head decondensation in sequence (Figure 3). This is an interesting concept that may refine our ability to diagnose male factor infertility. It is postulated that if an abnormality in a specific step of the binding–fertilization chain could be identified, the information gained could then be used in selecting optimal therapy for a specific patient. This may even be of value in identifying patients with the best prognoses for intracytoplasmic sperm injection and vice versa.
Sperm receptors

Sperm receptors, i.e. wheat germ agglutinin receptors, on the sperm head is an interesting development in the field of diagnostic andrology. Gabriel indicated a correlation between wheat germ agglutinin receptors at the equatorial region with semen parameters and, specifically, morphology (Table 9). Potentially, this observation can be of clinical value as well as part of the parameters evaluated in sequential analysis (see Chapter 24, 25).

Oxygen species

This aspect of semen evaluation and its clinical relevance will be covered by Aitken in Chapter 21 of this textbook.

CONCLUSION

The aim of this chapter is to highlight some of the exciting new developments in diagnostic andrology with emphasis on the latest ones in the field of sperm morphology. The concept of sequential diagnosis in this field seems to be important and can possibly prove to be of practical help in the future to decide on a specific treatment with assisted reproduction or assisted fertilization.

References


Table 9 Percentage wheat germ agglutinin receptor localization on human sperm membrane domains in P, G pattern and normal semen samples (mean ± SD). Reproduced with permission from Gabriel et al. with permission of Blackwell Wissenschafts Verlag

<table>
<thead>
<tr>
<th>Analysis % morphology</th>
<th>Patient group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P pattern 0-4</td>
</tr>
<tr>
<td>Acrosomal</td>
<td>6.46±14</td>
</tr>
<tr>
<td>Equatorial</td>
<td>20.13±9a</td>
</tr>
<tr>
<td>Post acrosome</td>
<td>93.54±15</td>
</tr>
<tr>
<td>Neck</td>
<td>20.92±13</td>
</tr>
<tr>
<td>Midpiece</td>
<td>2.85±15</td>
</tr>
<tr>
<td>Tail</td>
<td>3.4±7</td>
</tr>
</tbody>
</table>

*versus b = P<0.05; *versus * = P<0.05


HUMAN SPERMATOZOA IN ASSISTED REPRODUCTION

...morphology, sperm-zona pellucida binding, and fertilization rates. *Fertil. Steril.*, **58**, 1178–84


Applicable chapters in the following text book will be reflected on:

Male Infertility
diagnosis and treatment

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INTRODUCTION

Several semen parameters are used to discriminate the fertile male from the subfertile male. The most widely used parameters are sperm concentration, motility, progressive motility and sperm morphology. Of these parameters, sperm morphology is the single indicator most widely debated in the literature. A large number of classification systems have been used to describe the factors that constitute a morphologically normal/abnormal spermatozoon. The most widely accepted classification systems for sperm morphology are the World Health Organization (WHO) criteria of 1987 and 1992\textsuperscript{1,2} and the Tygerberg strict criteria, now also used by the WHO since 1999\textsuperscript{3-6}.

Although there is a positive correlation between normal semen parameters and male fertility potential, the threshold values for fertility/subfertility according to WHO criteria\textsuperscript{1,2} are of little clinical value in discriminating between the fertile and the subfertile male\textsuperscript{7-31}. If these criteria were to be applied, a great number of fertile males (partners having had pregnancies shortly before, after or at the time of a spermogram) would be classified as subfertile. The predictive values of sperm morphology using strict criteria in \textit{in vitro} fertilization (IVF) and intrauterine insemination (IUI) have been reviewed recently and proved to be useful\textsuperscript{12,13}.

Much less has been published on the use of this criterion regarding \textit{in vivo} fertility.

In this chapter, we evaluate the classification systems for semen parameters after review of the literature published in English on semen parameters and \textit{in vivo} fertility potential. We also use data from the literature to establish fertility/subfertility thresholds for semen parameters according to the WHO 1999 guidelines\textsuperscript{3-6}. These thresholds should be of clinical value and useful when assessing male fertility potential for \textit{in vivo} conditions, in order to identify those males with a significantly reduced chance of achieving success under these conditions.

WHO CRITERIA OF 1987 AND 1992 AND MALE FERTILITY POTENTIAL

The semen analysis is used in clinical practice to assess male fertility potential. To be of clinical value, the methods used should be standardized, and threshold values for fertility/subfertility should be calculated for the different parameters used in the standard semen analysis.

Because there are so many different methods for semen evaluation, it would be difficult to standardize the methods used in its analysis. This applies especially to the assessment of sperm
morphology. The two classification systems most widely accepted are the WHO\textsuperscript{1,2} and the Tygerberg strict criteria\textsuperscript{3-6}. Various methodological problems concerning sperm morphology have been identified. The variants among different methods of morphology assessment have been reported by Ombelet \textit{et al.}\textsuperscript{14-16} and others\textsuperscript{17,18}, and they recommend standardization of semen analysis methodologies. Some authors recommend that laboratories should adopt the accepted standards, such as those proposed by the WHO\textsuperscript{17,18}. Another problem identified is the variation in intra- and interindividual and interlaboratory sperm morphology assessment\textsuperscript{18,19}. This problem can be addressed by using the Tygerberg strict criteria, as Menkveld \textit{et al.} showed that comparable and reliable results between and within observers could be obtained when using this method\textsuperscript{19}. Franken \textit{et al.} delivered dedicated work on continuous quality-control programs for strict sperm morphology assessment, and demonstrated that consistent readings could be achieved; they hence stressed the need for global quality-control measurements in andrology laboratories\textsuperscript{20,21}. Cooper \textit{et al.}\textsuperscript{18} also urged the standardization of such quality-control programs and that quality control centers should reach agreement with each other.

Previous WHO thresholds of 50\% and 30\% for sperm morphology were empirical values and not based on any clinical data. Several authors found these values to be of little or no clinical value\textsuperscript{7,9,10}. These studies did, however, find a positive correlation between a high proportion of morphologically normal sperm and an increased likelihood of fertility and/or pregnancy. Other studies have confirmed this correlation\textsuperscript{22-25}.

Van Zyl \textit{et al.}\textsuperscript{23} were the first to show a faster than linear decline in fertilization rate when the proportion of normal forms dropped to less than 4\%. Eggert-Kruse \textit{et al.}\textsuperscript{23} found a higher \textit{in vivo} pregnancy rate for higher percentage normal forms at thresholds of 4, 7 and 14\% using strict criteria for morphology assessment. Zinaman \textit{et al.}\textsuperscript{26} confirmed the value of sperm morphology (strict criteria) by demonstrating a definite decline in pregnancy rate \textit{in vivo} when the normal morphology dropped below 8\% and sperm concentration below $30 \times 10^6$/ml. In a study performed by Slama \textit{et al.}\textsuperscript{27}, measuring the association between time to pregnancy and semen parameters, it was found that the proportion of morphologically normal sperm influenced the time to pregnancy up to a threshold value of 19\%. This value is somewhat higher than that calculated in other studies.

THE USE OF SEMEN PARAMETERS IN IVF AND IUI PROGRAMS

The percentage of normal sperm morphology (strict criteria) has a positive predictive value in IVF and IUI programs. Normal sperm morphology thresholds produced positive predictive values for IVF success when using the 5\% and 14\% thresholds, respectively, with the overall fertilization rate and overall pregnancy rate significantly higher in the group with normal morphology $\geq$ 5\% as compared with the < 5\% group\textsuperscript{12}. A meta-analysis of data from IUI programs showed a higher pregnancy rate per cycle in the group with normal sperm morphology $\geq$ 5\%. In the group with normal sperm morphology < 5\%, other semen parameters predicted IUI success\textsuperscript{13}. In the IUI meta-analysis, motility\textsuperscript{28}, total motile sperm count\textsuperscript{29} and concentration\textsuperscript{30} also played a role in some of the studies evaluated, while others\textsuperscript{31} stated that sperm morphology alone was enough to predict the prognosis. Because of the high cost of assisted reproduction, males with good or reasonable fertility potential under \textit{in vivo} conditions should be identified on the basis of semen quality. Conversely, males with a poor fertility potential should be identified, and introduced to assisted reproduction programs.
FERTILITY/SUBFERTILITY THRESHOLDS FOR SPERM MORPHOLOGY USING TYGERBERG STRICT CRITERIA, SPERM CONCENTRATION AND SPERM MOTILITY/PROGRESSIVE MOTILITY

In an effort to establish fertility/subfertility thresholds for the aforementioned parameters, we identified four articles in the published literature. It is our opinion that these articles constitute a representative sample of published studies of the predictive value of sperm morphology, sperm concentration and motility/progressive motility for in vivo fertility/subfertility. These articles compared the different semen parameters of a fertile and a subfertile group. They used either classification and regression tree (CART) analysis or receiver operating characteristic (ROC) curve analysis to estimate thresholds for the various semen parameters. The ROC curve was also used to assess the diagnostic accuracy of the different parameters and their ability to classify subjects into fertile and subfertile groups.

Using ROC curve analysis, Ombelet et al. calculated the following thresholds: proportion normal morphology 10%, proportion normal motility 45% and normal sperm concentration $34 \times 10^6$/ml. Sperm morphology was shown to be the parameter with the highest prediction power (area under the curve (AUC) 78%). Much lower thresholds were calculated using the 10th centile of the fertile population, these thresholds being 5% for normal morphology, 28% for motility and $14.3 \times 10^6$/ml for sperm concentration (Tables 18.1 and 18.2).

Günalp et al. also calculated thresholds using ROC curve analysis. These thresholds were: proportion normal morphology 10%, proportion normal motility 52%, proportion progressive motility 42% and sperm concentration $34 \times 10^6$/ml. The two parameters that performed best were progressive motility (AUC 70.7%) and

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<tr>
<th>Table 18.1</th>
<th>Thresholds: fertile vs. subfertile populations studied</th>
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<tbody>
<tr>
<td>Authors</td>
<td>Normal morphology (%)</td>
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<tr>
<td>Guzick et al. (2001)</td>
<td>9</td>
</tr>
<tr>
<td>Menkved et al. (2001)</td>
<td>4</td>
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<tr>
<td>Günalp et al. (2001)</td>
<td>10</td>
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<tr>
<td>Ombelet et al. (1997)</td>
<td>10</td>
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<table>
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<tr>
<th>Table 18.2</th>
<th>Possible lower thresholds for the general population to distinguish between subfertile and fertile men based on the assumed incidences of subfertile males in their populations</th>
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<tbody>
<tr>
<td>Authors</td>
<td>Normal morphology (%)</td>
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<tr>
<td>Menkved et al. (2001)</td>
<td>3</td>
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<tr>
<td>Günalp et al. (2001)</td>
<td>5</td>
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<tr>
<td>Ombelet et al. (1997)</td>
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</table>
morphology (AUC 69.7%). Assuming 50% prevalence of subfertility in the population, the
authors used the positive predictive value as an indicator to calculate a lower threshold for each
parameter. Values of 5% for proportion normal morphology, 30% for proportion normal motility,
14% for proportion progressive motility and 9 \times 10^6/ml for sperm concentration were calcu-
lated (Tables 18.1 and 18.2)\textsuperscript{33}.

In the most recent article of the four, Menkveld \textit{et al.}\textsuperscript{34} found much lower thresholds
than the others. Using ROC curve analysis, the following thresholds were calculated: 4% for
normal morphology and 45% for normal motility. Again, morphology showed good predictive
value with an AUC of 78.2%. Although a threshold for sperm concentration was not calculated (a sperm
concentration less than 20 \times 10^6/ml was used as inclusion criterion), the authors proposed that the
cut-off value of 20 \times 10^6/ml could be used with confidence, based on the resultant lower 10th cen-
tile of the fertile population. Adjusted cut-off points calculated on the assumption of 50%
prevalence of male subfertility were as follows: 3% for proportion normal morphology and 20% for
proportion normal motility (Tables 18.1 and 18.2)\textsuperscript{34}.

In the fourth article by Guzick \textit{et al.}\textsuperscript{35}, the authors used CART analysis and calculated two
thresholds for each semen parameter which allowed designation into three groups, namely
normal (fertile), borderline and abnormal (subfertile). The normal (fertile) group had values greater
than 12% for morphology, greater than 63% for motility and higher than 48 \times 10^6/ml for sperm
concentration. The abnormal (subfertile) group had values lower than 9% for morphology, lower
than 32% for motility and lower than 13.5 \times 10^6/ml for sperm concentration.

In these four articles, the predictive power of the different parameters was calculated as the
AUC, using the ROC curve. The AUC for sperm morphology ranged from 66 to 78.2%, confirm-
ing the high predictive power of this parameter. In fact, it had the best performance among the
different semen parameters in two articles\textsuperscript{32,35}. The thresholds calculated in these two articles
were 10% and 9%, respectively, while G"unalp \textit{et al.}\textsuperscript{33} calculated a threshold of 12% using sensi-
tivity and specificity to analyze their data, and the fourth study calculated a 4% predictive cut-off
value. Although sensitivity and specificity for the values are relatively high, the positive predictive
values are not. This will therefore result in classifying fertile males as subfertile, probably leading
to a degree of anxiety as well as unnecessary and costly infertility treatment. A second and much
lower threshold was calculated in three of the four articles. Ombelet \textit{et al.}\textsuperscript{32} calculated this much
lower threshold by using the 10th centile of the fertile population, while G"unalp \textit{et al.}\textsuperscript{33}
screened the population with the positive predictive value as indicator, and Menkveld \textit{et al.}\textsuperscript{34}
asumed a 50% prevalence of subfertility in their study population. The lower threshold ranged from 3 to 5%
(Table 18.2). These lower thresholds have a much higher positive predictive value than the higher
thresholds, with a negative predictive value not much lower.

We suggest that the lower threshold should be used to identify males with the lowest potential for
a pregnancy under \textit{in vivo} conditions. Values above the lower threshold should be regarded as
normal. These findings are in keeping with previous publications by Coetzee \textit{et al.}\textsuperscript{12} (IVF data) and
Van Waart \textit{et al.}\textsuperscript{13} (IUI data), which reported a significa-
cantly lower chance of successful pregnancy in males with normal morphology below their calcu-
lated thresholds.

The higher threshold values for percentage motile sperm as calculated in the four articles
(using ROC curve or CART analysis) ranged from 32 to 52%, while the lower threshold values
ranged from 20 to 30%. Motility also had a high predictive power, with an AUC of between 59 and
79.1%. G"unalp \textit{et al.}\textsuperscript{33} calculated thresholds for progressive motility: a higher threshold of 42%,
using the ROC curve, and a lower threshold of 14%, with the positive predictive value as
indicator. In this study, progressive motility
proved to be a marginally better predictor of subfertility than sperm morphology, with AUC values of 70.7 and 69.7%, respectively. Montanaro Gauci et al. found percentage motility to be a significant predictor of IUI outcome. The pregnancy rate was almost three times higher in the group with motility > 50% as compared with the group with motility < 50%.

