# SPERM DNA FRAGMENTATION:

## IMPLICATIONS IN ASSISTED REPRODUCTIVE TECHNOLOGIES

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Dissertation presented for the degree of Doctor of Philosophy at Stellenbosch University




**Promoter**

**Professor TF Kruger MD**

December 2007

## Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that it has not previously in its entirety or in part been submitted at any university for a degree.

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CF Hoogendijk

November 2007

# SUMMARY

Male fertility has for many years been defined *in vitro* as the ability of sperm to fertilize oocytes and to obtain early cleavage-stage embryos. Spermatozoa comprise of an extraordinary high percentage of polyunsaturated fatty acids in their plasma membrane. Due to an extremely low content of cytoplasm, sperm cells have a particularly low potential to scavenge reactive oxygen species (ROS), and are therefore highly sensitive to oxidative processes, which lead to sperm nucleus DNA damage/fragmentation. Normally, DNA fragmentation occurs in every ejaculate and can be induced by an excessive ROS production of active leukocytes or the spermatozoa themselves. Under distressed conditions, DNA fragmentation may also occur in the testis as a result of oxidative processes in the apoptotic cascade. These DNA fragmentations can be regarded as late signs of programmed cell death (apoptosis).

Clinically, DNA fragmentation in spermatozoa results in significantly decreased implantation and pregnancy rates especially in patients with oligo- and/or teratozoospermia. The p-pattern normal sperm morphology has been shown to give poorer fertilization rates *in vitro* than the g- and n-patterns. In this study there is reported on the significant correlation found between the p-pattern normal sperm morphology and sperm DNA fragmentation as measured with the terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling (TUNEL) assay. This finding further explains the lower fertility potential of patients presenting with p-pattern normal sperm morphology.

In addition, this study explores the intricate relations between ROS in the semen, DNA fragmentation of the spermatozoa, as measured with the TUNEL assay and the sperm chromatin structure assay (SCSA®), spermatozoa apoptotic status and sperm parameters as measured with a standard semen analysis. Positive correlations were found between ROS and the apoptotic status of the sperm, as well as between sperm with non-fragmented DNA and sperm concentration and percentage motility. The results emphasize the importance of sperm selection especially when the treatment of choice is intracytoplasmic sperm injection (ICSI).

An early sign of programmed cell death, also known as apoptosis, is the externalization of phosphatidylserine (PS) from the inner membrane leaflet to the outer leaflet. PS shows a high affinity to Annexin V. Apoptotic spermatozoa are able to fertilize oocytes, but embryo senescence may occur at the time when the paternal genes are activated. In this study there is reported on a novel method whereby spermatozoa can be separated on the basis of their apoptotic status through flow cytometry. Results showed that the normal sperm morphology, according to strict criteria, of the resultant non-apoptotic sperm fraction is significantly higher than that of the apoptotic counterpart. With refinement of this technique, it will be possible in future to use these separated non-apoptotic sperm cells during ICSI for fertilization.

From the above it is apparent that the spermatozoon has to play a vital role in the development of the embryo from fertilization to implantation and pregnancy. It is, however, important to note that besides the gametes, there are *other critical factors* which contribute to a successful *in vitro* fertilization (IVF) cycle, among these are the *in vitro* culture conditions. In this regard, this study compared two sequential embryo culture systems. It was found that the more complex medium resulted in better day three embryo quality and a better blastocyst formation rate and pregnancy rate.

These findings highlight the importance of a holistic perspective towards the complexity of the factors involved in affecting embryo quality and pregnancy outcome.

# OPSOMMING

Manlike fertiliteit is vir baie jare gedefinieer as die *in vitro* vermoë van ‘n spermsel om ‘n eiersel te bevrug om sodoende embrios te verkry. Die spermsel se plasmamembraan bestaan uit ‘n hoë persentasie poli-onversadigde vetsure. As gevolg van die klein hoeveelhede sitoplasma van die spermsel het dit ‘n beperkte weerstand teen reaktiewe suurstof spesies (ROS) en is gevolglik baie sensitief vir oksidasie. Oksidasie lei tot DNS skade/fragmentasie. DNS fragmentasie kom in spermselle van alle ejakulate voor en is gewoonlik die gevolg van ROS produksie deur die leukosiete in die semen of vanaf die spermselle self. Onder sekere omstandighede kan DNS fragmentasie ook voorkom in die testis waar dit deel vorm van apoptose. Hierdie tipe DNS skade word gesien as laat tekens van geprogrammeerde seldood (apoptose).

In oligo- en/of teratozoospermiese mans lei DNS fragmentasie tot verlaagde implantasie- en swangerskapssyfers. Die p-patroon normale sperm morfologie groep gee laer *in vitro* bevrugting en swangerskapsyfers as die g- en n-patrone. In hierdie studie doen ons verslag oor die statisties betekenisvolle korrelasie wat gevind is tussen die p-patroon normale sperm morfologie en DNS fragmentasie soos gemeet met die ‘terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling’ of te wel TUNEL toets. Hierdie bevinding is ‘n verdere verklaring vir die laer fertiliteits potensiaal van pasiënte wat voordoen met p-patroon sperm morfologie.

‘n Verdere doel van die studie was om die moontlike verband tussen ROS in die semen, spermatozoa DNS fragmentasie, apoptotiese status van die sperms en die motiliteits parameters van die spermatozoa te bepaal. ‘n Positiewe korrelasie is gevind tussen ROS en sperm apoptotiese status. Sperms met ongeframenteerde DNS is ook positief gekorreleer met sperm konsentrasie en motiliteit. Die resultate beklemtoon die belangrikheid van spermseleksie veral in pasiënte waar die keuse van behandeling intrasitoplasmiese sperm inspuiting (ICSI) is.

‘n Vroeë teken van apoptose is die eksternalisering van ‘phosphatidylserine’ (PS) vanaf die interne oppervlakte van die plasmamembraan na die eksterne oppervlak. PS het ‘n hoë affiniteit vir Annexin V. Apoptotiese sperms het die vermoë om ‘n oösiet te bevrug,

maar kan lei tot die staking van embrio deling wanneer die vaderlike gene ‘n rol begin speel in embrio ontwikkeling. In hierdie studie het ons ‘n nuwe metode ontwikkel waarvolgens die spermatozoa in die ejakulaat op grond van hul apoptotiese status geskei kan word in apoptotiese en nie-apoptotiese fraksies. Die normale sperm morfologie van die nie-apoptotiese fraksie is betekenisvol beter as dié van die apoptotiese fraksie. Verdere verfyning van die tegniek kan daartoe lei dat dit in die toekoms toegepas kan word om vir nie-apoptotiese sperms te selekteer veral voor die uitvoering van ICSI.

Uit die bogenoemde is dit duidelik dat die spermsel ‘n baie belangrike rol in die ontwikkeling van ‘n embrio, vanaf bevrugting tot implantasie en swangerskap, speel. Dit is egter ook belangrik om in gedagte te hou dat daar ander bydraende faktore tot ‘n suksesvolle *in vitro* swangerskap is, soos laboratorium toestande en embrio kultuursisteem. Om hierdie rede is daar ook twee kultuurmedia in hierdie studie vergelyk. Daar is bevind dat die meer komplekse medium beter kwaliteit embrios op dag drie lewer, asook meer blastosiste en ‘n hoër swangerskapsyfer.

Dit is dus duidelik dat dit uiters belangrik is om ‘n holistiese perspektief te hê op die komplekse faktore wat ‘n invloed mag hê op bevrugting, embrio kwaliteit asook die swangerskapsyfer.

*To my family with love*

"Great is the art of beginning, but greater is the art of ending."
*Lazurus Long*

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# CHAPTER HIGHLIGHTS

## Summary

Human Fertilization represents an essential, utmost complex process which is influenced by numerous oocyte and spermatozoal parameters *in vivo* and *in vitro.* Furthermore, if the *in vitro* situation is examined, the conditions provided by the culture system should also be taken into account. Since different studies repeatedly showed that functional parameters of spermatozoa have considerate influence on this whole process, scientific interest mainly focuses on parameters like motility, morphology, acrosome reaction or the ability of male germ cells to bind to the zona pellucida. Only recently, the integrity of sperm DNA has been recognized as an important contributing factor to fertilization and pregnancy, and is therefore been invetisgated. It was shown that sperm DNA integrity is not only correlated with the fertilization process and pregnancy but also with sperm concentration in the ejaculate, as well as sperm motility. This knowledge is of eminent importance as damages of the paternal DNA or genome can result in early embryonic death. Moreover, on the basis of a lack of repair mechanisms of the male genome there are reasons to assume that the fertilization of oocytes with spermatozoa derived from ejaculates with an increased percentage of sperm DNA fragmentation can result in an elevated risk of early childhood cancer, especially in the male offspring.

As possible causes for these sperm DNA fragmentations three different hypotheses are discussed in the literature, namely oxidative stress, insufficient DNA packaging during spermatogenesis and apoptosis, and for each of these mechanisms good reasons are given. In many of the articles favoring the apoptosis hypothesis, the determination of sperm DNA fragmentation is normally performed by means of employing the TUNEL or COMET assay, which is both regarded as late markers of apoptosis. However, DNA fragmentation can also be caused by other factors like reactive oxygen species (ROS). Therefore, in order to verify apoptosis as being the process causing sperm DNA fragmentation, additional apoptotic parameters like FAS expression, the externalization of phosphetidylserine or the presence of activated key enzymes of the apoptotic process, caspases, especially the effector caspases 2, 3 and 7, should be determined.

Within the scope of this doctoral thesis it was the aim to link sperm morphology to sperm DNA fragmentation as male fertility predictors in order to obtain a better understanding of the origin and predictive value of these paramount parameters in andrological diagnosis. The development of a method to select spermatozoa based on sperm morphology and DNA fragmentation that are most capable to fertilize and lead to a healthy pregnancy was also investigated. In addition, another aim of this study was to find ways of improving blastocyst formation rate and therefore pregnancy rate by choosing an appropriate culture medium for assisted reproduction.

# Chapter I

## A: Anatomy and molecular morphology of the spermatozoon

This chapter summarizes light- and electron-microscopic features that outline the basic characteristics of the anatomy of the human spermatozoon. Furthermore, sperm chromosomes are discussed by means of the highly ordered and specific structure and packaging of the chromatin, together with the potential relationship between the increased incidence of numerical chromosomal aberrations and abnormal sperm morphology observed in infertile men.

This chapter is an extension of an original chapter by Kruger TF, Menkveld R and Oehninger S (1996) Anatomy of the mature spermatozoon. In Acosta AA and Kruger TF (eds) Human spermatozoa in assisted reproduction. 2nd edn, The Parthenon Publishing Group, New York, USA, pp.13-17.

*This review was published*
*Hoogendijk CF, Kruger TF and Menkveld R (2007) Anatomy and molecular morphology of the spermatozoon. In Oehninger SC and Kruger TF (eds) Male Infertility – Diagnosis and Treatment. Informa UK Ltd., Oxon, UK, pp.3-11.*

## **B:** The pathophysiology and genetics of human male reproduction

This chapter is a review of the process of spermatogenesis and also the role of the spermatozoon in embryogenesis. In order to understand the physiology of fertilization, understanding the mechanism of spermatogenesis and its morphological and genetic processes is of paramount importance. Spermatozoa are produced in a unique process named spermatogenesis. During this process, spermatogenic stem cells undergo reduction of the genome from diploid cells to haploid cells, and also unequaled morphological and functional changes. Thus, spermatozoa are not only the smallest (length of sperm head: 4-5 µm) and most polarized cells (sperm head in front; flagellum at rear) in the body, but also the only cells that fulfill their function outside the body and even in a different individual. The final destination is the female reproductive tract. Therefore, spermatozoa are highly specialized cells, simply a “means of transportation”, that transport genetic information from the male to the female. For this purpose, the oocyte and sperm cell require specific physiological functions. In order for the sperm cells to acquire these functions, morphological and physiological development of the spermatozoa has to take place. In addition, a proper chromosomal and genetic constitution is mandatory, i.e. chromosomal and DNA integrity must be given.

During spermatogenesis, spermatozoa which eventually have to mature during epididymal maturation, acquire the morphological and physiological foundations for normal sperm function. This means, that if the processes taking place in the course of spermatogenesis are defective, dysfunctional male germ cells, will be formed.

*This review was published*

*Hoogendijk CF and Henkel R (2007) The pathophysiology and genetics of human male reproduction. In Oehninger SC and Kruger TF (eds) Male Infertility – Diagnosis and Treatment. Informa UK Ltd., Oxon, UK, pp.35-48.*

## Chapter II

### The correlation of DNA fragmentation, apoptosis and reactive oxygen species (ROS) with functional semen parameters

In this chapter, the results of the above mentioned study was reported and discussed. DNA fragmentation of spermatozoa was determined with two assays; firstly the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay and secondly the sperm chromatin structure assys (SCSA®). The apoptotic status of the spermatozoa was determined with binding of biotinylated Annexin V to phosphatidylserine (PS). The aforementioned three assays were performed with flow cytometry. Reactive oxygen species (ROS) in the semen was also determined. All of these parameters were correlated with routine semen analysis parameters as well as computer assisted semen analysis (CASA) motility and velocity parameters. The results showed significant negative correlations between ROS and sperm concentration ($P < 0.0001$) and non-apoptotic sperm ($P < 0.01$). Non-apoptotic sperm had significant positive correlations with sperm motility ($P < 0.05$) and concentration ($P < 0.0001$). TUNEL-negative sperm had significant positive correlations with sperm motility ($P < 0.001$) and concentration ($P < 0.0001$). The application of these findings in clinical practice can ultimately increase implantation and pregnancy rates in patients where ICSI is the treatment of choice.

*The results from this chapter were submitted for publication to Human Reproduction.*
***Manuscript ID: HUMREP-07-0777.***

## Chapter III

### Human spermatozoa with P-pattern normal sperm morphology according to strict criteria show increased levels of DNA fragmentation

In this chapter the results of the above mentioned study are reported and discussed. Since one semen parameter, normal sperm morphology, which has repeatedly been shown to have a strong correlation with the fertilization potential of spermatozoa appears to have the highest predictive power for fertilization *in vitro,* this study aimed to

investigate the correlation between normal sperm morphology and sperm DNA fragmentation and apoptosis, respectively. The results of the study showed a significant positive correlation for the p-pattern (0 – 4%) morphology group and the percentage of sperm with intact DNA (according to the terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling (TUNEL) assay). 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 normal appearing spermatozoa to ultimately increase implantation and pregnancy rates in these patients as well as ensure the resulting offspring's health.

*The results from this chapter were submitted for publication to Human Reproduction* ***Manuscript ID: HUMREP-07-0778.***

## Chapter IV

### The selection of non-apoptotic sperm using annexin V binding and flow cytometry

In this chapter, a non-invasive method whereby apoptotic and non-apoptotic sperm can be separated was developed. Using this method the resultant non-apototic sperm subgroup can still be used for fertilization with ICSI. In addition, after separation, normal sperm morphology according to Tygerberg's strict criteria was assessed and the sperm morphology profile between non-apoptotic and apoptotic sperm charted. The results showed that separating non-apoptotic and apoptotic sperm by means of flow cytometry proved to be successful as there was a significant enrichment of both apoptotic and non-apoptotic sperm in the respective subpopulations. Furthermore, there was a significant increase in the median normal sperm morphology between the apoptotic and the non-apoptotic sperm subpopulations. The non-apoptotic sperm subpopulation had morphologically superior quality sperm compared to apoptotic sperm.

*The results from this chapter were submitted for publication to Fertility and Sterility.* ***Manuscript ID: F and S4252.***

# Chapter V

## A study of two sequential culture media – impact on human embryo quality and pregnancy rates

All the above mentioned studies (Chapters 2, 3, and 4) concentrated on the spermatozoon's contribution towards the development of an embryo and a pregnancy. It is, however, important to realize that embryo development is dependent on many other factors. Of these, the *in vitro* culture conditions are very important, as these are artificially created to simulate the physiological *in vivo* environment.

This study assessed pregnancy outcomes after transfer of day 3 embryos and day 5 blastocysts obtained in Sydney IVF Medium and Quinn's Advantage Sequential Culture Medium. The *in vitro* development of embryos in these sequential media using embryo cleavage rate, embryo morphology (day 2 and 3) and blastocyst formation rate as end-points, were compared. The results indicated that the sequential range of Quinn's Advantage Sequential Culture Media was more beneficial for *in vitro* embryo culture as each of the media in the range contributed collectively, resulting in more embryos with a better quality. The reason for the significant increase in embryo developmental parameters and increase pregnancy rate can possibly be attributed to the differences in the composition between the two media.

*The results of this chapter were published*
*Hoogendijk CF, Kruger TF, Windt ML, Siebert TI and Henkel R (2007) A study of two sequential culture media – impact on embryo quality and pregnancy. SAJOG, 13, 52-58.*

# Conclusion

The results of this study showed significant relationships between sperm DNA damage and concentration, motility and normal sperm morphology. In addition, the study found a significant relationship between ROS and Annexin-V binding. The <5% normal sperm morphology group (p-pattern) had significantly more DNA damage than the ≥5% normal sperm morphology group (g- and n-pattern). This was also the first study reporting on the separation of p- and g-pattern spermatozoa by means of flow cytometry on the basis of fluorescence labeling with Annexin V. Furthermore, this study showed that the two assays referred to for detecting sperm DNA damage, i.e. the TUNEL assay and the sperm chromatin structure assay (SCSA), are in fact detecting not the same but different aspects of sperm DNA damage. These are important findings that contribute to both the treatment and the diagnostics of male infertility. The importance of these findings lies in the fact that sperm DNA damage can significantly affect the infant's, particularly boys, health as such damage can lead to early childhood cancer. It is therefore not only important to identify patients with an elevated risk but also to increase the number of "healthy' sperm that can be used for the fertilization process. This study also clearly shows that poor sperm morphology is closely linked to these DNA damages and it is therefore mandatory to carefully assess sperm morphology. Finally, since the environment of the embryo is essential for its healthy growth, the study results furthermore showed the contribution of the correct culture conditions towards embryo development and the onset of pregnancy.

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# CHAPTER I

## Introduction

# PART A

## Anatomy and molecular morphology of the spermatozoon

## INTRODUCTION

This chapter summarizes light- and electron-microscopic features that outline the basic characteristics of the anatomy of the human spermatozoon. Furthermore, sperm chromosomes are discussed at the hand of the highly ordered and specific structure and packaging of the chromatin, together with the potential relationship between the increased incidence of numerical chromosomal aberrations and abnormal sperm morphology observed in infertile men.

## LIGHT AND ELECTRON MICROSCOPIC MORPHOLOGICAL CHARACTERISTICS OF SPERMATOZOA

Mature spermatozoa are highly specialized and condensed cells that do not grow or divide. A spermatozoon consists of a head, containing the paternal heredity material (DNA), and a tail, which provides motility (Figures 1.1 and 1.2).

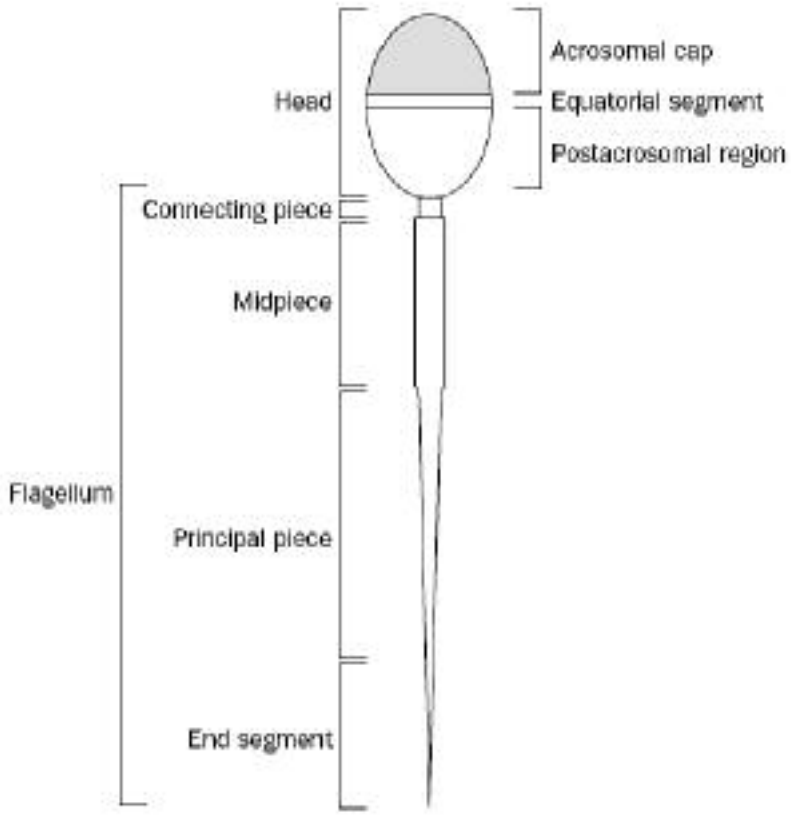


**Figure 1.1** Schematic drawing of light microscopic human spermatozoon

The spermatozoon is endowed with a large nucleus but lacks the large cytoplasm that is characteristic of most somatic cells. Men are unique among mammals in the degree of morphological heterogeneity of spermatozoa found in the ejaculate (Hafez, 1976; Kruger *et al.*, 1986; Menkveld *et al.*, 1990).

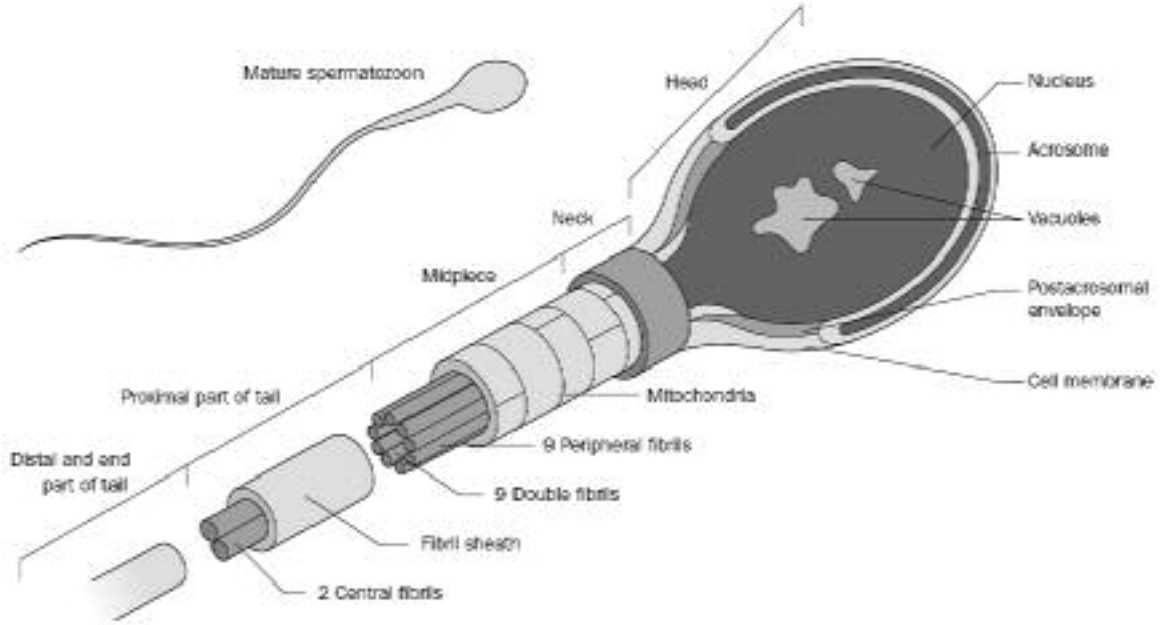


Figure 1.2 Light and electron microscopic diagrams of human spermatozoon

## SPERM HEAD

### Light microscopy

Human spermatozoa are classified using bright field microscope optics on fixed, stained specimens (Kruger *et al.*, 1986; Menkveld *et al.*, 1990). 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 (Katz *et al.*, 1986). The normal head should be oval in shape. Allowing for slight shrinkage that the 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 (Katz *et al.*, 1986). Two slightly different types of normal spermatozoa head forms have been described based on spermatozoa found in endocervical canal mucus after coitus (Menkveld *et al.*, 1990). 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 (Menkveld *et al.*, 1990). Since diversity is a fact of all biological systems, trivial variations must be regarded as normal (Menkveld *et al.*, 1990).

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 (WHO, 1992; 1999). Human spermatozoa have a well-defined acrosomal region comprising about two-thirds of the anterior head area (Kruger *et al.*, 1986; Menkveld *et al.*, 1990; WHO, 1992). They do not exhibit an apical thickening like many other species, but show a uniform thickness/thinning towards the end forming the equatorial segment. Because of this thinning, the area appears more intensely stained when examined with the light microscope. Depending on the 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 the three-dimensional image of SEM, 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 (Hafez, 1976). 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 (Hafez, 1976).

Transmission electron microscopy

The electron microscopic morphological characteristics of human spermatozoa are presented in Figures 1.2–1.6. The sperm head is a flattened ovoid structure consisting primarily of the nucleus. The acrosome is a cap like structure covering the anterior two-thirds of the sperm head (Figures 1.2 and 1.3), which arises from the Golgi apparatus of the spermatid as it differentiates into a spermatozoon. Unlike that in 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 (Hafez, 1976).

During fertilization of the egg, the enzyme-rich contents of the acrosome are released at the time of acrosome reaction. During fusion of the outer 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 (Barros and Franklin, 1986). The equatorial segment of the acrosome persists more or less intact since it does not participate in acrosome reaction (Figure 1.3).

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 1.3). The nucleus (Figure 1.3), 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 (Hafez, 1976).

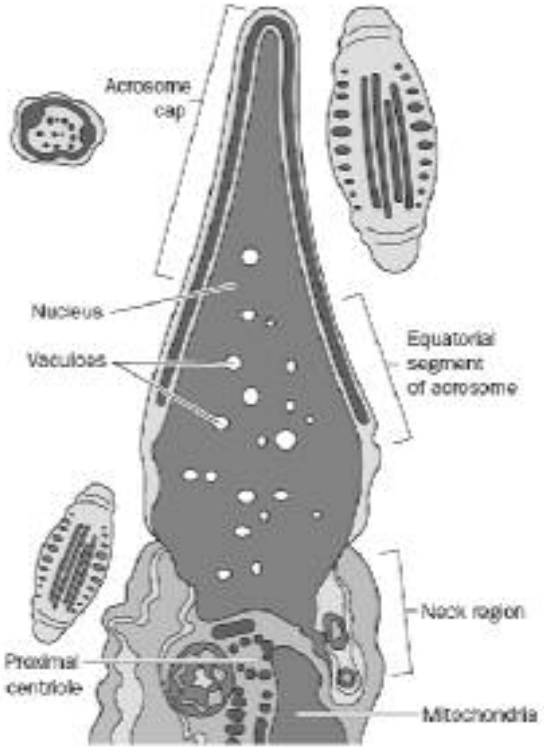


Figure 1.3 Schematic drawing of longitudinal section of sperm head

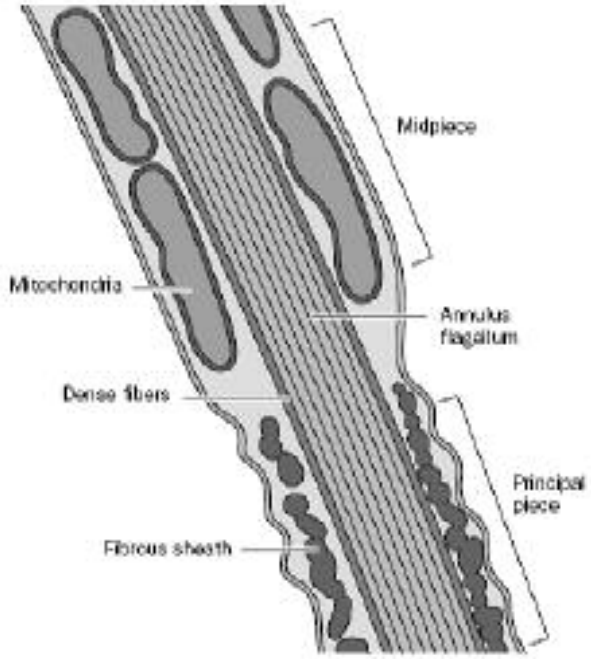


Figure 1.4 Longitudinal section of region between the midpiece and principal piece of human spermatozoon

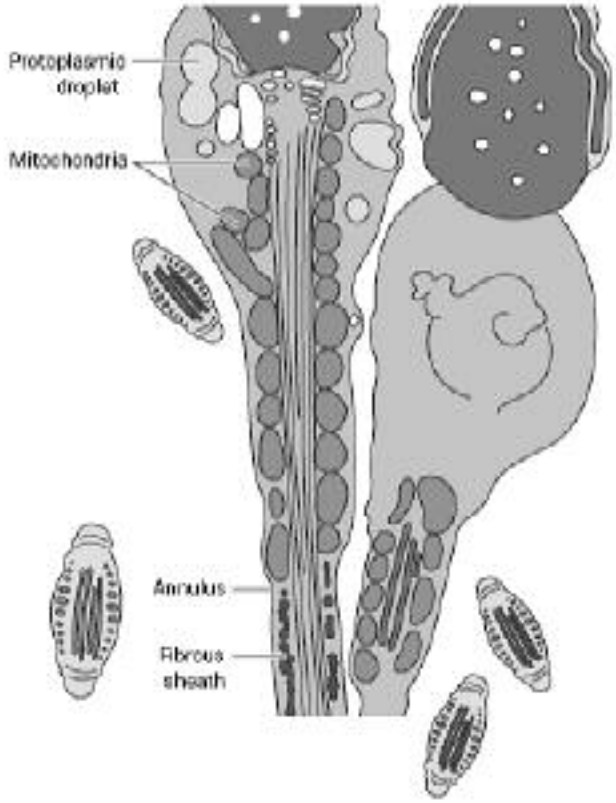


Figure 1.5 Longitudinal section through mid-piece

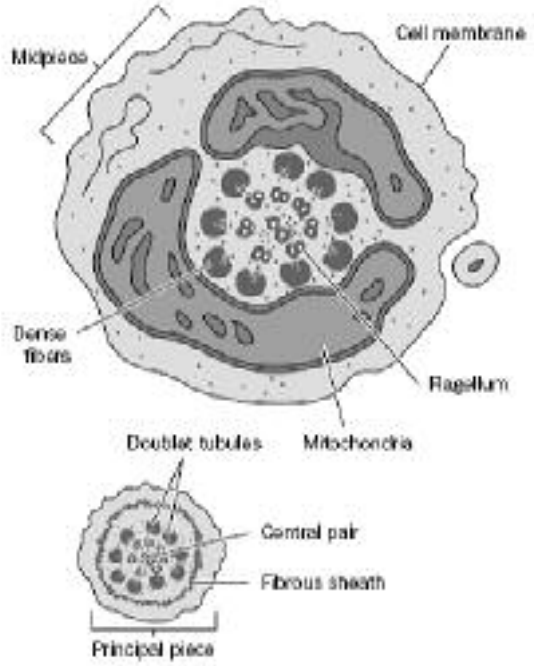


Figure 1.6 Cross-section of human sperm tail

## Molecular morphology

The sperm chromosome structure is very complex. Some of the attributes are similar to somatic cell DNA organization and others are unique to spermatogenic cells. Sperm DNA packaging can be subdivided into four levels.

### Level I: Chromosomal Anchoring by the Nuclear Annulus

The two strands of naked DNA, which makes up each chromosome, are attached to a sperm specific structure, the nuclear annulus. This represents a novel type of DNA organization, termed chromosomal anchoring, that is only found in spermatogenic cells. The nuclear annulus is shaped like a bent ring, and is about 2 µm in length. It is found only in sperm nuclei, although it is currently unknown at what stage of spermiogenesis it is first formed. So far there is no evidence for a nuclear annulus-like structure in any somatic cell type. In contrast, there is evidence of its existence in hamster (Ward and Coffey, 1989), human (Barone *et al*., 1994), mouse, and *Xenopus* sperm nuclei. Its existence in a wide variety of species suggests a fundamental role in sperm function.

Unique DNA sequences were found to be associated with the nuclear annulus. Ward (1993) termed these sequences NA-DNA. The existence of these unique sequences suggests that the nuclear annulus anchors chromosomes by particular sequences and not by random DNA binding. By organizing the chromosomes so that the NA-DNA sites of each chromosome are aggregated onto one structure, the nuclear annulus may also affect the determination of sperm nuclear shape. For example, in the hamster spermatozoon the longer chromosomes may extend into the thinner hook of the nucleus, while a portion of every chromosome is located at the nuclear annulus. This is supported by image analysis of the distribution of DNA throughout the hamster sperm nucleus, which demonstrated that the highest concentration of DNA in the packaged sperm nucleus is at the base where the nuclear annulus is located; in contrast the lowest concentration of DNA is in the hooked portion (Ward *et al*., 1996).

The hypothesis of the possible structure of sperm chromatin packaging is further supported by electron microscopic evidence that the chromatin near the implantation

fossa is one of the first areas to condense during spermiogenesis (Loir and Courtens, 1979). Thus, the nuclear annulus may represent the only known aspect of sperm chromatin condensation that is specific for individual chromosome sites.

Level II: Sperm DNA loop domain organization

Anchored chromosomes are organized into DNA loop domains. Parts of the nuclear matrix, protein structural fibers, attach to the DNA every 30 to 50 kb by specific sequences termed matrix attachment regions (MARs). This arranges the chromosome strands into a series of loops. This type of organization can be visualized experimentally in preparations; known as nuclear halos. Halos consist of loops of naked DNA, 25 to 100 kb in length, attached at their bases to the matrix. Each loop domain visible in the nuclear halo consists of a structural unit of chromatin that exists in vivo in a condensed form.

The organization of DNA into loop domains is the only type of structural organization resolved thus far that is present in both somatic and sperm cells. In somatic cells, DNA is coiled into nucleosomes, then further coiled into a 30 nm solenoid fiber, and then organized into DNA loop domains. The corresponding structures in sperm chromatin have a very different appearance. Protamine binding causes a different type of coiling, and DNA is folded into densely packed toroids, but still organized into loop domains. Mammalian sperm nuclei do contain a small amount of histones that are presumably organized into nucleosomes (Tanphaichitr *et al*., 1978; Choudhary *et al.*, 1995) but most of the DNA is reorganized by protamines. This means that with the evolutionary pressure to condense sperm DNA, all aspects of chromatin structure is sacrificed other than the organization of DNA into loop domains. This suggests DNA loop domains play a crucial role in sperm DNA function.

Level III: Protamine decondensation

The binding of protamines condenses the DNA loops into tightly packaged chromatin. DNA protamin binding forms toroidal or doughnut-shaped structures in which the DNA is very concentrated (Hud *et al.,* 1995). During spermiogenesis, histones, the DNA binding proteins of somatic spermatogenic precursor cells, are replaced by protamines. Since histone bound DNA requires much more volume than the same amount of DNA bound to protamines (Ward and Coffey, 1991), this change in

chromatin structure probably accounts for some of the nuclear condensation that occurs during spermiogenesis. Protamines bind DNA along the major groove; this completely neutralizes DNA so that neighboring DNA strands bind to each other by Van der Waal's forces. Protamine binding leads to the condensation and preservation of the DNA loop domain organization present in the round spermatid (Ward, 1993).

Level IV: Chromosome organization

The results of several studies (Zalensky *et al*., 1995; Haaf and Ward, 1995; Ward *et al*., 1996) have led to the proposal of a model (Ward and Zalensky, 1996) in which there are limited constraints on the actual position of the chromosomes in the sperm nucleus. The NA-DNA sequences are located at the base of the nucleus, centromeres are located centrally, and the telomeres located peripherally. Outside of these three constraints, the folding of the chromosomal p and q arms is flexible.

## SPERM TAIL

### Light Microscopy

The sperm tail formation arises at the spermatid stage. During spermatogenesis the centriole is differentiated into three parts: midpiece, main or principle piece and endpiece (Figures 1.1 and 2.1). The midpiece has a similar length to the head, and is separated from the tailpiece by a ring, the annulus (Figure 1.5). The following tail aberrations can be observed:

(1) Neck and midpiece aberrations, including their absent (seen as 'free' or 'loose' heads), non-inserted 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 (WHO, 1992);

(2) Tail aberrations include short, multiple, hairpin, broken (angulation >90°) tails, irregular width, coiling tails with terminal droplets, or any combination of these (WHO, 1992);

(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 (Menkveld, 1990; WHO, 1992). The endpiece is not distinctly visualized by light microscopy.

Scanning electron microscopy

With SEM the tail can be subdivided into three distinct parts, i.e. midpiece, principle piece and end piece. 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 end piece has a small diameter due to the absence of outer dense fibers (ODF) (Hafez, 1976).

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 1.2, 1.5 and 1.6). The midpiece provides the sperm with the energy necessary for motility. The central axial core of eleven fibrils is surrounded by an additional outer ring of nine coarser fibrils (Figures 1.2 and 1.6). Individual mitochondria are wrapped around these ODF in a spiral manner to form the mitochondrial sheath, which contains the enzymes involved in the oxidative metabolism of the sperm (Figures 1.2 and 1.4-1.6). The mitochondrial sheath of the midpiece is relatively short, slightly longer than the combined length of the head and neck (Hafez, 1976).

The principle piece (main piece), the longest part of the tail, provides most of the propellant machinery. The ODF 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 principle piece (Figure 1.2) (White, 1974). 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 (Hafez, 1976). The tail terminates in the end piece 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 distal fading of microtubules.

## SPERM MORPHOLOGY AND CHROMOSOMAL ANEUPLOIDIES

Many authors have studied the association between abnormal sperm shape and increased frequency of aneuploidies. The conclusions of these studies are inconsistent; this is most probably because the sperm attributes were evaluated in the same semen sample, but not in the same sperm. As early as 1991, Martin studied sperm karyotypes (Martin, 1991). This author demonstrated that all chromosomes undergo nondisjunction during spermiogenesis but that the G group chromosomes (Spriggs *et al.*, 1996; Bernardini *et al.*, 1998) and the sex chromosomes have a significantly increased frequency of aneuploidy. Using FISH, Spriggs and co-workers (1996) determined that most chromosomes have a disomy frequency of approximately 0.1% (1/1000); in contrast the sex chromosomes and chromosomes 21 and 22 have a significantly increased frequency of aneuploidy. Thus, the sex chromosome bivalent and the G group chromosomes are more susceptible to nondisjunction during spermatogenesis.

Bernardini *et al.* (1998) suggested a relationship between increased frequencies of aneuploidy and diploidy in semen samples containing spermatozoa with enlarged heads. Several other studies have concluded that morphologically abnormal sperm may also have a significantly increased risk for being aneuploid (Colombero *et al*., 1999; Calogero *et al*., 2001; Rubio *et al.*, 2001; Yakin and Kahraman, 2001; Templado *et al.*, 2002). An interesting report, based on examination of sperm injected into mouse oocytes, suggested that in semen samples with high incidences of amorphous, round and elongated sperm heads, there was an increased proportion of structural chromosome abnormalities. These abnormalities include chromosome and chromatid fragments, dicentric and ring chromosomes, but no increase in numerical chromosomal aberrations was observed (Lee *et al*., 1996). In another study Ryu *et al.* (2001) reported on 120 normal and abnormal sperm (according to Tygerberg strict criteria) in eight men each, and concluded however that normal morphology is not a valid indicator for selection of sperm with haploid nuclei. Rives

*et al*. (1999) showed that although the disomy frequencies of infertile males were directly related to the severity of oligozoospermia, there was no relationship between aneuploidy frequency and abnormal morphology. In men with increased levels of globozoospermia, shortened flagella syndrome or sperm with acrosomal abnormalities, no association was found between sperm shape and numerical chromosomal aberrations (Viville *et al*., 2000).

In another study, De Vos and co-workers (2003) determined the influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome after ICSI. With regard to the different morphologic defects observed, they found the following fertilization rates: 63.4% (52 of 82) for spermatozoa with elongated heads, 63.3% (124 of 196) for spermatozoa with cytoplasmic droplets, 59.6% (223 of 374) for spermatozoa with amorphous heads, and 34.1% (15 of 44) for spermatozoa with broken necks. One hundred and one injected spermatozoa showed a combination of two morphologic defects (overall fertilization rate, 57.4%). No fertilization ensued from 6 round-headed spermatozoa lacking acrosomes, and 12 spermatozoa showing vacuoles in their acrosomes provided a fertilization rate of 66.6%. They concluded that sperm morphology assessed at the moment of ICSI correlated well with fertilization outcome but did not affect embryo development. Furthermore, the implantation rate was lower when only embryos resulting from injection of an abnormal spermatozoon were available.

Recently, Celik-Ozenci and co-workers (2004) studied the relationship between sperm shape and numerical chromosomal aberrations in individual spermatozoa, using FISH, objective morphometry, sperm dimension and shape assessment, along with Tygerberg strict criteria. The results indicate that numerical chromosomal aberrations can be present in sperm heads of any size or shape, but the risk is greater with amorphous sperm. Even the most normal appearing sperm with normal head and tail size can be disomic or diploid, although diploidy is less prevalent with normal sperm dimensions and shape.

## CONCLUSION

Although many of the structures described here, especially the ultrastructure characteristics based on electron microscopy studies, are not visible by standard and daily light microscopic examinations, a basic knowledge of these structures is very important for the correct evaluation and interpretation of sperm morphology. In turn, this information will assist the clinician in the estimation of male fertility potential.

From the molecular structure of the sperm, it is evident that the sperm DNA is packaged within the nucleus in an extremely complex and ordered fashion; there is, however, some degree of flexibility to this organization. A detailed model for how chromosomes are packaged in the sperm nucleus is gradually emerging; implications of this knowledge are already impacting on the study of fertility, particularly in preparations of nuclei for ICSI, diagnosis of semen samples, and for understanding the fate of sperm DNA after fertilization. As our knowledge of sperm chromatin increases, the more evident it is that visual assessment is an unreliable method for ICSI selection of sperm. More specific methods for sperm selection, such as hyaluronic acid binding (Huszar *et al*., 2003) may alleviate the problem of fertilization with sperm of diminished maturity and genetic integrity during ICSI.

Original light and electron microscopy photographs, as well as schematic morphology/anatomy drawings (light- and electron microscopy), are depicted in Figures 1.1-1.6. If followed carefully, a clear picture of the basic sperm anatomy will be obtained.

## REFERENCES

Barone JG, De Lara J, Cummings KB and Ward WS (1994) DNA organization in human spermatozoa. J Androl 15, 139-144.

Barros C and Franklin LE (1986) Behavior of the gamete membranes during sperm entry into the mammalian egg. J Cell Biol 37, C13-18.

Bernardini L, Borini A, Preti S, Conte N, Flamigni C, Capitanio GL and Venturini PL (1998) Study of aneuploidy in normal and abnormal germ cells from semen of fertile and infertile men. Hum Reprod 13, 3406–3413.

Calogero AE, De Palma A, Grazioso C, Barone N, Romeo R, Rappazzo G and D'Agata R (2001) Aneuploidy rate in spermatozoa of selected men with abnormal semen parameters. Hum Reprod 16, 1172–1179.

Celik-Ozenci C, Jakab A, Kovacs T, Catalanotti J, Demir R Bray-Ward P, Ward D and Huszar G (2004) Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum Reprod 19, 2052-2059.

Choudhary SK, Wykes SM, Kramer JA, Mohamed AN, Koppitch F, Nelson JE and Krawetz SA (1995) A haploid expressed gene cluster exists as a single chromatin domain in human sperm. J Biol Chem 270, 8755-8762.

Colombero LT, Hariprashad JJ, Tsai MC, Rosenwaks Z and Palermo GD (1999) Incidence of sperm aneuploidy in relation to semen characteristics and assisted reproductive outcome. Fertil Steril 72, 90–96.

De Vos A, Van De Velde H, Joris, H, Verheyen, G, Devroey, P and Van Steirteghem A (2003) Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 79, 42 –48.

Haaf T and Ward WS (1995) Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers. Exp Cell Res 219, 604-611.

Hafez ESE (1976) Human semen and fertility regulation in men. CV Mosby, St. Louis.

Hud NV, Downing KH and Balhorn R (1995) A constant radius of curvature model for the organization of DNA in toroidal condensates. Proc Natl Acad Sci USA 92, 3581-3585.

Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E and Vigue L (2003) Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 79, 1616–1624.

Katz DF, Overstreet JW, Samuels SJ, Niswander PW, Bloom TD and Lewis EL (1986) Morphometric analysis of spermatozoa in the assessment of human male fertility. J Androl 7, 203-210.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA and Smith K (1986) Sperm morphological features as a prognostic factor in in vitro fertilization. Fertil Steril 46, 1118-1123.

Lee JD, Kamiguchi Y and Yanagimachi R (1996) Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. Hum Reprod 11, 1942–1946.

Loir M and Courtens JL (1979) Nuclear reorganization in ram spermatids. J Ultrastruct Res 67, 309-324.

Martin RH (1991) Cytogenetic analysis of sperm from a man heterozygous for a pericentric inversion, inv(3)(p25q21). Am J Hum Genet 48, 856–861.

Menkveld R, Stander FSH, Kotze TJ vW, Kruger TF and Van Zyl JA (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 5, 586-592.

Rives N, Saint CA, Mazurier S, Sibert L, Simeon N, Joly G and Mace B (1999) Relationship between clinical phenotype, semen parameters and aneuploidy frequency in sperm nuclei of 50 infertile males. Hum Genet 105, 266–272.

Rubio C, Gil-Salom M, Simon C, Vidal F, Rodrigo L, Minguez Y, Remohi J and Pellicer A (2001) Incidence of sperm chromosomal abnormalities in a risk population: relationship with sperm quality and ICSI outcome. Hum Reprod 16, 2084–2092.

Ryu HM, Lin WW, Lamb DJ, Chuang W, Lipshultz LI and Bischoff FZ (2001) Increased chromosome X, Y, and 18 nondisjunction in sperm from infertile patients that were identified as normal by strict morphology: implication for intracytoplasmic sperm injection. Fertil Steril 76, 879–883.

Spriggs EL, Rademaker AW and Martin RH (1996) Aneuploidy in human sperm: the use of multicolour FISH to test various theories of nondisjunction. Am J Hum Genet 58, 356–362.

Tanphaichitr N, Sobhon P, Taluppeth N and Chalermisarachai P (1978) Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. Exp Cell Res 117, 347-356.

Templado C, Hoang T, Greene C, Rademaker A, Chernos J and Martin R (2002) Aneuploid spermatozoa in infertile men: teratozoospermia. Mol Reprod Dev 61, 200–204.

Viville S, Mollard R, Bach ML, Falquet C, Gerlinger P and Warter S (2000) Do morphological anomalies reflect chromosomal aneuploidies? Case report. Hum Reprod 15, 2563–2566.

Ward WS (1993) DNA loop domain tertiary structure in mammalian spermatozoa. Biol Reprod 48, 1193-1201.

Ward WS and Coffey DS (1989) Identification of a Sperm Nuclear Annulus: A Sperm DNA Anchor. Biol Reprod 41, 361-370.

Ward WS and Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells (Review). Biol Reprod 44, 569-574.

Ward WS and Zalensky A (1996) The unique, complex organization of the transcriptionally silent sperm chromatin. Crit Rev Euk Gene Exp 6, 139-147.

Ward WS, McNeil J, de Lara J and Lawrence J (1996) Localization of the three genes in the assymetric hamster sperm nucleus by fluorescent in situ hybridization. Biol Repro 54, 1271-1278.

White IG (1974) Mammalian semen. In Hafez, ESE (eds) Reproduction of farm animals, 3rd edn. Lea & Febiger, pp. 102-122.

World Health Organization (1999) WHO Laboratory Manual for Examination of Human Semen. 4th edn Cambridge University Press, London, UK.

World Health Organization (1992). WHO Manual for the examination of human semen and sperm cervical mucus interaction. 2nd edn, Cambridge University Press, London, UK.

Yakin K and Kahraman S (2001) Certain forms of morphological anomalies of spermatozoa may reflect chromosomal aneuploidies. Hum Reprod 16, 1779–1780.

Zalensky AO, Allen MJ, Kobayashi A, Zalenskya IA, Balhorn R and Bradbury EM (1995) Well-defined genome architecture in the human sperm nucleus. Chromos 103, 577-590.

# PART B

# The pathophysiology and genetics of human male reproduction

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## INTRODUCTION

The male germ cells, the spermatozoa, are produced in a unique process named spermatogenesis. During this process, spermatogenic stem cells undergo the reduction of the genome from diploid cells to haploid cells, as well as unequaled morphological and functional changes. In this respect, spermatozoa are not only the smallest (length of sperm head: 4-5 µm) and most polarized cells (sperm head in front; flagellum at rear) in the body, but also the only cells that fulfill their function outside the body, even in a different individual, the female reproductive tract. Therefore, spermatozoa are highly specialized cells, simply a “means of transportation”, that transport the genetic information from the male to the female, the oocyte, for which specific physiological functions of these cells are required. In order for the sperm cells to acquire these functions, the morphological and physiological development of the spermatozoa has to take place. In addition, a proper chromosomal and genetic constitution is mandatory, i.e. chromosomal and DNA integrity must be given.

During spermatogenesis, spermatozoa which eventually have to mature during epididymal maturation, acquire the morphological and physiological foundations for normal sperm function. This means, that if the processes taking place in the course of spermatogenesis are defective, dysfunctional male germ cells, will be formed. Therefore, in order to understand the physiology of fertilization, the understanding of spermatogenesis and its morphological and genetic processes is of paramount importance.

### A: GENETIC CONTROL OF SPERMATOGENESIS

The relationship between structurally abnormal spermatozoa and genetically defective spermatozoa poses a crucial unknown. The long sequence of events involved in spermatogenesis, from germ cell differentiation to functionally mature spermatozoa, is fraught with the possibility of both structural and genetic damage. Spermatogenesis consists of three distinct intervals: (i) proliferation and differentiation of diploid spermatogonial stem cells, (ii) meiosis where chromosome pairs and genetic recombination occurs, and (iii) spermiogenesis. Spermiogenesis is

a unique series of events in which the rather commonplace appearing, albeit haploid, round spermatids, differentiate into species-specific shaped spermatozoa. Collectively, these intervals consist of many developmental events, which offer numerous opportunities for the introduction of damage into the genome of the male gamete. These concerns are exacerbated by the ability of scientists and embryologists to use differentiating male germ cells, prior to the completion of spermatogenesis, for fertilization. This raises the question: are we not introducing "incomplete" male gametes into oocytes?

*Spermatogonial differentiation*

The intricate mechanisms whereby stem cells maintain a population of proliferating and differentiating cells are only beginning to be unraveled (Morrison *et al.*, 1997). In the mammalian testis, spermatogonial type A stem cells proliferate producing three classes of spermatogonia: (i) a group of presumably identical spermatogonial stem cells, (ii) a population of differentiating spermatogonia, and (iii) a large number of cells that undergo cell death by apoptosis (Dym, 1994). The originators of this developmental cascade, the type A spermatogonia, represent a mixed population of cell types designated type $A_0$, $A_1$, $A_2$, $A_3$, or $A_4$ spermatogonia. Among these cells, the identity of "true" stem cells is yet to be definitively established. Although multiple stem cell renewal models have been proposed, one commonly accepted model proposes that type $A_0$ spermatogonia represent a reserve population of stem cells, which divide slowly and can repopulate the testis after damage (Dym and Clermont, 1970). Thus, the types $A_1$ to $A_4$ spermatogonia are believed to be the renewing stem cell spermatogonia and it is these cells that maintain the fertility of a man. The type A spermatogonia differentiate into intermediate and type B spermatogonia, which in turn divide and enter the differentiating pathway leading to spermatozoa. These cellular programming events appear to be irreversible, because once committed to differentiation, the spermatogonia appear incapable of re-entering the pathway that produces stem cells. The implications of genetically defective spermatogonia are substantial, since it is these cells that will function as the precursors of spermatozoa throughout the life of the individual. The large number of spermatogonial stem cells that undergo apoptosis suggests that a sophisticated monitoring system has evolved in which "defective" stem cells are removed. Currently, much effort is being directed towards studies defining mechanisms of apoptosis in somatic cells. Research efforts

need to be extended to define the mechanisms by which specific populations of stem cells are selected to be targets for cell death. Specifically in the testis, an understanding of how the differentiating germ cells are continually being assessed, presumably by a self monitoring system, will help greatly to minimize the production of genetically defective germ cells.

*Meiosis*

Meiosis represents a fascinating interval of spermatogenesis in which genetic alterations, including genetic damages are intentionally introduced into the genome, which in turn contribute to the evolutionary change of species. In addition to its essential role in producing haploid gametes from diploid stem cells, the extended interval of meiotic prophase has evolved to provide the critical cellular milieu for precise genetic recombination (Stern, 1993).

Meiotic prophase commences with preleptotene primary spermatocytes, the cell type in which the last semiconcervative DNA replication of the male germ cell occurs. All subsequent DNA synthesis in the differentiating male germ cells represents DNA repair synthesis. Chromosome condensation initiates concomitantly with the movement of the leptotene and zygotene spermatocytes to the adluminal compartment of the seminiferous tubule from the basal membrane region. Alignment and complete pairing of the chromosomal homologues are completed in pachytene spermatocytes. As the chromosomes condense, axial elements appear between the two sister chromatids of each chromosomal homologue. The addition of a visible central element to the chromosomes produces the synaptonemal complex, a highly conserved structure in the meiotic cells of organisms ranging from the water mould to humans that is needed for effective synapsis. Because synapsis of chromosomes represents an event unique and critical to genetic recombination, meiotic cells contain many novel structural proteins and enzymes needed for chromosome and DNA alignment, DNA breakage, recombination and DNA repair. Among the proteins recently shown to be important in the genetic recombination process are Rad 51, a human homologue of a bacterial recombination protein (Scully *et al.*, 1997); BRCA1, a tumor suppresser gene implicated in familial breast and ovarian cancers (Scully *et al.*, 1997); ATM-related genes, members of a gene family proposed to prevent DNA damage (Zakian, 1995; Barlow *et al.*, 1996; Keegan *et al.*, 1996); a ubiquitin

conjugating repair enzyme believed to be involved in protein turnover (Kovalenko *et al.*, 1996; Roest *et al.*, 1996); a mammalian homologue to a meiosis specific DNA double strand breaking enzyme (Keeney *et al.*, 1997), DNA recombination genes (Edelman and Kucherlapati, 1996; Edelman *et al.*, 1996) and a meiotic specific heat shock protein (Dix *et al*., 1996). Since meiosis is crucial for survival of a species, an elaborate series of safeguards have evolved to pair, break, and repair the chromosomal DNA. Despite such regulatory mechanisms, it is well known that translocations and aneuploidy are regularly introduced during the meiotic divisions. Moreover, in a sizable population of infertile men, germ cell differentiation arrests during meiosis (Reijo *et al.*, 1995). Anomalies in pairing and chromosome segregation are likely to contribute to this population of infertile men. Moreover, the many specific molecular processes essential for meiosis provide many targets for both genetic damage and for the introduction of structural defects leading to the arrest of germ cell development. Our rapidly advancing knowledge of the mechanisms of meiosis in both males and females will provide substantial insights into a significant cause of male infertility.

*Spermiogenesis*

Spermiogenesis represents an interval of spermatogenesis that appears exceptionally susceptible to the introduction of both genetic and structural defects in the maturing male gamete as the round spermatid is transformed into the highly elongated and polarized (sperm head in front, flagellum at rear) spermatozoon at a time of reduced repair capabilities. Moreover, during spermiogenesis, a major reorganization of the cell occurs. The nucleus elongates and an acrosome containing a group of proteolytic enzymes develops. At the chromosomal level, the histones, the predominant chromatin proteins of somatic cells, are replaced by the highly basic transition proteins, which in turn are replaced by the protamines, producing a tightly compacted nucleus with extensive disulfide bridge crosslinking. In fact, sperm chromatin condensation during spermiogenesis results in a DNA taking up about 90% of the total volume of the sperm nucleus. In contrast, in normal somatic cells, the DNA takes up only 5% of the nucleus volume, while in mitotic chromosomes DNA takes up about 15% of the nuclear volume (Ward and Coffey, 1991).

The displacement of the histones from the nucleosomes during spermiogenesis may leave the DNA of the haploid genome especially susceptible to damage at a time of limited repair capabilities. Although unscheduled DNA repair has been demonstrated to occur in early stages of spermatid development (Sega, 1979), as spermiogenesis proceeds, unscheduled DNA synthesis diminishes and it is not known whether any of the sophisticated DNA repair mechanisms that function during meiosis are still operational. In addition to the major nuclear restructuring during spermiogenesis, the axoneme and tail of the developing male germ cell are produced requiring synthesis of many structural proteins, including those of the fibrous sheath (Morales *et al.*, 1995) and the outer dense fiber proteins (Carrera *et al.*, 1994). These cellular changes require extensive gene expression from the actively transcribed haploid genome before it matures into a genetically quiescent nucleus. In fact, transcription of RNA ceases during mid-spermiogenesis (Kierszenbaum, 1994), and translational regulation plays the prominent regulatory role in the extensive protein synthesis throughout the latter half of spermiogenesis that is required to produce spermatozoa (Spirin, 1994; Hecht, 1995; Schäfer *et al.*, 1995).

The major reorganizational events of the differentiating spermatid are accompanied by significant alterations in the energy suppliers of the cell, the mitochondria. Mitochondria exhibit several distinct morphologies as germ cell differentiation proceeds (De Martino *et al.*, 1979). Spermatogonia and somatic testicular cells contain the “cigar-shaped” mitochondria found in most somatic tissues. During meiosis, mitochondria with diffuse and vacuolated matrices start replacing the “somatic” mitochondria. By the beginning of spermiogenesis, the “somatic” mitochondria have been totally replaced by “germ cell” mitochondria, which in turn are replaced by the crescent-shaped mitochondria of spermatozoa. These structural changes in mitochondria are accompanied by major changes in protein composition (Hecht and Bradley, 1981; Hake *et al.*, 1990). Although human spermatocytes and spermatids are estimated to contain over 103 mitochondria, each spermatozoa midpiece contains only approximately 75 uniquely helically shaped mitochondria. This requires a reduction or possibly a selection of mitochondria as the germ cells differentiate (Hecht and Liem, 1984). At the conclusion of spermiogenesis, most of the cytoplasm of the elongated spermatid is removed as the residual body is pinched off, leaving spermatozoa with little cytoplasm, and no cytoplasmic ribosomes.

Although cytoplasmic protein synthesis does not occur in spermatozoa, cytoplasmic mitochondrial protein synthesis continues (Alcivar *et al.*, 1989).

Considering the massive changes that occur during spermiogenesis, it is not surprising that many cases of germ cell blockage during spermiogenesis lead to infertility in men. Defects in the synthesis of the midpiece, axoneme, mitochondria or tail assembly would result in structurally abnormal spermatozoa often with poor motility, while mutations in proteins needed for the compaction of sperm nuclei or sperm head shaping would lead to spermatozoa with abnormal heads. Despite the presence of aberrant appearing spermatozoa, it is premature to equate morphological aberrations with genetic aberrations. More disquieting, minor base pair substitutions in critical genes that would not alter spermatozoan morphology would lead to genetically defective but normal appearing spermatozoa!

Our inability to detect genetically defective male gametes is of great concern when round spermatid nucleus injections (ROSNI) and round spermatid injections (ROSI) are used to overcome the sterility of men incapable of completing spermatogenesis (Ogura *et al.*, 1994; Fishel *et al.*, 1996). The success of ROSNI and ROSI has demonstrated that although spermiogenesis is essential for the reorganization of the male germ cell to become a motile cell, it is not needed for fertilization. Thus, the normal physiological selection processes leading to fertilization can be bypassed in mice and men. Unfortunately, morphological examination of the spermatids tells little of any underlying genetic defects in the spermatid chosen for injection. A major research effort must be undertaken with a mammalian model system such as the mouse in which a large population of progeny produced by ROSNI and ROSI are produced and evaluated. Among the concerns raised by these procedures is whether we are circumventing gene imprinting in the male genome. The detection of DNA methylation of spermatozoa in the epididymis also raises questions (Ariel *et al.*, 1994). Without a detailed analysis of this approach in an animal system, we could be facing major genetic dangers introduced by the ROSNI and ROSI technologies (Tesarik, 1996; Silber and Johnson, 1998; Sofikitis *et al*., 1998).

## B: GENETICS OF THE SPERMATOZOON

During the past few years many exciting discoveries, previously unsuspected by scientists, have been made about the structure and function of sperm DNA. For example, the paternal genome has been shown to contain endogenous nicks, probably as a normal part of spermiogenesis (Bianchi *et al.*, 1993). In patients in whom these nicks are left unrepaired during the final stages of spermiogenesis, fertility is decreased (Sakkas *et al.*, 1996). Topoisomerases, the enzymes that are thought to be responsible for these nicks, are present throughout spermatogenesis; but are not present in spermatozoa (Morse-Gaudio and Risley, 1994; Chen and Longo, 1996). Evenson *et al.* (1980; 1983; Evenson & Jost, 1994) have developed the sperm chromatin structure assay (SCSA®) that assesses the potential of sperm DNA to denature under certain conditions. This potential also correlates with reduced fertility (Sailer *et al.*, 1996). Perhaps most surprising of all, evidence published by Spadafora and colleagues (Lavitrano *et al.*, 1989; Zoraqi and Spadafora, 1997) shows that fully mature mouse spermatozoa have the potential to incorporate exogenous DNA sequences into the paternal genome. Finally, since a sheep has been cloned from an adult cell (Wilmut *et al.*, 1997) and this technique also succeeded in several other mammalian species like cattle, mouse, goat, pig, cat, and rabbit and even in a primate, the Rhesus monkey, this has raised the question of the importance of the paternal genome and its unique structure in embryogenesis (Wilmut *et al*., 1997).

The above mentioned discoveries have forced us to rethink the idea of sperm DNA structure in which we visualize the paternal genome as being so tightly packaged into an almost crystalline state that it is virtually inert until it is unfolded during fertilization. The sperm chromosome structure is, in fact, very complex – some attributes are similar to somatic cell DNA organization, and others are unique to spermatogenic cells.

When discussing sperm chromatin packaging, several different aspects of structure need to be addressed. These can be divided into different levels of complexity based on the length of DNA being discussed. Each chromosome consists of one double stranded DNA molecule, containing telomeric repeats at both ends, and centromeric

repeats somewhere along its length. Chromosomes are in the order of several million base pairs in length, and, when fully decondensed, are each many times longer than the sperm nucleus itself. At the other end of the spectrum are sperm-specific protamines each of which bind to only a few base pairs of DNA.

Sperm DNA packaging can be subdivided into four levels. In the following paragraphs, the structural relationship between the different levels of DNA packaging in the mature sperm nucleus, will be discussed.

Level I: Chromosomal Anchoring by the Nuclear Annulus

In the first step of the assembly of sperm chromatin, the two strands of naked DNA that make up the chromosomes are attached to a sperm specific structure, the nuclear annulus. This represents a novel type of DNA organization, termed chromosomal anchoring, that is only found in spermatogenic cells. Spermatozoa that are washed with non-ionic detergents such as NP-40, and then treated with high salt and reducing agents to extract the protamines, will decondense completely, leaving no trace of nuclear structure. The DNA, however, remains anchored to the base of the tail, so that the sperm chromatin appears as a broom, with the tail acting as the handle (Ward and Coffey, 1989). Since this chromosomal anchoring is maintained in sperm nuclei from which protamines have been extracted, it is independent of protamine binding. Ward and Coffey (1989) have isolated a small structure that is located at the implantation fossa in hamster spermatozoa, which they have termed the nuclear annulus, to which the DNA is attached in these decondensed nuclei. The nuclear annulus is shaped like a bent ring, and is about 2 µm in length. It is found only in sperm nuclei, although it is currently unknown at what stage of spermiogenesis it is first formed. Thus, so far no evidence for a nuclear annulus-like structure in any somatic cell type has been found. In contrast there is evidence of its existence in hamster (Ward and Coffey, 1989), human (Barone *et al.*, 1994), mouse (Markova, 2001), and *Xenopus* sperm nuclei (Cordes *et al*., 1997). Its existence in a wide variety of species suggests a fundamental role in sperm function.

Unique DNA sequences were found to be associated with the nuclear annulus. Ward and co-workers termed these sequences NA-DNA (Ward *et al*., 1989). The existence of these unique sequences suggests that the nuclear annulus anchors chromosomes

by particular sequences and not by random DNA binding. They also hypothesized that NA-DNAs on different chromosomes become associated early during spermiogenesis, to initiate chromatin condensation by aggregating specific sites of each chromosome to one point.

This hypothesis is supported by the work of Zalensky *et al.* (1995) who suggested that sperm chromosomes are packaged as extended fibers along the length of the nucleus. Each chromosome so far examined has only one site at the base of the nucleus, where the nuclear annulus is located. By organizing the chromosomes so that the NA-DNA sites of each chromosome are aggregated onto one structure, the nuclear annulus may also affect the determination of sperm nuclear shape. For example, in the hamster spermatozoon the longer chromosomes may extend into the thinner hook of the nucleus, while a portion of every chromosome is located at the nuclear annulus. This is supported by image analysis of the distribution of DNA throughout the hamster sperm nucleus, which demonstrated that the highest concentration of DNA in the packaged sperm nucleus is at the base where the nuclear annulus is located; in contrast the lowest concentration of DNA is in the hooked portion (Ward *et al.*, 1996).

The hypothesis is further supported by electron microscopic evidence that the chromatin near the implantation fossa is one of the first areas to condense during spermiogenesis (Loir and Courtens, 1979). Thus, the nuclear annulus may represent the only known aspect of sperm chromatin condensation that is specific for individual chromosome sites.

Level II: Sperm DNA loop domain organization

DNA loop domain organization

At this level anchored chromosomes are organized into DNA loop domains. Parts of the nuclear matrix, protein structural fibers, attach to the DNA every 30 to 50 kb by specific sequences termed matrix attachment regions (MARs). This arranges the chromosome strands into a series of loops. This type of organization can be visualized experimentally in preparations; known as nuclear halos. Nuclear halos are made up of the nuclear matrix with a halo of DNA surrounding it. This halo consists of loops of naked DNA, 25 to 100 kb in length, attached at their bases to the matrix

(Ward *et al*., 1989; Nadel *et al*., 1995). Each loop domain visible in the nuclear halo consists of a structural unit of chromatin that exists *in vivo* in a condensed form.

As with chromosomal anchoring, DNA loop domain formation is independent of protamine binding. The organization of DNA into loop domains is the only type of structural organization resolved thus far that is present in both somatic and sperm cells. In somatic cells, DNA is coiled into nucleosomes, then further coiled into a 30 nm solenoid fiber, and then organized into DNA loop domains. The corresponding structures in sperm chromatin have a very different appearance. Protamine binding causes a different type of coiling, and DNA is folded into densely packed toroids, but still organized into loop domains. Mammalian sperm nuclei do contain a small amount of histones which are presumably organized into nucleosomes (Tanphaichitr *et al.*, 1978; Choudhary *et al.*, 1995), but most of the DNA is reorganized by protamines. This means that with the evolutionary pressure to condense sperm DNA, all aspects of chromatin structure are sacrificed other than the organization of DNA into loop domains. This suggests DNA loop domains play a crucial role in sperm DNA function.

### DNA loop domain function

In somatic cells, DNA loop domain organization has been implicated in both the control of gene expression and in DNA replication. Each DNA loop domain replicates at a fixed site on the nuclear matrix, by being reeled through the enzymatic machinery located at the base of the loop (Vogelstein *et al.*, 1980; Jackson *et al.*, 1990). DNA replication origins have been localized to the nuclear matrix in mammals (Vaughn *et al.*, 1990), and the varying sizes of replicons in different species have been correlated with the sizes of loop domains (Buongiorno-Nardelli *et al.*, 1982). A replicon can be thought of as the distance between two regions of replication. The attachment sites of individual genes to the nuclear matrix vary between cell types, and are also involved in transcription. Active genes are tightly associated with nuclear matrix, but inactive genes are usually located within the extended part of the DNA loop (Robinson *et al.*, 1982; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Mirkovitch *et al.*, 1987; Gerdes *et al.*, 1994). In this manner, the three dimensional organization of DNA plays an important role in DNA function.