The higher threshold values for sperm concentrations calculated by Omelet et al. and Guzik et al. ranged from 13.5 to 34,000 sperm/ml, while the lower threshold values ranged from 9.0 to 14.5,000 sperm/ml. An AUC value of between 55.5 and 69.4% served as confirmation of the predictive power of this parameter. Although Menkveld et al. did not calculate a threshold value for sperm concentration (because values of less than 20,000 sperm/ml served as inclusion criteria in their study), they suggested a threshold value of 20,000 sperm/ml to be used with confidence, because it did not influence the results from their fertile population. The clinical value of motility and sperm concentration serves as confirmation of findings reported in numerous other publications.

Although the various parameters had good predictive power, independent of each other, the clinical value of semen analysis was increased when the parameters were used in combination. Omelet et al. found that differences between the fertile and subfertile populations only became significant when two or all three semen parameters were combined. Bartoo et al. concluded that fertility potential is dependent on a combination of different semen characteristics. Eggert-Kruse et al. found a significant correlation between the three parameters reviewed in their study. Although the different semen parameters demonstrate good individual predictive power, the clinical value of the semen analysis increases when the parameters are used in combination. We therefore suggest that no parameter should be used in isolation when assessing male fertility potential. The lower thresholds as discussed in this chapter have a much higher positive predictive value and a high negative predictive value. Therefore, we suggest that these lower thresholds should be used in identifying the subfertile male.

As suggested by the WHO in 1999, each group should develop their own thresholds, based on the population they are working in. It seems as if the sperm morphology threshold of 0–4% normal forms indicates a higher risk group for subfertility, and fits the IVF and IUI data calculated previously. The four articles discussed above showed the same trends, and can serve as guidelines to distinguish fertile from subfertile males.

As far as concentration and motility are concerned, the thresholds are not clear, but a concentration lower than 10⁶ sperm/ml and a motility lower than 30% seem to fit the general data. However, more, preferably multicenter, studies are needed to set definitive thresholds.

**SEmen Profile of the General Population: Partners of Women with Chronic Anovulation**

In general, there is quite a poor level of understanding and evidence regarding the semen analysis profile of the general population. Many male populations have been proposed to mirror the general population in terms of semen analysis. Using donors in a semen-donation program for normality is certainly not the best option, since this population is positively biased for fertility. Army recruits are biased by age. Husbands of tubal-factor patients can be biased by a positive history of infection (tubal factor due to pelvic infection) or a good fertility history (women with tubal sterilization). Therefore, we believe that possibly the best reference group for studying the semen profile in a general population includes partners of women who have been diagnosed with chronic anovulation/PCOS (polycystic ovarian syndrome) (maximum of three menstrual periods per year). We would thus like to propose employing the lower thresholds to indicate patients with subfertility, and, by using the cohort of
anovulatory women, we obtain a reflection of the semen profile in a general population.

Two different studies, one retrospective and one prospective, evaluating the semen analysis of partners of women presenting with anovulation were selected.

**Retrospective study of partners of women presenting with chronic anovulation (≥ 35 days) at Tygerberg Fertility Clinic**

Included in this study were all male partners of patients diagnosed as anovulatory at the Tygerberg Fertility Clinic. Methods used to examine the semen were according to WHO guidelines, and for sperm morphology Tygerberg strict criteria were used. The laboratory personnel initially evaluated all slides, and each slide was then evaluated by one observer (TFK) according to strict criteria. Sixty-two samples were eventually selected and included in the study (Table 18.3).

**Prospective study of partners of women presenting with PCOS at Tygerberg Fertility Clinic**

Tygerberg Fertility Clinic conducted a study in patients with PCOS. The patients were diagnosed with PCOS according to the recent Rotterdam consensus statement. The aim of this study was to establish factors influencing ovulation induction in this group.

The semen of the partners of all these women was examined. Methods used to examine the semen were according to WHO guidelines, and for sperm morphology Tygerberg strict criteria were used. The laboratory personnel initially evaluated all slides, and all P-pattern morphology slides were re-evaluated by one observer (TFK) (Table 18.4). The thresholds used for subfertility were those suggested by Van der Merwe et al. in their recent review: 0–4% normal forms, < 30% motility, < 10⁶/ml, outlined in the first section of this chapter.

<table>
<thead>
<tr>
<th>Table 18.3 Retrospective study of partners of women presenting with chronic anovulation (≥ 35 days) at Tygerberg Fertility Clinic (&lt; 10⁶/ml cut-off)</th>
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<td><strong>Patients</strong></td>
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<tr>
<td>Normozoospermia</td>
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<td>Sperm abnormality</td>
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<td>Single-parameter defect</td>
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<td>azoospermia</td>
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<td>oligozoospermia (O)</td>
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<tr>
<td>Triple-parameter defect</td>
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<td>OAT</td>
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Threshold values used: concentration < 10⁶/ml, motility < 30%, morphology < 4% normal forms

**DISCUSSION**

In the two studies (Table 18.3, retrospective; Table 18.4, prospective) ± 50% of patients had a normal semen analysis. The most common single abnormality was that of teratozoospermia (25.8% retrospective, 27.8% prospective). Azoospermia occurred in 1.4–4.8% of patients, with triple-parameter defects found in only 1.4–3.2% of cases (Tables 18.3 and 18.4).

The thresholds as calculated above were used in a group of anovulatory women. These thresholds reflect the prevalence of male factor infertility in the general population. It is interesting to note that in both the retrospective and prospective studies, the prevalence of teratozoospermia (< 4%
should be performed and the necessary treatment offered. In the case of PCOS, the female factor (anovulation) should obviously be corrected, starting, as first-line approach, with weight loss in women with a body mass index > 25. Although 50% of these patients had a male factor according to the definition used, it is also important to note that only ± 5% of these factors were serious (azoospermia and the triple-parameter defects), with 7–9.7% with a double defect.

To our knowledge, this is the first attempt to use the specific suggested lower thresholds to define prevalence of the subfertile male in the general population by using an anovulatory group of women. These thresholds will guide the clinician towards a more directive management where indicated.

REFERENCES

Clinical management of male infertility

Murat Arslan, Sergio Oehninger, Thinus F Kruger

INTRODUCTION

It is estimated that male subfertility is present in up to 40–50% of infertile couples, alone or in combination with female factors. There has been extensive progress in the diagnosis and treatment of male factor infertility since the inception of assisted reproductive technologies (ART). Moreover, the advent of intracytoplasmic sperm injection (ICSI) has resulted in a dramatically increased likelihood of pregnancy in couples suffering from most causes of male infertility. Fundamental advances have been made in the genetics of male disorders. Nevertheless, and at the same time, we are now witnessing a steady state in the development of assays that can be predictive of sperm functional capacities, both under in vivo and in vitro conditions.

Therefore, it is evident now, as it was a few years ago, that more research is needed to establish the causes and pathogenic mechanisms involved in male disorders leading to abnormal sperm function. The correct approach for male infertility evaluation should include a rational program composed of careful evaluation of the patient’s history, a complete physical examination, laboratory tests of basic/extended semen analysis and a urological, endocrinological and genetic work-up, as appropriate.

A comprehensive semen analysis following the World Health Organization (WHO) guidelines is fundamental at the primary-care level to make a rational initial diagnosis and to select the appropriate clinical management. Collection and analysis of the semen must be undertaken by properly standardized procedures in appropriately qualified and accredited laboratories. The ‘basic’ semen evaluation should include: (1) assessment of physical semen characteristics (volume, liquefaction, appearance, consistency, pH and agglutination); (2) evaluation of sperm concentration, grading of motility and analysis of morphological characteristics (using strict criteria); (3) determination of sperm vitality (viability), testing for sperm autoantibodies (using the mixed antigen test and/or the direct immunobead test), presence of leukospermia and immature sperm cells; and (4) bacteriological studies. The identification and separation of the motile sperm fraction is also an integral part of the initial semen evaluation.

Clinicians and scientists are still searching for semen parameter thresholds in the so-called ‘normal fertile populations’ in order to be able to define fertility, subfertility and infertility more accurately. Recent publications have appropriately readdressed these issues as part of both European and American studies. In a recent publication, van der Merwe et al., reassessed
fertility/subfertility thresholds for normal basic sperm parameters by a thorough, structured review of the current literature. Results demonstrated new and lower threshold levels for fertility/subfertility. These cut-off values included a sperm concentration <15 million/ml, progressive motility <30% and <5% normal morphology. These thresholds also fit data from the in vitro fertilization (IVF)\textsuperscript{12} and intrauterine insemination (IUI)\textsuperscript{13} settings.

There are multiple structural and biochemical sperm alterations that are present in subfertile men. Anatomically, they can be divided into: membrane alterations (that can be assessed by tests of resistance to osmotic changes, translocation of phosphatidylserine and others), nuclear alterations (abnormal chromatin condensation, retention of histones and presence of DNA fragmenta
tion), cytoplasmic lesions (excessive generation of reactive oxygen species, loss of mitochondrial membrane potential and retention of cytoplasm— with excessive creatine kinase content or the presence of active caspases) and flagellar disturbances (disturbances of the microtubules and fibrous sheath). Some of these alterations are indicative of immaturity, the presence of an apoptosis phenotype, infection-necrosis or other unknown causes\textsuperscript{14-24}.

Attention has shifted to the examination of sperm nuclear abnormalities. Currently, various tests are available for the detection of chromatin/DNA defects, including aniline blue staining\textsuperscript{25}, acridine orange\textsuperscript{26}, the sperm chromatin structure assay (SCSA)\textsuperscript{27}, the assessment of DNA fragmentation\textsuperscript{16,28,29} and fluorescence in situ hybridization (FISH) for aneuploidy\textsuperscript{30}.

Notwithstanding their occurrence and correlation with clinical outcomes, it is not clear how these abnormalities directly influence sperm function, particularly gamete transportation, fertilization and contribution to embryogenesis. Furthermore, most such assays are still experimental, and more research is needed to validate their results in the clinical setting and to determine their true capacity to predict male fertility potential.

On the other hand, there are other specific and critical sperm functional capacities that can be more reliably examined in vitro. These functions include: motility, competence to achieve capacitation, zona pellucida binding and the acrosome reaction. The assessment of these features is what is typically considered as sperm functional testing.

The extended semen analysis should include the preferential examination of these essential sperm functional attributes. These assays have been categorized into: (1) tests that examine defective sperm function indirectly through the use of biochemical means (i.e. measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others); (2) bioassays of gamete interaction (i.e. the heterologous zona-free hamster-oocyte test and homologous sperm–zona pellucida binding assays) and induced acrosome-reaction scoring; and (3) computer-aided sperm motion analysis (CASA) for the evaluation of sperm motion characteristics\textsuperscript{3,31-41}.

We reported an objective, outcome-based examination of the validity of the currently available assays based upon the results obtained from 2906 subjects evaluated in 34 published and prospectively designed, controlled studies. The aim was carried out through a meta-analytical approach that examined the predictive value of four categories of sperm functional assays (computer-aided sperm motion analysis or CASA, induced acrosome-reaction testing, sperm penetration assay or SPA and sperm–zona pellucida binding assays) for IVF outcome\textsuperscript{42}.

Results of this meta-analysis demonstrated a high predictive power of the sperm–zona pellucida binding and induced acrosome-reaction assays for fertilization outcome under in vitro conditions\textsuperscript{42}. On the other hand, the findings indicated a poor clinical value of the SPA as predictor of fertilization, and a real need for standardization and further investigation of the potential clinical utility of CASA systems. Although this study provided objective evidence based on which clinical
management and future research may be directed, the analysis also pointed out limitations of the current tests and a need for the standardization of present methodologies and the development of novel technologies.

Typically, male infertility presents clinically as an abnormal basic or extended semen analysis. Abnormalities in sperm indices may occur as an isolated parameter or as a combination of various parameters. Oligozoospermia and teratozoospermia are the most frequently observed isolated defects in our clinical practices, but more frequently, various degrees of oligoasthenoteratozoospermia (OAT) are present. Here, it is our aim to examine the causes and clinical management of the various single and multiple sperm defects.

ISOLATED SPERM ABNORMALITIES

Decreased sperm concentration (azo-/oligozoospermia)

Pathologies classified as ‘decreased sperm concentration’ range from mild oligospermia (<15 million sperm/ml) to azoospermia (no sperm in the ejaculate). On a simplistic basis, the clinically known causative entities can be subdivided into those of pretesticular, testicular and post-testicular origin.

A variety of endocrinopathies that disrupt the hypothalamic–pituitary–testicular axis constitute pretesticular etiologies of oligozoospermia. These endocrinopathies might be congenital (Kallmann’s syndrome) or acquired (prolactinoma, other hypothalamic–pituitary tumors and pathologies), and require the measurement of serum prolactin levels together with follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone for differential diagnosis in a patient with decreased sperm concentration. Further evaluation with assessment of other pituitary hormones (thyroid stimulating hormone (TSH), growth hormone, cortisol) and intracranial imaging systems (computed tomography (CT), magnetic resonance imaging (MRI)) is crucial in cases of hypogonadotropic hypogonadism.

Six to 24 months of treatment in patients with idiopathic hypogonadotropic hypogonadism, either with gonadotropins or pulsatile gonadotropin-releasing hormone (GnRH), frequently results in sperm indices sufficient for fertility in these patients. Patients with a diagnosis of prolactinoma respond rapidly to antidiopaminergic agents. Because of their impressive therapeutic effects in patients with prolactinoma, these agents have also been tried in idiopathic oligoasthenozoospermia to improve sperm parameters. However, it has recently been shown in a meta-analysis that although they decrease serum prolactin levels further within the normal range, they are not helpful in improving sperm indices or fertility.

Post-testicular etiologies resulting in reduced or absent sperm output include a variety of obstructive lesions of the genital tract (inflammatory, infectious, congenital or iatrogenic, such as vasectomy) and ejaculatory disorders, particularly retrograde ejaculation. Retrograde ejaculation should be suspected in any case of azoospermia with low seminal volume, and might be congenital, acquired (prostatic and bladder-neck surgery, diabetes mellitus, inguinal lymph node excision) or idiopathic in origin.

Testicular causes include hypospermatogenesis due to a reduction in the number of germ cells, incomplete/complete maturation arrest of germinal cell differentiation and/or germinal cell aplasia. These entities are characterized by disturbances of spermatogenesis and/or an aberrant apoptotic process occurring during mitosis, meiosis and/or spermiogenesis/spermiation. Some of these pathologies are end results or the sequelae of viral infections, iatrogenic agents (chemo- and radiotherapy) and varicoceles, as well as disturbances secondary to genetic/chromosomal/environmental aberrations. Nonetheless, it is our experience that in almost all such cases oligozoospermia is associated with moderate to severe
degrees of astheno- and teratozoospermia (see below).