Possible function of sperm DNA loop domains

It has been demonstrated that the specific configurations of DNA loop domains are markedly different in sperm and somatic cells (Barone *et al.*, 1989; Nadel *et al.*, 1995). In somatic cells, DNA replication and transcription are the major functions in which DNA loop domain structures are involved (Tanphaichitr *et al.*, 1978; Vogelstein *et al.*, 1980; Buongiorno-Nardelli *et al.*, 1982; Gasser and Laemmli, 1986; Mirkovitch *et al.*, 1987; Jackson *et al.*, 1990; Vaughn *et al.*, 1990; Gerdes *et al.*, 1994; Choudhary *et al.*, 1995). However, since mature sperm nuclei perform neither process (Stewart *et al.*, 1984) it is not clear what the function of sperm DNA loop domain organization is. Two possibilities exist. First, the DNA loop domain structures in spermatozoa may be residual structures that were required for transcription or DNA replication that occurred during spermatogenesis. Secondly, they may be involved in regulating these functions during embryonic development, if the embryo inherits them. If, for example, paternal genes in the male pronucleus of a newly fertilized egg were organized into the same DNA loop configurations they have in sperm nuclei, it would suggest that this organization might help to regulate transcription and DNA replication in early embryonic development. This would have the exciting implication that the sperm nucleus provides the embryo with a specific chromosomal architecture that may be functional during embryogenesis (Emery and Carrell, 2006).

Level III: Protamine decondensation

In the third step of the assembly of sperm chromatin structure, the binding of protamines condenses the DNA loops into tightly packaged chromatin. Hud *et al.* (1995) have demonstrated that when protamines bind DNA, they form toroidal, or doughnut-shaped structures in which the DNA is very concentrated. During spermiogenesis, histones, the DNA binding proteins of somatic spermatogenic precursor cells, are replaced by protamines. Since histone bound DNA requires much more volume than the same amount of DNA bound to protamines (Ward and Coffey, 1991) this change in chromatin structure probably accounts for some of the nuclear condensation that occurs during spermiogenesis. Histones package DNA by organizing it into nucleosomes, in which the DNA is wrapped around an octamer of histone proteins. Protamines, on the other hand, bind DNA in a markedly different manner. These positively charged proteins bind the DNA along the major groove,

completely neutralizing the DNA so that neighboring DNA strands bind to each other by Van der Waal's forces. Protamines are believed to coil the DNA into doughnut like structures in which the DNA exists in an almost crystalline like state (Hud *et al.*, 1995). If each toroid is a single DNA loop domain (Ward, 1993), protamine binding will lead to the condensation and preservation of the DNA loop domain organization present in the round spermatid.

Level IV: Chromosome organization

The next level of sperm chromatin packaging is the spatial arrangement of the condensed chromosomes within the mature sperm nucleus. This has been investigated in several different ways. First, Zalensky and co-workers (Zalensky *et al.*, 1995) have demonstrated that in human sperm nuclei the centromeres of all chromosomes are aggregated in the center of the nucleus, while the telomeres are located at the periphery. In a second approach, Haaf and Ward (1995) have analysed whole chromosomes and found similar results. Finally, Ward and co-workers (Ward *et al.*, 1996) mapped the three dimensional location of three genes in the hamster sperm nucleus and found that while each one tended to be located in the outer third of the nucleus, there was otherwise little specificity to the positioning of the genes. This data lead to the proposal of a model (Ward and Zalensky, 1996) in which there are limited constraints on the actual position of the chromosomes in the sperm nucleus. The NA-DNA sequences are located at the base of the nucleus, centromeres are located centrally, and the telomeres located peripherally. Outside of these three constraints, the folding of the chromosomal p and q arms is flexible. Interestingly, this type of organization does not seem to be present in monotreme mammal spermatozoa. In these species chromosomes are aligned end to end (Watson et al., 1996). In most eutherian mammals examined, however, the centromeres are organized in a central location, making such an end-to-end arrangement impossible.

## C: Role of spermatozoa in embryogenesis

For many years, male fertility has been defined in vitro as the possibility of sperm to fertilize the oocyte, and to obtain early cleavage stage embryos. In human in vitro fertilization (IVF), the gold standard/test for sperm fertility potential was the ability of a fertilized egg to develop into a 2 to 4 cell embryo. It was assumed that all the embryos obtained had the same developmental potential independent of the quality of sperm. Thereafter, several authors (Yovitch et al., 1984; Ron-El et al., 1991; Parinaud et al., 1993) observed that a poor morphological embryonic quality and poor embryonic developmental ability are associated with severe sperm morphology defects and oligo-asthenozoospermia.

Janny and Ménézo (1994) observed a negative relationship between sperm quality and the ability to reach blastocyst stage. We now know that the difference in sperm fertilizing capacity is not simply related to sperm penetration failure. The following is an analysis of the chronology of the steps involved in these embryonic failures.

### C1: Early defects at the time of fertilization

#### The centrosome

The first epigenetic contribution of the spermatozoon is the centrosome, the microtubule-organizing center of the cell. Correct assembly and function of microtubules is fundamental for the separation of chromosomes at meiosis and migration of the male and female pronuclei. The maternal inheritance of the centrosome observed in mice has brought about confusion until the work of Schatten (1994) and Simerly et al. (1995). Considering the semi-conservative form of this organelle and its critical role in mitosis, it seems obvious that a functionally imperfect centrosome borne by a subnormal spermatozoon induces problems in early embryogenesis, i.e. formation of cytoplasmic fragments and abnormal distribution of the chromosomes (Palermo et al., 1994; Simerly et al., 1995). Asch et al. (1995) reported that up to 25% of the non-segmented eggs are in fact fertilized but submitted to cell division defects. Centrin and $\gamma$- tubulin could be involved in this pathology of the centrosome (Navara et al., 1997).

In bovine oocytes, Navara et al. (1993) have observed a positive relationship between the size and the quality of the sperm-aster and the reproductive performances in bulls.

Oocyte activation factors

The process of meiosis re-initiation probably goes through an exit from M-phase due to cyclin B degradation and re-phosphorylation of $p34^{cdc2}$ following a decrease in cytostatic factor CSF (Murray, 1996). It is generally accepted that intracellular $Ca^{2+}$ is the universal signal for triggering oocytes into metabolic activity. It is still not clear how the spermatozoon causes this calcium oscillation. A heat-sensitive (Dozortsev et al., 1995) and soluble protein, in sperm called oscillin, acting through the inositol phosphate pathway could be at the origin of these calcium oscillations (Swann, 1993).

Defects in oscillin (or other soluble activating factors) could account for delays in zygote formation, as described by Ron-El et al. (1991). However, for Eid et al. (1994), based on their observations in bovine zygotes, this hypothesis could not be the only one. In a group of embryos sired by low fertility bulls, they did not observe any delay in pronuclear formation but a delayed initiation and reduced length of zygotic S-phase correlated with reduced embryonic development in vitro. A longer S-phase was correlated with higher fertility in vivo.

Poor chromatin packaging and/or anomalies in DNA packaging could contribute to failure of sperm decondensation, independently of any activation problems (Sakkas et al., 1996).

C2: Developmental arrests between fertilization and the beginning of Genomic Activation

It is quite surprising to expect a paternal derived influence between fertilization and genomic activation, i.e. before the appearance of the first products resulting from the first massive transcriptions involving the paternal genome. It is now well documented that the longest cleavage stage is linked to embryonic genome activation (Sakkas et al., 1989). There is obviously a race against the clock between, firstly, the ineluctable turnover of the maternal mRNA and, on the other hand, the first massive synthesis of the embryonic transcripts. The cumulative delays observed, cycle after cycle, due to epigenetic defects brought by sub optimal spermatozoa, lead to developmental arrests, the maternal stores being exhausted before the beginning of transcription. One third of the human IVF embryos blocks, in vitro, around the time of genomic activation (Janny and Ménézo, 1994).

Antisperm antibodies may also have a deleterious effect on early preimplantation development. Naz (1992) observed that antibodies against very special epitopes might block embryos: especially if cleavage signal proteins (CS-1) or regulatory products of the OCT-3 gene are immunoneutralized.

C3: Developmental arrests between Genomic Activation and Implantation

After genomic activation the very sensitive transition between morula and blastocyst follows. Complex remodeling within the embryo occurs with the first differentiation. Janny and Ménézo (1994) observed a loss of blastocysts at this point; which was significantly increased in men with poor sperm quality (31% vs. 22% for control group). They concluded that poor quality sperm has a negative role on preimplantation development even after genomic activation.

The lesson from Intra Cytoplasmic Sperm Injection (ICSI)

One of the most exciting breakthroughs in the treatment of male infertility is ICSI (Palermo et al., 1992). The success that we observe in ICSI, considering the poor quality of the sperm, can partially be ascribed to the following. In human IVF with poor quality sperm the cumulative effect of the delay in the fertilization process (Ron-El et al., 1991), and the delays associated with epigenetic problems (Ron-El et al., 1991) may cause prolonged cell cycles and late divisions (2-cell embryos on Day 2, 4-cell on day 3), leading to developmental arrest around genomic activation, in relation to the depletion of the mRNA maternal store (Ron-El et al., 1991). In contrast the fertilization process in ICSI is shorter since the sperm is introduced into the cytoplasm (Ron-El et al., 1991).

Van Landuyt and co-workers (2005) showed that the blastocyst formation rate after ICSI compares to the rate after regular IVF. An in depth analysis, however, demonstrates that more patients have embryos which are unable to reach the blastocyst stage (Van Landuyt et al., 2005). Interestingly there seems to be an <<all or nothing>> trend regarding blastocyst development. If one blastocyst is obtained, then all embryos from the patient in question normally develop into blastocysts, whilst if no blastocysts are seen at day 5, it is highly unlikely that any embryos will go on to develop into blastocysts (Van Landuyt et al., 2005).

ICSI is of no use if performed 24 hours after failed fertilization (Marek et al., 1995): The maternal mRNA reserves are already at this point too depleted to allow a development from fertilization to genomic activation (Ron-El et al., 1995). It is very likely that major sperm defects cannot be cured by ICSI (Ron-El et al., 1995).

The ICSI process itself carries other genetic related problems such as the genetic link between oligo-astheno teratozoospermia and sperm genetic disorders. Some of these features include microdeletions of the Y-chromosome (Kremer et al., 1997). The negative influence of suboptimal spermatozoa is linked to the integrity and quality of the paternal DNA. In 1985, Bourouillou and co-workers (Bourrouillou et al., 1985) observed an increase in chromosomal abnormalities as a function of sperm count; in 1995 Moosani and co-workers (Moosani et al., 1995) clearly demonstrated increased chromosomal disorders in the sperm population of infertile men with idiopathic infertility.

In this context it is also important mentioning the consequences of fertilization of oocytes with sperm deriving from an ejaculate containing a high incidence of disturbed DNA integrity in IVF and especially ICSI patients. According to present knowledge, sperm DNA fragmentation might not only cause an impaired embryonic development and early embryonic death (Asch et al., 1995; Jurisicova et al., 1996; Simerly et al., 1997) but also an increased risk of childhood cancer in the offspring (Ji et al., 1997; Aitken et al., 1998). The latter is due to the vulnerability of human sperm DNA during late stages of spermatogenesis and epididymal maturation. At this stage, DNA repair mechanisms have been switched off, resulting in a genetic instability of the male germ cells (Aitken and Krausz, 2001), especially on the Y chromosome resulting in male-specific cancers (McElreavey and Quintana-Murci, 2003). However, this DNA damage is not only caused by these intrinsic factors, but can also be triggered by extrinsic factors like excess amounts of oxidants produced by leukocytes in the ejaculate (Henkel et al., 2005).
The influence of the spermatozoon-carried mitochondria during ICSI, on early or late embryogenesis, is, however, still a matter of debate (Sutovsky and Schatten, 2000).

C4: Genomic imprinting

Experimental manipulations of mouse zygotes have clearly proved the necessary complementary relationship between the maternal and paternal genome to ensure normal embryonic development. Even if implantation and late development can be observed in rabbit and mouse, parthenogenesis never leads to live births (Surani et al., 1987). Surani and co-workers (1987) observed that hypertrophy of the inner cell mass and hypotrophy of the extraembryonic tissue is related to gynogenesis. On the contrary, androgenesis performed by removal of the female pronucleus followed by a duplication of the paternal genome, leads to hypertrophy of extraembryonic tissues. This is due to genomic imprinting which occurs as early as the pronuclear stage. Genomic imprinting seems to be directly related to variations in the methylation pattern of some genes. One of the most important systems in genomic imprinting is IGF2/IGF2-R (De Groot and Hochberg, 1993). The ligand is contributed by the paternal genome and the receptor by the maternal one. The maternal and the paternal X chromosomes are submitted to differential inactivation, related to different methylation patterns of Xist locus, in the preimplantation period. Xist is the initiator of methylation carried by the X chromosome (De Groot and Hochberg, 1993).

The H19 gene, a tumor suppressor, is expressed in the placenta but not in the mole (Goshen et al., 1994). The potential invasiveness of the placenta and/or placental tumors is directly related to the paternal genome qualitatively and quantitatively (Goshen et al., 1994). Disorganized imprinting may have harmful effects on early preimplantation and late postimplantation development (Goshen et al., 1994).

Conclusion

As discussed, it is clear that paternal factors have major effects on early embryogenesis. In the last decade, major advances have been made in assisted reproduction technologies. ICSI has been proposed as a tool for overcoming the sperm deficiencies observed at the time of fertilization. This technology can assist in overcoming some of the defects affecting early preimplantation development. Time gained by the direct sperm insertion into the cytoplasm may help in avoiding delays that impair early preimplantation development.

However, it is unlikely that ICSI can universally compensate for male factor defects. Moreover, it raises questions regarding the genetic basis of some of the defects observed, and on some other hidden genetic links. The growing number of children that have been produced by ICSI is beginning to provide us with a good base to evaluate transmission of genetic defects. To date, there is evidence showing that infertility in fathers due to microdeletions in the Y chromosome is transmitted from one male generation to the next (Chandley et al., 1989; Kent-First et al., 1996). These examples of male infertility are believed to be due to the deletion of genes such as the DAZ (Deleted in Azoospermia) and RBM (RNA-binding motif) genes (Vogt, 2004). These genes show mapping to the Y chromosome linked microdeletions (Reijo et al., 1995; Najmabadi et al., 1996; Reijo et al., 1996; Pryor et al., 1997).

## REFERENCES

Aitken RJ and Krausz C (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122, 497-506.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z and Irv DS (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 59, 1037-1046.

Alcivar AA, Hake LE, Millette CF, Trasler JM and Hecht NB (1989) Mitochondrial gene expression in male germ cells of the mouse. Dev Biol 135, 263-271.

Ariel M, Cedar H and McCarrey J (1994) Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. Nature Genet 7, 59-63.

Asch R, Simerly C, Ord T and Schatten G (1995) The stages at which human fertilization arrests: microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. Hum Reprod 10, 1897-1906.

Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, Liyanage M, Eckhaus M, Collins F, Shiloh Y, Crawley JN, Ried T, Tagle D and Wynshaw-Boris A (1996) Atm-deficient mice: A paradigm of ataxia telangiectasia. Cell 86, 159-171.

Barone JG, De Lara J, Cummings KB and Ward WS (1994) DNA organization in human spermatozoa. J Androl 15, 139-144.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U and Sakkas D (1993) Effect of DNA protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Repro 49, 1083-1088.

Bourrouillou G, Dastugue N and Colobies P (1985) Chromosome studies in 952 infertile males with sperm count below 10 million/ml. Hum Genet 71, 366-367.

Buongiorno-Nardelli M, Micheli G, Carri MT and Marrilley M (1982) A relationship between size and supercoiled loop domains in the eukaryotic genome. Nature 298, 100-102.

Carrera A, Gerton GL and Moss B (1994) The major fibrous sheath polypeptide of mouse sperm: Structural and functional similarities to the A-kinase anchoring proteins. Dev Bio 165, 272-284.

Chandley AC, Gosden JR, Hargreave TB, Spowart G, Speed RM and McBeath S (1989) Deleted Yq in the sterile son of a man with a sattelite Y chromosome (Yqs) J Med Genet 26, 145-153.

Chen JL and Longo FJ (1996) Expression and localisation of DNA topoisomerase II during rat spermatogenesis. Mol Reprod Dev 45, 61-71.

Choudhary SK, Wykes SM, Kramer JA, Mohamed AN, Koppitch F, Nelson JE and Krawetz SA (1995) A haploid expressed gene cluster exists as a single chromatin domain in human sperm. J Biol Chem 270, 8755-8762.

Cockerill PN and Garrard WT (1986) Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell 44, 273-282.

Cordes VC, Reidenbach S, Rackwitz HR, Franke WW (1997) Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. J Cell Biol 136, 515-529.

De Martino C, Floridi A, Marcante ML, Malorni W, Scorza Barcellona P, Bellocci M and Silvestrini B (1979) Morphological, histochemical, and biochemical studies on germ cell mitochondria of normal rats. Cell Tissue Res 196, 1-22.

De-Groot N and Hochberg A (1993) Gene imprinting during placental and embryonic development. Mol Reprod Dev 36, 390-406.

Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, Goulding EH and Eddy EM (1996) Targeted gene disruption of Hsp 70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc Natl Acad Sci USA 93, 3264-3268.

Dozortsev D, Rybouchkin A, De Sutter P, Qian C and Dhont M (1995) Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. Hum Reprod 10, 399-407.

Dym M (1994) Spermatogonial stem cells of the testis. Proc Natl Acad Sci USA 91, 11287-11289.

Dym M and Clermont Y (1970) Role of spermatogonia in the repair of the seminiferous epithelium following X radiation of the rat testis. Amer J of Anat 128, 265-282.

Edelmann W and Kucherlapati R (1996) Role of recombination enzymes in mammalian cell survival. Proc Natl Acad Sci USA 93, 6225-6227.

Edelmann W, Cohen PE, Kane M, Lau K Morrow B, Bennett S, Umar A, Kunkel T, Cattoretti G, Chaganti R, Pollard JW, Kolodner RD and Kucherlapati R (1996) Meiotic pachytene arrest in MLH1-deficient mice. Cell 85, 1125-1134.

Eid LN, Lorton SP and Parrish JJ (1994) Paternal influence on S-Phase in the first cell cycle of the bovine embryo. Biol Reprod 51, 1232-1237.

Emery BR and Carrell DT (2006) The effect of epigenetic sperm abnormalities on early embryogenesis. Asian J Androl 8, 131-142.

Evenson DP and Jost LK (1994) Sperm chromatin structure assay: DNA denaturability. In Darzynkiewicz Z, Robinson JP and Crissman HA (eds) Methods in Cell Biology, Vol. 42, Flow Cytometry, 2nd edn, Academic Press Inc., Orlando, pp.159–176.

Evenson DP and Melamed MR (1983) Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 31, 248–253.

Evenson DP, Darzynkiewicz Z and Melamed MR (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210, 1131–1133.

Fishel S, Aslam I and Tesarik J (1996) Spermatid conception: a stage too early, or a time too soon? Hum Reprod 11, 1371-1375.

Gasser SM and Laemmli UK (1986) Cohabitation of scaffold binding regions with upstream- enhanser elements of three developmentally regulated genes of D. melanogaster. Cell 46, 521-530.

Gerdes MG, Carter KC, Moen PT, Jr and Lawrence JB (1994) Dynamic changes in the higher-level chromatin organization of specific sequences revealed by in situ hybridization to nuclear halos. J Cell Biol 126, 289-304.

Goshen R, Ben Rafael Z, Gonik B, Lustig O, Tannos V, de-Groot N and Hochberg AA (1994) The role of genomic imprinting in implantation. Fertil Steril 62, 903-910.

Haaf T and Ward WS (1995) Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers. Exp Cell Res 219, 604-611.

Hake LE, Alcivar AA and Hecht NB (1990) Changes in length accompany translocational regulation of the somatic and testis-specific cytochrome c genes during spermatogenesis in the mouse. Development 110, 249-257.

Hecht NB (1995) The making of a spermatozoon: A molecular perspective. Dev Gen 16, 95-103.

Hecht NB and Bradley FM (1981) Changes in mitochondrial protein composition during testicular differentiation in mouse and bull. Gam Res 4, 433-449.

Hecht NB and Liem H (1984) Mitochondrial DNA is synthesized during meiosis and spermiogenesis in the mouse. Exp Cell Res 154, 293-298.

Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB and Kruger TF (2005) Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. Fertil Steril 83, 635-642.

Hud NV, Downing KH and Balhorn R (1995) A constant radius of curvature model for the organization of DNA in toroidal condensates. Proc Natl Acad Sci USA 92, 3581-3585.

Jackson DA, Dickinson P and Cook PR (1990) The size of chromatin loops in HeLa cells. EMBO J 9, 567-571.

Janny L and Ménézó YJR (1994) Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. Mol Reprod Develop 38, 36-42.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM and Jin F (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. J of the Nat Cancer Inst 89, 238–244.

Jurisicova A, Casper RF, MacLusky NJ and Librach CL (1996) Embryonic human leukocyte antigen-G expression: possible implications for human preimplantation development. Fertil Steril 65, 997-1002.

Keegan KS, Holtzman DA, Plug AW, Christenson ER, Brainerd EE, Flaggs G, Bentley NJ, Taylor EM, Meyn MS, Moss SB, Carr AM, Ashley T and Hoekstra MF (1996) The Atr and Atm protein kinases associate with different sites along meiotically pairing chromosomes. Genes and Dev 10, 2423-2437.

Keeney S, Giroux CN and Kleckner N (1997) Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell 88, 375-384.

Kent-First MG, Koi S, Muallem A, Blazer S and Itskovitz-Eldor J (1996) Infertility in intracytoplasmic-sperm-injection-derived sons. Lancet 348, 332.

Kierszenbaum AL (1994) Mammalian Ssermatogenesis in vivo and in vitro: A partnership of spermatogenic and somatic cell lineages. Endocr Rev 15, 116-134.

Kovalenko OV, Plug AW, Haaf T, Gonda DK, Ashley T, Ward DC, Radding CM and Golub EI (1996) Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes. Proc Natl Acad Sci USA 93, 2958-2963.

Kremer JAM, Tuerlings JHAM, Meuleman EJH, Schoute F, Mariman E, Smeets DF, Hoefsloot LH, Braat DD and Merkus HM (1997) Microdeletions of Y chromosome and intracytoplasmic sperm injection: from gene to clinic. Hum Reprod 12, 687-691.

Lavitrano M, Camaino A, Fazio VM, Dolci S, Farace MG and Sadafora C (1989) Sperm Cells as vectors for introducing foreign DNA into eggs; Genetic transformation of mice. Cell 57, 717-723.

Loir M and Courtens JL (1979) Nuclear reorganization in ram spermatids. J Ultrastruct Res 67, 309-324.

Marek D, Doherty D, Doogy KM, Doogy KJ (1995) Second day intracytoplasmic sperm injection (ICSI) for failed in-vitro fertilization (IVF): a comparative analysis of ICSI done on the day of retrieval vs ICSI done on the day after retrieval [abstract]. In: Abstracts of the Scientific Oral and Poster Sessions Program. Supplement to Fertility & Sterility. Seattle: ASRM, S100–1.

Markova MD (2001) Electron microscopic observations of mouse sperm whole mounts after extraction for nuclear matrix and intermediate filaments. Arch Androl 47, 37-45.

McElreavey K and Quintana-Murci L (2003) Male reproductive function and the human Y chromosome: is selection acting on the Y? RBM Online 7, 17-23.

Mirkovitch J, Gasser SM and Laemmli UK (1987) Relation of chromosome structure and gene expression. Philos Trans R Soc Lond Biol 317, 563-574.

Moosani N, Pattinson N, Carter MD, Cox DM, Rademaker AW and Martin RH (1995) Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluresence in situ hybridisation. Fertil Steril 64, 811-817.

Morales CR, Oko R and Clermont Y (1995) Molecular cloning and developmental expression of an mRNA encoding the 27kDa outer dense fiber protein of rat spermatozoa. Mol Reprod and Dev 37, 229-240.

Morrison SJ, Shah NM and Anderson DJ (1997) Regulatory mechanisms in stem cell biology. Cell 88, 287-298.

Morse-Gaudio M and Risley MS (1994) Topoisomerase II expression and VM-26 induction of DNA breaks during spermatogenesis in Xenopus laevis. J Cell Sci 107, 2887-2898.

Murray A (1996) Creative blocks: cell cycle checkpoints and feed-back controls. Nature 359, 599-604.

Nadel B, de Lara J, Finkernagel SW and Ward WS (1995) Cell specific organization of the 5S rRna gene cluster DNA loop domains in spermatozoa and somatic cells. Biol Reprod 53, 1222-1228.

Najmabadi H, Huang V, Yen P, Subbarao MN, Bhasin D, Banaag L, Naseeruddin S, de Kretser DM, Baker HW and McLachlan RI (1996) Substantial prevalence of microdeletions of the Y chromosome in infertile men with idiopathic azoospermia and oligozoospermia detected using a sequence-tagged site-based mapping strategy. J of Clin Endo Met 81, 1347-1352.

Navara CS, First N and Schatten G (1993) Individual bulls affect sperm aster size and quality: relationship between the sperm centrosome and development. Mol Biol Cell 4, 828.

Navara CS, Hewitson LC, Simerly CR, Sutovsky P and Schatten G (1997) The implications of a paternally derived centrosome during human fertilization: consequences for reproduction and treatment of male factor infertility. AJRI 37, 39-49.

Naz RK (1992) Effect of antisperm antibodies on early cleavage of fertilized ova. Biol Reprod 46, 130-139.

Ogura A, Matsuda J and Yanagimachi R (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci USA 91, 7460.

Palermo G, Joris H, Devroey P and Van Streitegheim AC (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 2, 17-18.

Palermo G, Munné S and Cohen J (1994) The human zygote inherits its mitotic potential from the male gamete. Hum Reprod 9, 1220-1225.

Parinaud J, Mieusset R, Vieitez G, Labal B and Richoilley G (1993) Influence of sperm parameters on embryo quality. Fertil Steril 60, 888-892.

Pryor JL, Kent-First M, Muallem A, Van Bergen AH, Nolten WE, Meisner L and Roberts KP (1997) Microdeletions in the Y chromosome of infertile men. NEJM 336, 534-539.

Reijo R, Alagappan RK, Patrizio P and Page DC (1996) Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. Lancet 347, 1290-1293.

Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, de la Chapelle A, Silber S and David C (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 10, 383-393.

Robinson SI, Nelkin BD and Vogelstein B (1982) The Ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell 28, 99-106.

Roest HP, van Klaveren J, de Wit J, van Gurp CG, Koken MHM, Vermey M, van Roijen JH, Hoogerbrugge JW, Vreeburg JT, Baarends WM, Bootsma D, Grootegoed JA and Hoeijmakers JH (1996) Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell 86, 799-810.

Ron-El R, Nachum H, Herman A, Golan A, Caspi E and Soffer E (1991) Delayed fertilization and poor embryonic development associated with impaired semen quality. Fertil Steril 55, 338-344.

Sailer BL, Jost LK and Evenson DP (1996) Bull sperm head morphometry related to abnormal chromatin structure and fertility. Cytom 24, 167-173.

Sakkas D, Batt PA and Cameron AWN (1989) Development of pre-implantation goat (Capra hircus) embryos in vivo and in vitro. J Reprod Fert 87, 359-365.

Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G and Campana A (1996) Sperm chromatin anomalies can inluence decondensation after intracytoplasmic sperm injection. Hum Reprod 11, 837-843.

Schäfer M, Nayerinia K, Engel W and Schäfer U (1995) Translocational control in spermatogenesis. Dev Bio 172, 344-352.

Schatten G (1994) The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev Biol 165, 299-335.

Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Xiao Y, Weaver D, Feunteun J, Ashley T and Livingston DM (1997) Association of BRCA1 with Rad 51 in mitotic and meiotic cells. Cell 88, 256-275.

Sega GA (1979) Unscheduled DNA synthesis (DNA repair) in germ cells of male mice- its role in the study of mammalian mutagenesis. Genetics 92, 49-58.

Simerly C, Hewitson LC, Sutovsky P and Schatten G (1997) The inheritance, molecular dissection and reconstitution of the human centrosome during fertilization: consequences for infertility. In Barratt C, De Jonge C, Mortimer D, and Parinaud J (eds) Genetics of Human Male Fertility. EDK Press, Paris, pp.58-286.

Simerly C, Wu G-J, Zoran S, Ord T, Rawlins R, Jones J, Navara C, Gerrity M, Rinehart J and Binor Z (1995) The paternal inheritance of the centrosome, the cells microtubule- organizing center, in humans, and the implications for infertility. Nature Medicine 1, 47-52.

Silber SJ and Johnson (1998) Are spermatid injections of any clinical value? ROSNI and ROSI revisited. Round spermatid nucleus injection and round spermatid injection. Hum Reprod 13, 509-515.

Sofikitis N, Miyaqawa I, Yamamoto Y, Loutradis D, Mantzavinos T, Tarlatzis V (1998) Micro- and macro-consequences of ooplasmic injections of early haploid male gametes. Hum Reprod Update 4, 197-212.

Spirin AS (1994) Storage of messanger RNA in eukaryotes: Envelopment with protein, translocation barrier at 5' side, or conformational masking by 3' side? Mol Reprod Dev 38, 107-117.

Stern H (1993) The process of meiosis. In Ewing L and Desjardin C (eds) Cell and molecular biology of the testis. Oxford University Press, New York, pp.296-331.

Stewart TA, Bellvé AR and Leder P (1984) Transcription and promoter usage of the myc gene in normal somatic and spermatogenic cells. Science 226, 707-710.

Surani MAH, Barton SC and Norris ML (1987) Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. Biol Reprod 36, 1-16.

Sutovsky P and Schatten G (2000) Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. Int Rev Cytol 195, 1-65.

Swann K (1993) The soluble sperm oscillogen hypothesis. Zygote 1, 273-276.

Tanphaichitr N, Sobhon P, Taluppeth N and Chalermisarachai P (1978) Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. Exp Cell Res 117, 347-356.

Tesarik J (1996) Fertilization of oocytes by injecting spermatozoa, spermatids and spermatocytes. Hum Reprod 1, 149-152.

Van Landuyt L, De Vos A, Joris H, Verheyen G, Devroey P and Van Steirteghem A (2005) Blastocyst formation in in vitro fertilisation versus intra cytoplasmic injection cycles: influence of the fertilisation procedure. Fert Steril 83, 1397-1403.

Vaughn JP, Dijkwel PA, Mullender LHF and Hamlin JL (1990) Replication forks are associated with the nuclear matrix. Nucl Acids Res 18, 1965-1969.

Vogelstein B, Pardoll DM and Coffey DS (1980) Supercoiled loops and eucaryotic DNA replication. Cell 22, 79-85.

Vogt PH (2004) Molecular genetics of human male infertility: from genes to new therapeutic perspectives. Curr Pharm Des 10, 471-500. Review.

Ward WS (1993) DNA loop domain tertiary structure in mammalian spermatozoa. Biol Reprod 48, 1193-1201.

Ward WS and Coffey DS (1989) Identification of a sperm nuclear annulus: A sperm DNA anchor. Biol Reprod 41, 361-370.

Ward WS, Partin AW and Coffey DS (1989) DNA Loop Domains in Mammalian Spermatozoa. Chromo 98, 153-159.

Ward WS and Coffey DS (1991) DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cells (Review). Biol Reprod 44, 569-574.

Ward WS and Zalensky A (1996) The unique, complex organization of the transcriptionally silent sperm chromatin. Crit Rev Euk Gene Exp 6, 139-47.

Ward WS, McNeil J, de Lara J and Lawrence J (1996) Localization of three genes in the hook-shaped hamster sperm nucleus by fluorescent in situ hybridisation. Biol Repro 54, 1271-1278.

Watson JM, Meyne J and Graves JA (1996) Ordered tandem arrangement of chromosomes in the sperm heads of monotreme mammals. Proc Natl Acad Sci USA 93, 10200-10205.

Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS (1997) Viable off-spring derived from fetal and adult mammalian cells. Nature 385, 810-813.

Yovitch JL and Stanger JD (1984) The limitations of in vitro fertilization from males with severe oligospermia and abnormal sperm morphology. J In vitro Fert Embryo Transf 1, 172-179.

Zakian VA (1995) ATM-related genes: What do they tell us about functions of the human gene? Cell 82, 685-687.

Zalensky AO, Allen MJ, Kobayashi A, Zalenskya IA, Balhorn R and Bradbury EM (1995) Well-defined genome architecture in the human sperm nucleus. Chromos 103, 577-590.

Zoraqi G and Spadafora C (1997) Integration of foreign DNA sequences into mouse sperm genome. DNA Cell Biol 16, 291-300.

# CHAPTER II

## The correlation of DNA fragmentation, apoptosis and reactive oxygen species (ROS) with functional semen parameters

Abstract

Introduction

Materials and Methods

- *Semen samples*
- *Quantification of seminal leukocytes*
- *Measurement of ROS production in the ejaculate*
- *Apoptosis*
- *DNA fragmentation*
- *Sperm Chromatin Structure Assay (SCSA®)*
- *Statistical analysis*

Results

Discussion

References

**ABSTRACT**

**Introduction:** Sperm DNA fragmentation is characterized by strand breaks and is particularly frequent in the ejaculates of sub fertile men, (males with genital tract inflammations) as well as in semen of patients after receiving radiation or chemotherapy. Sperm DNA fragmentation originates from three potential sources: deficiencies in recombination and chromatin packaging during spermiogenesis, oxidative stress or abortive apoptosis.

**Aim:** To detect sperm DNA fragmentation, sperm apoptosis and ROS production in semen. To determine the inter relation between these measured parameters.

**Materials and Methods:** A total of 79 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. Semen parameters were assessed using WHO criteria for normal concentration and motility, as well as with CASA, morphology was assessed using strict criteria. Seminal leukocyte concentrations were determined with a myeloperoxidase test. Spermatozoa DNA integrity was investigated with the TUNEL assay and the sperm chromatin structure assay (SCSA®). Spermatozoa apoptosis was determined by binding of Annexin V to phosphatidylserine. ROS was determined with a chemiluminescent technique.