**Decreased sperm motility (asthenozoospermia)**

Asthenozoospermia is defined as the presence of progressive motility <30%\(^{11}\). Its origin can be iatrogenic, structural, functional, genetic or environmental. Possible causes of isolated asthenozoospermia include: iatrogenic reasons (improper handling of the semen sample), anti-sperm antibodies, infections, partial axonemal defects, sperm-tail fibrous sheath defects and poor development of the outer dense fibers, the presence of fewer mitochondria in the midpiece or even aplasia, sperm centriole dysfunction, carboxymethyl transferase enzyme deficiency and epididymal pathologies (typically associated with inflammation-infection)\(^{36-62}\).

The autosomal recessive-inherited immotile cilia syndrome\(^{63}\) and sperm mitochondrial DNA mutations\(^{64-67}\) have been identified as two gene-related causes of isolated sperm motility disorders. Recently, Baccetti et al.\(^{68}\) reported a patient with severe isolated asthenozoospermia characterized by an absence of the fibrous sheath in the principal-piece region of the tail in the whole sperm population, which strongly suggests a genetic origin.

In patients with documented asthenozoospermia, the diagnosis work-up should emphasize repeated semen analyses in order to exclude inappropriate handling of the specimen as the cause. Repeated semen and urine cultures together with immunological tests should also be performed. Structural analysis of the sperm tail (flagellum) under transmission electron microscopy is the method of choice for diagnosis of immotile cilia syndrome in suspected patients with isolated severe asthenozoospermia.

It is worth mentioning that for isolated asthenozoospermia, many different sperm preparation techniques, with or without *in vitro* motility enhancers, have been tried. These agents have included pentoxifylline, 2-deoxyadenosine, kallikrein, platelet-activating factor and some antioxidants\(^{69,70}\). Although different levels of improvement have been reported with these agents, none of them has truly gained acceptance for routine use in clinical practice.

**Decreased normal morphology (teratozoospermia)**

The importance of sperm morphology in male factor infertility has been demonstrated in multiple reports\(^{5,12,71-76}\) even though there is no complete uniformity in the definition of normal sperm morphology and teratozoospermia\(^{3,71,77,78}\). After the introduction and validation of strict criteria by Kruger et al.\(^{2}\), sperm morphology gained acceptance as the most important sperm parameter in the prediction of IVF outcome\(^{72-79}\). Later on, many studies demonstrated good correlation between sperm morphology and sperm functional tests such as zona pellucida binding assays\(^{34,80-83}\) and the zona-free hamster-oocyte penetration assay\(^{84,85}\). Poor morphology also correlates with abnormal sperm calcium influx\(^{86}\) and an abnormal acrosome reaction\(^{87}\). Its prognostic value has also been validated in IUI cycles\(^{3,88-90}\).

On the other hand, the pathophysiology of teratozoospermia is not completely understood. Numerical and structural chromosomal defects have been claimed in its pathogenesis. Investigations of spermatozoa from somatically normal men during meiosis using the FISH technique resulted in findings of a higher percentage of disomy, trisomy or tetrasomy for chromosome 1\(^{91}\), chromosome 7\(^{92}\), chromosome 8\(^{93}\), chromosome 13\(^{94,95}\), chromosome 18\(^{92,93,96}\), chromosome 21\(^{94}\) and the sex chromosomes\(^{91-93,95,96}\). Importantly, these abnormalities occurred mostly in populations with combined defects of sperm parameters (OAT) and infertility. The authors of these studies proposed that the effects of factors that impair sperm indices during gametogenesis extend to the cytogenetic constitution of spermatozoa. Conversely, some other studies could not find any
correlation between sperm chromosomal abnormality and fertility97-99.

Harkonen et al.92 focused on isolated teratozoospermia and demonstrated higher frequencies of disomies 7, 18, YY and XY and diploidy in patients having <10% normal morphology. Calogero et al.93 found higher incidences of disomies 8, 18, X and Y in patients with isolated teratozoospermia and OAT, compared with men with normozoospermia. These authors suggested that teratozoospermia might be the critical sperm parameter associated with aneuploidy. The same group also showed an increase in sperm aneuploidy rate in patients with OAT, particularly in the presence of an elevated percentage of spermatozoa with enlarged heads100.

On the other hand, Gole et al.101 found a higher incidence of sex chromosomal disomy in patients with OAT compared with teratozoospermic patients. Recently, Burrello et al.102 reported a higher aneuploidy rate for spermatozoa with abnormal head shapes from OAT patients, compared with normally shaped spermatozoa from normal men. Their results showed that normal morphology in patients with OAT does not rule out the presence of aneuploidy in selected sperm for ICSI. These results weaken the possibility of a direct causal relationship between isolated teratozoospermia and sperm chromosomal abnormalities. However, there is consensus in the literature that infertile men and/or men with poor sperm indices carry a higher frequency of aneuploidy in their spermatozoa. More studies are needed to identify the effects of different chromosomal aberrations on different sperm parameters/functions.

There is also substantial evidence in the literature supporting that deregulation of specific genes might play a role in the appearance of morphological abnormalities in ejaculated spermatozoa. It has been shown in a mouse model that aeh mutations (abnormal spermatozoon head shape) on chromosome 4 might cause specific structural changes in the sperm head103,104. Adham et al.105 showed the development of sperm head abnormalities in mice containing Tnp2 (transition protein

2) gene disruption, which takes part in the nuclear organization of spermatozoa. Xu et al.106 also demonstrated that male mice lacking a regulatory protein in the process of spermatogenesis (protein casein kinase 2 α, Csnk2a) due to Csnk2a gene disruption performed by transgenesis were infertile, with globozoospermia (acrosomeless sperm). In addition, the altered expression and arrangement of some cytoskeletal proteins (calcin, protein 4.1) has been associated with aberrant morphological changes during spermiogenesis107,108. Recently, Milatiner et al.109 demonstrated a correlation between the severity of teratozoospermia in infertile men and changes in the nucleotide structure of the androgen receptor gene.

COMBINED SPERM ABNORMALITIES: OLIGOASTHENOTERATOZOOSPERMIA

As mentioned above, OAT is the most common clinical presentation of male infertility. It is typically the reflection of abnormal (testicular) spermatogenesis but it can also be due to post-testicular etiologies. Approximately half of clinical cases, however, still remain idiopathic.

There are numerous known spermatogenesis defects leading to OAT20,54,110-114. They include: germ cell anomalies (depletion, aberrant apoptosis, defective differentiation), mitotic and meiotic defects and alterations of spermiogenesis/spermiation. Aberrant apoptosis has been observed at the primary spermatocyte and spermatid levels115,116 and also in Sertoli cells117. Arrest or quantitatively abnormal spermatogenesis at any stage may result in oligozoospermia. Meiotic alterations and spermiogenesis defects are probably associated with teratozoospermia.

The concept of sperm immaturity has gained acceptance. Retention of cytoplasm (including retention of organelles and enzymes participating in metabolism, apoptosis and other functions that become exaggerated) is probably the result of an abnormal Sertoli cell–late spermatid interaction, leading to the release of dysmorphic, dyskinetic
and dysfunctional spermatozoa. Abnormalities of sperm release from the seminiferous tubules (or spermiaciem) are also probably present in some cases. Epididymal dysfunctions or pathologies can also influence sperm membrane domain constitution and may induce morphogenic/dysfunctional changes.

**CLINICAL MANAGEMENT**

The treatment plan should be constructed based upon complete identification of both male and female factors (Figure 21.2). In the presence of pure male infertility (no identifiable female factors), therapy may be: (1) medical (endocrine such as in hypogonadism or hyperprolactinemia, antibiotics in case of infections); (2) urological (surgical or non-surgical treatments, such as conventional, microsurgical or laparoscopic surgery, including correction of varicocele, epididymo-vasovasostomy and modern approaches for ejaculatory disorders); and/or (3) low- or high-complexity assisted reproductive technologies (ART). The severity of male subfertility and some important prognostic risk factors in the female (e.g. age, duration of infertility, presence of endometriosis and other pathologies) may accelerate the indication for ART.

It is our opinion that, at the present time, there is no clinical role for the empirical use of medical treatments of normogonadotropic subfertile men with idiopathic OAT. In the absence of a defined medical indication, there are no evidence-based data to support the use of gonadotropins, anti-estrogens, antioxidants, multivitamins or other unproven therapies.

Currently recommended ART options include: 'low-complexity' IUI therapy, 'standard' IVF and embryo transfer, and IVF augmented with ICSI. If the female partner is aged <35 years, typically 4–6 cycles of IUI using the husband's sperm in combination with controlled ovarian hyperstimulation are recommended as a simple (low-complexity) ART approach, particularly if >1 million motile sperm can be recovered.

Preliminary data suggest that in order to increase cost-efficiency and loss of valuable time, IUI should not be performed if the total motile recoverable fraction is low, if the hemizona index (HZI) is <31%122, if the calcium ionophore-induced acrosome reaction is ≤22%123, if the zona pellucida-induced acrosome reaction is <16%123 and/or if the proportion of sperm depicting DNA fragmentation is >12%.124

Patients with a motile sperm fraction of <5 million motile spermatozoa following swim-up or gradient centrifugation, but with mild to moderate teratozoospermia (in the range 4–14% normal forms by strict criteria), may be offered 'standard' IVF therapy. In those cases, good fertilization and pregnancy rates are achieved with an increase in the sperm insemination concentration.125 However, nowadays, these patients are offered ICSI in an effort to eliminate any risk of low or failed fertilization, or a combination of IVF and ICSI (in sibling oocytes) in the group with sperm morphology > 14% normal forms, dependent on the individual IVF unit.
In our programs, patients are selected for ICSI according to the following indications:\textsuperscript{7,127}

- Poor sperm parameters (i.e., $<5 \times 10^6$ total spermatozoa with adequate progressive motility after separation and/or severe teratozoospermia, with $<4\%$ normal forms in the presence of a borderline to low total motile fraction);
- Poor functional abilities, including a defective sperm–zona pellucida binding capacity with a hemizona assay index $<30\%$\textsuperscript{82,128} and/or a low ($<16\%$) zona pellucida-induced acrosome reaction or ZIAR\textsuperscript{87,129,130};
- Previous failed fertilization in IVF;
- Failure of IUI therapy in cases presenting with moderately abnormal sperm parameters ($5-10 \times 10^6$ total spermatozoa with adequate progressive motility after separation or morphology in the range of 5–14%), and also

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**Figure 21.2** Algorithm for clinical management of the subfertile man. COH, controlled ovarian hyperstimulation; IUI, intrauterine insemination; ART, assisted reproductive technologies; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; TESE, testicular sperm extraction; MESA, microsurgical epididymal sperm aspiration.
including the presence of antisperm antibodies;

- Presence of obstructive or non-obstructive azoospermia, where ICSI is combined with sperm extraction from the testes or the epididymis;\(^{127,131-134}\),

- In the presence of severe oligoasthenoteratozoospermia or if the outcome of sperm function testing indicates a significant impairment of fertilizing capacity, couples should be immediately directed to ICSI. This approach is probably more cost-effective and will avoid loss of valuable time, particularly in women >35 years.

Based on currently available data, we estimate that ICSI should be indicated when male infertility is properly diagnosed based upon a state-of-the-art extended evaluation of the male partner, and also in cases with previous failed fertilization. Published prospective, randomized studies have demonstrated that it is not beneficial to perform ICSI in non-male infertility or unexplained infertility cases. Altogether, there are no data to suggest that ICSI should be performed in all cases of in vitro conception (reviewed in references 135 and 136). Consequently, to perform ICSI in all cases on a purely pragmatic basis appears to be a significant departure from principles of evidence-based medicine.

Greco \textit{et al}.\(^ {137}\) recently reported that ICSI with testicular spermatozoa provides the first-line ART option for men with high levels of DNA damage in ejaculated sperm. Nonetheless, more studies are needed clinically to validate methods of assessing DNA damage and the impact of DNA abnormalities on clinical outcomes.

Sperm cryopreservation represents a valuable therapeutic option in the management of male infertility. Current indications include: (1) mandatory use in artificial insemination programs with donor semen; (2) patient's convenience (i.e., partner's absence where IUI is performed in the presence of normal sperm parameters); (3) preservation of reproductive capacity in men with various types of neoplasias before undergoing radical surgery and/or radio-chemotherapy\(^ {138}\); (4) aiding in the management of infertile men undergoing vasectomy reversal (vasovasostomy) or epididymovasostomy, when 'banking' may provide a future sperm source for possible use in IUI or ICSI therapies; and (5) because of the outstanding success with ICSI, even infertile men with different degrees of oligoasthenoteratozoospermia can now be offered the use of cryopreserved-thawed spermatozoa for assisted fertilization. Today, this applies not only to ejaculated but also to testicular and epididymal spermatozoa recovered for the purpose of ICSI\(^ {139,140}\).

Interesting and challenging concepts to be applied to future treatment modalities of male infertility are germ cell transplantation and \textit{in vitro} spermatogenesis\(^ {141,143}\). Further progress in the identification of spermatogonial stem cells and techniques of germ cell transplantation\(^ {144}\), in addition to the optimization of culture systems for \textit{in vitro} spermatogenesis\(^ {145}\), may give new options to patients with azoospermia.

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c. The impact of training on the scientists’ knowledge and understanding of sperm morphology.

i. Scientists’ knowledge and understanding of sperm morphology

The impact of the sperm morphology findings was most obvious in the andrology laboratories worldwide. Scientists were trained using the following methods:

- Practical courses in semenology with emphasis on sperm morphology training.
- The use of sperm morphology atlases to illustrate sperm morphology classification practically (Menkveld et al., 1991 and Kruger et al., 2004).
- The acceptance of the new classification by WHO also led to illustrations in the WHO manual in 1999 helping with the training and acceptance of the concepts of Strict Criteria (WHO 1999).
- It was further emphasized by the acceptance by the WHO in 2010. The classification of sperm morphology was performed by the author and co-workers in the 2010 WHO semen manual (WHO 2010).