**Results:** CASA velocity parameters did not correlate with the other parameters measured in this study. ROS correlated significantly negative with sperm concentration ($P < 0.0001$) as well as the percentage of non-apoptotic (Annexin V negative) sperm ($P < 0.01$). The percentage of non-apoptotic sperm had significant positive correlations with sperm motility ($P < 0.05$) and concentration ($P < 0.0001$). The percentage of TUNEL negative sperm had significant positive correlations with sperm motility ($P < 0.001$) and concentration ($P < 0.0001$). In addition, it was also shown, with the Bland and Altman plot, that although there was a significant negative correlation between the percentage of TUNEL-negative sperm and the DFI as determined with the SCSA® ($P < 0.0001$) these two assays essentially measure different aspects of DNA damage.

**Conclusion:** Sperm motility and concentration had significant positive correlations with the percentage of non-apoptotic and DNA non-fragmented sperm. Application of these findings in clinical practice can ultimately increase implantation and pregnancy rates in ICSI patients.

## INTRODUCTION

Sperm DNA fragmentation damage is characterized by strand breaks and is particularly frequent in ejaculates of sub fertile men (Irvine *et al*., 2000) as well as in patients after radiation or chemotherapy (Stahl *et al*., 2006) and male genital tract inflammations (Henkel *et al*., 2003; Henkel *et al*., 2005a). Although sperm DNA fragmentation does not constitute a mutation in its own, it still is a promutagenic change of the male genome. Under *in vivo* conditions, these defective, abnormal sperm would have been restrained by physiological barriers (cervical mucus, uterine environment, cumulus oophorus, zona pellucida and the oolemma) and thus been prevented from fertilizing an oocyte. However, with the advent and implementation of assisted reproductive techniques (ART) into patient treatment regimes, particularly with the introduction of intra-cytoplasmic sperm injection (ICSI), such defective sperm have the chance to fertilize an oocyte and possibly generate mutations in the offspring (Twigg *et al*., 1998a; Aitken and Krausz, 2001; Aitken and Sawyer, 2003; Alvarez, 2003).

Sperm DNA fragmentation originates from three potential sources: deficiencies in natural processes such as recombination and chromatin packaging which takes place during spermiogenesis (late spermatid stage) (McPherson and Longo, 1992; McPherson and Longo, 1993), oxidative stress (Aitken *et al*., 1998; Agarwal and Saleh, 2002; Henkel *et al*., 2005b) or abortive apoptosis (Sakkas *et al*., 1999).

During spermatogenesis a complex and dynamic process of proliferation and differentiation occurs as spermatogonia are transformed into spermatozoa. This process involves a series of meioses and mitoses, changes in cytoplasmic architecture, replacement of histones with protamines (protamination) leading to a highly packed chromatin (Kumaroo *et al*., 1975; Goldberg *et al*., 1977; Poccia, 1986; Balhorn *et al*., 1999). During protamination nicks are created to provide relief of torsional stress and aid chromatin rearrangements (McPherson and Longo, 1993a). These nicks disappear completely at the time when chromatin packaging is completed (McPherson and Longo, 1992; 1993a; 1993b; Ward and Coffey, 1991; Sakkas *et al*., 1995). McPherson and Longo (1992) hypothesized that chromatin packaging requires an endogenous nuclease, topoisomerase II, to create and ligate

nicks in order to facilitate protamination. In accordance with this hypothesis, it has recently been shown that topoisomerase II plays a major role in linking DNA replication to chromosome condensation and that it interplays with a large protein complex i.e. condensation, which has key functions in mitotic chromosome assembly and organization (Hirano, 2002; Cuvier and Hirano, 2003). In addition, it has also been shown that topoisomerase II is present in the human seminiferous tubules (Sakkas *et al*., 2003) which are the location of spermatogenesis.

The DNA remodeling that occurs during spermatogenesis is unique in that it produces a cell type in which the nucleus is transcriptionally inactive and a large part of the cell is stripped off. It is therefore not surprising that the human ejaculate normally contains a heterogeneous population of spermatozoa that possess a variety of abnormalities at the nuclear, cytoskeleton, and organelle levels. In the human, it is well known that the chromatin of the mature sperm nucleus can be abnormally packaged (Evenson *et al*., 1980). Furthermore; there is a strong relation between abnormal chromatin packaging and sperm nuclear DNA damage (Evenson, 1990; Bianchi *et al*., 1993; Gorczyca *et al*., 1993; Manicardi *et al*., 1995), and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters (McPherson and Longo, 1993a; Lopes *et al*., 1998a).

The second hypothesis on the origins of DNA fragmentation in ejaculated spermatozoa describes oxidative stress as the underlying factor for sperm DNA damage. Whilst Barrosso and co-workers (2000) correlated sperm DNA fragmentation with the endogenous generation of ROS, other studies showed correlations with the endogenous generation as well as exogenous stimulus of ROS (Argawal et al., 2003). In addition to sperm DNA fragmentation, oxidative stress has been implicated in impaired sperm functional competence, including poor fertilization rates in IVF (Sun *et al*., 1997; Aitken *et al*., 1998; Lopes *et al*., 1998a; 1998b; Henkel *et al*., 2004; Henkel, 2007). Reactive oxygen species (ROS) cause peroxidative damage to the plasma membrane (Alvarez *et al*., 1987; Aitken *et al*., 1989a, 1989b; Iwasaki and Gagnon, 1992; Agarwal and Saleh, 2002), furthermore, ROS are also known to attack DNA, inducing strand breaks and other oxidative based damage in human spermatozoa (Hughes *et al*., 1996; Kodoma *et al*., 1997; Twigg *et al*., 1998a;

1998b; Hughes *et al*., 1999; Barroso *et al*., 2000). DNA damage of mammalian spermatozoa, induced by free radicals, has also been associated with antioxidant depletion in the seminal plasma (Rhemrev *et al*., 2001; Agarwal *et al*., 2003), presence of transition metals in the sperm culture medium, leukocyte contamination, redox cycling xenobiotics and testicular heating (Fraga *et al*., 1996; Lloyd *et al*., 1997; Shen *et al*., 1997; Hughes *et al*., 1998; Jarow, 1998; Twigg *et al*., 1998a, 1998b; Henkel et al., 2005a).

The third hypothesis, trying to explain sperm nuclear damage and ultimately DNA fragmentation refers to DNA fragmentation as a consequence of apoptosis (Sakkas *et al*., 1999). Apoptosis, also called 'programmed cell death' is the controlled disassembly of cells from within (Kerr *et al*., 1972). Apoptosis is characterized by a cascade of events which include condensation and fragmentation of the chromatin, compaction of cytoplasmic organelles, reduced mitochondrial transmembrane potential (Luo *et al*., 1998), mitochondrial release of cytochrome c (Bossy-Wetzel *et al*., 1998), production of reactive oxygen species (ROS) (Whittington *et al*., 1999), dilatation of the endoplasmic reticulum and a decrease in cell volume (Arends *et al*., 1991). Animal studies have shown that apoptosis is the underlying mechanism of germ cell homeostasis during normal spermatogenesis (Sinha *et al*., 1998; Print and Loveland, 2000).

Apoptosis is characterized by changes of the plasmamembrane. An early sign of apoptosis is the translocation of the negatively charged phospholipid phosphatdylserine (PS) from the normal location on the inner leaflet of the plasmamembrane to the outer side thereof. PS is thus exposed on the external membrane surface (Martin *et al*., 1995; Vermes *et al*., 1995). PS has a high affinity to Annexin V (AV), a phospholipid-binding protein of about 35kDa (Van Heerde *et al*., 1995). This binding is an early marker of apoptosis and its detection has been reported to precede other alterations such as DNA digestion, changes in nuclear and cytoplasmic organization, and cellular fragmentation into apoptotic bodies (Van Blerkom and Davis, 1998).

Another factor that has been shown to be closely involved in sperm apoptosis is Fas (Lee *et al*., 1997). Fas (CD95; APO-1) is a type I transmembrane receptor protein

that belongs to the tumor necrosis factor/nerve growth factor receptor family and mediates apoptosis (Watanabe-Fukunaga *et al*., 1992; Krammer *et al*., 1994; Schulze-Osthoff *et al*., 1994). Fas contains an intracellular death domain; binding of Fas ligand (FasL) to Fas induces apoptosis (Suda *et al*., 1993; Nagata and Suda, 1995). In the testis the Sertoli cells express FasL mRNA (Suda *et al*., 1993), and therefore this system might be involved in the regulation of spermatogenesis with regard to limiting the number of sperm cells that can be supported by the Sertoli cells (Lee *et al*., 1997; Rodriguez *et al*., 1997).

Closely involved in the calculated disassembly of cells into so-called apoptotic bodies, are the 'cytosolic aspartate-specific proteases' (caspases) (Thornberry and Lazebnik, 1998). Caspases have been detected in their active form in human spermatozoa (Weng *et al*., 2002). During the cascade of apoptotic events, the active effector caspase-3 irreversibly activates specific DNases that degrade the DNA (Earnshaw *et al*., 1999) leading to DNA fragmentation and disassembly of the spermatozoa (Steller, 1995).

DNA fragmentation can be detected by means of different test systems. Using an *in situ* end-labeling technique, TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling) various authors have observed relatively high rates of apoptosis in testicular biopsies from infertile men with various degrees of testicular insufficiency (Lin *et al*., 1997a; 1997b; Jurisicova *et al*., 1999). Human sperm as well as various other cell types in which apoptosis is occurring, or has been induced, show a TUNEL signal that has been associated with endonuclease digestion of DNA into oligonucleosomal fragments which exhibit a characteristic ladder-like pattern when separated by gel electrophoresis (Gavrielli *et al*., 1991; Kerr, 1993).

The objectives of the current study were: (i) to detect spermatozoa DNA fragmentation by means of the (TUNEL assay and SCSA®), the translocation of phosphatidylserine (AV binding) and the presence of leukocyte infiltration (ROS) in semen samples from patients attending the unit's andrology laboratory; and (ii) to analyze the relationship between DNA damage and phosphatidylserine redistribution and the exogenous production of ROS.

## MATERIALS AND METHODS

*Semen Samples*

These studies were performed upon approval of the Committee for Human Research at the Faculty of Health Sciences, Stellenbosch University, Tygerberg Academic Hospital, Tygerberg, South Africa (project number 99/137). A total of 79 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. The only exclusion criterion was the presence of less than a total of 10 million sperm in the original sample; this was determined in order to have sufficient numbers of sperm cells to perform all planned experiments. The men collected semen by masturbation into sterile cups following 2-4 days of sexual abstinence. Semen parameters were assessed using the criteria for normal concentration and total motility according to the World Health Organization (WHO) (1999). The speed of forward progression was estimated according to the arbitrary criteria of Hotchkiss (Hotchkiss et al., 1938), namely, the designation of 4 as the best quality of progression and declining quality as 3, 2, 1, and 0. The level of 3 is considered as a good average progression, with 2+ as the line of demarcation between acceptable and poor motility. Normal sperm morphology was assessed using the Tygerberg strict criteria (Kruger *et al.*, 1986; Menkveld *et al.*, 1990; WHO, 1999), whereby normal sperm morphology is divided into three groups, p- (0-4% normal morphology), g- (5-14% normal morphology) and n-pattern (>14% normal morphology).

*Computer assisted semen analysis (CASA)*

Sperm movement characteristics were assessed using the Hamilton-Thorne Motility Analyzer with the Integrated Visual Optical System (HTMA-IVOS version 10.5; Hamilton Thorne Research Inc., Beverly, MA). In outline, this unit comprised an internal microscope system with a heated stage (37°C) and strobic light source that illuminated the specimen with a series of phase-locked light flashes, acquiring frames at 25 Hz. The images were fed into an integrated IBM-compatible computer that analyzed the data according to a series of preprogrammed algorithms.

The HTMA-IVOS settings employed in the study were minimum contrast, 10; minimum cell size, 4 pixels; low and high size gates, 0.4 and 1.6, respectively; low and high

intensity gates, 0.5 and 2.0, respectively; non-motile head size, 12 pixels; non-motile cell intensity, 130; slow cells immotile; and magnification factor, 2.33. Thirty frames (1.2 sec) were collected for analysis.

The samples were loaded onto a 20 µm deep Microcell chamber (Conception Technologies, La Jolla, CA), which had been pre-heated to 37°C, and analyzed. Accurate identification of non-motile sperm cells versus debris and round cells was achieved by replaying the stored video frames and adjusting the static intensity, elongation and size limits appropriately. Data from at least 200 motile spermatozoa were collected for each specimen analyzed.

The motility parameters assessed included curvilinear velocity (VCL; a measure of the total distance traveled by a given sperm during the acquisition divided by the time elapsed, µm/sec), straight line velocity (VSL; the straight line distance from beginning to end of track divided by time taken, µm/sec), average path velocity (VAP; the spatially average path that eliminates the wobble of the sperm head, µm/sec), amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation, µm), and % motile cells. Data from each individual cell track was recorded and analyzed.

*Quantification of seminal leukocytes*

Leukocyte concentrations in the semen were quantified by a myeloperoxidase test (Shekarriz *et al.*, 1995). A 40 µL volume of liquefied semen specimen was placed in a 1.5 mL reaction tube (Eppendorf UK Limited, Cambridge, UK) with 40 µL of 4,4'-diaminobiphenyl (benzidine) solution (6.8 mmol/L). The solutions were mixed and allowed to stand at room temperature (RT) for 5 minutes. Peroxidase-positive leukocytes are staining brown and were counted using a Neubauer counting chamber (Marienfeld, Lauda-Königshofen, Germany) under phase contrast (magnification, X40). The average of 16 fields was calculated. The results, after correction for dilution, were recorded as $X10^6$ peroxidase-positive cells per milliliter of semen.

Leukocytospermia was defined as concentrations of $>1x10^6$ peroxidase-positive cells per milliliter of semen (WHO, 1999). Peroxidase-positive cells include polymorpho-nuclear leukocytes, which represent 50% to 60% of all seminal leukocytes, and

macrophages, which represent another 20% to 30% (Thomas *et al.*, 1997). The term *leukocytes* used throughout the text refers to polymorphonuclear (PMN) leukocytes and macrophages.

*Measurement of ROS production in the ejaculate*

ROS generation was monitored with the chemiluminescent technique described by Iwasaki and Gagnon (1992) using luminol (5-amino-2,3 dihydro-1,4 phalazinedione; Sigma Chemical Co., St. Louis, MO, USA) as the probe. In brief, 100 µL of raw semen was diluted 1:5 with Quinn's sperm wash medium (Sage In-Vitro Fertilization, a CooperSurgical Company, Trumbull, CT, USA) containing 5% human serum albumin (HSA), and centrifuged for 10 min at 300xg and RT. The resultant pellet was resuspended in 400 µL fresh Quinn's sperm wash medium, aliquots of 100 µL were transferred into 4 wells of a white microtitre plate (Dynatech, Chantilly, VA, USA), the first well of each row contained Quinn's sperm wash medium only and served as negative control. Before each measurement 10 µL of luminol, prepared as 5 mM stock in dimethyl sulfoxide (Sigma) was added to the well.

For each well, the levels of ROS were assessed by measuring the luminol-dependent chemiluminescence in the integrated mode for three minutes (both in control conditions and after exposure to luminol) with a Luminometer (MicroLumat Plus, Perkin Elmer instruments, Bad Wildbad, Germany). Background luminescence (obtained from Quinn's sperm wash medium negative control) was subtracted from the test values. The concentration of ROS was expressed as counts per million viable sperm, as determined by means of the eosin test.

*Apoptosis*

An early sign of apoptosis is the translocation of the negatively charged phospholipid phosphatidylserine, which has a high affinity to Annexin V (AV) (van Heerde *et al.*, 1995) from the inner leaflet of the plasma membrane to the outer surface (Vermes *et al.*, 1995). To determine the sperm's binding ability to AV, the sperm suspension in Quinn's sperm wash medium with 10 x $10^6$ sperm (total number) was diluted 1:10 with PBS (Sigma), washed, and centrifuged for 10 minutes at 500xg and RT. The supernatant was discarded and the remaining pellet resuspended in 195 µL binding

buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4). A negative control sample was analyzed by using binding buffer without $Ca^{2+}$. Afterwards, 5 µL AV labeled with FITC (Annexin V FITC Kit; Bender MedSystem Diagnostics, Vienna, Austria; catalog no. BMS306FI) were added and the samples were incubated in the dark at RT for 15 minutes. Subsequently, 800 µL binding buffer were added, and the spermatozoa were analyzed in a flow cytometer (Becton Dickinson, Lincoln Park, NY, USA). A minimum of 5,000 spermatozoa was examined for each test. The sperm population was gated by using forward-angle light scatter; side-angle light scatter was used to exclude electronic noise and debris. The FITC-labeled AV-positive sperm cells were measured in the FL1 channel of the flow cytometer. Flow cytometry data for each sample collected were; the percentage of AV-positive/-negative sperm in each gate as well as the mean channel fluorescence in each gate (AV-positive/-negative). From this data the relative activity (RA) in each gate could be calculated (% gated sperm x mean channel fluorescence).

*DNA fragmentation*

DNA fragmentation can be measured by the method of terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling assay (TUNEL assay) (Gavrielli *et al.*, 1991). This assay evaluates late stages of apoptotic events and detects both apoptotic and necrotic cells (Frankfurt *et al.*, 1996).

The characteristics of the TUNEL assay have been described elsewhere (Sun *et al.*, 1997). The assay was performed for the most part according to the method described by (Gavrielli *et al.*, 1991). One million washed spermatozoa were fixed with 4% [w/v] Para formaldehyde for 30 minutes at RT. The cells were then washed once with phosphate-buffered saline (PBS), pH 7.4, (Oxoid, Basingstoke, Hampshire, UK). The wash entailed the addition of 1 mL PBS to the fixed cell suspension with subsequent centrifugation at 800 x g for 10 minutes at room temperature. After permeabilization with 0.2% [v/v] Triton X-100 (Calbiochem, Merck, Darmstadt, Germany) in PBS for 2 minutes on ice, the samples were washed twice with PBS. A positive control sample was analyzed by incubating the spermatozoa with 50 U DNase (RNase Free; Boehringer Mannheim, Mannheim, Germany) in 0.2% [w/v] $MgCl_2$ 0.1% [w/v] $CaCl_2$ solution for 10 minutes at RT. Control samples were then washed twice more with PBS. Fifty microliters of TUNEL mix (TdT and FITC-labeled dUTP in a 1:9 ratio)

(Boehringer Mannheim) were added. To the negative control, no dUTP was added. The samples were incubated for 60 minutes at 37°C and then washed twice with PBS. They were then resuspended in 500 µL PBS. For each determination, at least 5,000 spermatozoa were examined using flow cytometry. The sperm population was gated by using forward-angle light scatter, and side-angle light scatter dot plot to exclude electronic noise and debris. The FITC-labeled dUTP-positive spermatozoa were measured in the FL1 channel of the flow cytometer (Becton Dickinson), these were TUNEL positive sperm. Flow cytometry data for each sample collected were; the percentage of TUNEL positive/negative sperm in each gate as well as the mean channel fluorescence in each gate (TUNEL positive/negative). From this data the RA in each gate were calculated (% gated sperm x mean channel fluorescence).

*Sperm Chromatin Structure Assay (SCSA®)*

In contrast to the TUNEL assay, which detects real DNA damage, the SCSA determines potential DNA damage (Henkel 2007). Single-stranded and double-stranded DNA content in the sperm head are measured with this standardized method (Evenson *et al.*, 2002). A portion of the cell suspension (5 $X10^6$) was treated with a low pH (pH 1.2) detergent containing 0.1% Triton X-100 (Calbiochem, Merck), 0.15 mol/L NaCl (Sigma) and 0.08 N HCl for 30 seconds, and then stained with 6 mg/L purified acridine orange (AO) (Molecular Probes, Eugene, OR, USA) in a phosphate-citrate buffer, pH 6.0. Cells were analyzed using the FACscan flow cytometer (Becton Dickinson). Under experimental conditions, when excited with a 488 nm light source, AO that is intercalated with double-stranded DNA will emit green fluorescence, while AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by flow cytometric (FCM) measurements of the metachromatic shift from green (native double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (damaged DNA) versus green (normal DNA) fluorescence intensity cytogram patterns. More than 5000 spermatozoa were evaluated for each semen sample. The ratio of red/(red+green) yields the percentage of DNA fragmentation, referred to as DFI.

*Statistical Analysis*

After testing for normal distribution by means of the Chi-square test, a non-parametrical test (Spearman Rank Correlation) was performed as the data were not normally distributed. Results are presented as mean ± SD as well as the median and the range. $P < 0.05$ was considered significant.

Furthermore, statistical comparison of the methods applied to test for DNA damage was performed according to Bland and Altman (1986). In addition, a mountain plot (Krouwer and Monti, 1995), which is created by computing a percentile for each ranked difference between a method and a reference method, was performed.

The two latter statistical methods are methods that compare two or more measurement techniques. The Bland and Altman plot is a simple graphic method by which the differences between two techniques are plotted against the average of the two techniques. This procedure identifies methods that are interchangeable if the differences within the mean ± 1.96 SD are not clinically important. The plot is useful in revealing relationships between the differences and the averages, looking for systematic biases and identifying possible outliers. In comparison with the Bland and Altman plot, the mountain plot has the advantages of comparing distributions more easily and of finding the central 95% of the data, even in cases where the data is not normally distributed.

The software used for statistical analyses was MedCalc version 9.0 obtained from MedCalc Software, Mariakerke, Belgium.

**RESULTS**

The summary statistics of all analyzed parameters are compiled in Table I. The high variation of values, reflected by the high standard deviation (SD) is expected, as biological parameters were measured in this study.

Table I: Summary statistics of parameters analyzed in this study

| Parameter | n | Mean ± SD | Range | Median |
|---|---|---|---|---|
| Age | 75 | 32.03 ± 7.40 | 22.00 - 58.00 | 32.00 |
| Volume (mL) | 79 | 3.44 ± 1.49 | 1.00 - 7.00 | 3.50 |
| Sperm motility (%) | 79 | 46.58 ± 12.80 | 10.00 - 70.00 | 50.00 |
| Forward Progression | 79 | 2.67 ± 0.34 | 1.00 - 3.50 | 2.75 |
| Sperm concentration ($x10^6$/mL) | 79 | 49.11 ± 36.95 | 0.80 - 205.50 | 43.00 |
| pH | 79 | 7.37 ± 0.23 | 7.00 - 8.00 | 7.20 |
| Normal sperm morphology | 79 | 6.70 ± 4.35 | 0.00 - 18.00 | 6.00 |
| White blood-cells concentration ($x10^6$/mL) | 79 | 1.04 ± 0.90 | 0.08 - 5.00 | 0.80 |
| Peroxidase-positive cells ($x10^6$/mL) | 79 | 0.18 ± 0.16 | 0.00 - 0.90 | 0.15 |
| CASA sperm motility (%) | 63 | 46.81 ± 14.25 | 11.00 - 75.00 | 49.00 |
| VCL (µm/sec) | 63 | 136.90 ± 20.08 | 101.10 - 193.50 | 137.50 |
| VSL (µm/sec) | 63 | 77.21 ± 9.57 | 48.30 - 92.60 | 80.10 |
| VAP (µm/sec) | 63 | 88.98 ± 11.12 | 59.60 - 109.50 | 91.30 |
| ALH (µm) | 63 | 4.84 ± 0.81 | 3.20 - 7.00 | 4.80 |
| Luminescence | 73 | 3145.15 ± 5940.93 | -16.75 - 42341.25 | 1234.75 |
| ROS count/million viable sperm | 73 | 401.56 ± 1365.15 | 0.00 - 8900.00 | 93.00 |
| SCSA® (green) | 64 | 88.10 ± 41.27 | 48.41 - 237.87 | 73.46 |
| DFI (%) | 64 | 0.36 ± 0.23 | 0.04 - 0.85 | 0.30 |
| % AV-negative sperm | 68 | 71.71 ± 15.68 | 27.01 – 99.83 | 72.87 |
| % TUNEL negative sperm | 59 | 65.01 ± 24.34 | 14.59 – 97.65 | 70.78 |
| TUNEL negative MCF | 59 | 412.01 ± 81.44 | 312.79 – 609.16 | 384.00 |

**CASA:** Computer assisted semen analysis; **VCL:** curvilinear velocity; **VSL:** straight line velocity; **VAP:** average path velocity; **ALH:** amplitude of lateral head displacement; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded DNA; **MCF:** mean channel fluorescence; **DFI:** DNA fragmentation index; **AV:** Annexin V; **TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling

Table II represents correlation statistics of reactive oxygen species (ROS) present in the semen with different parameters measured in this study. While ROS in the neat semen did not correlate with age, sperm motility, forward progression and normal sperm morphology, a significant negative correlation was found for sperm concentration (n = 73; r = -0.505; P < 0.0001), as well as for the percentage of AV-negative sperm (non-apoptotic sperm) in the neat semen (n = 63; r = -0.374; P = 0.0032). On the other hand, there was a significant positive correlation of ROS in the neat semen with the concentration of peroxidase positive cells in the neat semen (n = 73; r = 0.403; P = 0.0006). Furthermore, the data from the current study show that there is no relationship between the ROS measured in the neat semen and the DNA fragmentation of the sperm as measured by the SCSA® and TUNEL assay.

Table II: Correlation of reactive oxygen species (ROS), as measured in the neat semen, with different parameters

| Parameter | n | Spearman's ρ | *P* |
|---|---|---|---|
| Age (years) | 69 | -0.064 | 0.5950 |
| Sperm motility (%) | 73 | -0.189 | 0.1096 |
| Forward progression | 73 | 0.075 | 0.5257 |
| Sperm concentration (x$10^6$/mL) | 73 | -0.504 | <0.0001 |
| Morphology | 73 | -0.154 | 0.1915 |
| % AV-negative sperm | 63 | -0.374 | 0.0032 |
| % TUNEL negative sperm | 57 | -0.124 | 0.3526 |
| SCSA® (green) | 59 | -0.139 | 0.2892 |
| DFI (%) | 59 | 0.134 | 0.3080 |
| Leukocytes (x$10^6$/mL) | 73 | 0.403 | 0.0006 |

**AV:** Annexin V; **TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded/native DNA; **DFI:** DNA fragmentation index

In this study, correlations of the percentage of non-apoptotic sperm in the neat semen sample with the age of the patient, normal sperm morphology, the percentage of TUNEL negative sperm, the native DNA content of the sperm nucleus as well as the DFI did not reach significance (Table III). However, significant positive

correlations were found between the percentage of Annexin V-negative sperm and sperm motility (n = 67; r = 0.267; *P* = 0.0302) as well as the sperm concentration (n = 67; r = 0.411; *P* = 0.0008) in the neat semen.

Table III: Correlation of percentage Annexin V-negative sperm with different parameters

| Parameter | n | Spearman's ρ | *P* |
|---|---|---|---|
| Age (years) | 64 | -0.019 | 0.8822 |
| Sperm motility (%) | 67 | 0.267 | 0.0302 |
| Forward progression | 67 | 0.221 | 0.0729 |
| Sperm concentration (x$10^6$/mL) | 67 | 0.411 | 0.0008 |
| Morphology | 67 | 0.177 | 0.1513 |
| SCSA® (green) | 57 | 0.058 | 0.6630 |
| DFI (%) | 57 | -0.232 | 0.0820 |

**SCSA®:** sperm chromatin structure assay; **(green):** double-stranded/native DNA; **DFI:** DNA fragmentation index

Table IV: Correlation of the percentage of TUNEL-negative sperm with different parameters

| Parameter | n | Spearman's ρ | *P* |
|---|---|---|---|
| Age (years) | 55 | -0.097 | 0.4779 |
| Sperm motility (%) | 59 | 0.446 | 0.0007 |
| Forward progression | 59 | 0.155 | 0.2389 |
| Sperm concentration (x$10^6$/mL) | 59 | 0.537 | <0.0001 |
| Morphology | 59 | 0.143 | 0.2753 |
| % AV-negative sperm | 53 | 0.212 | 0.1271 |
| SCSA® (green) | 52 | 0.325 | 0.0201 |
| DFI (%) | 52 | -0.994 | <0.0001 |
| Leukocytes (x$10^6$/mL) | 59 | 0.058 | 0.6596 |

**AV:** Annexin V; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded/native DNA; **DFI:** DNA fragmentation index

Table IV is a summary of the relationship between the percentage of TUNEL-negative sperm (sperm with intact DNA) and the different parameters measured. Correlations between sperm with intact (non-fragmented) DNA and the patient's age, percentage normal sperm morphology, sperm apoptotic status as well as the concentration of peroxidase positive cells in the neat semen, did not reach significance.

However, there were significant positive correlations between sperm with intact DNA and the percentage of motile sperm (n = 59; r = 0.446; *P* = 0.0007), the sperm concentration (n = 59; r = 0.537; *P* < 0.0001) and the SCSA (green) native DNA content of the sperm nucleus (n = 52; r = 0.325; *P* = 0.0201). In contrast, there was a significant negative correlation between sperm with intact DNA (TUNEL-negative) and the sperm DNA fragmentation index (DFI) as measured with the SCSA® (n = 52; r = -0.994; *P* < 0.0001).

Correlations between the sperm movement attributes, as measured with CASA, and ROS, the percentage AV-negative and TUNEL-negative spermatozoa as well as the DNA fragmentation index (DFI) are presented in Table V. The percentage motile spermatozoa as determined with CASA had significant positive correlations with the percentage AV-negative (n = 57; r = 0.263; *P* = 0.0479) and the percentage TUNEL-negative (n = 46; r = 0.397; *P* = 0.0063) spermatozoa. Furthermore, there was a significant negative correlation between the DFI and the percentage motile spermatozoa (n = 51; r = -0.305; *P* = 0.0296). None of the other velocity measurements (VCL, VSL, VAP and ALH) correlated with any of the parameters measured.

Table V: Correlation of CASA motility parameters with reactive oxygen species (ROS) production in the semen, apoptotic status of spermatozoa, percentage of TUNEL-negative spermatozoa and the sperm DNA fragmentation index (DFI)

| CASA Parameter | | Spearman's n | Spearman's ρ | Spearman's *P* |
|---|---|---|---|---|
| Motility (%) | ROS | 57 | -0.156 | 0.2444 |
| | % AV-negative sperm | 57 | 0.263 | 0.0479 |
| | % TUNEL-negative sperm | 46 | 0.397 | 0.0063 |
| | DFI (%) | 51 | -0.305 | 0.0296 |
| VCL (µm/sec) | ROS | 57 | -0.032 | 0.8129 |
| | % AV-negative sperm | 57 | -0.0593 | 0.6615 |
| | % TUNEL-negative sperm | 46 | -0.073 | 0.6302 |
| | DFI (%) | 51 | -0.087 | 0.5445 |
| VSL (µm/sec) | ROS | 57 | 0.141 | 0.2919 |
| | % AV-negative sperm | 57 | -0.123 | 0.3640 |
| | % TUNEL-negative sperm | 46 | 0.027 | 0.8593 |
| | DFI (%) | 51 | -0.083 | 0.5618 |
| VAP (µm/sec) | ROS | 57 | 0.072 | 0.5921 |
| | % AV-negative sperm | 57 | -0.064 | 0.6372 |
| | % TUNEL-negative sperm | 46 | -0.026 | 0.8616 |
| | DFI (%) | 51 | -0.070 | 0.6246 |
| ALH (µm) | ROS | 57 | -0.129 | 0.3360 |
| | % AV-negative sperm | 57 | -0.043 | 0.7490 |
| | % TUNEL-negative sperm | 46 | -0.102 | 0.4988 |
| | DFI (%) | 51 | -0.081 | 0.5714 |

**AV:** Annexin V; **VCL:** curvilinear velocity (a measure of the total distance traveled by a given sperm during the acquisition divided by the time elapsed); **VSL:** straight line velocity (the straight line distance from beginning to end of track divided by time taken); **VAP:** average path velocity (the spatially average path that eliminates the wobble of the sperm head); **ALH:** amplitude of lateral head displacement (the mean width of sperm head oscillation); **DFI:** DNA fragmentation index; **ROS:** reactive oxygen species

Assessing agreement between the two methods applied to test DNA fragmentation, SCSA® (green) and the TUNEL assay (mean channel fluorescence), by performing a Bland-Altman plot (Figure 1) resulted in a graph where the data points are not evenly distributed along the abscissa.

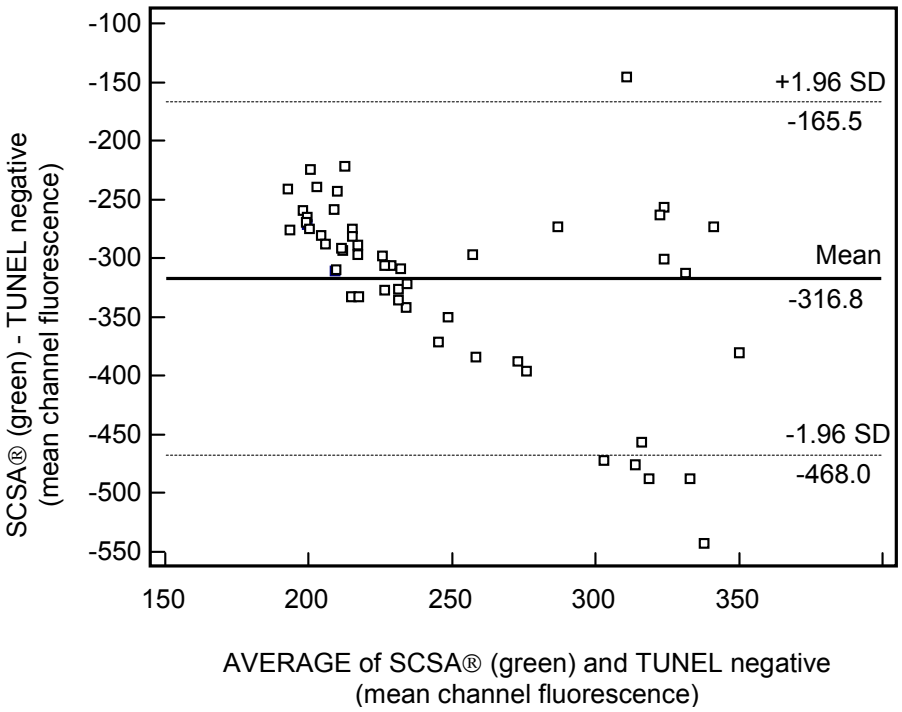


Figure 1: A Bland and Altman plot for the comparison of the two test systems, i.e. TUNEL (mean channel fluorescence) and SCSA® (green), used in this study to evaluate DNA damage in the sperm nucleus. The graph displays a scatter diagram of the differences plotted against the averages of the two test systems and shows that the variation of one of the methods is dependant on the magnitude of the measurements.