This fact led to the development of courses that was given by the author (TFK) as well as by Prof R Menkveld and Prof DR Franken at different places worldwide. A number of courses in Boston, USA and in Bern Switzerland followed that were the responsibility of the author (TFK). The courses in Switzerland ran over a 10 year period. The last course was as recently as August 2010 in Bern. Careful data of the findings during the courses were stored and this resulted into a number of articles by Prof DR Franken (Franken et al., 2003; Franken et al., 2006; Franken et al., 2010) as first author which made an excellent scientific contribution in the field of education. It was shown that sperm morphology training is worthwhile and that continuous quality control measures following a course and will bring accurate reading of sperm morphology. This will lead to reliable reports by the laboratories involved (Franken et al., 2003; Franken et al., 2006; Franken et al., 2010).
The sperm morphology atlases (Menkveld et al., 1991; Kruger et al., 2004) were thus initiated to help in the training of scientists' and technologists worldwide as it was impossible to reach all laboratories interested. In the first atlas by Menkveld (Menkveld et al., 1991) a chapter dealing with the clinical impact explaining the concept of fertile and sub-fertile to scientists will be reflected on, in this section. In the 2004 atlas by Kruger and Franken there were clinical chapters but also contributions on the interpretation of normal and abnormal forms with the aim to use the atlas as a laboratory training manual on a day to day basis (Kruger et al., 2004).
Applicable chapters in the following text book will be reflected on:

Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA, Oehninger S. Atlas of Human
Atlas of Human Sperm Morphology

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CHAPTER 2

Clinical Application

T.F. Kruger, R. Menkveld, E.E. Oettlé, R.J. Swanson, A.A. Acosta, S. Oehninger

2.1 INTRODUCTION

Today in vitro fertilization is playing an important role in the management of infertile couples as well as substantiating old theories and developing new ones in all areas of human reproduction. Of great interest to the authors has been those patients with pure teratozoospermia (normal spermatozoa concentration and motility but below normal sperm morphology). As a prognostic tool, the value of evaluating pure teratozoospermia has been shown in an analysis of our IVF results (1). A clear threshold occurred when the morphology reached a value equal to or less than 14% normal forms with the fertilization rate per oocyte dropping to only 37% without the occurrence of a pregnancy. When the value of morphologically normal spermatozoa was greater than 14% the fertilization rate was 82.5% at a concentration of 50,000 sperm per milliliter per oocyte at insemination.

2.2 PREDICTIVE ROLE OF SPERM MORPHOLOGY

2.2.1 CLINICAL DATA

In a prospective study in Tygerberg, performed on 129 patients over 190 cycles (1), patients were divided into four groups based on the percentage of normal morphology. All these male patients had a sperm concentration of more than 20 million/ml with the normal motility greater than 30%.

The patients were divided into four groups based on the percentage of normal morphologic features. Group I, normal morphology, 0 to 14%; group II, 15% to 30%; group III, 31 to 45%; group IV, 46% to 60%. In group I, 104 oocytes were obtained from which 37% fertilized. In group II, 324 oocytes were retrieved with a fertilization rate of 81%; in group III, 309 oocytes were retrieved with a fertilization rate of 82%; in group IV, 69 oocytes were retrieved with a fertilization rate of 91% (Table 2.1). A clear fertilization threshold was observed in the sperm samples having
Table 2.1.
Fertilization Rate per Oocyte

<table>
<thead>
<tr>
<th>% Normal Forms</th>
<th>Group I (0-14%)</th>
<th>Group II (15-30%)</th>
<th>Group III (31-45%)</th>
<th>Group IV (46-60%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>104</td>
<td>324</td>
<td>309</td>
<td>69</td>
<td>806</td>
</tr>
<tr>
<td>%</td>
<td>13</td>
<td>40</td>
<td>38</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Oocytes fertilized</td>
<td>38</td>
<td>264</td>
<td>252</td>
<td>63</td>
<td>617</td>
</tr>
<tr>
<td>Fertilization rate/oocyte (%)</td>
<td>37</td>
<td>81</td>
<td>82</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>


Table 2.2.
Observed Proportion of Pregnancies Below and Above the Threshold of Percentage of Normal Sperm Morphologic Features

<table>
<thead>
<tr>
<th>% Normal Sperm Morphologic Features</th>
<th>Observed Proportion of Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>&lt;14%</td>
<td>1-13</td>
</tr>
<tr>
<td>&gt;14%</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td>32/124</td>
</tr>
</tbody>
</table>


A percent of normal morphology of 14% or lower. No pregnancy was achieved in group I, regardless of the number of oocytes retrieved. In groups II, III and IV combined, if three or more oocytes were retrieved, a pregnancy rate per embryo transferred of 25.8% was obtained (Table 2.2). In those cases where only one or two oocytes were retrieved, a pregnancy rate per embryo transferred of 11.4% was obtained. In a similar study at the Jones Institute (2), this threshold was used with a fertilization rate of

Table 2.3.
Norfolk Program: Sperm Morphology as a Predictor of Human IVF

<table>
<thead>
<tr>
<th>Fertilization Rate</th>
<th>Group I (26 patients)</th>
<th>Group II (71 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Normal Forms &lt;14%)</td>
<td>(Normal Forms &gt;14%)</td>
</tr>
<tr>
<td>Per oocyte (%)</td>
<td>49.4</td>
<td>88.3</td>
</tr>
<tr>
<td>MI, MII (preovulatory)</td>
<td>0 = 28%</td>
<td>0 = 1.4%</td>
</tr>
<tr>
<td></td>
<td>&lt; 50 = 36%</td>
<td>&lt; 50 = 11.2%</td>
</tr>
<tr>
<td></td>
<td>&gt; 50 = 36%</td>
<td>&gt; 50 = 87.4%</td>
</tr>
<tr>
<td>Cleavage Rate (%)</td>
<td>47.5</td>
<td>87.0</td>
</tr>
</tbody>
</table>


*96 patients, concentration >20 x 10^6/ml, motility >30%.
Table 2.4.
Probable Causes of Failed Fertilization in 52 patients Using Routine (Classic) and New Criteria to Evaluate Sperm Morphology*

<table>
<thead>
<tr>
<th></th>
<th>Old Criteria</th>
<th>New Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Cases</td>
<td>%</td>
</tr>
<tr>
<td>Oocyte anomalies</td>
<td>10</td>
<td>19.2</td>
</tr>
<tr>
<td>Sperm anomalies</td>
<td>17</td>
<td>32.6</td>
</tr>
<tr>
<td>Combination (oocyte + sperm)</td>
<td>4</td>
<td>7.7</td>
</tr>
<tr>
<td>Unexplained</td>
<td>21</td>
<td>40.4</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>100</td>
</tr>
</tbody>
</table>


49.4% in the group with normal morphology below 14% and a fertilization rate of 88.3% in the group with >14% normal morphology ($P < 0.0001$) (Table 2.3).

Selecting such low value as an accurate threshold is a consequence of defining stringent criteria for evaluating the morphology. With the advent of in vitro fertilization, the ability to judge human egg-sperm interaction is limited only by moral/ethical responsibilities. A more comprehensive understanding of the "normal morphology" is unfolding.

Oehninger et al. (3) reported a retrospective study on 52 couples in whom fertilization failed during IVF treatment. In the initial evaluation, 40.4% of fertilization failures could not be explained, and sperm abnormalities were present in 40.3% of cases with no correlation between the two events. After reassessing the sperm morphology according to the strict criteria, sperm abnormalities could be predictably responsible for fertilization failure in 74.9% of the cases, and in only 11.5% of the cases was fertilization failure truly unexplained (Table 2.4).

2.2.2 FUNCTIONAL TESTS

2.2.2.1 Hemizona Assay (HZA)

The human Hemizona Assay (HZA) was used by Burkman et al. (4) to assess tight sperm binding to the hemizona using fertile versus infertile semen, as judged by in vitro fertilization. Spermatozoal samples from fertile men showed significantly greater capacity for zona binding compared to spermatozoal samples from infertile men. The only other significant difference between the two groups was the lower percentage of normal morphology in the infertile spermatozoa according to our strict criteria (5) (Table 2.5).

In addition, Franken et al., utilizing the hemizona assay (HZA), showed that semen samples classified as morphologically poor (<4% normal forms) exhibited a significant impairment of sperm binding to the human zona pellucida (6). Moreover, Oehninger et al. reported that IVF cases with poor fertilization rates (male factor patients) had lower sperm binding ability in the HZA as compared with patients with successful fertilization (7). These results are discussed in greater detail elsewhere (8).
Table 2.5.
Number of Sperm Which Bound Tightly to Hemizonae During the HZA and Semen
Parameters for Three IVF Patient Groups Classified by the Percentage of Sperm with Strictly
Defined Morphology (Mean ± SE)a

<table>
<thead>
<tr>
<th>Morphology Group</th>
<th>HZA: No. Tightly Bound Sperma</th>
<th>Concentration (x 10⁶/ml)</th>
<th>Motility (%)</th>
<th>% Normal Morphologyb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N = 7f)</td>
<td>46.4 ± 11 (a)f</td>
<td>108.0 ± 14 (b)f</td>
<td>57.0 ± 5</td>
<td>18.2 ± 1 (c,d)f</td>
</tr>
<tr>
<td>Borderline (N = 14)f</td>
<td>31.7 ± 11</td>
<td>48.0 ± 9 (b)f</td>
<td>39.0 ± 7</td>
<td>8.1 ± 1 (d)f</td>
</tr>
<tr>
<td>Poor (N = 15)f</td>
<td>20.0 ± 7 (a)f</td>
<td>74.0 ± 15</td>
<td>37.3 ± 4</td>
<td>1.4 ± 0.3 (c,f)</td>
</tr>
</tbody>
</table>


b Based on the method of Kruger et al.

c Excellent morphology in >14%, of evaluated spermatozoa.

d Percentage of sperm with excellent morphology was >4%, but <14%.

e Excellent morphology in ≤4%, of sperm.

f Values followed by the same letters in parentheses (a–d) are significantly different (P < 0.05).

2.2.2.2 Hamster Ovum Penetration Assay (SPA)

The existence of a correlation between SPA and the conventional semen parameters has been examined by numerous authors, but no consensus has been achieved, mainly because of the varied experimental conditions and assessment criteria of various laboratories. Most early investigators found no correlation between SPA and any traditional semen parameters: sperm density, motility and morphology (9–12). Cohen et al. (13, 14) found low but significant correlations with the total motile sperm count and the percentage of morphologically normal sperm, with the former as the better predictor of the SPA. Rogers et al. (15) also reported a correlation with motility and morphology but found the latter to be more important.

In Norfolk, 70 patients with a sperm concentration > 20 x 10⁶/ml, a motile sperm fraction > 30%, and normal sperm morphology ranging from 1 to 39% were evaluated prospectively in the SPA (16). BWW was utilized as the incubation medium and 3% bovine serum albumin (BSA) as the protein source during a short incubation (6 hours) protocol (17). Results showed a statistically significant relationship between the percent of sperm with normal forms > 14% and penetration rate in the SPA (p = 0.001). Furthermore, the outcome of the SPA was correlated with in vitro fertilization, retrospectively, in 84 patients. Thirty-eight patients had an SPA < 10%, with no fertilization in vitro in 13 patients (33.3%), and successful fertilization in 25 (66.7%). Forty-five had an SPA > 10%, with fertilization in 37 patients (82.2%) and no fertilization in 8 (17.8%). Based on these results, it was concluded that the SPA seems to have a good predictive value for IVF when it is normal but is less reliable when results are < 10%. Even patients with a sperm penetration assay of 0% had 66.7% fertilization in the human system. Overall, using the new criteria for sperm morphology and our SPA standards, morphology seems to be a better predictor of IVF outcome and a more useful adjunct for counseling patients.
When using strict criteria, morphology is an excellent predictor of hamster penetration and human fertilization. Although the conventional semen parameters may not be linearly correlated with the sperm-fertilizing potential (1, 18), these factors may reflect the underlying physiological status of the sperm.

### 2.3 Morphologic Patterns in Normal Morphology Below 14%

Even with severe impairment in the fertilization rate in the below-14% normal sperm morphology group, some of these patients still fertilize human eggs. Patients with normal sperm morphology less than 14% were studied at the Jones Institute to establish morphologic patterns to try to identify subgroups with different fertilization ability.

Forty-five patients were allocated to this prospective study. To avoid the impact of count and motility on fertilization all male patients had to have a sperm concentration greater than or equal to 20 million per milliliter and a motile sperm fraction greater than or equal to 30% in the semen analysis. Patients were divided into two groups, those that fertilized no oocytes at all (group I) and those that fertilized at least one oocyte (group II). A significant difference was demonstrated ($P < 0.0001$) between the percentage of normal morphology in group I, 1.8% (SEM = 2.4), as compared to group II, 7.7% (SEM = 3.3). Slightly amorphous head abnormalities in group I (14.8%; SEM = 9.7) were significantly different ($P < 0.0001$) from those of group II (28.4%; SEM = 7.8).

The predictive value was better for normal morphology ($r^2 = 0.44$) than for slightly amorphous forms ($r^2 = 0.36$). No other sperm morphological abnormalities were significantly different between the two groups (19).

Based on the significant difference between normal morphology and the slightly amorphous forms in groups I and II, we propose two distinct patterns in the below-14% group. The poor prognosis, or P pattern, has a mean normal morphology below 4%. The good prognosis, or G pattern, has a normal morphology of 5 to 14%. At a sperm concentration of 50,000 sperm/ml/ovum, the G pattern predicts a significantly better chance for egg fertilization ($P < 0.0001$) than the P pattern. G pattern fertilization rate was 63.9% per oocyte while P pattern produced a 7.6% fertilization rate (Table 2.6).

<table>
<thead>
<tr>
<th>Table 2.6. Abnormal Morphology as a Predictor of Human IVF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal Morphology as a Predictor of Human IVF*</td>
</tr>
<tr>
<td>45 Patients (Concentration &gt; 20 × 10⁶/ml, motility &gt; 30%)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Group I (n = 13)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P Pattern</td>
</tr>
<tr>
<td>Mean no. of embryos (per patient)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Group II (n = 32)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P Pattern</td>
</tr>
<tr>
<td>Mean no. of embryos (per patient)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>7.6</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>63.9*</td>
</tr>
<tr>
<td>2.6*</td>
</tr>
</tbody>
</table>


* $P = 0.0001$. 

---

418
2.4 GAMETE MANIPULATION FOR IMPROVED FERTILIZATION

Once fertilization and cleavage takes place in the group of patients with less than 14% normal morphology the chance of a pregnancy is good. This may have higher validity for the G pattern patients. Increasing the spermatozoal concentration to 500,000 per ml/egg when in the presence of ova has improved the fertilization rate significantly. In a subsequent study, Oehninger et al. (20) showed that increasing the number of inseminating spermatozoa 2- to 10-fold in a group of poor morphology patients, the fertilization rate increased from 14.5 to 62.2%. Strikingly, however, the term pregnancy outcome of this group did not improve (Table 2.7).

Another factor of utmost importance in the handling of patients with teratozoospermia and perhaps all patients with a male factor diagnosis is the maturity of oocytes. To test this hypothesis patients were divided into two groups: those with a normal sperm morphology less than 5% normal forms—P pattern—and those with normal sperm morphology between 5 to 14%—G pattern. Oocytes were classified into metaphases I and II according to the principles laid down by Veeck et al. (21) 6 hours after retrieval and fertilized at that time with 100,000 sperm per milliliter.

The fertilization rate in the P pattern group for metaphase I oocytes was 8 of 36 oocytes (22.2%) and 17 of 29 oocytes (58.6%) for the metaphase II category ($P < 0.01$). In the G pattern group the fertilization rate in the metaphase I category was 54 of 90 oocytes (60%) and 10 of 66 oocytes (60.6%) in the metaphase II category. The conclusion drawn from these observations was that in the P pattern group selection of oocytes according to the criteria of Veeck et al. (21) can influence the

<table>
<thead>
<tr>
<th>Table 2.7.</th>
<th>Fertilization Rates and Pregnancy Outcome in the Different Groups and Subgroups$^{a,b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
</tr>
<tr>
<td></td>
<td>$n = 41$</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>94.3</td>
</tr>
<tr>
<td>Pregnancy rate/cycle</td>
<td>43.9</td>
</tr>
<tr>
<td>Pregnancy rate/transfer</td>
<td>43.9</td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td>29.7</td>
</tr>
<tr>
<td>Ongoing pregnancy rate/cycle</td>
<td>31.7</td>
</tr>
<tr>
<td>Ongoing pregnancy rate/transfer</td>
<td>31.7</td>
</tr>
</tbody>
</table>


$^{b}$ All results are expressed in %.

$^{c}$ $P < 0.0001$ compared with groups 1 and 2.

$^{d}$ $P < 0.005$.

$^{e}$ $P < 0.0003$ compared with group 1; $P < 0.01$ compared with group 2.

$^{i}$ $P < 0.003$ compared with groups 1 and 2.

$^{j}$ $P < 0.001$ compared with group 1; $P < 0.05$ compared with group 2.

$^{k}$ $P < 0.006$ compared with group 1; $P < 0.04$ compared with group 2.
prognosis of the patients regarding fertilization and thus also pregnancy outcome. In the G pattern group the fertilization rate did not differ significantly.

Even more important is the work of Van der Merwe et al. (22), in which they evaluated this concept in a prospective way in patients undergoing gamete intrallopian transfer treatment for their infertility problem because of teratozoospermia. In the 292 cycles evaluated, no significant difference in pregnancy outcome was observed when predominantly metaphase I (M-I) oocytes were used, compared with those patients receiving predominantly metaphase II (M-II) oocytes, although a trend was evident favoring M-II oocytes. This finding was true of (a) the P pattern—use of 3 to 4 M-I oocytes (total of 4 transferred in each trial) produced 1 ongoing pregnancy from 17 transfer cycles (5.9%) versus use of 3 to 4 M-II oocytes, which produced 4 ongoing pregnancies from 33 transfer cycles (12.1%); (b) the G pattern—use of 3 to 4 M-I oocytes produced 5 ongoing pregnancies from 39 transfer cycles (12.8%) versus use of 3 to 4 M-II oocytes, which produced 16 ongoing pregnancies from 103 transfer cycles (15.5%); and (c) the idiopathic group—use of 3 to 4 M-I oocytes produced 3 ongoing pregnancies from 31 transfer cycles (9.7%) versus use of 3 to 4 M-II oocytes produced 18 ongoing pregnancies from 69 transfer cycles (26.1%).

TO CONCLUDE

Based on the data discussed, normal sperm morphology is definitely a cornerstone in the evaluation of the infertile couple. When the strict criteria are applied as illustrated in the next sections, valuable information can be obtained regarding prediction of fertilization in vitro as well as pregnancy outcome. With this information, the scientist can manipulate the semen by increasing the concentration in vitro and, at least, improve the chances of fertilization and, in some cases, even pregnancy outcome.

REFERENCES


Applicable chapters in the following text book will be reflected on:

WHO LABORATORY MANUAL

for the examination of human semen and sperm–cervical mucus interaction

FOURTH EDITION

Published on behalf of the
WORLD HEALTH ORGANIZATION
by

CAMBRIDGE UNIVERSITY PRESS
The UNDP/UNFPA/WHO/World Bank Special Programme of Research, Development and Research Training in Human Reproduction wishes to acknowledge the participation, in the preparation and editing of the fourth edition of the WHO Laboratory Manual for the Examination of Human Semen and Sperm—Cervical Mucus Interaction, of the following:

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Torrance, CA 90509
United States of America
Fig. 2.8. Schematic drawings of some abnormal forms of human spermatozoa. (Adapted from Kruger et al., 1995.)

A. Head defects
- (a) Tapered
- (b) Pyriform
- (c) Round; small and acrosomal either absent or present.
- (d) Amorphous
- (e) Vacuolated
- (f) Acrosomal area small
- (g) Neck and midpiece defects
- (h) Bent neck
- (i) Asymmetrical insertion of mid-piece
- (j) Thick mid-piece
- (k) Thin mid-piece
- (l) Tail defects
- (m) Short tail
- (n) Bent tail
- (o) Coiled tail
- (p) Cytoplasmic droplet defect

B. Neck and midpiece defects
- (g) Bent neck
- (h) Asymmetrical insertion
- (i) Thick mid-piece
- (j) Thin mid-piece

C. Tail defects
- (x) Short
- (y) Bent
- (z) Coiled

D. Cytoplasmic droplet
- (r) >1/3rd head
Applicable chapters in the following text book will be reflected on:

Atlas of
HUMAN SPERM
MORPHOLOGY EVALUATION

Edited by

Edited by Thinus F. Kruger MD, FRCOG
and Daniel R. Franken PhD
Tygerberg Hospital
Tygerberg
Republic of South Africa

Taylor & Francis
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LONDON AND NEW YORK
A PARTHENON BOOK
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3 Relationship between sperm morphology and binding capacity to the zona pellucida: a critical step leading to fertilization 27
   S.C. Oehninger

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Preface

Our 25 years of experience in the evaluation of sperm morphology assisted us in developing a teaching module that contributed to the establishment of morphology as a clinically important measurement. This module also forms the cornerstone of a training program that has been responsible for setting up a global training and quality control program that has been functional since 1993. The module consists basically of the current *Atlas of Human Sperm Morphology Evaluation* and a CD-ROM training program (Strict 1-2-3®). The rapid improvements in computer software programs during the last few years have accommodated the development of this teaching method. Strict 1-2-3® contains stored photographic images consisting of a testing and quality control program. The trainee is requested to first familiarize him/herself with the morphometric configurations of the normal sperm cell present on the CD-ROM. Thereafter, the test phase of Strict 1-2-3® includes photomicrographs of sperm cells that are randomly selected by the computerized program which can be used for training or quality control purposes.

Sperm morphology evaluation, according to strict criteria, is the end-product of ideas and research involving many scientists and clinicians over a period of decades. The mainstay of strict criteria is based on clear definition of the normal form and clinical correlation. Strict criteria are used to assist the scientist in the IVF laboratory and the clinician guiding the patient with infertility problems. The *Atlas of Human Sperm Morphology Evaluation* is an attempt to describe normal and abnormal forms. However, sperm morphology evaluation skills will only become reliable and accurate after acquiring training using the interactive section of the Atlas. The authors hope that this book and interactive Atlas will assist scientists in a meaningful way.
The Tygerberg strict criteria: what are the clinical thresholds for in vitro fertilization, intrauterine insemination, and in vivo fertilization?

T.F. Kruger, F. Van der Merwe and J. Van Waart

Clinical thresholds to distinguish between fertile and infertile or subfertile patients were first attempted by our unit in the early 1970s\(^1\). Normal forms were defined as follows, based on criteria laid down by cervical mucus selection.

A spermatozoon is considered normal when the head has a smooth, oval configuration with a well-defined acrosome comprising about 40–70% of the sperm head. In addition, there must be no neck, mid-piece, or tail defects and no cytoplasmic droplets of more than one-half the size of the sperm head. We consider borderline forms abnormal. At least 100, but preferably 200, spermatozoa with tails were classified into one of seven groups: normal (head and tail normal), normal head but with another abnormality present, large heads, small heads, tapering heads, duplicated heads, or amorphous heads all with or without tail, neck or mid-piece defects. Tail, neck, and mid-piece defects, loose head, immature germinal cells, and unknown cells were recorded separately and reported per 100 spermatozoa. The size of the spermatozoa was evaluated in five different areas to ensure a more randomized evaluation.

In vitro fertilization (IVF) led to the development of a model where certain variables could be studied in more detail. In 1986, a study was performed correlating normal sperm morphology with IVF\(^2\).

In this study all male patients had a sperm concentration of \(20 \times 10^9\) ml and motility parameters of \(>30\%\) to negate the possible impact of the other parameters. One hundred and ninety-nine cycles were studied using logistic regression analysis. A threshold of 14% was calculated with a 37% fertilization rate in the \(<14\%\) normal morphology group and \(>82\%\) fertilization rate in the group \(>14\%\) normal forms. A second prospective study followed, evaluating the fertilization rate in the sperm morphology group with \(<14\%\) normal forms\(^3\). In this study it was shown that, with a sperm morphology of \(<5\%\) normal forms, the fertilization rate was only 7.6% (previously defined as the 0–4% group, i.e. P-pattern or poor-prognosis group). For the sake of convenience, we refer to this group as the 5% threshold group. The fertilization rate in the 5–14% normal forms group was 63% (G-pattern or good-prognosis group). The difference was highly significant. Three morphologic patterns were thus defined, namely the P-pattern group, or poor-prognosis group (0–4% normal forms); the G-pattern group, or good-prognosis group (5–14% normal forms); and the N-pattern group (those with normal forms \(>14\%\)).

Structured literature reviews on sperm morphology (strict criteria)

This was an attempt to evaluate the results of different authors studying the impact of sperm morphology on pregnancy outcome in IVF and intrauterine insemination (IUI), and to record clinical thresholds of semen parameters for in vivo fertilization.
In vitro fertilization

To study the impact of strict criteria on IVF outcome, a structured literature review was undertaken in 1998. Published literature in which normal sperm morphology was used to predict fertilization and pregnancy during the period 1978 to 1996 was reviewed. The statistical outcomes and conclusions of the studies were tabulated, and where sufficient data were available, the odds ratios for fertilization (per oocyte) and pregnancy (per cycle) were calculated. A total of 216 articles were identified by the sourcing methodology, but only 49 provided data that could be tabulated and analyzed. The majority (82%) of the analyzed studies concluded that normal sperm morphology (including acrosomal morphology) had a role to play in the determination of male fertility potential. Eighteen of the analyzed studies provided sufficient data for statistical analysis. Fifteen studies used the strict criteria to evaluate sperm morphology, two used WHO guidelines, and one used both the strict criteria and the WHO guidelines.

Using a 5% threshold (strict criteria), ten studies provided data that could be analyzed for the prediction of fertilization\(^5\)\(^-\)\(^14\) (Figure 1.1) and eleven studies for the prediction of pregnancy (Figure 1.2). All the studies showed a positive predictive value for fertilization in vitro, with only one\(^6\) (odds ratio 1.42 (CI: 0.9–2.25)) not reaching significance (Figure 1.1). In the prediction of pregnancy (per cycle)\(^2\)\(^,\)\(^6\)\(^,\)\(^13\)\(^-\)\(^15\)\(^,\)\(^16\), nine studies obtained a positive predictive value. The predictive value of the studies by Oehninger et al.\(^6\), Enginsoy et al.\(^9\), and Grow et al.\(^12\) reached significance (Figure 1.2).

Using a 14% threshold (strict criteria), five studies provided data that could be analyzed for the prediction of fertilization\(^2\)\(^,\)\(^6\)\(^-\)\(^8\)\(^,\)\(^14\) (Figure 1.1), and eight studies for the prediction of pregnancy (Figure 1.2). Similar to the 5% analyses, all these studies showed positive and significant predictive value with regard to fertilization in vitro (Figure 1.1). In the prediction of pregnancy, two studies\(^7\)\(^,\)\(^8\) did not obtain a positive predictive value, while two\(^5\)\(^,\)\(^6\) studies were positive and significant. This proved the value of the IVF model to study sperm–oocyte interaction and fertilization in vitro.

Not only did the structured review prove the value of strict criteria, but it also set a new threshold for the patient with poorer prognosis in IVF, not only for fertilization rate in vitro but also for pregnancy rate per embryo transfer. The ≤ 5% threshold clearly

\[\text{Figure 1.1} \quad \text{Odds ratio and confidence intervals for the predictive value of normal sperm morphology (strict criteria) for fertilization in vitro}\]
indicated a patient group with lower potential to fertilize in vitro.

It was concluded by Coetzee et al.⁴ that normal sperm morphology may not be absolute in its prediction of fertilization and pregnancy, but remains the most cost-effective means of diagnosing male infertility or subfertility and assisting in the formulation of a treatment regime. It was concluded that the selection of the correct treatment regime would help to maximize fertilization, transfer, and ultimately pregnancy probability.

**Intrauterine insemination**

In a recent study by Van Waart et al.¹⁹, a structured literature review was performed, evaluating the predictive value of normal sperm morphology in IUI programs as a predictor of male fertility potential. Literature where sperm morphology was used to predict IUI outcome during 1984 and 1998 was reviewed.

Of the 421 articles identified, only eight provided sufficient data for statistical analysis. Two studies used the 1987 and 1992 WHO criteria, but had insufficient data to provide conclusive analysis.

Six studies used the Tygerberg strict criteria²⁰⁻²⁵. A meta-analysis of these six studies yielded a risk difference (RD) between the pregnancy rates achieved in the patients below and above the 5% strict-criteria threshold of –0.07 (95% CI: –0.11 to –0.03; p < 0.001). This meta-analysis thus showed a significant improved pregnancy rate above or equal to the 5% strict-criteria threshold (Table 1.1).

It was concluded that normal sperm morphology evaluation by strict criteria should be an integral part of male factor evaluation.