A folded empirical distribution function curve, more commonly known as a mountain plot (Figure 2), comparison of the SCSA® (green) with TUNEL as the reference method, revealed a marked deviation from zero, indicating that the two methods are not unbiased. Furthermore, the long tail to the right-hand side in the plot is indicative of large differences between the two methods.

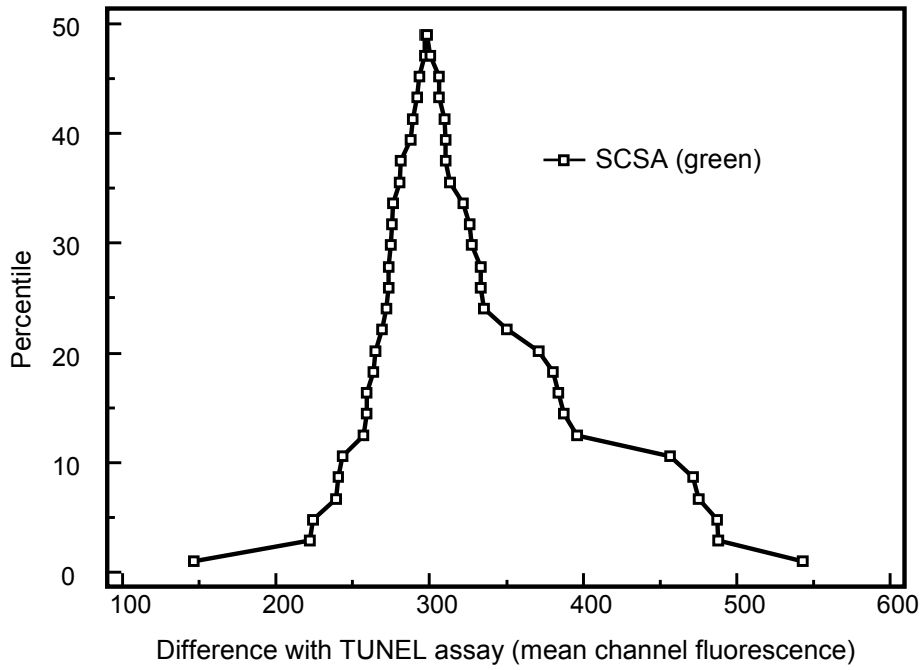


Figure 2: A Mountain plot for the comparison of the sperm chromatin structure assay (SCSA® (green)) with the terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling (TUNEL) assay (mean channel fluorescence).

**DISCUSSION**

In this study, a variety of biological assays were carefully applied to detect reactive oxygen species (ROS) in the neat semen, DNA fragmentation in the sperm nucleus and sperm cell apoptosis (programmed cell death). Except for the detection of ROS, which was based on luminescence, DNA fragmentation and apoptosis was detected using flow cytometry. Automated detection of fluorescence, with flow cytometry, gives some advantages over the manual alternative, fluorescence microscopy. Flow cytometry is more accurate because of a higher number of cells evaluated, particularly more discriminative, allow for automatic, therefore rapid, analysis of a high number of cells and provide less subjective and statistically more reliable results than microscopic examination (Telford *et al.*, 1994; Darzynkiewics *et al.*, 1997).

In the current study, we assessed three different cytofluorometric methods detecting firstly, DNA fragmentation of the sperm nucleus with the terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling (TUNEL) assay and the sperm chromatin structure assay (SCSA®) and secondly sperm cell apoptosis with biotinylated Annexin V binding to phosphatidylserine. The presence of these cellular alterations, as detected with luminescence and flow cytometry, were compared with each other as well as conventional sperm parameters. The conventional sperm parameters (motility, forward progression, sperm concentration, white blood cell concentration, supra vital stain, quantification of seminal leukocytes) were all assessed according to the World Health Organization (WHO) criteria (WHO, 1999), and normal sperm morphology was assessed according to strict criteria (Kruger *et al.*, 1986; WHO, 1999). Furthermore, sperm motility and velocity parameters were assessed with computer assisted semen analysis (CASA). All above mentioned conventional semen parameters, as well as ROS, were assessed in neat semen, for all other parameters measured in this study (sperm DNA fragmentation and apoptotic status and sperm chromosome packaging) the seminal plasma fraction of the neat semen was washed away.

When considering the ROS production in the neat semen, the results of the current study (Table II) show a significant negative correlation between the production of ROS and the sperm concentration ($n = 73$; $r = -0.505$; $P < 0.0001$), this result

confirms previous findings (Ochsendorf *et al.*, 1994; Henkel and Schill, 1998; Henkel *et al.*, 2003). Furthermore, there was a significant positive correlation between the production of ROS and the concentration of leukocytes (n = 73; r = 0.403; *P* = 0.0006). These results suggest that the ROS measured in this study was produced mainly by the leukocytes in the neat semen (Aitken *et al.*, 1992; Kessopoulou *et al.*, 1992; Griveau *et al.*, 1995; Ford *et al.*, 1997). ROS affect the functional competence and DNA integrity of sperm cells at different rates and in different ways. The effect of ROS is concentration dependant. At low levels of oxidation, these effects are beneficial and not detrimental (Godeas *et al.*, 1997). Furthermore, a role for ROS in the induction of sperm chromatin compaction has been suggested (Bianchi *et al.*, 1993; Manicardi *et al.*, 1998); this is thought to be of physiological importance in protecting the genome from oxidative attack (Bianchi *et al.*, 1993; Manicardi *et al.*, 1998).

On the other hand, it has been demonstrated that exogenous ROS generation causes an increase in DNA fragmentation in human sperm and that the administration of antioxidants prevents the amount of DNA damage (Lopes *et al.*, 1998a). The question remains, where in the life cycle sperm of sperm cells do leukocytes affect spermatozoa; is it possibly during spermatogenesis or epididymal maturation or maybe after ejaculation? Due to the limited and short-term exposure, a direct and immediately mediated effect of leukocytes appears unlikely. In this regard, the data of this study are consistent with those of Henkel *et al.* (2003) in that the results of this study show a poor correlation between the ROS production in the ejaculate and percentage of DNA fragmentation as measured with either SCSA® or the TUNEL assay (Table II) as well as a non-significant correlation between the number of leukocytes and the percentage of TUNEL-negative spermatozoa (Table IV).

Furthermore, the results of this study showed a significant negative correlation between ROS in the neat semen and the percentage of non-apoptotic (Annexin V-negative) spermatozoa (n = 63; r = -0.124; *P* = 0.0032) (Table II). There therefore seems to be a very close relationship between the presence of leukocytes in the semen and apoptosis of the sperm. Two possible hypotheses are suggested: the leukocytes might be responsible for apoptosis, or, alternatively, the apoptotic sperm

might induce a chemotactic response. Various studies (Aitken *et al.*, 1992; Kessopoulou *et al.*, 1992; Griveau *et al.*, 1995; Ford *et al.*, 1997) support the first hypothesis. As mentioned previously, leukocytes are considered the main source of ROS in sperm suspensions. ROS are a known inductor of apoptosis in somatic cells (Ratan *et al.*, 1994) as well as in mature spermatozoa (Ramos and Wetzels, 2001). In an experimental study, Ramos and Wetzels (2001) showed that Annexin V (AV) binding of spermatozoa increases shortly after addition of $H_2O_2$ and after just 60 minutes incubation of sperm with $H_2O_2$ almost all cells were in apoptosis. However, a clear *in vivo* demonstration of the relationship between leukocyte contamination and male fertility potential has not yet been provided.

The second hypothesis is that leukocytes are attracted by apoptotic spermatozoa. Apoptotic cells are believed to undergo heterophagic elimination by phagocytes without releasing pro-inflammatory mediators or ROS (Rossi and Aitken, 1997). It has been hypothesized that a similar mechanism probably provides for senescent or abnormal sperm removal (Rossi and Aitken, 1997). It has been demonstrated that many spermatogenetic cells die by apoptosis and are subsequently phagocytosed by Sertoli cells and that translocation of phosphatedylserine to the outer part of the plasma membrane is the signal for phagocytosis (Shiratsuchi *et al.*, 1997).

In addition to the significant correlation of the percentage of non-apoptotic spermatozoa with ROS production in the semen, significant positive correlations were found between non-apoptotic spermatozoa and percentage sperm motility [WHO criteria: (n = 67; r = 0.267; $P = 0.0302$); CASA: (n = 57; r = 0.263; $P = 0.0479$)] (Tables III and V) and sperm concentration (n = 67; r = 0.411; $P = 0.0008$) (Table III). However, these sperm parameters not only correlated significantly with the apoptotic status of the sperm but also with the DNA fragmentation status (as measured by the TUNEL assay) of the sperm nucleus. Significant positive correlations were found between the percentage of spermatozoa with intact DNA and percentage motile sperm [WHO criteria: (n = 59; r = 0.446; $P = 0.0007$); CASA: (n = 46; r = 0.397; $P = 0.0063$)] (Tables IV and V) as well as the sperm concentration (n = 59; r = 0.537; $P < 0.0001$) (Table IV). The above mentioned results are in accordance with the findings of other studies (Barroso et al., 2000; Oosterhuis *et al.*, 2000; Henkel *et al.*, 2003) and not only confirm that a low sperm concentration might be caused by a high rate

of apoptosis, but also that this apoptotic process takes place just before or just after ejaculation because the TUNEL assay, which measures apoptosis at a relatively late stage of the process, is more strongly correlated with sperm concentration than was the AV binding assay, which measures apoptosis during an early stage of the process. In addition, the results of the current study confirm the relation between non-apoptotic spermatozoa and sperm motility (Oosterhuis *et al.*, 2000) as well as TUNEL-negative spermatozoa and sperm motility (Sun *et al.*, 1997; Henkel *et al.*, 2003).

Although this study showed no correlation between measured parameters (ROS, AV binding, SCSA® and TUNEL assay) and the sperm velocity parameters (VCL, VSL, VAP and ALH) (Table V) as determined with CASA, Aitken *et al.* (1998) showed that these parameters appeared to be much more sensitive to the detrimental effects of oxidative stress than the percentage motility, which was not impaired at high levels of oxidative stress, or the rate of sperm-oocyte fusion, which was also determined in the study (Aitken *et al.*, 1998).

In relation to the AV binding assay, none of the other sperm parameters measured reached significance when correlated with the percentage of non-apoptotic spermatozoa (Table III). Contrary, there was a significant positive correlation between the native DNA (sperm with green fluorescence as measured with the SCSA®) content of the sperm nucleus and the percentage of TUNEL-negative (non-fragmented DNA) spermatozoa ($n = 52$; $r = 0.325$; $P = 0.0201$) (Table IV), as well as a significant negative correlation between TUNEL-negative spermatozoa and the DNA fragmentation index (DFI), as measured with the SCSA® ($n = 52$; $r = -0.994$; $P < 0.0001$) (Table IV). Both SCSA® and the TUNEL assay measure sperm DNA fragmentation, and although the data from this study show a significant correlation between these two assays ($r = -0.994$; $P < 0.0001$) (Table IV) they essentially measure different aspects of DNA fragmentation as can be seen from the Bland and Altman plot (Bland and Altman, 1986) (Figure 1) as well as the Mountain plot (Krouwer and Monti, 1995) (Figure 2). The correlation coefficient (r) measures the strength of the relation between the two sperm DNA fragmentation methods and not the agreement between them (Bland and Altman, 1986). The SCSA®, developed by

Evenson *et al.* (1999), measures potential DNA damage, and is based on the same principle of the metachromatic shift of the colour of acridine orange as the acridin orange test (Tejada *et al.*, 1984), the detection method, however, is flow cytometry. On the other hand, the TUNEL assay measures real DNA damage, i.e. DNA strand breaks (Gorczyca *et al.*, 1993). During the assay, labeled DNA precursors (dUTP; deoxyuridine triphosphate) are bound to single- and double-strand DNA breaks by means of an enzymatically catalyzed reaction using the template-independent terminal deoxynucleotidyl transferase (TdT). Biotinylated or fluorescinated dUTP is incorporated at the 3'-OH ends of the DNA, which increases as the number of strand breaks increase resulting in an equivalent increase in the signal strength (Gorczyca *et al.*, 1993).

To summarize, the results of the current study confirm those found by many other authors. With relation to reactive oxygen species (ROS) a significant negative correlation was found with sperm concentration ($P < 0.0001$) as well as non-apoptotic (AV-negative) sperm ($P = 0.0032$) (Table II). In addition a positive correlation was found with the concentration of leukocytes ($P = 0.0006$) (Table II). Not only did non-apoptotic sperm have significant positive correlations with both sperm motility ($P = 0.0302$) and sperm concentration ($P < 0.0001$) (Table III), TUNEL-negative sperm showed the same significant positive relation with the percentage of motile sperm ($P = 0.0007$) as well as with the sperm concentration ($P < 0.0001$) (Table IV). In addition, it was also shown that although there was a significant correlation between TUNEL-negative sperm and the DNA fragmentation index (DFI) as determined with the SCSA® ($r = -0.994$; $P < 0.0001$) these two assays essentially measure different aspects of DNA fragmentation (Figures 1 and 2) and are therefore not directly comparable or exchangeble.

From the literature it is evident that high rates of apoptotic spermatozoa have been associated with decreased fertilization rates (Sun *et al.*, 1997; Host *et al.*, 2000), on the other hand, Henkel *et al.* (2003) showed that sperm with DNA fragmentation are able to fertilize an oocyte but that these embryos will result in lower implantation and pregnancy rates. Furthermore, oxidative stress in the ejaculate and DNA damaged sperm has been implicated as the cause of elevated levels of childhood cancer in the offspring (Fraga *et al.*, 1996; Ji *et al.*, 1997; Sorahan *et al.*, 1997). The tests applied

in this study fall outside the capabilities of many andrology laboratories, especially those in developing countries. As they are specialized, they require skilled and trained technologists, and expensive equipment to perform. However, the results of this study contribute towards the necessary knowledge which can assist the embryologist performing assisted reproductive technologies (ART) to make informed decisions, based on scientific evidence, which could lead ultimately to improved implantation and pregnancy rates. When the treatment of choice is intracytoplasmic sperm injection (ICSI) selecting the best sperm for fertilization is crucial; therefore, a step-wise selection protocol for ICSI sperm is recommend. Firstly, the careful selection of progressively motile spermatozoa and thereafter a morphology assessment, according to strict criteria, of those selected. Based on the results of this study, as well as those of other authors mentioned, these spermatozoa should result in increased fertilization, implantation and ultimately pregnancy rates as well as a lower risk of developing childhood cancer.

In conclusion, the results of the study showed that sperm motility and concentration had significant positive correlations with non-apoptotic and DNA non-fragmented sperm. The application of these findings in clinical practice can ultimately increase implantation and pregnancy rates in patients where ICSI is the treatment of choice.

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**REFERENCES**

Agarwal A and Saleh RA (2002) Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 29, 817-827.

Agarwal A, Saleh RA and Bedaiway, MA (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 4, 829-843.

Aitken RJ, Clarkson JS and Fishel S. (1989a) Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biology of Reproduction 41,183–187.

Aitken RJ, Clarkson S, Hargreave TB, Irvine DS and Wu FCW (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl 10, 214-220.

Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K and Richardson DW (1992) Differential contribution of leukocytes and spermatozoa to the high levels of reactive oxygen species recorded in the ejaculates of oligozoospermic patients. J Reprod Fertil 94, 451-462.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z and Irvine DS (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biology of Reproduction 59, 1037–1046.

Aitken RJ and Krausz C (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122, 497-506.

Aitken RJ and Sawyer D (2003) The human spermatozoon – not waving but drowning. Adv Exp Med Biol 518, 85-98.

Alvarez JG, Touchstone JC, Blasco L and Storey BT (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl 8, 338-348.

Alvarez JG (2003) DNA fragmentation in human spermatozoa: significance in the diagnosis and treatment of infertility. Minerva Ginecol 55, 233-239.

Arends M and Wyllie A (1991) Apoptosis: mechanism and roles in pathology. Int. Rev Exp Pathol 32, 223-254.

Balhorn R, Cosman M, Thornton K, Krishnan VV, Corzett M, Bench G, Kramer C, Lee J and Hud NV (1999) Protamine mediated condensation of DNA in mammalian

sperm. In Gagnon C (ed), The Male Gamete: From Basic Science to Clinical Applications. Cache River Press , Vienna, IL, pp.55-78.

Barroso G, Morshedi M and Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 15, 1338-1344.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U and Sakkas D (1993) Effect of deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick-translation of murine and human mature spermatozoa. Biol Reprod 49, 1083–1088.

Bland JM and Altman DG (1986) Statistical method for assessing agreement between two methods of clinical measurement. The Lancet i, 307-310.

Bossy-Wetzel E, Newmeyer DD and Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 17, 37-49.

Cuvier O and Hirano T (2003) A role of topoisomerase II in linking DNA replication to chromosome condensation. J Cell Biol 160, 645-655.

Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Muratami T and Traganos F (1997) Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 27, 1–20.

Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68, 383-424.

Evenson DP, Darzynkiewicz Z and Melamed MR (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210, 1131-1133.

Evenson DP (1990) Flow cytometric analysis of male germ cell quality. Methods Cell Biol 33, 401–410.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P and Claussen OP (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 3, 1039–1049.

Evenson DP, Larson KL and Jost LK (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 23, 25–43.

Ford WC, Whittington K and Williams AC (1997) Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of NADPH to protect sperm against their effects. Int J Androl 20, (Suppl 3), 44-49.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM and Ames BN (1996) Smoking and low antioxidant levels increase oxidative damage to DNA mutation. Research 351, 199–203.

Frankfurt OS, Robb JA, Sugarbaker EV and Villa L (1996) Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. Exp Cell Res 226, 387-397.

Gavrielli Y, Sherman Y and Ben-Sasson AB (1991) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119, 493-501.

Godeas C, Tramer F, Micali F, Soranzo M, Sandri G and Panfili E (1997) Distribution and possible novel role of phopholipid hydroperoxide glutathione peroxidase in rat epididymal spermatozoa. Biol Reprod 57, 1502-1508.

Goldberg RB, Geremia R and Bruce WR (1977) Histone synthesis and replacement during spermatogenesis in the mouse. Differentiation 7, 167–180.

Gorczyca W, Traganos F, Jesionowska H and Darzynkiewicz Z (1993) Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Exp Cell Res 207, 202–205.

Griveau JF, Dumont E Renard P, Callegari JP and Le Lannou D (1995) Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J Reprod Fertil 103, 17-26.

Henkel R and Schill WB (1998) Sperm separation in patients with urogenital infections. Andrologia 30, (Suppl 1), 91-97.

Henkel R, Kierspel E, Hajimohammed M, Stalf T, Hoogendijk CF, Mehert C, Menkveld R, Schill W-B and Kruger TF (2003) DNA fragmentation of spermatozoa and assisted reproductive technology. RBM Online 7, 477-484.

Henkel R, Hajimohammed M, Stalf T, Hoogendijk CF, Mehnert C, Menkveld R, Gips H, Schill W and Kruger TF (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 4, 965-972.

Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB and Kruger TF (2005a) Effect of reactive oxygen species produced by spermatozoa

and leukocytes on sperm functions in non-leukocytospermic patients. Fertil Steril 83, 635-642.

Henkel R, Maaß G, Bödeker R-H, Scheibelhut C, Stalf T, Mehnert C, Schuppe H-C, Jung A and Schill W-B (2005b) Sperm function and assisted reproduction technology. Rep Med Biol 4, 7-30.

Henkel R (2007) DNA fragmentation and its influence on fertilization and pregnancy outcome. In Oehninger SC and Kruger TF (eds) Male Infertility: Diagnosis and treatment. Informa UK Ltd., Oxon, UK, pp.277-290.

Hirano T (2002) The ABCs of SMC proteins: two-armed ATPases for chromosome condensation, cohesion, and repair. Genes Dev 16, 399-414.

Host E, Lindenberg S and Smidt-Jensen S (2000) DNA strand breaks in human spermatozoa: correlation with fertilization *in vitro* in oligozoospermic men and in men with unexplained infertility. Acta Obstet Gynecol Scand 79, 189-193.

Hotckiss, RS et al., (1938) Semen analyses of two hundred fertile men. Am J Med 196, 362-384.

Hughes CM, Lewis SEM, McKelvey-Martin J and Thompson W (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod 2, 613–619.

Hughes CM, Lewis SE, McKelvey-Martin VJ and Thompson W (1998) The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum Reprod 13, 1240-1247.

Hughes CM, McKelvey-Martin VJ and Lewis SE (1999) Human sperm DNA integrity assessed by the Comet and ELISA assays. Mutagenesis 14, 71-75.

Irvine DS, Twigg J, Gordon E, Fulton N, Milne P and Aitken RJ (2000) DNA integrity in human spermatozoa: relationship with semen quality. J Androl 21, 33–44.

Iwasaki A and Gagnon C (1992) Formation of reactive oxygen species in spermatozoa of infertile patients. Fertl Steril 57, 409-416.

Jarow JP (1998) Detection of oxidative RNA damage in human sperm and the association with cigarette smoking. J Urol 159, 1774-1775.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM and Jin F (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. J Natl Cancer Inst 89, 238–244.

Jurisicova A, Lopes S, Meriano J, Oppedisano L, Casper RF and Varmuza S (1999) DNA damage in round spermatids of mice with targeted disruption of the Pplcγ

gene and in testicular biopsies of patients with non-obstructive azoospermia. Mol Hum Reprod 5, 323-330.

Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26, 239-257.

Kerr JF (1993) Definition of apoptosis and overview of its incidence. In Lavinand M and Watters D (eds), Programmed Cell Death. Harwood Academic Publishers, Langhorne, PA, USA, pp.1-15.

Kessopoulou E, Tomlinson MJ, Barratt CL, Bolton AE and Cooke ID (1992) Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? J Reprod Fertil 94, 463-470.

Kodama H, Yamaguchi R, Fukuda J, Kasai H and Tanaka T (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril 68, 519–524.

Krammer PH, Behrmann I, Daniel P, Dhein J and Debatin KM (1994) Regulation of apoptosis in the immune system. Curr Opin Immunol 6, 279-289.

Krouwer JS and Monti KL (1995) A simple, graphical method to evaluate laboratory assays. Eur J Clin Chem Clin Biochem 33, 525-527.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA and Smith K (1986) Sperm morphologic features as a prognostic factor in *in vitro* fertilization. Fertil Steril 46, 1118-1123.

Kumaroo KK, Jahnke G and Irvin JL (1975) Changes in basic chromosomal proteins during spermatogenesis in the mature rat. Arch Biochem Biophys 168, 413–424.

Lee J, Richburg JH, Youkin SC and Boekelheide K (1997) The FAS system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138, 2081-2088.

Lin WW, Lamb DJ, Wheeler TM, Lipschultz LI and Kim ED (1997a) *In situ* en-labeling of human testicular tissue demonstrates apoptosis in conditions of abnormal spermatogenesis. Fertil Steril 68,1065-1069.

Lin WW, Lamb DJ, Wheeler TM, Abrams J, Lipshultz LI and Kim ED (1997b) Apoptotic frequency is increased in spermatogenic maturation arrest and hypospermatogenic states. J Urol 158, 1791-1793.

Lloyd DR, Phillips DH and Carmichael PL (1997) Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. Chem Res Toxicol 10, 393-400.

Lopes S, Sun JG, Jurisicova A, Meriano J and Casper RF (1998a) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 69, 528-532.

Lopes S, Jurisicova A, Sun JG and Casper RF (1998b) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 13, 896-900.

Luo X, Budihardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94, 481-490.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U and Sakkas D (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod 52, 864–867.

Manicardi GC, Tombacco A, Bizzaro D, Bianchi U, Bianchi PG and Sakkas D (1998) DNA strand breaks in ejaculated human spermatozoa: Comparison of susceptibility to the nick translation and terminal transferase assays. Histochem J 30, 33–39.

Martin S, Reutelingsperger C, McGahon AJ, Rader AJ, van Schie RC, LaFace DM and Green DR (1995) Early redistribution of plasma membrane phophatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition of overexpression of Bcl-2 and Abl. J Exp Med 182, 1545-1556.

McPherson SM and Longo FJ (1992) Localization of DNAse I-hypersensitive regions during rat spermatogenesis: stage dependent patterns and unique sensitivity of elongating spermatids. Mol Reprod Dev 31, 268-279.

McPherson SM and Longo FJ (1993a) Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids. Eur J Histochem 37, 109-128.

McPherson SM and Longo FJ (1993b) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol 158, 122-130.

Menkveld R, Stander FSH, Kotze TJvW, Kruger TF and Van Zyl JA (1990) The evaluation of morphological characteristics of human spermatozoa according to strict criteria. Hum Reprod 5, 586-592.

Nagata S and Suda T (1995) Fas and Fas ligand: lpr and gld mutations. Immunol Today 16, 39-43.

Ochsendorf FR, Thiele J, Fuchs J, Schuttau H, Freisleben HJ, Buslau M and Milbradt R (1994) Chemiluminescence in semen of infertile men. Andrologia 26, 289-293.

Oosterhuis GJ, Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J and Vermes I (2000) Measuring apoptosis in human spermatozoa: a biological assay for semen quality? Fertil Steril 74, 245-250.

Poccia D (1986) Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. Int Rev Cytol 105, 1–65.

Print CG and Loveland KL (2000) Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 22, 423-430.

Ramos L and Wetzels AM (2001) Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. Hum Reprod 16, 1703-1707.

Ratan PR, Murphy TH and Barban JM (1994) Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem 62, 376-379.

Rhemrev JP, Vermeiden JP, Haenen GR, De Bruijne JJ, Rekers-Mombarg LT and Bast A (2001) Progressively motile human spermatozoa are well protected against *in vitro* lipid peroxidation imposed by induced oxidative stress. Andrologia 33, 151-158.

Rodriguez I, Ody C, Araki K, Garcia I and Vassali P (1997) An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J 16, 2262-2270.

Rossi AG and Aitken RJ (1997) Interactions between leukocytes and the male reproductive system. The unanswered questions. Adv Exp Med Biol 424, 245-252.

Sakkas D, Manicardi GC, Bianchi PG, Bizzaro D and Bianchi U (1995) Relationship between the presence of endogenous nicks and sperm chromatin packing in maturing and fertilizing mouse spermatozoa. Biol Reprod 52, 1149-1155.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG and Bianchi U (1999) Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 4, 31-37.

Sakkas D, Seli E, Bizzaro D, Tarozzi N and Manicardi GC (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. RBM Online 7, 428-432.

Shekarriz M, Sharma RK, Thomas AJ Jr and Agarwal A (1995) Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. J Assist Reprod Genet 12, 70-74.

Shen HM, Chia SE, Ni ZY, New AL, Lee BL and Ong CN (1997) Detection of oxidative DNA damage in human sperm and association with cigarette smoking. Reprod Toxicol 11, 675-680.

Shiratsuchi A, Umeda M, Ohba Y and Nakanishi Y (1997) Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. J Biol Chem 272, 2354-2358.

Sinha Hikim AP, Wang C, Lue Y, Johnson L, Wang XH and Swerdloff RS (1998) Spontaneous germ cell apoptosis in humans: Evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. J Clin Endocrinol Metab 83, 152-156.

Sorahan T, Lancashire RJ, Hulten MA, Peck I and Stewart AM (1997) Childhood cancer and parental use of tobacco: deaths from 1953 to 1955. Br J Cancer 75, 134-138.

Stahl O, Eberhard JE, Jepson K, Spano M, Cwikiel M, Cavallin-Stahl E and Giwercman A (2006) Sperm DNA integrity in testicular cancer patients. Hum Reprod 21, 3199-3205.

Steller H (1995) Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

Schulze-Osthoff K, Krammer PH and Droge W (1994) Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBO J 13, 4587-4596.

Suda T, Takahashi T, Golstein P and Nagata S (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75, 1169-1178.

Sun JG, Jurisicova A and Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization *in vitro*. Biol Reprod 56, 602-607.

Tejada RI, Mitchell JC, Norman A, Marik JJ and Friedman S (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril 42, 87-91.

Telford WG, King LE and Fraker PJ (1994) Rapid quantification of apoptosis in pure and heterogeneous cell populations using flow cytometry. J Immunol Meth 172, 1–16.

Thomas J, Fishel SB, Hall JA, Green S, Newton TA and Thornton SJ (1997) Increased polymorphonuclear granulocytes in seminal plasma in relation to sperm morphology. Hum Reprod 12, 2418-2421.

Thornberry NA and Lazebnik Y (1998) Caspases: enemies within. Science 281, 1312-1316.

Twigg J, Fulton N, Gomez E, Irvine DS and Aitken RJ (1998a) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 13, 1429–1437.

Twigg J, Irvine DS, Houston P, Fulton N, Michael L and Aitken RJ (1998b) Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. Mol Hum Reprod, 4, 439–445.

Van Blerkom J and Davis PW (1998) DNA strand breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis. Hum Reprod 13, 1317-1324.

Van Heerde WL, Degroot PG and Reutelingsperger CPM (1995) The complexity of the phospholipid binding protein annexin V. Thromb Haemost 73, 172-179.

Vermes I, Haanen C and Reutelingsperger CPM (1995) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labelled annexin V. J Immunol Methods 180, 39-52.

Ward WS and Coffey DS (1991) DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cells (Review). Biol Reprod 44, 569-574.

Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA and Nagata S (1992) The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148, 1274-1279.

Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S and Oehninger S (2002) Caspase activity and apoptotic markers in ejaculated human sperm. Mol Hum Reprod 8, 984-991.

Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG and Ford WC (1999) Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. Int J Androl 22, 236-242.

World Health Organization (1999) World Health Organization Laboratory Manual for Examination of Human Semen. Cambridge University Press, Cambridge, UK.

# CHAPTER III

## Human spermatozoa with P-pattern normal sperm morphology according to strict criteria show increased levels of DNA fragmentation

Abstract

Introduction

Materials and Methods

- *Semen samples*
- *Apoptosis*
- *DNA fragmentation*
- *Sperm Chromatin Structure Assay (SCSA®)*
- *Statistical Analysis*

Results

Discussion

Acknowledgements

References

**ABSTRACT**

**Introduction:** Fertilization is dependent on a sequential series of functional sperm parameters. One parameter, normal sperm morphology, has been found to be predictive of fertilization *in vitro*. Another parameter, DNA integrity, has been shown to be positively correlated with pregnancy but not fertilization. The fact that this parameter is significant for pregnancy but not fertilization implies that sperm with DNA fragmentation have the potential to fertilize an oocyte, but at the time when the paternal genes are essential for further embryo development, this is not possible and results in embryo senescence.

**Aim:** In this study, the aim was to investigate the rate of sperm DNA fragmentation in the different normal sperm morphology groups (<5% and ≥5% normal forms).

**Materials and Methods:** A total of 79 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. Sperm DNA integrity was investigated firstly with the TUNEL assay (DNA fragmentation) and secondly with the sperm chromatin structure assay (SCSA®) (single- and double-stranded DNA). Apoptosis was investigated by means of Annexin V (AV) binding. These data were correlated with normal sperm morphology.

**Results:** The 79 men were divided into two groups according to their normal sperm morphology; 24 had a normal sperm morphology < 5% (p-pattern) and 55 had a normal sperm morphology ≥5% (g- and n-pattern collectively). In the p-pattern group, there was a significant positive correlation of TUNEL-negative relative activity with morphology (n = 14; r = 0.782; $P$ = 0.0048). The Mann-Whitney test for independent samples revealed a significant difference for double-stranded DNA between the two defined morphology groups ($P$ = 0.0109).

**Conclusion:** A correlation between the p-pattern and DNA fragmentation can assist the embryologist to choose those sperm with intact DNA during ICSI and thus lead to increased implantation and pregnancy rates.

## INTRODUCTION

A semen analysis is an important component of the male fertility evaluation. Although the conventional semen parameters (sperm concentration, motility, and morphology) may help to differentiate infertile from sub fertile men (Van der Merwe *et al.*, 2005), none of these parameters is powerful enough to discriminate effectively, as there is a significant overlap between the semen parameters of infertile and fertile men (Guzick *et al.*, 2001). Infertile men with a normal semen profile are therefore said to have unexplained infertility. Furthermore, conventional semen parameters exhibit a high degree of variability from sample to sample and this high biological variability of the functional sperm parameters even contributes to the complexity of this already multifactorial event fertilization (Amann 1989; Henkel *et al.*, 2005).

Thus, in order to obtain a most conclusive impression of a particular patient's sperm fertilizing capacity there is a definite need for the identification and application of new and additional markers of male fertility potential. These should better discriminate infertile from fertile men than conventional semen parameters do. Functional parameters such as the hemizona assay (HZA) or the acrosome reaction (AR) have allowed us to better understand sperm function and dysfunction (Henkel *et al.*, 1993; Oehninger *et al.*, 1994) and predict the outcome with natural or assisted reproduction. However, they are rather large-scaled procedures and considering that for the hemizona assay human zona material has to be used, are not suitable as 'easy to perform' routine tests. Therefore, new markers may help better characterize a subgroup of infertile men previously diagnosed as having unexplained infertility. During recent years, sperm DNA damage has been a major focus for male fertility research and has been proven to be a potential new marker of fertility (Agarwal *et al.*, 2002; Henkel *et al.*, 2004; Bungum *et al.*, 2007) and a number of tests have been developed (for review see: Henkel, 2007a). This is also because serious concerns about the impact of sperm DNA damage on the health of the embryo as well as the offspring, particularly the male, have been risen (Ji *et al.*, 1997; Aitken and Krausz, 2001; Ozmen *et al.*, 2007).

Sperm DNA integrity has been shown to correlate with male fertility potential *in vivo* and *in vitro*, and some evidence suggests that sperm DNA integrity may be a better

discriminator of male fertility potential than conventional semen parameters (Lopes *et al.*, 1998; Evenson *et al.*, 1999; Larson *et al.*, 2000; Spano *et al.*, 2000) also because it appears that this parameters seems to have a lower variability than the conventional sperm parameters (Zini *et al.*, 2001; Smit *et al.*, 2007). Independent investigators have demonstrated that couples in whom the husband's semen exhibits a high percentage of damaged spermatozoa with denatured DNA (greater than 30%) have very low potential for natural fertility (Evenson *et al.*, 1999; Spano *et al.*, 2000). In addition, it is well known that sperm DNA fragmentation has a negative influence on pregnancy rates after *in vitro* fertilization (IVF) (Henkel *et al.*, 2004) and ICSI (Henkel *et al.*, 2003; Bungum *et al.*, 2007).