**Studies on fertile versus infertile/subfertile patients**

Reviewing the current literature on semen parameters and in vivo fertility potential, Van der Merwe et al.²⁶ recently aimed to conduct a structured review and to establish fertility/subfertility thresholds for semen parameters. Published literature comparing fertile and subfertile populations between 1983 and 2002 was reviewed. A total of 265 articles were iden-
Table 1.1  Pregnancy rate per cycle: risk difference for pregnancy rate (strict criteria, 4% threshold). Value not included (whole population) in final meta-analysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>≤ 4%</th>
<th>&gt; 4%</th>
<th>Weight</th>
<th>Risk difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>idiopathic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montanaro-Gauci et al.</td>
<td>6/36</td>
<td>35/274</td>
<td>77.9</td>
<td>-0.10 (-0.17 to -0.04)</td>
</tr>
<tr>
<td>Matorres et al.</td>
<td>13/120</td>
<td>3/23</td>
<td>22.1</td>
<td>-0.02 (-0.17 to 0.13)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td>100</td>
<td>-0.08 (-0.16 to -0.01)</td>
</tr>
<tr>
<td>Whole population</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toner et al.22</td>
<td>6/86</td>
<td>35/309</td>
<td>20.9</td>
<td>-0.04 (-0.11 to 0.02)</td>
</tr>
<tr>
<td>Ombelet et al.23</td>
<td>40/335</td>
<td>76/469</td>
<td>24.7</td>
<td>-0.05 (-0.09 to 0.00)</td>
</tr>
<tr>
<td>Karabinus et al.24</td>
<td>3/53</td>
<td>44/485</td>
<td>20.3</td>
<td>-0.03 (-0.10 to 0.03)</td>
</tr>
<tr>
<td>Lindheim et al.25</td>
<td>1/99</td>
<td>15/77</td>
<td>15.5</td>
<td>-0.19 (-0.28 to -0.09)</td>
</tr>
<tr>
<td>Matarros et al.21</td>
<td>18/172</td>
<td>10/99</td>
<td>18.6</td>
<td>-0.00 (-0.07 to 0.08)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td>100</td>
<td>-0.06 (-0.11 to -0.01)</td>
</tr>
</tbody>
</table>

| Total                  |      |      | 11.79 (df = 5) |                          |

ified by the sourcing methodology, but only four articles provided data that could be tabulated and analyzed. Using ROC (receiver operating characteristic) curves, morphology (strict criteria) proved to be the best predictor of subfertility in three of the four articles, with concentration and motility/progressive motility also showing good predictive power. Calculated thresholds ranged from 4 to 10% for morphology, 13.6 x 10⁶/ml and 34 x 10⁶/ml for concentration, and from 31.8 to 52% for motility. A second set of much lower thresholds was calculated in three of the articles. The adjusted lower thresholds were between 3 and 5% for morphology, 9 x 10⁶/ml for concentration, and between 20 and 30% for motility. Because these lower thresholds have a much higher positive predictive value, it was suggested that they should be used to identify the subfertile male. The lower thresholds for morphology also fit IVF and IUI data calculated previously. It was concluded that using the parameters in combination increases the clinical value of a semen analysis.

Study of a fertile population: three continents, four centers

In recent publications, thresholds of 8–10% for normal sperm morphology have been suggested to distinguish between fertile and infertile men in in vitro situations. It was also shown that sperm morphology is the semen parameter with the best prognostic value in these couples. The aim of this study on the fertile population was to compare the basic semen parameters of proven fertile patients from four different international fertility centers (three continents).

Men who had impregnated their wives within 1 year of unprotected sexual intercourse were recruited to give a semen sample. The wives of these men were all pregnant at the time of evaluation. Semen parameters (concentration, motility) were evaluated according to WHO 1999 guidelines, while the sperm morphology was evaluated according to the strict criteria.

In this study, there were 57 patients in the Argentinian center, of which 18 were from one center (Argen1) and 39 from a second center (Argen2), 36 in the South African center (Tygerberg), and 68 in the Turkish center. Cross-tabulations of the morphology groups by pattern were done. There was a significant difference in the morphologic profile as expressed by P, G, and N categories (p < 0.0001). The Tygerberg center had 19% in the P-pattern group and 6% in the N-pattern group. The reason for this finding is still uncertain, and further studies in different population groups are under way.

When the four centers were added together, there were 9/161 (5.6%) P patterns, 91/161 (56.5%) G patterns, and 61/161 (37.9%) N patterns. The percentage motility in the Tygerberg center was the lowest as well as in the percentage normal morphology. The Argen1 center had the highest concentration per/ml compared with the other three centers (Table 1.2).
### Table 1.2 Descriptive statistics of semen parameters

<table>
<thead>
<tr>
<th>Center</th>
<th>Concentration (ml)</th>
<th>Motility (%)</th>
<th>Morphology</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argen 1</td>
<td>138.3 (74.4)</td>
<td>64.5 (12.9)</td>
<td>12.2 (3.7)</td>
<td>2.7 (1.5)</td>
</tr>
<tr>
<td>Argen 2</td>
<td>65.4 (56.8)</td>
<td>58.1 (17.1)</td>
<td>16.1 (6.8)</td>
<td></td>
</tr>
<tr>
<td>Tygerberg</td>
<td>66.7 (54.8)</td>
<td>47.6 (16.3)</td>
<td>6.8 (3.8)</td>
<td>2.6 (1.8)</td>
</tr>
<tr>
<td>Turkey</td>
<td>50.4 (30.9)</td>
<td>59.8 (14.6)</td>
<td>14.9 (6.1)</td>
<td>3.9 (1.9)</td>
</tr>
</tbody>
</table>

The concentration per/ml and percentage motility were in the same range for all the centers, except for Argen 1 regarding concentration per/ml. The difference in percentage morphology in the P-pattern group of Tygerberg needs further evaluation. The majority of patients were, however, in the G-pattern sperm morphology group or higher. Patients in the G-pattern group can therefore be considered as fertile, while those below 5% should be regarded with a higher potential for subfertility.

### Conclusion

From the reviews mentioned, the main problem was one of obtaining sufficient data from the old WHO criteria (1992 and earlier) in order to look at the morphology impact on IVF and IUI results. Not only did the threshold differ in different papers, but also insufficient data meant that a meta-analysis could not be performed. On the other hand, thresholds on strict criteria and sufficient papers with data resulted in a consensus opinion on the thresholds. This is the reason why the World Health Organization accepted the strict criteria as an international guideline[31].

It seems as if the P-pattern group (normal morphology 4–14%) is the group with the lowest pregnancy success rate in IUI, IVF, and in *in vitro* fertilization. It is, however, important to view these thresholds in perspective. We do not claim that a pregnancy is not possible within the P-pattern group, but only that there is a significantly lower chance of a pregnancy. It is, however, important to note that all the other semen parameters are also contributing, and that if the other parameters are below suggested thresholds, the chance of success will be even lower.

On the other hand, when there is an excellent concentration/ml and percentage motility >50%, these parameters will compensate for patients with P-pattern morphology, with a significantly better chance of pregnancy than IUI[20].

It is also suggested that these morphological thresholds can be used to direct a patient towards controlled IVF or IVF-intracytoplasmic sperm-injection (ICSI) treatment if sufficient oocytes are available during stimulation.

### References


What is a normal spermatozoon?

D.R. Franken and T.F. Kruger

Introduction

A fundamental knowledge and understanding of the configurations and bright-field microscopic appearance of the ideal sperm cell has proven to be the basis of the strict-criteria evaluation method for sperm morphology. Studies have indicated that once an andrology-laboratory technologist has mastered the morphometric concept(s) of the 'ideal' sperm cell, he/she has shown to be extremely consistent in morphology-reading skills\(^1,2\). Once sperm morphology assessments become consistent, reliable and repeatable, the concept of sperm morphology being the single most important semen parameter that is correlated with the fertility potential during both in vitro\(^3,4,5\) and in vivo\(^6\) studies becomes a reality.

Furthermore, clinically significant results from the andrology laboratory can only become a reality when all technologists responsible for the analysis of semen have received training and are enrolled on a internal, as well as an external, continuous sperm morphology quality-control program\(^1,2\). The laboratory director should have access to the performance records of each individual in his/her laboratory. Previous studies have indicated that the sperm morphology-reading skills of a specific individual are directly influenced not only by the experience level of the technologist, but also by the combination of experience level with the methodology used to evaluate morphology\(^5\).

Sperm morphology training

Training can be presented through a variety of approaches, of which the one-to-one workshop method is the ideal. The advantage of this method is that the trainee has direct communication and input on a one-to-one basis with an experienced worker. This method, however, has a disadvantage in that only a small number of trainees can be trained per session. Our experience has indicated that a maximum of ten students per teacher can be trained per session\(^1,2\) with this method. A second and also valuable teaching method is the so-called consensus technique. With this method, the trainer (usually an experienced sperm morphology technician) uses electronic images which are projected onto a screen. During teaching sessions, numbered sperm images can be discussed with a group of trainees. Typically, the group moves on to a new image only when all the trainees have reached consensus about a specific image. The advantage of this method lies in the fact that large numbers of students can be trained during a single session. The disadvantage of this method, however, lies in the mass-communication style when the individual is often lost during group discussions.

A third training method is the use of an interactive CD-ROM program known as Strict 1-2-3\(^\circledR\). Strict 1-2-3 contains a large number of high-quality images of numbered spermatozoa. The trainee is requested to first familiarize him/herself with the
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morphometric configurations of the normal sperm cell present on the CD-ROM. Thereafter, the test phase of Strict 1-2-3 includes photomicrographs of sperm cells that are randomly selected by the computerized program, and which can be used for training or quality-control purposes.

How to acquire and maintain sperm morphology evaluation skills

Our 25 years of experience in the evaluation of sperm morphology have assisted us in developing a teaching module that has contributed to the establishment of morphology as a clinically important measurement. This module also forms the cornerstone of a training program that has been responsible for setting up a global training and quality-control program; the latter has been functional since 1993. The module consists basically of the current Atlas of Human Sperm Morphology Evaluation and a CD-ROM training program (Strict 1-2-3®). The rapid improvements in computer software programs during the last few years have accommodated the development of this teaching method.

Strict 1-2-3 contains stored photographic images consisting of a testing and quality-control program. A self-training session, the test phase of the program can be activated, during which the trainee is requested to evaluate randomly selected sperm images for normality. One of the advantages of the Strict 1-2-3 program is that training can be done at the leisure and available time of the technician. Also, the trainee receives direct information from the reference laboratory that is responsible for the program. The program also allows for the possibility of setting up a continuous communication system (in some cases a quality-control program) between the reference laboratory and the trainee. This teaching approach forms the cornerstone of the World Health Organization’s training program that has been presented at Tygerberg Hospital since 1997.

Human spermatozoa

Many attempts to standardize the methodology of sperm morphology assessment have been launched since 1951. A prominent contribution was initiated by MacLeod and Gold, who introduced the first classification for sperm morphology. This classification was based on the size and shape of sperm cells, and subsequently divided spermatozoa into six different groups. In 1966, Freund published a classification that not only evaluated six groups of sperm heads, but also addressed sperm tail defects. Eliasson was the first to describe and specify the use of morphometric values for sperm head length and width. He rejected the counting of only one defect and basically advocated counting all defects, i.e., head, neck, mid-piece, and tail. David et al. described a whole new concept in sperm morphology assessment using a multiple-entry system, considering all defects and their combinations. All abnormalities in David’s system were equally important. The system is widely in use in France, but its main disadvantage lies in its complexity.

In 1980, the WHO classification supplied a very basic description of sperm morphology evaluation and was obviously not yet regarded as a very useful and valuable semen parameter. In the second WHO manual, the morphology-classification system was altered from a single- to a multiple-entry classification system. Three categories of abnormalities (cytoplasmic droplets, mid-piece, and tail defects) were removed, and two new classes were added (round and pin heads), thus resulting in nine classes describing sperm head. In addition, two other categories were created, i.e., mid-piece and tail defects.

In the meantime, Hofmann and Haider published a unique system in sperm morphology assessment known as the Düsseldorf classification. This classification emphasizes the association between specific sperm head defects and specific clinical manifestations. This classification system focused on the elongated sperm forms as well as on acrosomal defects.

The third WHO manual focused mainly on sperm morphology assessment. Five classes of abnormalities are provided, and in case a spermatozoon has more than one defect; all these abnormalities are scored separately under each class and therefore a teratozoospermic index could be calculated. Using only empirical reasons, a cut-off value for normality of 30-50% was chosen. In the fourth WHO manual, sperm morphology was officially to be assessed according to 'strict criteria'. The lower cut-off value for normality was left open, but with a comment that the data suggested that a value <15% reflected lower in vitro fertilization results.

Based on the work by Eliasson and according to 'strict criteria', a spermatozoon is considered normal if the sperm head, neck, mid-piece, and tail adhere to
published criteria\textsuperscript{12,13,21}. In addition, there should be a well-defined acrosomal region comprising 40–70\% of the head area. The mid-piece should be slender, less than 1 \textmu m in width, about one and a half times the length of the head, and attached axially to the head. The tail should be straight, uniform, thinner than the mid-piece, uncoiled, and approximately 45 \textmu m long\textsuperscript{19}. Our training program employed Papanicolaou staining, as that is our standard staining method\textsuperscript{22}. Apart from the above descriptions of normality for spermatozoa, emphasis was placed during the training period on sperm head morphology.

The descriptions require that all ‘borderline’ forms be considered abnormal\textsuperscript{21}. It also included spermatozoa adhering to the following criteria and measurements: (a) in principle, the head is oval-shaped with a smooth contour; (b) allowing for the slight shrinkage that fixation induces, the head should be 4–5 \textmu m in length and 2.5–3.5 \textmu m in width. The total length-to-width ratio should be 1.5–1.75 (width \times length = 5.0 \times 3.5 \textmu m, Figure 6.1).

When the evaluation is based on the above measurements after Papanicolaou staining, a normal spermatozoon can also be defined as normal oval shaped with a smooth contour, when slightly tapered or V-shaped in the posterior region (width \times length = 5.0 \times 3.5 \textmu m, Figure 6.2); head slightly narrower (width \times length = 5.0 \times 3.0, Figure 6.3); slightly narrower head and slightly tapered equatorial region (width \times length = 5.0 \times 3.0 \textmu m, Figure 6.4); these are all regarded as normal forms. Likewise, the smaller oval-shaped sperm cell (width \times length = 4.0 \times 2.5 \textmu m) and smaller sperm cell and slightly tapered posterior region (width \times length = 4.0 \times 2.5 \textmu m) are all considered normal forms. These minor variations of spermatozoa represent the forms that are considered to be normal. Borderline forms, or slightly amorphous, are those forms with configurations that fall outside the range of normal variation\textsuperscript{20}. Strict-criteria morphology therefore allows, within the classification for normality, spermatozoa with head configurations that fall outside the range provided of normal cells (oval shaped), but in which the abnormality is not sufficiently pronounced to be classified as abnormal.

**Slide preparation**

The use of pre-cleaned glass slides, prepared according to the standard method, is recommended\textsuperscript{21}. In order to ensure a thin smear with evenly spread sperm cells, the semen drop volume is determined by the sperm concentration. When the sperm cell concentration is $<20 \times 10^9$/ml, a 10–20 \textmu l drop is used, and when the concentration is $>20 \times 10^9$/ml, a 5–10 \textmu l drop is used.

The size of drop selected is placed in the middle of one end of the slide. The edge of a second slide is placed on the drop with a 30\° angle, thus allowing the semen droplet to spread evenly across the width of the first slide. Holding both the slides firmly, the second slide is pulled gently towards the operator, after which the slide is firmly pushed forwards across the entire length of the first slide. This technique, known as the feather technique\textsuperscript{19}, will ensure thin, evenly spaced sperm slides. Lacquet et al.\textsuperscript{24} defined a sperm cell concentration of 100 \times 10^9/ml, suspended in a 25-\textmu l droplet, to be optimum for providing 3–10 sperm cells/frame on the screen of the Hamilton Thorne Version 10HTM-IVOS (Dimension V2.01).

For manual reading, we found a 10-\textmu l sperm droplet with a cell concentration of 35 \times 10^9 cells/ml to be optimal for producing 8–15 sperm evenly spread over the surface of a pre-cleaned slide. This provided the ‘ideal’ microscopic field for morphology evaluation; namely, it ensures that all sperm are on the same focal plane and that no overlapping of any part of the cell occurs. Furthermore, owing to uniform staining, the resultant equal brightness over the entire slide enhances the quality of the slide. Contrast and background are important factors that could interfere with the technician’s ability to distinguish normal from abnormal cells. For instance, superposition of an object, or unclear outlining of the sperm cell, may result in an abnormal shape or size interpretation. Washing the sperm sample once or twice produced slides with high-contrast resolution and with few background particles. When performed, these often neglected, critical technical points will enable the technician to reach a sensitivity and specificity comparable with that published\textsuperscript{25}.

Several staining methods exist to produce sperm morphology slides of high contrast and quality. The Papanicolaou method\textsuperscript{22} produces good definition of both sperm cells and other cells (precursors, epithelium, and white blood cells). Staining characteristics of Papanicolaou stain results in the light-blue acrosome. The post-acrosomal region of the head stains dark blue, while the mid-piece and tail stain green or red, respectively. However, owing to limited laboratory time, photography and, in certain cases, due to the costs involved, other staining methods have
become popular over the past few years. These methods include Diff-Quik, Test Simplest, Shorr, and Spermac. The staining characteristics of Diff-Quik stain, for example, differ from that of Papanicolaou staining, i.e. the acrosome stains pale purple, while the post-acrosomal part of the head, mid-piece, and tail stain dark purple. Spermac staining, on the other hand, stains the nuclear portion of the head red, while the acrosome, mid-piece, and tail stain green.

The head width and length varies with the staining method, i.e. for Papanicolaou staining, the morphometric dimensions of the head are given as: length 4.5 µm and width 2.5-3.5 µm. Diff-Quik staining, however, results in a slightly swollen head, namely; 5-6 µm for the length and 2.5-3.5 µm for the width.

Normal spermatozoa

Figures 6.1 to 6.4 present examples of spermatozoa that fall within the limits given for length and width of normality.

Normal spermatozoa with dimensions 5.0 x 3.5 µm (Figure 6.1)

Normal human spermatozoa (boxed, figure 6.1) possess a smooth oval head shape with a well-defined acrosome covering 40-70% of the apical part of the head. The base of the head should be broad with only one tail (flagellum), which is symmetrically attached. Immediately behind the head, the first part of the tail (known as the mid-piece) should be somewhat thicker (but still slender in appearance) compared with the tail (maximum width = 1 µm) and 7.0-8.0 µm long. The tail diameter should be between 0.4 and 0.5 µm and ~50 µm in length. It should not be coiled or uneven in diameter. The tail should not be abnormally bent, i.e. more than 90°. The tail should have a well defined end-piece.

All borderline forms should be classified as abnormal in order to conform to the 'strict criteria' for morphological normality.

Normal spermatozoa with dimensions 5.0 x 3.5 µm (slightly tapered) (Figure 6.2)

The boxed spermatozoon in Figure 6.2 adheres to the criteria supplied for normality according to strict criteria. The post-acrosomal part of the head, however, is slightly narrower in the area of the mid-piece attachment.

Normal spermatozoa with dimensions 5.0 x 3.0 µm (Figure 6.3)

The boxed sperm cell in Figure 6.3 is regarded as normal since its configurations are within the limits of normality. The only deviation from the cell shown in Figure 6.1 is the width.

Normal spermatozoa with dimensions 5.0 x 3.0 µm (tapered) (Figure 6.4)

The boxed sperm cell in Figure 6.4 is also slightly narrower at the base of the post-acrosomal region.

Abnormal spermatozoa

John MacLeod defined a mature spermatozoon as a cell possessing a head, mid-piece, and tail, regardless of the irregularities of these structures. Despite this definition, the structural defects of the head were limited to large, small, tapering, double-headed, and amorphous. Additionally, he recognized sperm cells with a large cytoplasmic droplet and spermatozoids as a single class called 'immature' forms. These aberrations of the 'normal' cell were often referred to as pathological forms. Sperm morphology was reported by placing each spermatozoon into one of two groups, namely, 'normal' or 'pathological' (not normal).

Schirren described pathological forms of spermatozoa in the ejaculate as tapered, large head, change-in-middle portion, double head, deformed head, phantom, axial-implant defects, or double tail. Counting abnormal sperm relates only to the main regions of the spermatozoon, i.e. head, neck/mid-piece, and tail. Differentiation between different abnormalities within the head, or between different tail defects, is normally not performed. The sperm morphology classification system that originated in the 1970s developed through the years, so that today we have a system that recognizes not only the morphometrical importance of head length and width, but also includes a multi-score system to record the abnormalities.

The World Health Organization (WHO) strict criteria use a set of strict guidelines to identify the 'ideal' or normal sperm cell population in a given specimen. Originally, the shape
Figure 6.1  Normal spermatozoa with dimensions 5.0 × 3.5 μm

Figure 6.2  Normal spermatozoa with dimensions 5.0 × 3.5 μm, but slightly tapered in the post-acrosomal region
Figure 6.3  Normal spermatozoa with dimensions $5.0 \times 3.0\mu m$

Figure 6.4  Normal spermatozoa with dimensions $5.0 \times 3.0\mu m$, but slightly tapered in the post-acrosomal region
of the ‘ideal’ spermatozoan was identified and described by Menkveld et al.\textsuperscript{21}. In the late 1980s, the clinical importance of the ‘ideal’ sperm cell was highlighted by Kruger et al.\textsuperscript{3}, who correlated strict criteria with in vitro fertilization. The so-called strict criteria system includes critical evaluation of the sperm head morphology to determine whether a sperm head falls within the defined normal range\textsuperscript{23}.

This classification system recognizes the following aberrations:

- **Head**: large, small-tapering, duplicate, or amorphous;
- **Others**: neck defects, a bend of >30 degrees, mid-piece defects, tail defects (double or coiled), loose heads, immature forms (cytoplasmic droplets).

The multiple-entry classification developed in France by David\textsuperscript{14} is a complex scheme which identifies 15 defects in the sperm cell. A single cell may show up to a maximum of four defects. Each defect is separately evaluated and counted. The region and defects of the multiple-entry classification includes:

- **Head**: tapered, thin, small, large, multiple (including double), abnormal base (more or less similar to pyriform), and abnormal or absent acrosome;
- **Mid-piece**: cytoplasmic droplets, thin mid-piece, or bent tail;
- **Tail**: absent, short, irregular width, coiled, or multiple.

**Strict criteria**

**Head defects**

Head defects among sperm cells can be sub-divided into a number of abnormalities. Head defects include macrocephalic (large), microcephalic (pin heads), tapered (i.e. length-to-width ratio >2), pyriform (dumbbell), round, amorphous, vacuolated (>20% of the head area is occupied by unstrained vacuolar areas), heads with a small acrosomal area (<40% of the head area), and double heads. Usually most of these abnormalities are easy to identify, and the technologist has no problem in recording the different types of abnormalities.

**Macrocephalic head** This type of sperm adheres to dimensions outside the limits provided for normal sperm, i.e. length is greater than 5 μm and width is greater than 4 μm (Figure 6.5).

**Round sperm head** The abnormality is classified in the borderline group owing to the fact that these forms are seldom found in the reference population (cervical mucus or on zona pellucida). When using strict criteria, stringent adherence to measurements will lead to accurate reporting (Figure 6.6).

**Amorphous head** This abnormality is easy to identify since the configuration of the sperm cells are usually grossly abnormal and can be described as shapeless or amorphous. The staining Papanicolaou pattern of this abnormality is usually very dark blue or black (Figure 6.7).

**Irregular surface** By definition, a normal sperm cell should have a smooth pre- and post-acrosomal surface area. Any irregularity of the surface area is considered abnormal. In the boxed cell in Figure 6.8, the defect is small and the sperm can fall into the borderline group (Figure 6.8).

**Tapered head** It is believed that the tapering or elongated forms are caused by testicular stress, often due to environmental factors\textsuperscript{17,22} acting via the Sertoli cells on the latter stages of spermatogenesis. For example, mild Sertoli cell disorder is manifested as a simple elongation of the head (boxed sperm, Figure 6.9a) Slightly more pronounced forms show a cone-shape head, (Figure 6.9b), whereas in the most severe cases of this disorder the sperm head appears as a dumbbell.

The two boxed sperm cells in Figure 6.9a represent typical tapered forms (elongated forms) with mild aberrations in the length-to-width ratio. The tapered shape can be restricted to a specific area of the sperm head, for example on the pre- or post-acrosomal region. In some instances, the head typically stains darker than the normal cell and can be seen in certain specimens as spear-shaped cells. The tapered shape or elongated head forms can be divided into mild, moderate, and severe forms\textsuperscript{22}. All these forms are classified as abnormal.

**Tapered head** The boxed spermatozoan in Figure 6.9b shows a more pronounced form of narrowing and tapering, particularly in the post-acrosomal region, giving the sperm cell a cone-shaped appearance, and slightly more tapering or elongation of the
Figure 6.5  Macrocephalic head

Figure 6.6  Round sperm head
Figure 6.7  Amorphous head

Figure 6.8  Irregular surface
Figure 6.9a  Tapered spermatozoon (mild form)

Figure 6.9b  Tapered head (moderate form)
post-acrosomal region. Although the acrosome is normal in this case, the aberration is classified as abnormal.

Tapered head (pyriform or dumbbell) These cells are easily detected under bright field microscopy due to the dark blue/black stain with Papanicolaou stain. Abnormality of the post acrosomal region is often referred to as dumbbell shaped spermatozoa (Figures 6.10 and 6.11).

Tapered head (severe pyriform) The sperm is extremely elongated (Figure 6.11) and narrowed, with a markedly compressed acrosome. This most severe form of tapering (elongation) affects the middle of the sperm, often with a portion of nucleus or cytoplasm bulging out on either side. This is probably caused by a severe disorder of the Sertoli cell function, due to stress, and is irreversible.

Large acrosomal area An acrosomal area that comprises more than 70% of the head area should be regarded as abnormal in spite of the oval shape (Figure 6.12).

Small acrosomal area Since the acrosome develops from the Golgi complex of the germinal cell, the disorder of the acrosome development is caused by a defect at the level of the germinal cell. Acrosome defects can be classified into slight (<40% of head diameter) or the severe type where no acrosomal area is visible. The spermatozoa are usually misshapen or round when dealing with a severe form. Both forms are classified as abnormal.

Similar to the tapered sperm cells, small acrosomal areas can vary from a mild aberration (Figure 6.14) to a very severe disorder (Figure 6.13). Figure 6.13 contains two examples of spermatozoa with no acrosomal area visible, and classified as abnormal. Acrosomal abnormalities probably occur early in spermatid differentiation owing to abnormal function of the Golgi complex.

Small acrosomal area (mild form) The boxed spermatozoa in Figure 6.14, with a distinct small acrosomal area, shows a mild aberration.

Small or no acrosomal area (severe form) The boxed spermatozoa in Figure 6.13, with a distinct small acrosomal area, has a second structural defect in that it is also not oval with a thickened mid-piece.

Vacuoles When a spermatozoa has more than two small vacuoles in the head area, it should be regarded as an abnormality. Single large vacuoles are also abnormal, or even a vacuole in the post-acrosomal area is considered abnormal (Figure 6.15).

Neck/mid-piece defects

Mid-piece abnormalities are usually associated with structural defects with mitochondria, centrioles, microtubule formation, and retention of abnormal large cytoplasmic droplets. Mid-piece defects may occur with both head and/or tail defects. The mid-piece develops from the interaction of the centrioles with the spermatid nucleus. During early spermatogenesis, the sperm flagellum develops from the centriolar complex, which approaches the nucleus and attaches itself to its caudal pole, ensuring a linear alignment of the tail with the longitudinal axis of the head.

Under this category of abnormalities, the defects can be organized according to the locality of the problem; for example, a bent neck is defined when the neck and tail of the cell forms an angle greater than 90° to the long axis of the head. Further typical examples of this group of abnormalities include asymmetrical insertion of the mid-piece into the head, thick, or irregular mid-piece, or even an abnormally thin mid-piece. The latter defect is usually due to the absence of the mitochondrial sheath. Any combination of these defects can also be observed in semen.

Recent studies by Ollero et al. and Gil-Guzman et al. have shown that levels of reactive oxygen species (ROS) production in semen could be negatively correlated with the percentage of normal sperm forms as determined by World Health Organization classification and by Tygerberg strict criteria. The link between seminal ROS and high percentages of abnormal forms may be causal, and could be related to the greater capacity of morphologically-abnormal spermatozoa to produce ROS.

Furthermore, deficiencies of either glutathione or selenium substance can lead to instability of the mid-piece, resulting in defective motility. Glutathione and selenium substance are essential to the formation of phospholipid hydroperoxide glutathione peroxidase, an enzyme which is normally present in spermatids and which becomes a structural protein in the mid-piece of mature spermatozoa.
Figure 6.10  Tapered: pyriform (dumbbell) (severe)

Figure 6.11  Tapered: pyriform (severe)
Figure 6.12 Large acrosomal area

Figure 6.13 Small acrosomal area (severe form)
Figure 6.14  Small acrosomal area (mild form)

Figure 6.15  Vacuoles
Bent mid-piece

In the boxed sperm cells (Figures 6.16 and 6.17), the mid-piece is folded back on itself and, at the attachment of the mid-piece to the head, is kinked. This defect is characterized by severe axonemal disruption, often including rupture and disorganization of fibrils\textsuperscript{22}. Specific sperm morphological aberrations such as mid-piece defects are often typical of specific semen samples, and for that sperm morphology can therefore be regarded as the 'fingerprint image' of testicular function.

Thickened mid-piece

The boxed sperm cell (Figure 6.18) is regarded as a pseudo-droplet defect\textsuperscript{22} and is caused by a loosening of the mitochondria proximally, centrally, or distally, often leaving a denuded axonema. The arrowed sperm (Figure 6.18) is a typical example of a pseudodroplet defect. Usually, the mitochondria have loosened proximally and have clumped distally at the junction of the mid-piece and the principal piece of the tail\textsuperscript{22}. A damaged mid-piece suggests defective centrioles or segmental mitochondrial aplasia\textsuperscript{22}.

Irregular mid-piece

The irregular mid-piece (kinked mid-piece shown in Figure 6.19) is similar to the corkscrew defect described in bovine semen, in which there is a spiraling of mitochondria in the form of a helix\textsuperscript{22}.