Among other factors (for review see: Henkel, 2007b), sperm DNA fragmentation is caused by apoptosis. Apoptosis is the controlled disassembly of cells from within (Thornberry and Lazebnik, 1998) characterized by changes in the phospholipid content of the outer leaflet of the plasma membrane. A very early sign of apoptosis is the translocation of the negatively charged phospholipid phosphatidyl-serine from the inner to the outer leaflet of the plasma membrane (Martin *et al.*, 1995; Vermes *et al.*, 1995).

Since one semen parameter, which has repeatedly been shown to have a strong correlation with the fertilization potential of sperm, is normal sperm morphology (Kruger *et al.*, 1986; Coetzee *et al.*, 1998) and appears to have the highest predictive power (Henkel *et al.*, 2005), it was the aim of this study to investigate the correlation between normal sperm morphology and sperm DNA fragmentation and apoptosis, respectively.

## MATERIALS AND METHODS

Semen Samples

These studies were performed upon approval of the Committe for Human Research at the Faculty of Health Sciences, Stellenbosch University, Tygerberg Academic Hospital, Tygerberg, South Africa (project number 99/137). A total of 79 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. The only exclusion criterion was the presence of less than 10 x $10^6$ sperm in total in the original semen sample. This was determined in order to have sufficient numbers of sperm cells to perform all planned measurements.

The men collected semen by masturbation into sterile cups following 2-4 days of sexual abstinence. Semen parameters were assessed using the criteria for normal concentration and total motility according to the World Health Organization (WHO) (1999). The speed of forward progression was estimated according to the arbitrary criteria of Hotchkiss (Hotchkiss et al., 1938), namely, the designation of 4 as the best quality of progression and declining quality as 3, 2, 1, and 0. The level of 3 is considered as a good average progression, with 2+ as the line of demarcation between acceptable and poor motility. Normal sperm morphology was assessed using the Tygerberg strict criteria (Kruger *et al.*, 1986; Menkveld *et al.*, 1990; WHO, 1999), whereby normal sperm morphology is divided into three groups (poor prognosis, good prognosis and normal pattern), p- (0-4% normal morphology), g- (5-14% normal morphology) and n-pattern (>14% normal morphology).

Apoptosis was investigated by means of the early apoptotic parameter Annexin V (AV) binding and DNA fragmentation by means of the TUNEL assay.

*Apoptosis*

To determine the sperm's binding ability to AV, the sperm suspension in Quinn's sperm wash medium (Sage In-Vitro Fertilization Inc., a Cooper Surgical Company, Trumbull, CT, USA) with 10 x $10^6$ sperm (total number) was diluted 1:10 with phosphate buffered saline (PBS) (Sigma, St. Louis, MO, USA), washed, and

centrifuged for 10 minutes at 500xg and room temperature (RT). The supernatant was discarded and the remaining pellet resuspended in 195 µL binding buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4). A negative control sample was analyzed by using binding buffer without $Ca^{2+}$. Afterwards, 5 µL Annexin V labeled with FITC (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 RT. Subsequently, 800 µL binding buffer was added, and the spermatozoa were analyzed in a FACScalibur flow cytometer (Becton Dickinson, Lincoln Park, NY, USA). A minimum of 5000 spermatozoa was examined for each test. The sperm population was gated by using forward-angle light scatter; side-angle light scatter was used to exclude electronic noise and debris. The FITC-labeled AV-positive sperm cells were measured in the FL1 channel of the flow cytometer. Flow cytometry data for each sample collected were; the percentage of AV-positive/-negative sperm in each gate as well as the mean channel fluorescence in each gate (Annexin V positive/negative). From this data the relative activity (RA) in each gate were calculated (% gated sperm x mean channel fluorescence).

*DNA fragmentation*

DNA fragmentation can be measured by the method of terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling assay (TUNEL assay) (Gavrieli *et al.*, 1991). This assay evaluates rather late stages of apoptotic events and detects both apoptotic and necrotic cells (Frankfurt *et al.*, 1996).

The characteristics of the TUNEL assay have been described elsewhere (Sun *et al.*, 1997). The assay was performed for the most part according to the method described by (Gavrielli *et al.*, 1991). One million washed spermatozoa were fixed with 4% [w/v] paraformaldehyde for 30 minutes at RT. The cells were then washed once with PBS, pH 7.4, (Oxoid, Basingstoke, Hampshire, UK). The wash entailed the addition of 1 mL PBS to the fixed cell suspension with subsequent centrifugation at 800 x g for 10 minutes at room temperature. After permeabilization with 0.2% [v/v] Triton X-100 (Calbiochem, Merck, Darmstadt, Germany) for 2 minutes on ice, the samples were washed twice more with PBS. A positive control sample was analyzed by incubating the spermatozoa with 50 U DNase (RNase Free; Boehringer

Mannheim, Mannheim, Germany) in 0.2% [w/v] $MgCl_2$/0.1% [w/v] $CaCl_2$ solution for 10 minutes at RT. Control samples were then washed twice with PBS. Fifty microliters of TUNEL mix (TdT and FITC-labeled dUTP in a 1:9 ratio) (Boehringer Mannheim, Mannheim, Germany) were added. To the negative control, no dUTP was added. The samples were incubated for 60 minutes at 37°C and then washed twice with PBS. They were then resuspended in 500 µL PBS. For each determination, at least 5000 spermatozoa were examined using flow cytometry. The sperm population is gated by using forward-angle light scatter, and side-angle light scatter dot plot to exclude electronic noise and debris. The FITC-labeled dUTP-positive spermatozoa were measured in the FL1 channel of the flow cytometer (Becton Dickinson). These were TUNEL-positive sperm. Flow cytometry data for each sample collected were; the percentage of TUNEL-positive/negative sperm in each gate as well as the mean channel fluorescence in each gate (TUNEL-positive/-negative). From this data the RA in each gate was calculated (% gated sperm x mean channel fluorescence).

*Sperm Chromatin Structure Assay (SCSA®)*

Single-stranded and double-stranded DNA content in the sperm head are measured with this standardized method (Evenson *et al.*, 2002). A portion of the cell suspension (5 $X10^6$) was treated with a low pH (pH 1.2) detergent containing 0.1% Triton X-100 (Calbiochem, Merck), 0.15 mol/L NaCl (Sigma) and 0.08 N HCl for 30 seconds, and then stained with 6 mg/L purified acridine orange (AO) (Molecular Probes, Eugene, OR, USA) in a phosphate-citrate buffer, pH 6.0. Cells were analysed using the FACscan flow cytometer (Becton Dickinson). Under experimental conditions, when excited with a 488 nm light source, AO that is intercalated with double-stranded DNA will emit green fluorescence, while AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by flow cytometric (FCM) measurements of the metachromatic shift from green (native double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (damaged DNA) versus green (normal DNA) fluorescence intensity cytogram patterns. More than 5000 spermatozoa were evaluated for each semen sample. The ratio of red/(red+green) yields the percentage of DNA fragmentation, referred to as DFI.

*Statistical Analysis*

After testing for normal distribution by means of the Chi square test, non-parametrical tests (Spearman Rank Correlation, Mann-Whitney Test) was performed as the data was not normally distributed. Results are presented as median and range and $P < 0.05$ was considered significant. The software used was MedCalc version 9.2.0 obtained from MedCalc Software, Mariakerke, Belgium.

**RESULTS**

The summary statistics of all analyzed parameters are compiled in Table I. The high variation of values, reflected by the high standard deviation (SD) is expected, as biological parameters were measured in this study.

Table II shows the normal sperm morphology correlated with the results of semen parameters. While normal sperm morphology did not correlate with age, semen volume, the percentage of motile sperm, forward progression, early signs of apoptosis (AV binding) and DNA fragmentation as measured with TUNEL assay, a significant increase in the normal sperm morphology in men with a high sperm concentration could be revealed (n = 79; r = 0.317; $P = 0.0051$). Significance was reached when the sperm concentration in the fresh semen was higher than $60x10^6$/mL.

The correlation between the DNA fragmentation index (%DFI) and normal morphology did not reach significance. Furthermore, no correlations were found between percent DFI within the p- or g-pattern normal morphology groups.

On the other hand, the correlation between normal sperm morphology and double-stranded DNA content of the sperm (as analyzed by the SCSA® (green) in Table II) indicated a trend (n = 64; r = 0.231; P = 0.0667) (Figure 1).

Table I: Summary statistics of parameters analyzed in this study.

| Parameter | n | Median | Range |
|---|---|---|---|
| Age (years) | 75 | 32.00 | 22.00 - 58.00 |
| Volume (mL) | 79 | 3.50 | 1.00 - 7.00 |
| Motility (%) | 79 | 50.00 | 10.00 - 70.00 |
| Forward progression | 79 | 2.75 | 1.00 - 3.50 |
| pH | 79 | 7.20 | 7.00 - 8.00 |
| Sperm concentration ($x10^6$/mL) | 79 | 43.00 | 0.80 - 205.50 |
| Morphology (% normal forms) | 79 | 6.00 | 0.00 - 18.00 |
| AV-negative (% gated) | 66 | 3.45 | 0.00 - 57.02 |
| AV-negative (MCF) | 64 | 152.42 | 26.94 - 4189.00 |
| AV-negative RA | 64 | 629.25 | 2.54 - 96347.00 |
| TUNEL-negative (% gated) | 59 | 70.78 | 14.59 - 97.65 |
| TUNEL-negative (MCF) | 59 | 384.00 | 312.79 - 609.16 |
| TUNEL-negative RA | 59 | 28627.08 | 5315.79 - 55426.15 |
| SCSA® (Total events gated) | 64 | 4788.00 | 1833.00 - 4980.00 |
| SCSA® (green) | 64 | 73.46 | 48.41 - 237.87 |
| DFI (%) | 64 | 29.98 | 3.55 – 85.05 |

**AV:** Annexin V; **TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded DNA; **MCF:** mean channel fluorescence; **RA:** relative activity; **DFI:** DNA fragmentation index

Normal sperm morphology was divided into 2 groups; those with a normal morphology <5% (p-pattern; n = 24) and those where the normal morphology is ≥5% (g- and n-pattern; n = 55). Correlation of age, semen volume, AV-binding, and double-stranded DNA content (SCSA® green) within the p-pattern normal morphology group did not reveal any significance (Table III). However, a significant positive correlation was found for the percentage of TUNEL-negative RA sperm (sperm with intact DNA) and the p-pattern morphology group (n = 14; r = 0.782; *P* = 0.0048).

Table II: Correlation of normal sperm morphology with different parameters

| Parameter | n | Spearman's ρ | P |
|---|---|---|---|
| Age (years) | 75 | -0.080 | 0.4922 |
| Sperm motility (%) | 79 | 0.143 | 0.2068 |
| Sperm concentration (x$10^6$/mL) | 79 | 0.317 | 0.0051 |
| AV-negative RA | 64 | 0.029 | 0.8159 |
| SCSA (green) | 64 | 0.231 | 0.0667 |
| DFI (%) | 64 | -0.048 | 0.7041 |
| TUNEL-negative RA | 59 | 0.169 | 0.1978 |

**AV:** Annexin V; **TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded DNA; **MCF:** mean channel fluorescence; **RA:** relative activity; **DFI:** DNA fragmentation index

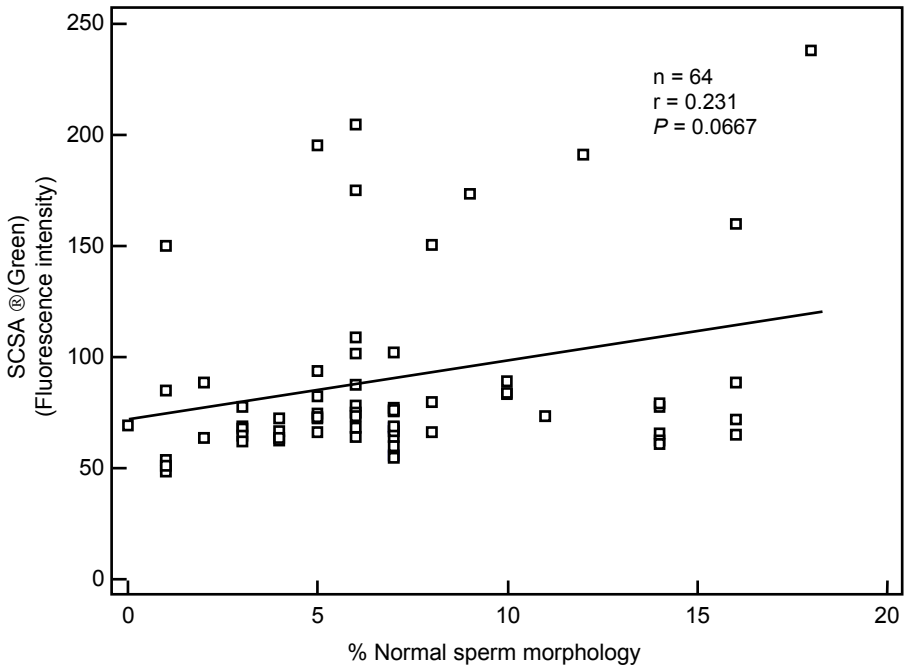


**Figure 1:** Relationship between the percentage normal sperm morphology according to strict criteria and the native DNA content (double-stranded DNA), as measured with the SCSA®, of the sperm nucleus. The SCSA® measures the intensity of acridin orange (AO) fluorescence using flow cytometry. AO fluoresces green when binding to native DNA.

Table III: Correlation of different sperm parameters within the two morphology groups

| Parameter | Morphology pattern | n | Spearman's ρ | *P* |
|---|---|---|---|---|
| Age (years) | <5% | 22 | -0.183 | 0.4028 |
| | ≥5% | 53 | 0.144 | 0.2976 |
| Semen volume (mL) | <5% | 24 | -0.030 | 0.8850 |
| | ≥5% | 55 | 0.125 | 0.3589 |
| Motility (%) | <5% | 24 | 0.492 | 0.0184 |
| | ≥5% | 55 | 0.065 | 0.6350 |
| Forward progression | <5% | 24 | 0.486 | 0.0197 |
| | ≥5% | 55 | 0.093 | 0.4963 |
| Sperm concentration ($x10^6$/mL) | <5% | 24 | 0.365 | 0.0803 |
| | ≥5% | 55 | 0.065 | 0.6350 |
| AV-negative RA | <5% | 20 | -0.211 | 0.3585 |
| | ≥5% | 44 | 0.245 | 0.1076 |
| TUNEL-negative RA | <5% | 14 | 0.782 | 0.0048 |
| | ≥5% | 45 | -0.109 | 0.4716 |
| SCSA® (green) | <5% | 17 | -0.030 | 0.9033 |
| | ≥5% | 47 | -0.019 | 0.8951 |
| DFI (%) | <5% | 17 | -0.227 | 0.3632 |
| | ≥5% | 47 | 0.173 | 0.2396 |

**AV:** Annexin V; **TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling; **SCSA®:** sperm chromatin structure assay; **(green):** double-stranded DNA; **RA:** relative activity; **DFI:** DNA Fragmentation index

Furthermore, significant correlations were found in the p-pattern group for both motility (n = 24; r = 0.492; *P* = 0.0184) and forward progression (n = 24; r = 0.486; *P* = 0.0197). In addition, when the sperm concentration was correlated with the p-pattern normal morphology group, there was no significance (n = 24; r = 0.365; *P* = 0.0803) but a possible trend was observed. In the ≥5% (g- and n-pattern) normal

morphology group no correlations were found for any of the parameters measured (Table III).

Table IV: Comparison of SCSA® (green), TUNEL-negative RA and sperm count between the two morphology groups

| Parameter | Morphology pattern | n | Range | Median | *P* |
|---|---|---|---|---|---|
| Sperm concentration ($x10^6$/mL) | <5% | 24 | 0.80 – 125.00 | 21.50 | 0.0097 |
| | ≥5% | 55 | 10.65 – 205.50 | 50.00 | |
| TUNEL-negative RA | <5% | 14 | 5315.79 – 49610.23 | 19526.80 | 0.0614 |
| | ≥5% | 45 | 6491.77 – 55426.15 | 32474.13 | |
| SCSA® (green) | <5% | 17 | 48.41 – 149.84 | 66.92 | 0.0082 |
| | ≥5% | 47 | 54.59 – 237.87 | 75.96 | |
| DFI (%) | <5% | 17 | 8.09 – 81.05 | 30.42 | 0.2098 |
| | ≥5% | 47 | 3.55 – 85.05 | 29.53 | |

**TUNEL:** terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling; **SCSA®:** sperm chromatin structure assay; **MCF:** mean channel fluorescence; **RA:** relative activity; **DFI:** DNA fragmentation index

The Mann-Whitney test for independent samples (Table IV) revealed significant comparisons between the two normal sperm morphology groups (<5% and ≥5%) for sperm concentration (*P* = 0.0100) as well as the percentage of sperm exhibiting double-stranded DNA (Figure 2) (*P* = 0.0082) between the two defined normal morphology groups (p-pattern vs. g-/n-pattern). Furthermore, the correlation of TUNEL-negative relative activity between these two morphology groups (Figure 3) did not reach significance but showed a tendency (*P* = 0.0614).

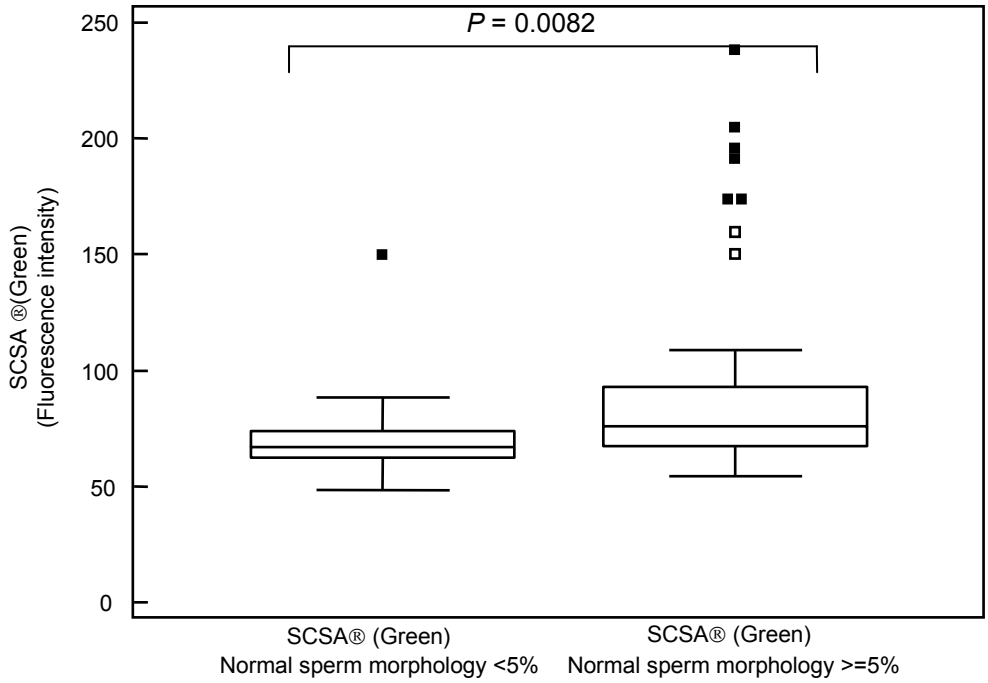


**Figure 2:** Comparison of SCSA® (green) (native/double stranded DNA) fluorescence within the <5% (n = 17) and the ≥5% (n = 47) normal sperm morphology group. Sperm in the ≥5% normal morphology group contained significantly more (*P* = 0.0082) native DNA than those in the <5% normal morphology group.

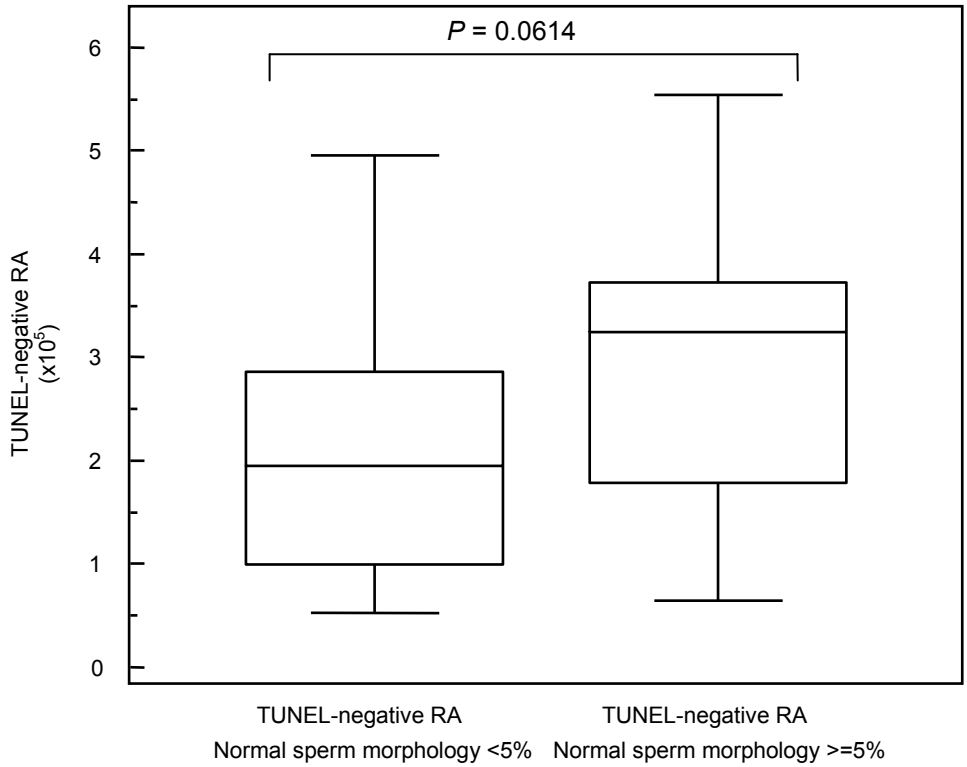


**Figure 3:** Comparison of TUNEL negative relative activity (RA) within the <5% (n = 14) and the ≥5% (n = 45) normal sperm morphology groups. This comparison did not reach significance (*P* = 0.0614).

**DISCUSSION**

We have investigated the possible relationship between normal sperm morphology, determined with strict criteria (Kruger *et al.*, 1988) and DNA damage, as measured with the terminal deoxynucleotidyl transferase-mediated dUDP-biotin end labeling (TUNEL) assay and the sperm chromatin structure assay (SCSA®), as well as an early parameter of apoptosis (Annexin-V binding). Considering that the size of the individual morphology groups were relatively small, the g-pattern group (5 – 14% normal morphology; n = 50) and the n-pattern group (≥ 15% normal morphology; n = 5), the g-pattern group and n-pattern group were treated as one morphology group, i.e. the g-pattern group. Another reason for combining the latter groups into one was that there is not a major difference between the fertilization rates *in vitro* (Kruger *et al.*, 1986; Coetzee *et al.*, 1998).

The results of the study showed a significant positive correlation for the p-pattern (0 – 4%) morphology group and the percentage of sperm with intact DNA (TUNEL-negative sperm). In this regard, no correlation could be found in the g-pattern group. Furthermore, with regard to the SCSA analyses, the results showed that the double stranded DNA content of the sperm nuclei in the p-pattern group is significantly less than that of the g-pattern group. No correlation with apoptosis could be found in or between any of the normal sperm morphology groups.

It is well known that normal sperm morphology plays an important role in fertilization and pregnancy rates in IVF. The strict morphology classification system has been refined with time [p (poor prognosis) pattern <5 %, g (good prognosis) pattern 5 – 14% and n (normal) pattern >14%] (Kruger *et al.*, 1988), and many studies (Oehninger *et al.*, 1988; Enginsu *et al.*, 1992; Ombelet *et al.*, 1994; Hernandez *et al.*, 1996) have confirmed the predictive value of normal sperm morphology within these established thresholds. In a structured literature review (meta-analysis) conducted to assess the importance of sperm morphology on fertilization and pregnancy rates in IVF, Coetzee *et al.* (1998) confirmed the importance of sperm morphology in relation to the positive predictive value for fertilization and pregnancy.

In an attempt to elucidate the possible correlation between normal sperm morphology and sperm physiological and genetic properties, in relation to fertilization, embryo quality and pregnancy, many authors have studied the correlations between normal sperm morphology and zona binding tests (Oehninger *et al.*, 1997), acrosome reaction (Bastiaan *et al.*, 2003), sperm nuclear DNA normality (Liu and Baker, 1992; Hammadeh *et al.*, 1998), double-stranded DNA content of the sperm nucleus (Claassens *et al.*, 1992) and sperm chromosome complement (Martin, 1998). The two most common zona binding tests currently utilized are the hemi-zona assay (HZA) and a competitive intact-zona binding assay, of these HZA has been shown to provide the highest discriminatory power for fertilization success/failure compared with sperm morphology. Furthermore, a structured literature review (Oehninger *et al.*, 1998) 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.

Acrosome reaction studies, using solubilized zona pellucida, showed that the sperm morphology, evaluated by strict criteria, correlated positively and highly significantly with the responsiveness of the acrosome reaction (r = 0.91; *P* = 0.0001) (Bastiaan *et al.*, 2003). At a morphology cut-off of 4%, the ROC curve analysis showed sperm morphology to be highly predictive of zona pellucida induced acrosome reaction (Bastiaan *et al.*, 2003). In addition, Liu and Baker (1994) also reported on patients with severe teratozoospermia and the inability of these patients' spermatozoa, in contrast to those from controls, to undergo the acrosome reaction in the presence of zona pellucida.

Sperm nuclear DNA normality is measured with the Aniline Blue stain, in this assay chromosome condensation was visualized by staining with acidic aniline blue which selectively stains persisting lysine-rich histones. The Aniline Blue stain has previously been shown to be positively correlated with normal sperm morphology, sperm zona pellucida binding and *in vitro* fertilization (Liu and Baker, 1992). In addition, Hofmann and Hilscher (1991) showed a significant positive correlation between aniline blue-staining and defects of acrosome formation. The Acridin Orange stain, which measures double stranded and single stranded DNA, has previously been positively correlated with normal sperm morphology (Claassen *et al.*,

1992; Angelopoulos *et al.*, 1998). Sperm morphology, however, proved to be a more significant predictor of fertilization *in vitro* compared to the Acridin Orange test (Claassens *et al.*, 1992).

Some studies have considered the possibility of a relation between chromosomal complement (aneuploidy rate) and normal sperm morphology. A clear correlation (although on the basis of a limited number of freshly ejaculated sperm cells) has been shown between individual morphology and chromosomal constitution of human sperm cells (Lee *et al.*, 1996). The incidence of structural chromosome aberrations is about four times higher in spermatozoa with amorphous heads than in those with morphologically normal heads (Lee *et al.*, 1996). These findings were confirmed by a more recent study where FISH analyses of macrocephalic spermatozoa showed that virtually all spermatozoa were chromosomally abnormal (Viville *et al.*, 2000).

In this regard, the important role of sperm DNA fragmentation in relation to fertilization and pregnancy outcome also has to be discussed. In the current study, we observed a significant positive correlation between normal sperm morphology and DNA intact sperm in the p-pattern group. Human sperm DNA fragmentation has a significant influence on male fertility (Sun *et al.*, 1997; Host *et al.*, 2000). This DNA fragmentation, as measured with the TUNEL assay, has previously been shown to be predictive of pregnancy in IVF and ICSI patients (Agarwal *et al.*, 2002; Henkel *et al.*, 2003; 2004; Bungum *et al.*, 2007). Although no direct correlation between the percentage of TUNEL-positive sperm and the fertilization rate was observed, patients with a high percentage of TUNEL-positive spermatozoa (>36.5%) showed a significantly lower mean pregnancy rate than those patients with a low percentage of TUNEL-positive sperm (<35.5%) (19.05% vs. 34.65%) (Henkel *et al.*, 2003). In contrast, no significant difference could be observed for the mean fertilization rates (63.49% vs. 57.42%).

The fertilization process is a complicated process dependant on many factors. With the introduction of ICSI, some of these factors are no longer barriers for successful fertilization. The potential relationship between sperm shape and the genetic integrity has become very pertinent, because ICSI sperm selection is not based on sperm–zona pellucida interaction as in conventional IVF, but rather on the visual

assessment of the shape of the sperm. In a study where the effect of individual sperm morphology in relation to fertilization and pregnancy outcome was observed, significantly lower pregnancy and implantation rates 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 (de Vos *et al.*, 2003).

The assays, as well as the equipment, utilized in this study are specialized and expensive. Not many laboratories, especially those in developing countries, assessing male fertility have the infrastructure to add these technologies to the standard male fertility potential screening. However, strict sperm morphology screening is an inexpensive and long-established method to determine male fertility potential. The fact that the p-pattern normal sperm morphology group had a significant positive correlation (n = 24; r = 0.782; *P* = 0.0048) with intact DNA in this study, adds information to the prognosis of a p-pattern diagnosis.

Human normal sperm morphology, especially in men falling in the p-pattern group, affects the fertilization rate *in vitro* (Coetzee *et al.*, 1998). Possible explanations for this observation can be poor binding to the zona pellucida (Oehninger *et al.*, 1998) and a reduced acrosome reaction (Liu and Baker, 1994; Bastiaan *et al.*, 2003). It is, however, interesting to observe that these severely deformed spermatozoa also present a higher DNA fragmentation rate (Sun *et al.*, 1997; Henkel *et al.*, 2003), and a higher incidence of chromosome abnormalities (Lee *et al.*, 1996). Furthermore, where morphologically abnormal spermatozoa were used for ICSI, independent of there origin (fresh ejaculated or testis biopsy), embryos with a lower potential for implantation were generated (de Vos *et al.*, 2003). Increasing evidence suggest that sperm morphology plays a significant role in ICSI outcome (Tesarik *et al.*, 2002; Bartoov *et al.*, 2003; Chemes and Rawe, 2003; De Vos *et al.*, 2003; Berkovitz *et al.*, 2005). Recently, Bartoov and co-workers (2003) introduced a modified IVF procedure – intracytoplasmic morphologically selected sperm injection (IMSI) – based on microinjection into retrieved oocytes of selected spermatozoa with strictly defined morphologically normal nuclei. The modified IMSI treatment results in

significantly higher pregnancy rates, compared with conventional IVF-ICSI (Bartoov *et al*., 2003)

The current study corroborates on the importance of sperm morphology by reporting for the first time on a significant positive correlation found between sperm morphology, according to strict criteria, and DNA integrity in men with a p-pattern normal morphology. This sheds new light on explaining the poorer prognosis in these patients in ICSI programs.

In conclusion, 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 and pregnancy rates in these patients as well as the offspring's health.

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**REFERENCES**

Agarwal A and Saleh RA (2002) Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 29, 817-827.

Amann RP (1989) Can the fertility potential of a seminal sample be predicted accurately? J Androl 10, 89-98.

Angelopoulos T, Moshel YA, Lu L, Macanas E, Grifo J and Krey LC (1998) Simultaneous assessment of sperm chromatin condensation and morphology before and after separation of procedures: effect on the clinical outcome after *in vitro* fertilization. Fertil Steril 69, 740-747.

Aitken and Krausz (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122, 497-506.

Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H, Artzi S, Gross M and Barak Y (2003) Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 80, 1413-1419.

Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A and Bartoov B (2005) The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum Reprod 20, 185-190.

Bastiaan HS, Windt M-L, Menkveld R, Kruger TF, Oehninger S and Franken DR (2003) Relationship between zona pellucida-induced acrosome reaction, sperm morphology, sperm-zona pellucida binding, and *in vitro* fertilization. Fertil Steril 79, 49-55.

Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J and Giwercman A (2007) Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 22, 174-179.

Chemes EH and Rawe YV (2003) Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. Hum Reprod Update 9, 405-428.

Claasens OE, Menkveld R, Franken DR, Pretorius E, Swart Y, Lombard CJ and Kruger TF (1992) The Acridine Orange test: determining the relationship between sperm morphology and fertilization *in vitro*. Hum Reprod 7, 242-247.

Coetzee K, Kruger TF and Lombard CJ (1998) Predictive value of normal sperm morphology: a structured literature review. Hum Reprod Update 4, 73-82.

De Vos A, Van De Velde H, Joris H, Verheyen G, Devroey P and Van Steirteghem A (2003) Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 79, 42-48.

Enginsu ME, Dumoulin JCM, Pieters MHEC, Bergers M, Evers JL and Geraedts JP (1992) Comparison between the hypoosmotic swelling test and morphology evaluation using strict criteria in predicting *in vitro* fertilization. J Assist Reprod Genet 9, 259-264.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P and Claussen OP (1999) Utility of the sperm chromatin assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14, 1039–1049.

Evenson DP, Larson KL and Jost LK (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 23, 25–43.

Frankfurt OS, Robb JA, Sugarbaker EV and Villa L (1996) Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. Exp Cell Res 226, 387-397.

Gavrieli Y, Sherman Y and Ben-Sasson AB (1991) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119, 493-501.

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D and Vogel DL (2001) Sperm morphology, motility, and concentration in infertile and fertile men. N Engl J Med 345, 1388–1393.

Hammadeh ME, Stieber M, Haidl G and Schmidt W (1998) Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. Andrologia 30, 29-35.

Henkel R, Müller C, Miska W, Gips H and Schill W-B (1993) Determination of the acrosome reaction inhuman spermatozoa is predictive of fertilization *in vitro*. Hum Reprod 8, 2128-2132.

Henkel R, Kierspel E, Hajimohammed M, Stalf T, Hoogendijk CF, Mehert C, Menkveld R, Schill W-B and Kruger TF (2003) DNA fragmentation of spermatozoa and assisted reproductive technology. RBM Online 7, 477-484.

Henkel R, Hajimohammed M, Stalf T, Hoogendijk C, Mehnert, Menkveld R, Gips H, Schill W-B and Kruger TF (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 4, 965-972.

Henkel R, Maaß G, Bödeker R-H, Scheibelhut C, Stalf T, Mehnert C, Schuppe H-C, Jung A and Schill W-B (2005) Sperm function and assisted reproduction technology. Rep Med Biol 4, 7-30.