Cytoplasmic droplets

Cytoplasmic droplets are a main source for the production of reactive oxygen species\textsuperscript{35}. When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm\textsuperscript{36}. Under these circumstances, the spermatozoa that are released during spermatiation are believed to be immature and functionally defective\textsuperscript{37}. Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme G6PD\textsuperscript{36,37}.

According to the World Health Organization's laboratory manual for the examination of human semen\textsuperscript{39}, cytoplasmic droplets greater than one-third of the area of a normal sperm head are considered to be abnormal. These droplets are usually located in the mid-piece area of the cell (Figures 6.20 and 6.21). Therefore, droplets with a smooth outline and a size not exceeding one-third of the sperm head are classified as normal. Sometimes cytoplasmic droplets can be identified in other regions, for example along the tail. A small amount of retained cytoplasmic material is regarded as normal, provided the droplet is less than one-third of the size of the head\textsuperscript{22}. A proper semen smear technique is essential so that mid-piece defects do not occur artificially.

Tail defects

A normal human sperm tail (flagellum) is a long, thin structure approximately 50\textmu m in length and 0.4-0.5\textmu m in diameter. The axonema forms the central element, which is a cylinder composed of a circumferential array of nine peripheral doublets surrounding a central pair of microtubules, and known as the 9+2 configuration. Each peripheral microtubule set is composed of two juxtaposed subunits, microtubules A and B; these microtubules are interconnected with dynein arms. The latter, a structural protein with ATPase activity, utilizes ATP energy to generate axonemal movement\textsuperscript{31}.

All mammalian sperm tails contain, apart from an axoneme specialized apparatus, i.e. outer dense fibers (ODF), which extend along the entire tail, and a fibrous sheath (FS), which initiates at the stack of mitochondria and surrounds the ODF until the terminal end of the sperm tail. Abnormalities in these structures are usually associated with reduced sperm tail motility and human infertility. Early studies indicated that ODF proteins are testis-specific and are synthesized in elongating spermatids\textsuperscript{38}.

Tail defects include spermatozoa with the following abnormalities short, multiple, hairpin, broken tails, bent tails (>90 degrees), tails with irregular width, or coiled tails.

Tail coiling

The most common tail defect was first identified among bovine sperm and is known as the 'Dog' defect. Characterized by various degrees of coiling of the tail, the defect probably develops in the epididymus and is associated with fibril rupture and axonemal disorganization\textsuperscript{22}. In cases of severe 'Dog' defects, sperm tails are coiled around both head and mid-piece.
Figure 6.16  Bent mid-piece

Figure 6.17  Mid-piece bent
Figure 6.18  Over-thick mid-piece. Arrowed sperm: pseudo-droplet defect

Figure 6.19  Irregular mid-piece
Figure 6.20  Cytoplasmic droplet

Figure 6.21  Cytoplasmic droplet
Haidl et al. investigated and described a new classification for flagellar disturbances with respect to sperm motility. This classification is an attempt to comprehend both the origin and the severity of tail defects and includes (a) disturbances of the mid-piece; (b) shaft irregularities as identified by abnormal staining during Papanicolaou’s or Shorr’s technique; (c) structural shaft defects; (d) breaking and coiling; and (e) short or rudimentary tails. Similar findings were reported by Chemes, who attributed flagellar structural abnormalities to be responsible for most cases of severe asthenozoospermia. Dag axonemal defect of mid-piece and tail (Figures 6.22 and 6.23) is characterized by sperm tails that are coiled within a stretched plasma membrane around the head. The sperm is immotile, and the axonemal structure is abnormal (disrupted, broken, loss of fibers). The defect is reported to originate in the epididymis and not testis. Coiled tails have also been associated with exposure to organic solvents (glues, paints, cleaning fluids). Tail coiling often results from a disturbance in epididymal function. Animal studies have revealed that scrotal heat drastically increases the number of sperm cells with this malformation.

**Coiled tail**

**Tail-up coiling**

Terminal tail tip coiling (Figure 6.24) is regarded as a minor defect, but these spermatozoa are classified as abnormal.

**Double tail**

Double tails in Figures 6.25 and 6.26 may arise from abnormal centriole formation or incomplete cytokinesis.

**Bent tail**

Figure 6.27 shows the difference between a normal curved tail (arrowed spermatozoon) and a defective bend in the tail (boxed). Kinking of the tail probably occurs in the early stages of spermiid differentiation; the growing tail cannot stretch the cell membrane and then continues to develop within it. Such malformations can later interfere with sperm release from the Sertoli cell; when they do, an enlarged cytoplasmic droplet remains associated with the head or principal tail piece.

**Stumped tail**

A 'stumped tail' spermatozoon (Figure 6.28) can be caused by dysplasia of the fibrous sheath or the result of disintegration in aging spermatozoa. Genetic origins can be suspected when more than 50% of the spermatozoa are identified with this tail defect. Severe asthenozoospermia or complete immotility has been reported among men with dysplasia of the fibrous sheath. These spermatozoa display characteristically short and irregular flagella.

**Abaxial implantation**

Common abnormalities of the head–mid-piece junction are identified by the abaxial implantation of the mid-piece. Scanning electron microscopy identified a vesicular structure that separates the head and mid-piece. During the initial stages of spermiogenesis, the sperm flagellum grows from the centriolar complex, which approaches the nucleus and attaches itself to the caudal pole ensuring a linear alignment of the tail with the longitudinal axis of the head.

Abnormalities of the head–mid-piece junction vary immensely and are dependent mainly upon the degree of alteration between the centriolar complex and nuclear structure. This is clearly illustrated in Figures 6.29 and 6.30. In Figure 6.29, a very subtle implantation aberration can be seen in the boxed sperm, whereas Figure 6.30 illustrates a cell with a major implantation disorder.

**Flat implantation**

Figure 6.31 shows a flat implantation site.

**Acephalic sperm**

Reports of semen that presents with varying numbers of 'decapitated sperm' (also known as 'acephalic sperm', Figure 6.32), to semen showing mainly abnormalities of the head–mid-piece junction and, in some cases, a mixture of these two conditions, have been published. Acephalic sperm are testicular in origin and develop from a failure of the centriole-tail structures to attach normally to the
Figure 6.22  Coiled tail (Dag defect)

Figure 6.23  Coiled tail (Dag defect)
**Figure 6.24** Tail-tip coiling

**Figure 6.25** Double tail
Figure 6.26  Double tail

Figure 6.27  Tail bent than more than 90°. Arrowed sperm: normal curvature
Figure 6.28 Stumped tail

Figure 6.29 Abaxial implantation
Figure 6.30  Abaxial implantation

Figure 6.31  Flat implantation site
spermatid nucleus. Consequently, the heads and tails develop independently and separate at the moment of spermiation; the heads are usually phagocytosed by the Sertoli cells.

References


34. Usmani F, et al. The role of PHGPX as a structural protein may explain the mechanical instability of the mitochondrial mid-piece that is observed in selenium deficiency. Science 1999;285:1393.


Applicable chapters in the following text book will be reflected on:

WHO laboratory manual for the Examination and processing of human semen

FIFTH EDITION

World Health Organization
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This edition of the manual is dedicated to the memory of the late Geoffrey Waites (1928–2005), former manager of the WHO Task Force on Methods for the Regulation of Male Fertility and co-editor of the second, third and fourth editions of this laboratory manual. The editorial committee’s devotion to its task was driven by its appreciation of Geoff’s honesty, fairness and concern for the underprivileged.
Plate 1

Micrographs courtesy of C. Brazil.
Morphology assessment of spermatozoa in Plate 1

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Micrographs courtesy of C Brazil.
Morphology assessment of spermatozoa in Plate 2

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ii. A concluding remark on the educational aspects of sperm morphology

The development of the Strict Criteria for sperm morphology (Menkveld et al., 1990) not only led to new criteria for the normal form internationally as outlined in 1986 (Kruger et al., 1986) and 1990 by Menkveld where the normal form was described in detail. The clinical findings and the description of the patterns of sperm morphology had an impact on clinical practice at pre-graduate level with definitive practical application as outlined, as well as at post-graduate level all over the world. This led to change in concepts, interpretations and often change in clinical practice. The new threshold values were and still are much debated but also accepted by those who repeated the research and attended courses to learn the technique to evaluate sperm morphology correctly.
2. **A chronologic summary of the impact of sperm morphology on clinical practice internationally and concluding remarks**

- The first publication in 1986 (Kruger et al., 1986) on strict sperm morphology and its impact on fertilization and pregnancy rate in assisted reproduction, had a huge effect on research and clinical practice in Assisted Reproductive Technology (ART) in the years to come.

- In 1988 the different sperm morphology patterns were described. In the 1988 article a fertilization rate of 7.6% was reported for the P-pattern group compared to the 63.9% for the G-pattern group (5 to 14% normal forms) (Kruger et al., 1988). The G-pattern was defined as those patients with a good prognosis. The term N-pattern was used for those with 14% and above normal forms and in previous publications had a fertilization rate of more than 80%.

- The abovementioned 1986 and 1988 (Kruger et al., 1986; Kruger et al., 1988) publications became the 2nd and 3rd most quoted publications in the history of Fertility and Sterility as reported in 2006 (Yang and Pan 2006).

- It took more than 10 years after the first publication on sperm morphology in 1986, with numerous publications and debates at international conferences to settle the sperm morphology ‘predictive’ issue for in vitro fertilization. A PhD student in the Department of Reproductive Medicine (Tygerberg Hospital), Dr Kevin Coetzee used a structured literature review to evaluate the question, whether there is a universal predictive value of normal morphology (Strict Criteria) in IVF? The positive findings were published in 1998 (Coetzee et al., 1998).

- The hemi-zona assay was developed at the Jones Institute in 1986 (Burkman et al., 1988) and numerous clinical (Oehninger et al., 2000; Franken et al., 1989) and laboratory related articles followed (Hodgen et al., 1988; Franken et al., 1990; Oehninger et al., 1991).

- A good correlation was observed between poor penetration of hamster oocytes as well as poor binding to the Hemi-zona assay (HZA) in patients with P-pattern sperm morphology. It was also reported that poor morphology correlates with abnormal calcium influx (Oehninger et al., 1994) and abnormal acrosome reaction (Bastiaan et al., 2003).

- The publications on computerized strict sperm morphology were novel in this field. In 1993 the first publication in the international literature followed after research at Stellenbosch University and Tygerberg Hospital on the computerized method. Excellent correlation between this method and the manual methods was reported (Kruger et al., 1993).
In 1993 a study (Kruger et al., 1993) on fertilization rates using computerized strict sperm morphology followed. Patients with sperm morphology < 14% and > 14% normal forms in 2 centers, showed a significantly lower fertilization rates in the group below 14% normal forms. These findings highlighted the fact that a new diagnostic tool to predict fertilization in vitro was now available.

In 1999 the WHO accepted the concept of Strict Criteria for the first time.

On sperm morphology and IUI it was shown in 2001 that the pregnancy rate in the P-pattern was significantly lower than the G-pattern (Van Waart et al., 2001).

It was the work of Bartoov in 2001 (Bartoov et al., 2001) that brought the concept of sperm selection to the forefront. The selection of spermatozoa with high magnification, selecting the ideal form, solved poor pregnancy rates in couples with repeated failures. This method was called IMSI. If no normal sperm could be found for sperm injection using the second best sperm, Berkovitz observed a poorer embryo quality and lower pregnancy rates with higher abortion rates (Berkovitz et al., 2006).

In 2005 a publication followed suggesting semen thresholds for the in vivo situation. The following semen values were suggested for the sub fertile male: Morphology < 5% normal forms, concentration below 10 million per ml. and motility below 30%. It was emphasized that by using the sperm morphology parameter in combination with the other semen values, the clinical value of the semen analysis should be improved (Van der Merwe et al., 2005).

The observation by Huzar (Huzar et al., 2007) that the normal forms bound to the zona were rich in hyaluronic acid (HA) and that there were less aneuploidy reported in the shapes observed were of great interest to the scientific community. This led to the publication by Prinosilova (Prinosilova et al., 2009). It was observed that a significant improvement in selection of the ideal forms took place in the hyaluronic acid group (test) compared to control.

In 2009 Christiaan Hoogendijk (Hoogendijk et al., 2009), one of the PhD students at Stellenbosch University and Tygerberg Hospital was encouraged to look at other methods to select sperm. He studied apoptosis in sperm populations. With annexin V binding he observed that non-apoptotic sperm had more normal forms compared to the apoptotic sperm confirming the observation that even the random selection of the ideal form will possibly assist in obtaining a ‘healthier’ sperm to be used for ICSI.

The findings of Bartoov (Bartoov et al., 2001) on IMSI/ICSI and sperm morphology correlates with the observations on strict morphology and the description of the normal form (Menkveld.
et al., 1990) as well as with the publications on the impact of low morphology on fertilization and pregnancy rates in vitro (Kruger et al., 1986).

- In 2010, the WHO accepted Strict Criteria as the international standard for sperm morphology evaluation. The suggested semen thresholds for sub fertility as proposed by us in 2005 (van der Merwe et al., 2005) was also endorsed using a large population based study (Cooper et al., 2010).

Conclusion:

Since the first publication in 1986, the international scientific community’s perception of sperm morphology was changed. Not only were new morphology patterns described with clinical application, but it was also shown that the P-pattern group had more DNA abnormalities, sperm function was defective and the potential to fertilize in vitro or in vivo was reduced. New semen fertility thresholds were suggested which was accepted in 2010 by the WHO. The use of a computerized method to evaluate sperm morphology brought a new objective tool to evaluate the male factor.

Initially an attempt to overcome the low fertilization chance in the P-pattern morphology group was to increase the sperm insemination concentration. With the development of the ICSI technique this problem was partially overcome but to improve pregnancy outcome required better sperm selection methods (strict evaluation). The prognosis of severe male factor patients and those with P-pattern morphology was thus further improved using sperm selection techniques. These methods were carefully researched looking at DNA fragmentation and aneuploidy rates within certain morphological types of spermatozoa.

The research on sperm morphology patterns led to a better understanding of sperm function and also improved prognosis in patients with severe teratozoospermia. It also had an impact on pre-graduate training of medical students and numerous publications and textbooks followed to assist postgraduate students.
The 1986 publication on strict sperm morphology, where it was indicted that this parameter affects fertilization *in vitro*, had a tremendous impact on research in the field of andrology and assisted reproduction (ART). This brought new insight in many aspects of sperm pathophysiology and thus pioneered solutions for many patients with male factor infertility and specifically men with severe teratozoospermia.
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Oehninger S, Veeck L, Franken D, et al. Human preovulatory oocytes have a higher sperm-binding ability than immature oocytes under hemizona assay (HZA) conditions: evidence supporting the concept of "zona maturation." Fertil Steril 1991;55:1165-1170