Henkel R (2007a) DNA based sperm assessment. In Kruger TF, van der Spuy Z and Kempers RD (eds) Advances in Fertility Studies and Reproductive Medicine – IFFS 2007. Juta, Cape Town, South Africa, pp.284-292.

Henkel R (2007b) DNA fragmentation and its influence on fertilization and pregnancy outcome. In Kruger TF and Oehninger S (eds) Male Infertility - Diagnosis and Treatment. Informa UK Ltd., Oxon, UK, pp.277-290.

Hernandez M, Molina R, Olmedo J, Olmedo SB, Coetzee K and Estofàn D (1996) Prognostic value of the strict criteria: an Argentinian experience. Arch Androl 37, 85-87.

Hofmann N and Hilscher B (1991) Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. Hum Reprod 7, 979-982.

Host E, Lindenberg S and Smidt-Jensen S (2000) The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. Acta Obstet Gynecol Scan 79, 559–563.

Hotckiss, RS et al., (1938) Semen analyses of two hundred fertile men. Am J Med 196, 362-384.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM and Jin F (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of non-smoking mothers. J Nat Can Inst 89, 238-244.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA and Smith K (1986) Sperm morphologic features as a prognostic factor in *in vitro* fertilization. Fertil Steril 46,1118-1123.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF and Oehninger S (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. Fertil Steril 49, 112-117.

Larson KL, DeJonge CJ, Barnes AM, Jost LK and Evenson DP (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15, 1717–1722.

Lee JD, Kamiguchi Y and Yanagimachi R (1996) Analysis of chromosome constitution of human spermatozoa with normal and abberant head morphologies after injection into mouse oocytes. Hum Reprod 9, 1942-1946.

Liu DY and Baker HWG (1992) Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates *in vitro*. Fertil Steril 58, 1178-1184.

Liu DY and Baker HWG (1994) Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization *in vitro*. Hum Reprod 9, 1694-1700.

Lopes S, Sun JG, Jurisicova A, Meriano J and Casper RF (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 69, 528–532.

Martin R (1998) Human sperm chromosome complements in chemotherapy patients and infertile men. Chromosoma 107, 523-527.

Martin S, Reutelingsperger C, McGahon A, Rader JA, van Schie RC, LaFace DM and Green DR (1995) Early redistribution of plasma membrane phophatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition of over expression of Bcl-2 and Abl. J Exp Med 182, 1545-1556.

Menkveld R, Stander FSH, Kotze TJvW, Kruger TF and van Zyl JA (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 5, 586-592.

Oehninger S, Acosta AA, Morshedi M, Veeck L, Swanson RJ, Simmons K and Rosenwaks Z (1988) Corrective measures and pregnancy outcome in *in vitro* fertilization in patients with severe sperm morphology abnormalities. Fertil Steril 50, 283-287.

Oehninger S, Sueldo C, Lanzendorf S, Mahony M, Burkman LJ, Alexander NJ and Hodgen GD (1994) A sequential analysis of the effect of progesterone on specific sperm functions crucial to fertilization *in vitro* in infertile patients. Hum Reprod 9, 1322-1327.

Oehninger S, Mahoney M, Ozgür K, Kolm P, Kruger TF and Franken D (1997) Clinical significance of human sperm-zona pellucida binding. Fertil Steril 67, 1121-1127.

Oehninger S, Sayed E and Kolm P (1998) Role of sperm function tests in subfertility treatment strategies. In Ombelet W, Bosmans E, Vandeput H (eds) Modern ART in the 2000's (Andrology in the Nineties) Parthenon Publishing Group, New York, pp.17-23.

Ombelet W, Fourie F, Vandeput H, Bosmans E, Cox A, Janssen M and Kruger TF (1994) Teratozoospermia and in-vitro fertilization: a randomized prospective study. Hum Reprod 9, 1479-1484.

Ozmen B, Koutlaki N, Youssry M, Diedrich K and Al-Hasani S (2007); DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. RBM Online 14, 384-395.

Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E and Leter G (2000) Sperm chromatin damage impairs human fertility. Fertil Steril 73, 43-50.

Smit M, Dohle GR, Hop WCJ, Wildhagen MF, Weber RFA and Romijn JC (2007) Clinical correlates of the biological variation of sperm DNA fragmentation in infertile men attending an andrology outpatient clinic. Int J Androl 30, 48-55.

Sun JG, Jurisicova A and Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization *in vitro*. Biol Reprod 56, 602-607.

Tesarik J, Mendoza C and Greco E (2002) Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. Hum Reprod 17, 184-189.

Thornberry NA and Lazebnik Y (1998) Caspases: Enemies within. Science 281, 1312-1316.

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

Vermes I, Haanen C and Reutelingsperger CPM (1995) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labelled annexin V. J Immunol Methods 180, 39-52.

Viville S, Mollard R, Bach ML, Falquet C, Gerlinger P and Warter S (2000) Do morphological anomalies reflect chromosomal aneuploidies?: case report. Hum Reprod 15, 2563-2566.

World Health Organization (1999) World Health Organization Laboratory Manual for Examination of Human Semen. Cambridge University Press, Cambridge, UK.

Zini A, Kamal K, Phang D, Willis J and Jarvi K (2001); Biologic variability of sperm DNA denaturation in infertile men. Urology 58, 258-261.

# CHAPTER IV

## The selection of non-apoptotic sperm using Annexin V binding and flow cytometry

Abstract

Introduction

Materials and Methods

- *Experimental design*
- *Annexin V staining*
- *Fluorescent cell sorting*
- *Sperm morphology assessment*
- *Statistical analysis*

Results

Discussion

Acknowledgements

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**ABSTRACT**

**Introduction:** Apoptosis is programmed cell death, based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide without eliciting an inflammatory response. Mature sperm cells have been reported to express distinct markers of apoptosis-related cell damage. There is an established consensus on the implication of apoptosis in male infertility, however, the exact mechanisms of its involvement remains to be elucidated.

**Aim:** This study aimed to assess the relationship between apoptosis in human ejaculated spermatozoa and the normal sperm morphology according to strict criteria.

**Method:** Semen specimens from 14 healthy donors were prepared by double density gradient centrifugation (DDGC). An aliquot of the DDGC sample was used as control and the remaining sample was incubated with Annexin V (AV) labeled with fluorescein isothiocyanate (FITC). Cell fluorescence signals were determined immediately after staining using a FACScalibur flow cytometer equipped with an argon laser emission of 488 nm, as well as a FACSSort fluidic sorting module. The sorting procedure delivered 2 sperm subpopulations: AV-negative and AV-positive. Of these subpopulations, as well as the control, morphology slides were made and the sperm morphology was assessed according to strict criteria.

**Results:** Separating non-apoptotic and apoptotic sperm by means of flow cytometry proved to be successful as there was a significant enrichment of both apoptotic and non-apoptotic sperm in the subpopulations ($P = 0.0001$). Furthermore, there was a significant increase ($P < 0.0001$) in the median normal sperm morphology between the apoptotic (median = 5.00) and the non-apoptotic (median = 12.00) sperm subpopulations.

**Conclusion:** The AV-negative sperm subpopulations have morphologically superior quality sperm compared to AV-positive sperm subpopulations. It is therefore important to select morphologically normal sperm during ICSI as it may contribute to increased implantation and pregnancy rates.

## INTRODUCTION

A conventional semen analysis includes the assessment of sperm concentration, motility and percentage of normal forms and it remains the standard procedure for evaluating the fertility potential of semen samples. Although it gives considerable information, such as the correlation of normal sperm morphology with fertilization potential (Kruger *et al*., 1986), the results of a semen analysis are only moderately predictive of an individual's fertility (Jeyendran, 2000).

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 (Kerr *et al.*, 1972). Apoptosis is characterized by a cascade of events which include condensation and fragmentation of the chromatin, compaction of cytoplasmic organelles, reduced mitochondrial transmembrane potential (Luo *et al.*, 1998), mitochondrial release of cytochrome c (Bossy-Wetzel *et al.*, 1998), production of reactive oxygen species (ROS) (Whittington *et al.*, 1999), dilatation of the endoplasmic reticulum and a decrease in cell volume (Arends and Wyllie, 1991).

Apoptosis is also characterized by changes in 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 plasma membrane to the outer side thereof. PS is thus exposed on the external membrane surface (Martin *et al.*, 1995; Vermes *et al.*, 1995). PS has a high affinity to the Annexin V (AV), a phospholipid-binding protein of about 35kDa (Van Heerde *et al.*, 1995), this binding is an early marker of apoptosis. Makers of terminal apoptosis include activated caspase-3, loss of the integrity of the mitochondrial membrane potential (MMP) and DNA fragmentation which are expressed by varying proportions of ejaculated sperm (Evenson *et al.*, 2002; Henkel *et al.*, 2004)

Apoptosis has been indicated as a cause of male infertility (Oosterhuis *et al.*, 2000; Oehninger *et al.*, 2003; Sakkas *et al.*, 2003; Taylor *et al.*, 2004). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (Lin *et al.*, 1997; Juriscova *et al.*, 1999). It has

been reported that the proportions of apoptotic sperm are higher in ejaculated semen samples from infertile men compared with fertile men (Taylor *et al.*, 2004). Furthermore, during cryopreservation, sperm caspases become more activated in patients with infertility than in healthy donors (Grunewald *et al.*, 2005). 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 (Sun *et al.*, 1997; Morris *et al.*, 2002; Henkel *et al.*, 2004).

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 (Marchetti *et al.*, 2002; Shen *et al.*, 2002; Weng *et al.*, 2002; Pena *et al.*, 2003; Liu *et al.*, 2004; Taylor *et al.*, 2004; Said *et al.*, 2005). In contrast, there are only a few studies on the relationship between sperm morphology, applying Tygerberg's strict criteria (Kruger *et al.*, 1986), and the proportion of apoptotic sperm (Siddighi *et al.*, 2004; Chen *et al.*, 2006; Aziz *et al.*, 2007). Dissimilarly, when applying the World Health Organization (WHO) (World Health Organization, 1999) criteria for sperm morphology, no correlations could be found between caspase-3 activation, intact MMP and PS externalization (Said *et al.*, 2005).

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 non-invasive methodologies are needed; these should enable the embryologist to select the healthiest sperm for fertilization during ICSI, which in turn will contribute positively towards better implantation and pregnancy rates.

By conjugating fluorescein to AV it has been possible to use the marker to identify 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 non-invasive method whereby AV-positive and AV-negative sperm can be separated in such a way that the resultant AV-negative sperm subgroup can still be used for fertilization with ICSI. In addition, after separation, normal sperm morphology according to Tygerberg's strict criteria was assessed and the sperm morphology profile between non-apoptotic and apoptotic sperm charted.

## MATERIALS AND METHODS

*Experimental design*

These studies were performed after approval from the Committee for Human Research 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 to 54 years. The only exclusion criterion was the presence of less than $20x10^6$/mL total motile spermatozoa in the original (post liquefaction) sample. This was determined in order to have sufficient numbers of sperm cells to perform the separation technique as well as subsequent motility and morphology analysis. Patients collected semen by masturbation into sterile cups following 2-4 days of sexual abstinence. The semen characteristics of volume, pH and agglutination were normal; all samples had less than $0.1x10^6$ round cells/mL.

Semen samples were allowed to liquefy for 30 min at room temperature followed by assessment of sperm parameters. Semen parameters were assessed using the WHO criteria (1999) for normal concentration and motility. Normal sperm morphology was assessed using the Tygerberg strict criteria (Kruger *et al.*, 1986; WHO, 1999), whereby normal sperm morphology is divided into three groups, p- (0-4% normal morphology), g- (5-14% normal morphology) and n-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., a Cooper Surgical Company, Trumbull, CT, USA). The samples were loaded onto the discontinuous gradient and centrifuged at 400xg for 12 minutes at room temperature. The resultant 90% pellet was washed by centrifugation (400xg) for an additional 10 min and re-suspended in Quinn's sperm wash medium (SAGE In-Vitro Fertilization Inc.) to a final concentration of 10 x $10^6$/mLsperm.

An aliquot of this suspension served as control, whereas the other aliquot was subjected to AV staining and fluorescent cell sorting.

*Annexin V staining*

Annexin V (AV) is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine. The translocation of phosphatidylserine residues to the outer layer of the plasma membrane was detected with AV labeled with fluorescein isothiocyanate (FITC). To label the sperm with AV the sperm suspension in Quinn's sperm wash medium (SAGE In-Vitro Fertilization Inc.) with 10 x $10^6$/mLsperm (total number) was diluted 1:10 with PBS (Sigma Chemical Co., St. Louis, MO, USA), washed, and centrifuged for 10 minutes at 500xg and room temperature. The supernatant was discarded and the remaining pellet resuspended in 195µL binding buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 2.5 mmol/L $CaCl^{2+}$, pH 7.4). Next, 5 µL AV labeled with FITC (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 binding buffer was added.

*Fluorescent cell sorting*

Cell fluorescence signals were determined immediately after staining using a FACScalibur flow cytometer (Becton Dickinson, Lincoln Park, NY, USA) equipped with an argon laser emission of 488 nm, as well as a FACSSort fluidic sorting module. FITC was identified by using a 530 band pass filter. The analysis was performed using CELLQUEST software (Becton Dickinson). A primary gate based on physical parameters (forward and side light scatter, FSC and SSC, respectively) was set to exclude dead cells or debris. The background level was estimated by omitting

the primary antibody. The FITC-labeled AV-positive sperm cells were measured in the FL1 channel of the flow cytometer. After determining the initial apoptotic status of each of the semen samples, the apoptotic and non-apoptotic sperm fractions were separated. A sort region of AV-positive events was set and these events were collected to represent the cells which had bound the fluorescent marker indicative of apoptotic cells. The sort region negative (AV-negative) were collected to represent non-apoptotic cells.

The fractions composed of apoptotic (annexin-V bound sperm) and non-apoptotic (unbound sperm) was retained separately in 50 mL polypropylene tubes (Beckton Dickinson, Lincoln Park, NY, USA). Prior to 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 500xg for 20 min at room temperature. The resultant pellet was re-suspended in 50 µL of binding buffer. An aliquot of 20 µL was used to make the morphology smear and the remaining sample was re-analyzed 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-dependant 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 Diagnostica, Darmstadt, Germany) according to a previously reported method (Lacquet *et al.*, 1996). 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 broken once the results were known.

*Statistical analysis*

After testing for normal distribution by means of the Chi square test, parametrical tests (paired samples t-test) for normally distributed data, as well as non-parametrical tests (Wilcoxon test for paired samples) for data not normally distributed were performed. Results are presented as mean ± SD and $P < 0.05$ was considered significant. The software used was MedCalc version 9.2.0 obtained from MedCalc Software, Mariakerke, Belgium.

**RESULTS**

Fourteen men were included in this study. Summary statistics of basic semen parameters are presented in Table I.

Table I: Summary statistics of basic semen parameters

| Parameter | n | Mean ± SD | Range | Median |
|---|---|---|---|---|
| Age | 14 | 34.57 ± 5.26 | 28.00 – 45.00 | 33.50 |
| Forward progression | 14 | 2.63 ± 0.24 | 2.00 - 2.75 | 2.75 |
| Motility (%) | 14 | 49.29 ± 13.28 | 20.00 - 70.00 | 50.00 |
| pH | 14 | 7.45 ± 0.26 | 7.20 - 8.00 | 7.50 |
| Sperm concentration ($x10^6$/mL) | 14 | 92.57 ± 43.54 | 41.00 - 205.50 | 87.75 |
| Volume (mL) | 14 | 3.50 ± 1.58 | 1.50 - 7.00 | 3.75 |

Table II presents the summary statistics for flow cytometry results of the double density gradient centrifugation (DDGC) (control), apoptotic and non-apoptotic sperm subgroups analyzed in this study. The high variation of values, reflected by the high standard deviation (SD) was expected, as biological parameters were measured in this study.

Table II: Flow cytometry summary statistics of the double density gradient centrifugation (DDGC) (control), apoptotic and non-apoptotic sperm subgroups analyzed

| Parameter | n | Mean ± SD | Range | Median |
|---|---|---|---|---|
| [a]Morphology | 14 | 8.36 ± 4.14 | 2.00 - 16.00 | 8.50 |
| [a]AV-negative | 14 | 76.21 ± 9.47 | 62.05 - 88.44 | 79.75 |
| [b]Morphology | 13 | 5.77 ± 3.49 | 1.00 - 13.00 | 5.00 |
| [b]AV-negative | 14 | 14.62 ± 14.10 | 1.25 - 61.53 | 11.86 |
| [c]Morphology | 13 | 11.92 ± 4.43 | 7.00 - 22.00 | 12.00 |
| [c]AV-negative | 14 | 95.59 ± 2.70 | 89.88 - 98.44 | 96.77 |

**AV:** Annexin V; **[a]:** double density gradient (DDG) sperm fraction (control); **[b]:** apoptotic sperm fraction; **[c]:** non-apoptotic sperm fraction

Table III presents the shift observed in the median normal sperm morphology after flow cytometric sperm cell sorting. After phosphatidyl serine (PS) externalization AV labeling, was performed on the DDGC sperm sample resulting in two sperm subgroups viz. apoptotic and non-apoptotic.

Table III: Shift in normal sperm morphology after flow cytometric cell sorting

| | Control sperm subgroup | Apoptotic sperm subgroup | Non-apoptotic sperm subgroup |
|---|---|---|---|
| n | 14 | 13 | 13 |
| Median morphology | 8.50 | 5.00 | 12.00 |

Results of the univariate comparisons, as performed with two-tailed t-tests, of the median normal sperm morphology in the different sperm subgroups are presented in Table IV.

Table IV: Comparison of sperm morphology between the control, apoptotic and non-apoptotic subgroups of sperm samples (paired sample t-test)

| Sperm subgroup | n | Median | 95% CI | Mean | P |
|---|---|---|---|---|---|
| [a]Control | 13 | 8.50 | 5.56 – 9.98 | 7.77 | < |
| [b]Apoptotic | 13 | 5.00 | 3.66 – 7.88 | 5.77 | 0.0001 |
| [a]Control | 13 | 8.50 | 5.70 – 10.91 | 8.31 | < |
| [c]Non-apoptotic | 13 | 12.00 | 9.25 – 14.60 | 11.92 | 0.0001 |
| [b]Apoptotic | 12 | 5.00 | 3.43 – 8.07 | 5.75 | < |
| [c]Non-apoptotic | 12 | 12.00 | 8.94 – 13.23 | 11.08 | 0.0001 |

Sperm morphology of the double density gradient centrifugation (DDGC) (control) and the subsequent two sperm subgroups resulting from flow-cytometric cell sorting were compared utilizing paired sample t-tests. All hypotheses testing were two-tailed; P < 0.05 was considered statistically significant. The 95% confidence intervals (CI's) are given for each case.

**[a]:** double density gradient centrifugation (DDGC) sperm subgroup (control); **[b]:** apoptotic sperm subgroup; **[c]:** non-apoptotic sperm subgroup

The non-apoptotic sperm subgroup had a significantly higher median (median = 12.00) normal sperm morphology compared to the median normal sperm morphology of the control sperm subgroup (median = 8.50) (n = 13; *P* < 0.0001) (Table III; Figure 1**B**) as well as the apoptotic sperm subgroup (median = 5.00) (n = 12; *P* < 0.0001) (Table III; Figure 1**C**). Furthermore, the apoptotic sperm subgroup had a significantly lower median (median = 5.00) of normal sperm morphology when compared with the control sperm subgroup (median = 8.50) (n = 13; P < 0.0001) (Table III; Figure 1**A**).

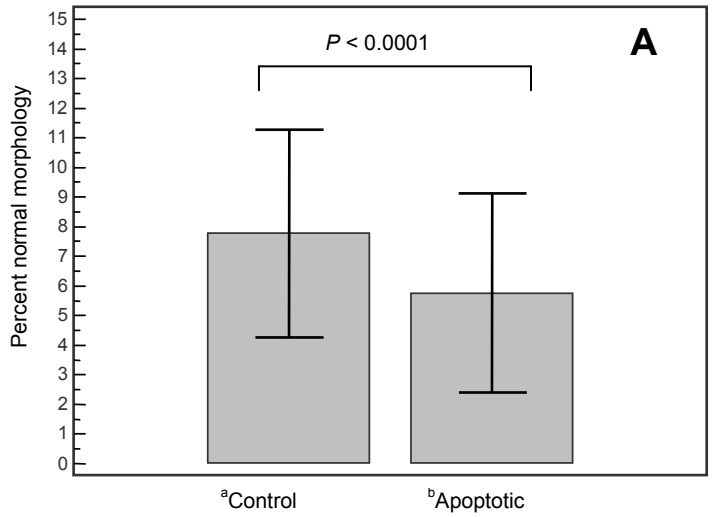


Figure 1**A**: Comparison between the control and the apoptotic subgroups ($P$ < 0.0001; n = 13). Bar plots of univariate comparisons, as performed with two tailed t-tests, of the percentages of normal sperm morphology in the different sperm subgroups ([a]control; [b]apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow cytometry.

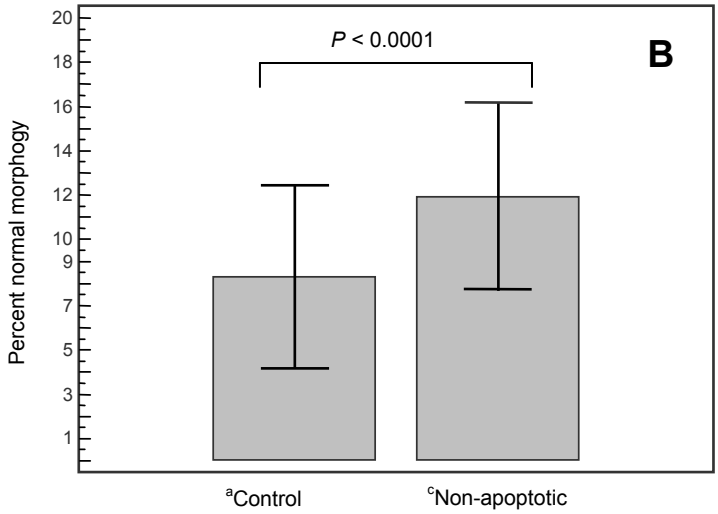


Figure 1B: Comparison between the control and non-apoptotic subgroups (P < 0.0001; n = 13). Bar plots of univariate comparisons, as performed with two tailed t-tests, of the percentages of normal sperm morphology in the different sperm subgroups ([a]control; [c]non-apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow cytometry.

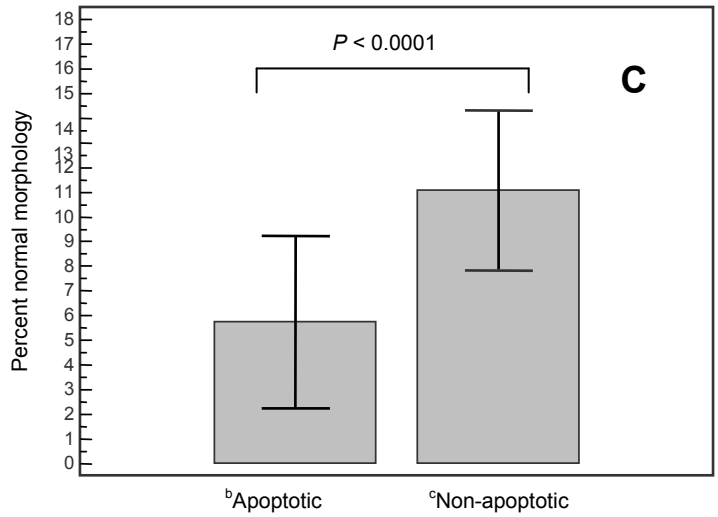


Figure 1C: Comparison between apoptotic and non-apoptotic subgroups (P < 0.0001; n = 12). Bar plots of univariate comparisons, as performed with two tailed t-tests, of the percentages of normal sperm morphology in the different sperm subgroups ([b]apoptotic subgroup; [c]non-apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow cytometry.

After fluorescent cell sorting, re-analysis of the resultant apoptotic and non-apoptotic subgroups were performed. The purpose for the re-analysis, as performed with the exact same instrument settings as for the original sample, was to determine the efficiency of the separation assay. Univariate comparisons, as performed with two tailed Wilcoxon's signed-rank tests, showed that in the non-apoptotic subgroup the median normal sperm morphology was significantly higher (n = 14; *P* = 0.0001), when compared with both the control and apoptotic subgroups (Figure 2B, C). In addition, these tests showed that the median normal sperm morphology in the apoptotic subgroup was significantly lower (n = 14; *P* = 0.0001) than in the control group (Figure 2A).

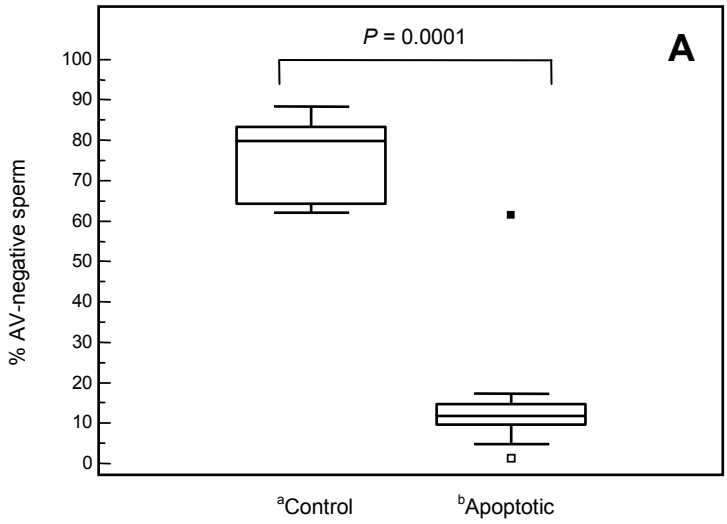


Figure 2A: Comparison between the control and the apoptotic subgroups (P = 0.0001; n = 14). Box-and-whisker plots of univariate comparisons, as performed with two tailed Wilcoxon's signed-rank tests, of the percentages of non-apoptotic sperm in the different sperm subgroups (a: control; b: apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow-cytometry.

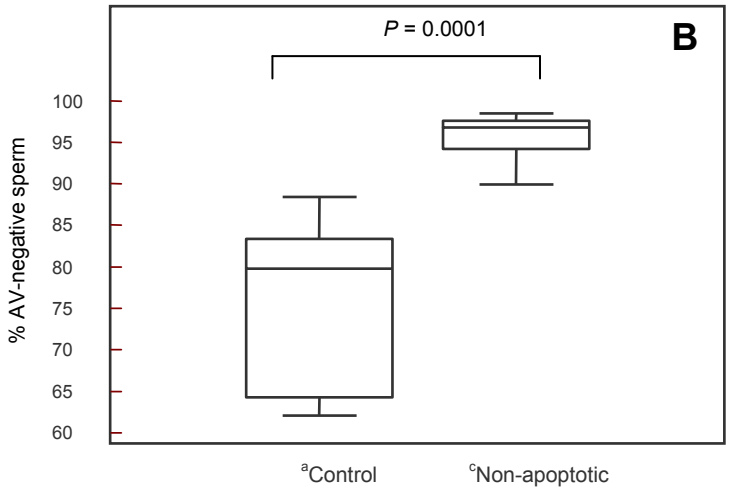


Figure 2B: Comparison between the control and non-apoptotic subgroups (P = 0.0001; n = 14). Box-and-whisker plots of univariate comparisons, as performed with two tailed Wilcoxon's signed-rank tests, of the percentages of non-apoptotic sperm in the different sperm subgroups (a: control; c: non-apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow-cytometry.

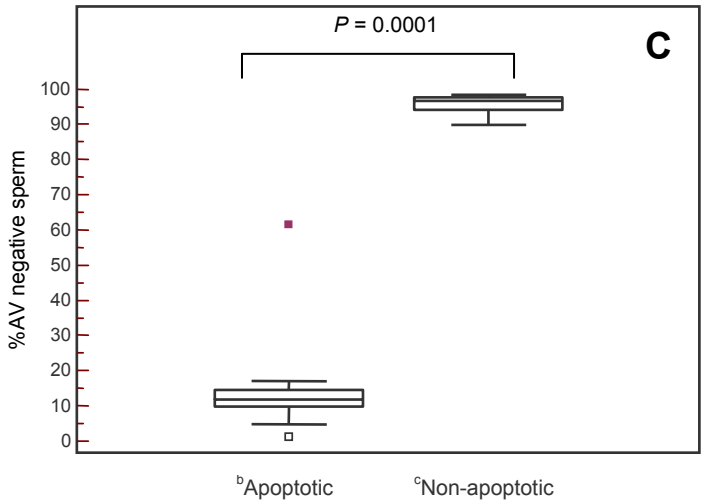


Figure 2C: Comparison between apoptotic and non-apoptotic subgroups (P = 0.0001; n = 14). Box-and-whisker plots of univariate comparisons, as performed with two tailed Wilcoxon's signed-rank tests, of the percentages of non-apoptotic sperm in the different sperm subgroups (b: apoptotic subgroup; c: non-apoptotic subgroup). Annexin V-bound apoptotic sperm separated from unbound non-apoptotic sperm by means of flow-cytometry.

**DISCUSSION**

The results of this study confirm the shift in the normal sperm morphological profile in sperm preparations, before and after the isolation of the non-apoptotic and apoptotic sperm (Table II and III). Isolation of non-apoptotic and apoptotic sperm was performed on the basis of phosphatidyl serine (PS) externalization using Annexin V (AV) labelling and flow cytometry. Normal sperm morphology was assessed according to strict criteria (Kruger *et al.*, 1986). Compared with the apoptotic sperm subgroup, the non-apoptotic subgroup had an improved normal sperm morphology profile as demonstrated by significantly higher proportions of sperm with normal morphology (Table IV). In addition, the normal sperm morphology of the non-apoptotic sperm subgroup had a similar favorable shift when compared with the double density gradient centrifugation (DDGC) (control) sperm subgroup. Furthermore, the study results demonstrated that the flow cytometric cell separation technique proved to be an adequate methodology for the separation of apoptotic and non-apoptotic 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 (Sakkas *et al.*, 1999; Gandini *et al.*, 2000; Shen *et al.*, 2000; Ricci *et al.*, 2002; Sakkas *et al.*, 2002; Siddighi *et al.*, 2004; Chen *et al.*, 2006). These studies, however, did not accurately distinct between moribund or necrotic sperm and motile sperm expressing apoptotic markers. In addition, there are studies which correlated the morphology in neat semen with apoptosis in selected motile sperm subpopulations after swim-up or double density gradient centrifugation techniques (Weng *et al.*, 2002; Benchaib *et al.*, 2003; Muratori *et al.*, 2003; Almeida *et al.*, 2005). All of the above mentioned studies applied different morphology assessment methodologies; WHO 1992 standards (Muratori *et al.*, 2003), WHO 1999 standards (Gandini *et al.*, 2000; Shen *et al.*, 2000; Ricci *et al.*, 2002; Benchaib *et al.*, 2003; Almeida *et al.*, 2005) and strict criteria (Sakkas *et al.*, 2002; Weng *et al.*, 2002; Siddighi *et al.*, 2004; Chen *et al.*, 2006). 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 the study of Aziz *et al.* (2007). In this study a highly motile sperm subpopulation was also selected through DDGC, but in contrast the resultant sperm subpopulation was sorted by means of a magnetic cell sorting (MACS) technique. This study found that the non-apoptotic sperm subpopulation had morphologically superior quality sperm compared with apoptotic sperm as reflected by significantly lower sperm deformity index (SDI) scores. The SDI score is a novel expression of the quality of sperm morphology and has previously been shown to be a more powerful predictor of male fertility and of *in vitro* fertilization outcome when compared to normal sperm morphology as predictor (Aziz *et al.*, 1996). In addition, there are two more studies which also found a positive correlation between normal sperm morphology and the apoptotic marker, percentage of PS externalization (Shen *et al.*, 2000; Ricci *et al.*, 2002). In these studies though, normal sperm morphology was assessed using WHO 1999 criteria.

In contrast to MACS, which is a liquid phase based cell sorting process (Aziz et al., 2007), a solid phase filter was recently developed (Grunewald et al., 2007). In this study the solid phase filter consisted of a glass wool column coated with AV. The solid phase filter was compared with conventional glass wool filtration as well as with AV MACS. AV-negative sperm filtered out by the newly developed molecular glass wool filtration (GWF) system displayed superior quality in terms of high mitochondrial membrane potential (MMP) integrity, as well as, to a small extent, caspase 3 activation and externalization of phosphatidylserine. This newly developed solid phase molecular filter system has proven to enrich spermatozoa free of apoptosis markers to the same extent as the AV magnetic separation technique (Grunewald et al., 2007).

In an attempt to elucidate the possible correlation between normal sperm morphology, as assessed with strict criteria (Kruger *et al.*, 1986), and sperm physiological, chromosomal and genetic properties, in relation to fertilization, embryo quality and pregnancy, many authors have studied the correlations between normal

sperm morphology and zona binding tests (Oehninger *et al.*, 1997), acrosome reaction (Bastiaan *et al.*, 2003), sperm nuclear DNA normality (Liu and Baker, 1992; Hammadeh *et al.*, 1998), double-stranded DNA content of the sperm nucleus (Claassens *et al.*, 1992), sperm chromosome complement (Martin, 1998) and sperm nucleus DNA fragmentation (see previous chapter). All of these studies have found that normal sperm morphology correlates positively with a physiologically more active, chromosomally more normal and genetically more intact sperm. In addition, this study confirms that normal sperm morphology has a significant positive correlation with non-apoptotic sperm ($P < 0.0001$). The importance of apoptosis in relation to fertilization, through IVF or intracytoplasmic sperm injection (ICSI) procedures, and pregnancy has previously been reported (Sun *et al.*, 1997; Levy *et al.*, 2001; Wang *et al.*, 2002). Wang *et al.* (2002) evaluated the relationship between sperm apoptosis and male infertility. They reported that sperm apoptosis by flow cytometry was significantly different between fertile and infertile groups ($P < 0.01$).

The importance of normal sperm morphology, as assessed with strict criteria (Kruger *et al.*, 1986), is that it has previously been shown to be predictive of fertilization and pregnancy rates *in vitro* (Oehninger *et al.*, 1988; Enginsu *et al.*, 1992; Ombelet *et al.*, 1994; Hernandez *et al.*, 1996). In a structured literature review (meta-analysis) conducted to assess the importance of sperm morphology on fertilization and pregnancy rates in, *in vitro* fertilization (IVF), Coetzee *et al.* (1998) confirmed the importance of sperm morphology in relation to the positive predictive value for fertilization and pregnancy.

From the literature it is evident, that spermatozoa with normal forms are per se 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 and 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 pregnancy and implantation rates were obtained after transfer of embryos resulting from morphologically abnormal sperm cells. This was especially true in those patients where there were spermatozoa with amorphous heads,

elongated heads, the presence of cytoplasmic droplets (reflecting incomplete maturity) or broken necks (de Vos *et al.*, 2003). In addition, another study found evidence suggesting that sperm morphology plays a significant role in ICSI outcome. Bartoov and co-workers (2003) introduced a modified IVF procedure – intracytoplasmic morphologically selected sperm injection (IMSI) – based on microinjection into retrieved oocytes of selected spermatozoa with strictly defined morphologically normal nuclei. The modified IMSI treatment results in significantly higher pregnancy rates, compared with conventional IVF-ICSI (Bartoov *et al*., 2003)

Rather than to rely only on the visual assessment for the normal form, Jakab *et al.* (2005) developed a sperm selection method based on the binding of mature sperm to hyaluronic acid (HA) (Huszar *et al.*, 1994). HA bound sperm has been shown to be devoid of persistent histones and apoptosis as evidenced by aniline blue staining and the absence of active caspase-3, respectively (Cayli *et al.*, 2004). In comparison, the current study has applied a novel method of non-apoptotic sperm selection based on the fact that apoptotic sperm externalizes PS, which can be bound with fluorescent AV, and thus be isolated from the unbound subpopulation by means of flow cytometric cell sorting. The importance of the significant positive correlation between non-apoptotic 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. The embryologist should be aware of these positive correlations and should subsequently pay special attention during the selection of sperm at the time of ICSI.

This study is based on the fact that apoptotic sperm are separated from non-apoptotic sperm through the binding of AV to the apoptotic marker; externalized phosphatidylserine. It is however important to realize that capacitated and acrosome reacted sperm also externalizes phosphatidylserine (Gadella and Harrison, 2002). Thus, this early sign of apoptosis is not a unique marker of abnormal/defective sperm. But, one of the terminal signs of apoptosis, i.e. DNA fragmentation, is a definite sign of abnormality as it has been shown too correlated negatively with

sperm concentration (Oosterhuis *et al*., 2000; Henkel *et al*., 2003) and sperm motility (Sun *et al.*, 1997; Henkel *et al.*, 2003). Furthermore, Henkel *et al*. (2003) confirmed that sperm with DNA fragmentation are able to fertilize oocytes but that these embryos will result in lower implantation and pregnancy rates. In general, damaged DNA carried into the zygote by the fertilizing spermatozoon will be repaired by the oocyte (Aitken *et al*., 2004). However, pathology can result when this DNA damage is converted into a genetic or epigenetic mutation as a consequence of aberrant DNA repair before the S phase of the first mitotic division. (Aitken and Krausz, 2001; Shimura *et al*., 2002; Aitken *et al*., 2004). From this it is clear that the selection method of choice is one that is able to exclude DNA fragmented sperm from the sample to be used for fertilization. In this regard, Ainsworth and co-workers (Ainsworth *et al.*, 2005) has developed a separation technique based on electrophoresis. The suspensions generated by the electrophoretic separation technique contained motile, viable, morphologically normal spermatozoa. The technique was comparable to discontinuous gradient centrifugation except that it took a fraction of the time and generated cells with significantly less DNA damage. The electrophoretic procedure holds promise as a convenient method for the rapid preparation of high-quality spermatozoa for assisted conception purposes (Ainsworth *et al.*, 2007).

In conclusion, the results of this study suggested that the non-apoptotic sperm subpopulation has morphologically superior quality sperm compared with the apoptotic 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, the sperm selection is a critical phase. Emphasis should be placed on selecting the morphologically most normal appearing spermatozoa to ultimately increase, implantation and pregnancy rates in these patients.

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[Papers by Ainsworth et al and Grunewald et al should be cited and discussed. In addition, while discussing this, emphasis should be on the selection of good quality sperm in order to improve outcome of ART.]

**REFERENCES**

Ainsworth C, Nixon B and Aitken RJ (2005) Development of a novel electrophoretic system for the isolation of human spermatozoa. Hum Reprod 20, 2261-2270.

Ainsworth C, Nixon B, Jansen RPS and Aitken RJ (2007) First recorded pregnancy and normal birth after ICSI using electrophoreticall isolated spermatozoa. Hum Reprod 22, 197-200.

Aitken RJ, Koopman P and Lewis SE (2004) Seeds of concern. Nature 432, 48-52.

Aitken and Krausz (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122, 497-506.

Almeida C, Cardoso F, Sousa M, Viana P, Goncalves A, Silva J and Barros A (2005) Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality. Hum Reprod 20, 1307-1313.

Arends MJ and Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 32, 223-254.

Aziz N, Buchan I, Taylor C, Kingsland CR and Lewis-Jones I (1996) The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization *in vitro*. Fertil Steril 66, 1000-1008.

Aziz N, Said T, Paasch U and Agarwal A (2007) The relationship between human sperm apoptosis, morphology and the sperm deformity index. Hum Reprod 22, 1413-1419.

Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H, Artzi S, Gross M and Barak Y (2003) Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 80, 1413-1419.

Bastiaan HS, Windt M-L, Menkveld R, Kruger TF, Oehninger S and Franken DR (2003) Relationship between zona pellucida-induced acrosome reaction, sperm morphology, sperm-zona pellucida binding, and *in vitro* fertilization. Fertil Steril 79, 49-55.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H and Guerin JF (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 18, 1023-1028.

Bossy-Wetzel E, Newmeyer DD and Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation

and independently of mitochondrial transmembrane depolarization. EMBO J 17, 37-49.

Cayli S, Sakkas D, Vigue L, Demir R and Huszar G (2004) Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bclx expression in mature and diminished maturity spermatozoa. Mol Hum Reprod 10, 365-372.

Chen Z, Hauser R, Trbovich AM, Shifren JL, Dorer DJ, Godfrey-Bailey L and Singh NP (2006) The relationship between human semen characteristics and sperm apoptosis: a pilot study. J Androl 27, 112-120.

Claasens OE, Menkveld R, Franken DR, Pretorius E, Swart Y, Lombard CJ and Kruger TF (1992) The Acridine Orange test: determining the relationship between sperm morphology and fertilization *in vitro*. Hum Reprod 7, 242-247.

Coetzee K, Kruger TF and Lombard CJ (1998) Predictive value of normal sperm morphology: a structured literature review. Hum Reprod Update 4: 73-82.

De Vos A, Van De Velde H, Joris H, Verheyen G, Devroey P and Van Steirteghem A (2003) Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 79, 42-48.

Enginsu ME, Dumoulin JCM, Pieters MHEC, Bergers M, Evers JL and Geraedts JP (1992) Comparison between the hypoosmotic swelling test and morphology evaluation using strict criteria in predicting *in vitro* fertilization. J Assist Reprod Genet 9, 259-264.

Evenson DP, Larson KL and Jost LK (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 23, 25–43.

Gadella BM and Harrison RAP (2002) Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophopholipids at the apical head plasma membrane of Boar sperm cells. (2002) Biol Rep 67, 340-350.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F and Lenzi A (2000) Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 15, 830-839.

Grunewald S, Paasch U, Wuendrich K and Glander HJ (2005) Sperm caspases become more activated in infertility patients than in healthy donors during cryopreservation. Arch Androl 51, 449-460.

Grunewald S, Miska W, Miska G, Rasch M, Reinhardt M, Glander HJ and Paasch U (2007) Molecular glass wool filtration as a new tool for sperm preparation. Hum Reprod 22, 1405-1412.

Hammadeh ME, Stieber M, Haidl and Schmidt W (1998) Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. Andrologia 30, 29-35.

Henkel R, Kierspel E, Hajimohammed M, Stalf T, Hoogendijk CF, Mehert C, Menkveld R, Schill W-B and Kruger TF (2003) DNA fragmentation of spermatozoa and assisted reproductive technology. RBM Online 7, 477-484.

Henkel R, Hajimohammed M, Stalf T, Hoogendijk C, Mehnert, Menkveld R, Gips H, Schill W, and Kruger TF (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 4, 965-972.

Hernandez M, Molina R, Olmedo J, Olmedo SB, Coetzee K and Estofàn D (1996) Prognostic value of the strict criteria: an Argentinian experience. Arch Androl 37, 85-87.

Huszar G, Vigue L and Oehninger S (1994) Creatine kinase immunocytochemistry of human sperm-hemizona complexes: selective binding of sperm with mature creatine kinase-staining pattern. Fertil Steril 61,136-142.

Jakab A, Sakkas D, Delpiano E, Cayli S, Kovanci E, Ward D, Ravelli A and Huszar G (2005) Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil Steril 84, 1665-1673.

Jeyendran RS (2000) Interpretation of semen analysis results: A practical guide. Cambridge Press, Bath, UK.

Jurisicova A, Lopes S, Meriano J, Oppedisano L, Casper RF and Varmuza S (1999) DNA damage in round spermatids of mice with a targeted disruption of the Pp1cgamma gene and in testicular biopsies of patients with non-obstructive azoospermia. Mol Hum Reprod 5, 323-330.

Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26, 239-257.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, Van Zyl JA and Smith K (1986) Sperm morphologic features as a prognostic factor in *in vitro* fertilization. Fertil Steril 46, 1118-1123.

Lacquet FA, Kruger TF, Du Toit TC, Lombard CJ, Sanchez Sarmiento CA, De Villiers A and Coetzee K (1996) Slide preparation and staining procedures for reliable results using computerized morphology (IVOS). Arch Androl 36, 133-138.

Levy R and Seifer-Aknin I (2001) Apoptosis during spermatogenesis and in ejaculated spermatozoa: importance for fertilization. Ann Biol Clin 59, 531-545.

Lin WW, Lamb DJ, Wheeler TM, Lipschultz LI and Kim ED (1997) *In situ* end-labeling of human testicular tissue demonstrates increased apoptosis in conditions of abnormal spermatogenesis. Fertil Steril 68, 1065-1069.

Liu DY and Baker HWG (1992) Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates *in vitro*. Fertil Steril 58, 1178-1184.

Liu CH, Tsao HM, Cheng TC, Wu HM, Huang CC, Chen CI, Lin DP and Lee MS (2004) DNA fragmentation, mitochondrial dysfunction and chromosomal aneuploidy in the spermatozoa of oligoasthenoteratozoospermic males. J Assist Reprod Genet 21, 119-126.

Luo X, Budihardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94, 481-490.

Marchetti C, Obert G, Deffosez A, Formstecher P and Marchetti P (2002) Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 17, 1257-1265.

Martin S, Reutelingsperger C, Mcgahon A, Rader JA, van Schie RC, LaFace DM and Green DR (1995) Early redistribution of plasma membrane phosphatedylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition of overexpression of Bcl-2 and Abl. J Exp Med 182, 1545-1556.

Martin R (1998) Human sperm chromosome complements in chemotherapy patients and infertile men. Chromosoma 107, 523-527.

Morris ID, Ilott S, Dixon L and Brison DR (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod 17, 990-998.

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G and Baldi E (2003) Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. J Androl, 24, 253-256.

Oehninger S, Acosta AA, Morshedi M, Veeck L, Swanson RJ, Simmons K and Rosenwaks Z (1988) Corrective measures and pregnancy outcome in *in vitro* fertilization in patients with severe sperm morphology abnormalities. Fertil Steril 50, 283-287.

Oehninger S, Mahoney M, Ozgür K, Kolm P, Kruger TF and Franken D (1997) Clinical significance of human sperm-zona pellucida binding. Fertil Steril 67, 1121-1127.

Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H and Beebe S (2003) Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. RBM Online 7, 469-476.

Ombelet W, Fourie F, Vandeput H, Bosmans E, Cox A, Janssen M and Kruger TF (1994) Teratozoospermia and *in-vitro* fertilization: a randomized prospective study. Hum Reprod 9, 1479-1484.

Oosterhuis GJ Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J and Vermes I (2000) Measuring apoptosis in human spermatozoa: a biological assay for semen quality? Fertil Steril 74, 245-250.

Pena FJ, Johannisson A, Wallgren M and Rodriquez-Martinez H (2003) Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Therio 60, 677-689.

Ricci G, Perticarari S, Fragonas E, Giolo E, Canova S, Pozzobon C, Guaschino S and Presani G (2002) Apoptosis in human sperm: its correlation with semen quality and presence of leukocytes. Hum Reprod 17, 2665-2672.

Said TM, Paasch U, Grunewald S, Baumann T, Li L, Glander HJ and Agarwal A (2005) Advantage of combining magnetic cell separation with sperm preparation techniques. RBM Online 10, 740-746.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG and Bianchi U (1999) Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 4, 31-37.

Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N and Bizzaro D (2002) Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 66, 1061-1067.

Sakkas D, Seli E, Bizzaro D, Tarozzi N and Manicardi GC (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. Reprod Biomed Online 7, 428-432.

Shen H and Ong C (2000) Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. Free Radicals in Biology and Medicine 28, 529–536.

Shen HM, Dai J, Chia SE, Lim A and Ong CN (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod 17, 1266-1273.

Shimura T, Inoue M, Taga M, Shiraishi K, Uematsu N, Takei N, Yuan ZM, Shinohara T and Niwa O (2002) p53-dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. Mol Cell Biol 22, 2220-2228.

Sun JG, Jurisicova A and Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization *in vitro*. Biol Reprod 56, 602-607.

Taylor SL, Weng SL, Fox P, Duran EH, Morshedi MS, Oehninger S and Beebe SJ (2004) Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. Mol Hum Reprod 10, 825-834.

Siddighi S, Patton WC, Jacobson JD, King A and Chan PJ (2004) Correlation of sperm parameters with apoptosis assessed by dual fluorescence DNA integrity assay. Arch Androl 50, 311-314.

Van Heerde WL, Degroot PG and Reutelingsperger CPM (1995) The complexity of the phospholipid binding protein annexin V. Thromb Haemost 73, 172-179.

Vermes I, Haanen C and Reutelingsperger CPM (1995) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labelled annexin V. J Immunol Methods 180, 39-52.

Wang GR, Zhou ZD, Ge ZM and Zhao MJ (2002) Preliminary investigation of relationship between sperm apoptosis and male infertility. Zhonghua Nan Ke Xue 8, 25-27.

Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S and Oehninger S (2002) Caspase activity and apoptotic markers in ejaculated human sperm. Mol Hum Reprod 8, 984-991.

Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG and Ford WC (1999) Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. Int J Androl 22, 236-242.

World Health Organization. (1999) World Health Organization Laboratory Manual for Examination of Human Semen. Cambridge University Press, Cambridge, UK.

# CHAPTER V

## A Study of two Sequential Media: Impact on Human Embryo Quality and Pregnancy rates

Abstract

Introduction

Materials and Methods

- Prospective Randomized control trial (April - May 2003)
- Retrospective study (January 2002 – March 2003 vs. June 2003 – May 2004)
- Ovarian Stimulation
- Oocyte retrieval
- Semen preparation
- Insemination
- Embryo culture
- Embryo Grading (modified from Veeck)
- Embryo Transfer and Pregnancy evaluation
- Statistical analysis

Results

- First phase of study: Prospective Randomized Control Trial
- Second phase of study: Retrospective study

Discussion

References

**ABSTRACT**

**Introduction:** In hindsight, the concept of culturing cells in an environment that resembles their in vivo milieu as much as possible seems self-evident. However, it has taken the last fifty years to embody this concept in the systems used for preimplantation mammalian embryo culture and further refinement is continuing. Now that the chemical composition of the fluid environment of the female reproductive tract is more accurately understood, emphasis is turning more to creating a dynamic culture environment that emulates the fluid environment of the reproductive tract along with macromolecular elements that interact with the embryo from fixed sites along the reproductive tract.

**Aim:** A comparative study of embryo quality and pregnancy outcome between SAGE QA Sequential medium (trial medium) and a readily available sequential embryo culture medium (control medium).

**Materials and Methods:** The study was designed to include two stages, firstly a randomized control trial and secondly a retrospective study. Standard stimulating protocols and embryo culture conditions were followed. In the randomized control trial *a*ll IVF patients attending our clinic from the beginning of April 2003 to the end of May 2003 were randomly allocated to either control (n = 31) or trial (n = 19) medium. In the retrospective study data from January 2002 to end of March 2003 (control medium; n = 179) were compared with data from June 2003 to end of May 2004 (trial medium; n = 197).

**Results:** *Prospective randomized trial:* In this study the only significant difference was in day 3 good quality embryos (33/79 (42%) v. 40/67 (60%) for Sydney IVF and Quinn's Advantage respectively, $p < 0.05$). *Retrospective study:* Significant difference ($p < 0.05$) for embryo development (early dividing embryos 156/786 (20%) v. 263/919 (29%)), day 3 good quality (234/639 (37%) v. 378/795 (48%)) and pregnancy rate (ongoing pregnancy rate 31/179 (17%) vs. 59/195 (30%)) between Sydney IVF Medium v. Quinn's Advantage Sequential Culture Medium.

**Conclusion:** The results of these two studies showed that the range of Quinn's Advantage Sequential Culture Media is more beneficial for in vitro embryo culture as each of the media in the range contribute collectively to more embryos with a better quality. The reason for the significant increase in embryo developmental parameters and pregnancy rate can possibly be attributed to the differences in the composition between the two media.

## INTRODUCTION

All the above mentioned studies (Chapters 2, 3, and 4) concentrated on the spermatozoon's contribution towards the development of an embryo and a pregnancy. It is, however, important to realize that embryo development is dependent on many other factors. Of these, the *in vitro* culture conditions are very important, as these are artificially created to simulate the physiological *in vivo* environment.

Optimization of culture media for the support of human embryos after *in vitro* fertilization (IVF) and before embryo transfer has been a source of considerable interest over the past decade. The first culture media used for human IVF, such as Ham's F10 and Earle's Balanced Salt Solution, were designed to support the development of somatic cells and other cell lines in culture. Other media, such as Tyrode's T6 and Whitten's WM1, have been used for IVF and embryo culture in laboratory animals. One of the first media specifically designed for human IVF was human tubal fluid (HTF) (Quinn *et al.*, 1985). Trying to imitate the *in vivo* environment to which the embryo is exposed, the formulation of HTF was based on the then known chemical composition of the fluids in the human fallopian tubes.

IVF culture media can generally be categorized into either the simple balanced salt solutions containing energy sources (e.g. Earle's, Tyrode's T6, WM1) or the more complex mixtures of inorganic salts, energy sources, amino acids, vitamins and other substances (e.g. Ham's F10, Ménézo's B2 and B3 media, Eagle's MEM) (Loutradis *et al.*, 2000). Before the mid 1990s, low production and accompanying low viability of blastocysts during human IVF due to the inability of any single culture medium to meet and sustain the changing nutritional requirements of pre-implantation embryos resulted in blastocyst transfer being abandoned (Gardner and Lane, 1997). Recent development of sequential media designed for human embryo culture has led to improvements in human IVF outcomes. These sequential media were formulated according to the carbohydrate composition of oviduct and uterine fluids and take into account the changing physiology and metabolic requirements of the human embryo (Gardner and Lane, 1997). The first stage of a sequential media system is comprised of a glucose-poor or even glucose-free medium designed to support the

development of the zygote stage to the eight-cell stage, followed by a second, more complex, medium suitable for development, following activation of the embryonic genome, up to the blastocyst stage.

Several studies have confirmed that high implantation rates can be achieved after transfer of blastocysts obtained with this new generation of media (Alves da Motta *et al.*, 1998; Racowsky *et al.*, 2000). In cases where patients have a good prognosis, implantation rates of up to 50% have been reported (Gardner *et al.*, 1998a; Gardner *et al.*, 2000). The percentage of blastocysts obtained as well as their viability is greater than that reported for culture systems using one medium throughout the culture, such as a mixture of Earle's and Ham's F-10 (Bolton *et al.*, 1991; Scholtes *et al.*, 1996) or minimal essential medium (MEM) (Noda *et al.*, 1994). Overall, the results achieved with sequential medium appear to be in the same range as those reported when embryos are co-cultured with feeder cells (Alves da Motta *et al.*, 1998; Fong and Bongso, 1998; Ménézo *et al.*, 1998). However, culture in sequential medium offers several advantages over co-culture. The risk of introducing pathogens is greatly reduced, culture handling is less time-consuming and the composition of the culture medium is less uncertain.

Several of these sequential media are commercially available, including G1/G2 medium (Barnes *et al.*, 1995; Gardner *et al.*, 1998b; Jones *et al.*, 1998a), G1.2/G2.2 medium (Gardner *et al.*, 1998a; Gardner *et al.*, 2000), S1/S2 medium (Marek *et al.*, 1999), P1/ Irvine Scientific Blastocyst medium (Behr *et al.*, 1999; Pantos *et al.*, 1999), IVF-50/G2 (Jones *et al.*, 1998a; 1998b), IVF 50-S2 (Fong and Bongso, 1998; Jones *et al.*, 1998b; Pantos *et al.*, 1999; Racowsky *et al.*, 2000), Sydney IVF Cleavage/Blastocyst medium (Parinaud *et al.*, 1998) and Quinn's Advantage Sequential Culture Medium (Cooke *et al.*, 2002). However, only a few studies have compared the proficiency of these media and assessed the viability of embryos cultured in sequential media (Van Langendockt *et al.*, 2001).

The aim of the present study was to assess pregnancy outcomes after transfer of day 3 embryos and day 5 blastocysts obtained in Sydney IVF Medium and Quinn's Advantage Sequential Culture Medium and to compare the *in vitro* development of

embryos in these sequential media using developmental speed, embryo morphology (day 2 and 3) and blastocyst formation rate as end-points.

## MATERIALS AND METHODS

The study was performed in two phases. In the first phase, both media were compared after conducting a prospective randomized control trial. The retrospective analysis comprises the second phase of the study.

*Prospective randomized control trial (April - May 2003)*

Inclusion criteria were as follows: female patient age < 38 years, day 3 FSH level < 12 mIU/mL (Ebrahim *et al.*, 1993), and the presence of at least 2 follicles ≥ 18 mm in diameter on the day of human chorionic gonadotrophin (hCG) administration. At oocyte retrieval, patients were randomly (alternately) assigned to one of two groups. In group A (N = 20) zygotes were cultured in Sydney IVF Cleavage/Blastocyst Medium (Cook, Brisbane, Australia) and in group B (N = 19) culture were performed in Quinn's Advantage Sequential Culture Medium (Sage In-Vitro Fertilization, a CooperSurgical Company, Trumbull, CT, USA). The composition of the two commercially available media is shown in Table I.

*Retrospective study (January 2002 - March 2003 v. June 2003 - May 2004)*

This study was conducted using the computer based data system of the Reproductive Biology Unit in the Department of Obstetrics and Gynaecology at Tygerberg Academic Hospital. For the above mentioned time periods the database was searched for all patients who had an intra cytoplasmic sperm injection (ICSI) cycle, with the following inclusion criteria: female patient age < 38 years, and > 1 embryo transferred. For the period January 2002 - March 2003, Sydney IVF Medium was used and 181 cycles adhered to the inclusion criteria (group A = Sydney IVF group). For the period June 2003 to May 2004, Quinn's Advantage Medium was used and 203 cycles adhered to the inclusion criteria (group B = Quinn's Advantage group).

*Ovarian stimulation*

For both studies controlled ovarian stimulation were achieved using a long protocol of gonadotrophin-releasing hormone (GnRH) agonist (Synarel; Montosano South Africa, Searle) followed by human menopausal gonadotrophin (hMG) (Pergonal; Serono, South Africa (Pty) Ltd) and/or pure FSH (Metrodin; Serono) from cycle day 3. Patients were followed up by performing oestradiol determinations as well as serial ultrasonographic measurement of all the follicles. Ovulation was induced by the administration of hCG (Profasi; Serono) as soon as the leading follicle reached 18 mm in diameter.

*Oocyte retrieval*

Follicle aspiration was done under conscious sedation (Dormicum; Roche Products (Pty) Ltd, South Africa, or Diprovan; Zeneca Pharmaceuticals, South Africa). Oocytes were recovered by transvaginal ultrasound-guided follicle aspiration 34 - 36 hours after hCG administration.

The cumulus oocyte complexes were isolated into Flushing Medium with 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) (Medi-Cult, Copenhagen, Denmark) (group A), or Quinn's Advantage Medium with HEPES (group B).

*Semen preparation*

In group A semen preparation was carried out in Medi-Cult sperm preparation medium with HEPES and in group B it was carried out in Quinn's sperm washing medium with HEPES. Where possible, motile sperm were isolated by a standard swim-up technique. This technique included two cycles of centrifugation (428 - 450 g for 10 minutes) and washing. Then 500 µl of the same medium was gently added to the resulting pellet and left in the incubator at 37ºC for 60 minutes to allow swim-up. In cases of oligozoospermia, asthenozoospermia and testis biopsy samples, motile spermatozoa were isolated using a discontinuous (45, 70, 90%) SilSelect (FertPro®, Beernem, Belgium ) gradient made with the HEPES buffered medium used for sperm preparation in each group. Ejaculated, testicular biopsy, cryopreserved ejaculated and cryopreserved testicular biopsy semen specimens were all included in the study.

*Insemination*

ICSI was used to inseminate retrieved oocytes. Cumulus cells were removed 3 - 5 hours after retrieval using ~40 IU/ml hyaluronidase (Sigma) in group A and ~30 IU/ml hyaluronidase in HEPES-HTF (SAGE In-Vitro Fertilization) in group B and denuding pipettes (Flexi pet (Cook)). Denuded oocytes were rinsed three times in 1 ml buffered medium. Metaphase II oocytes were identified and incubated in 50 µl droplets covered with paraffin oil (group A: Paraffin Oil (Medi-Cult); group B: Paraffin Oil for Tissue Culture (SAGE In-Vitro Fertilization)) until injection (37°C, 5% $CO_2$). ICSI was performed as soon as possible after denuding, i.e. $\geq$ 3 hours but $\leq$ 5 hours after retrieval. A standard ICSI method was followed. Prerequisites for successful injection were immobilization of the sperm cell in polyvinylpyrrolidone (PVP) (group A: PVP (Medi-Cult); group B: PVP (SAGE In-Vitro Fertilization)) and mild cytoplasmic aspiration.

ICSI was performed on a Nikon inverted microscope (Nikon Diaphot 300, Nikon Co., Tokyo, Japan) with Narishige micromanipulators (Narishige, Tokyo, Japan).

*Embryo culture*

For both studies and for all embryos, the culture conditions were identical. The only difference was the culture media used. Injected oocytes were rinsed and returned to the culture dish with either Sydney IVF Cleavage medium (group A) or Quinn's Advantage Cleavage medium (group B) for further incubation at 37°C. To ensure optimal culture conditions, manual temperature readings in the incubators (Forma 3164 (Forma Scientific, Marietty, Ohio, USA)) were performed every day, the pH of the media was checked every week and the $CO_2$ level was adjusted for the optimal pH range of 7.2 - 7.4. Using these criteria, the pH for both media during the study period was within the optimal range. None of the media used in this study underwent formulation changes during the course of the study. Oocytes were individually examined under a dissecting microscope at the following times: ~18 hours post insemination for the presence of two pronuclei and two polar bodies; 25 - 27 hours (~26 h) post insemination for 2-cell division (early division); ~45 hours post insemination for 4-cell division; and ~72 hours post insemination for 6 - 8-cell division.

*Embryo grading (modified from Veeck (Veeck, 2001))*

Embryo morphology was scored from 1 to 5 according to the shape of the blastomeres and the amount of detached anuclear fragments. Embryos were regarded as of 'good quality' when they were at the 4-cell stage at ~45 hours post insemination or at the 6 - 8-cell stage, ~72 hours post insemination with equal-sized blastomeres and minor (< 20%) or no cytoplasmic anuclear fragments. Whenever there was uncertainty regarding the embryo score, it was double-checked by a second or third embryologist until consensus was reached. Embryologists assessing embryo grading and selecting embryos for transfer were blinded as to which culture media was being used in the prospective study.

*Embryo transfer and pregnancy evaluation*

Embryos with the highest score were selected for transfer either on day 3 or day 5. A day 3 transfer was performed when there were 3 or less good quality embryos available on this day, on the other hand a day 5 blastocyst transfer was performed when there were 4 or more good quality embryos on day 3. For day 3 transfer, 3 embryos were transferred per patient. For day 5 transfer, 1 or 2 blastocysts were transferred per patient. Pregnancies were reported as positive when βhCG serum levels were > 10 IU/mL 10 days post transfer and increased to four times that value on day 14 post transfer. Clinical pregnancy was defined by the presence of a gestational sac, crown rump length of 2 - 4 mm, and fetal heartbeat at ultrasound performed 6 - 7 weeks after embryo transfer (ET).

*Statistical analysis*

Results are expressed as mean ± SD. Data were analyzed using the unpaired t-test and percentages were compared by $\chi^2$ analysis. $p < 0.05$ was defined as statistically significant.

**Table I**. Composition of Sydney IVF (Cook, Australia) and Quinn's Advantage Media (Coopersurgical, USA)

| | Sydney IVF Medium | | | Quinn's Advantage Medium | | |
|---|---|---|---|---|---|---|
| | *Fertilisation stage* | *Cleavage stage* | *Blastocyst stage* | *Fertilisation stage* | *Cleavage stage* | *Blastocyst stage* |
| pH | 7.3 - 7.5 | 7.3 - 7.5 | 7.3 - 7.5 | 7.3 | 7.2 | 7.3 |
| Phosphate | Low[a] | Low[a] | Low[a] | 0.01 mM | No | +[a] |
| $NaHCO_3$ | 25 mM | 25 mM | 25 mM | 20.2 mM | 15.6 mM | 22.6 mM |
| Protein | 1% (HSA) | 1% (HSA) | 1% (HSA) | 3 mg/ml HSA | 5 mg/ml SPS, $\alpha$- & $\beta$-globulins | 5 mg/ml SPS, $\alpha$- & $\beta$- globulins |
| Glucose | +[a] | No | | 2.8 mM | 0.1 mM | 2.8 mM |
| Magnesium | Low[a] | | | 0.2 mM | 2 mM | 2 mM |
| Na-pyruvate | 0.33 mM | | | 0.33 mM | 0.33 mM | 0.1 mM |
| Lactate | 21.4 mM (Na-lactate) | | | 2.04 mM (Ca-lactate) | | |
| Ala-Gln | no, (Ala + Gln)[a] | | | 1 mM | | |
| Amino acids | Taurine, glutamine, glycine, non-ess AAs[a] | | | 0.1 mM taurine + 0.5 mM non-ess AA (no Ala) + 1 mM ess AA[b] | | |
| Phenol red | no | | | yes | | |
| Basic salts | yes | | | yes | | |

[a] Exact concentration not provided; [b] Blastocyst Medium only; HSA : human serum albumin; SPS: serum protein substitute; Ala: alanine; Gln: glutamine; ess AA: essential amino acids; non-ess AA: non-essential amino acids

## RESULTS

**First phase of study:** Prospective randomised controlled trial

The study included 39 patients, who underwent 42 oocyte retrieval cycles. No statistically significant differences (Table II) were noted between the two groups with regard to age and number of previously failed cycles. In both the groups the indication for fertility treatment included tubal factor, male factor, idiopathic, anovulation, endometriosis and multiple factors.

Table II. Indication for fertility treatment in the prospective study for the control (group A; Sydney IVF Medium) and trial (group B; Quinn's Advantage Sequential Culture Medium) groups

| | Group A | Group B | p-value |
|---|---|---|---|
| Cycles | 23 | 19 | |
| Repeat cycles | 3 | 0 | p = 0.102 |
| Age (yrs) (mean ± SD) | 33 ± 5.0 | 35 ± 3.5 | p = 0.158 |
| Number of previously failed IVF cycles (mean ± SD) | 2.0 ± 1.6 | 2.5 ± 1.5 | p = 0.321 |
| Indication for IVF | | | |
| Tubal factor | 1/20 (5.0%) | 5/19 (26.3%) | p = 0.168 |
| Male factor | 6/20 (30.0%) | 9/19 (47.4%) | p = 0.446 |
| Idiopathic | 2/20 (10.0%) | 2/19 (10.5%) | p = 0.674 |
| Anovulation | 4/20 (20.0%) | 2/19 (10.5%) | p = 0.661 |
| Endometriosis | 3/20 (15.0%) | 1/19 (5.3%) | p = 0.605 |
| Multiple factors | 4/20 (20.0%) | 0 | p = 0.126 |

IVF: *in vitro* fertilization

Data on fertilisation, embryo quality and cleavage rates are presented in Table III. No significant difference was noted in the percentage of fertilised ova. Cleaving rates of embryos at 26 hours post insemination was not significantly different for the two

groups. Embryo quality, as assessed by morphological parameters, was also similar at day 2 post fertilisation. However, at day 3 post fertilisation a significantly higher number of good quality embryos were noted in group B (Group A: 33/79 (34%); group B: 40/67 (60%); $p < 0.05$). Blastulation rate included all embryos not transferred on day 3 that reached blastocyst stage; no significant difference between the two groups were noted.

Table III. Prospective randomised study: Fertilization, embryo development, embryo transfer and pregnancy rate for the control group (group A; Sydney IVF Medium) and the trial group (group B; Quinn's Advantage Sequential Culture Medium)

| | Group A | Group B | p-value |
|---|---|---|---|
| Cycles | 23 | 19 | |
| Retrieved oocytes | 134 | 122 | |
| Number of oocytes (mean ± SD) | 5.8 ± 3.2 | 6.4 ± 3.2 | |
| Fertilisation | 95/134 (70.9%) | 84/122 (69%) | p = 0.826 |
| Early dividing embryos | 26/95 (27.4%) | 20/84 (23.8%) | p = 0.705 |
| Embryo quality | | | |
| Day 2 good quality | 38/95 (40.0%) | 39/84 (46.4%) | p = 0.476 |
| Day 3 good quality | 33/79 (41.8%) | 40/67 (59.7%) | p = 0.031 |
| Blastulation rate | 11/32 (34.4%) | 14/31 (45.2%) | p = 0.536 |
| Embryo transfer (ET) | | | |
| Embryos transferred/cycle | 2.5 ± 0.8 | 2.5 ± 0.9 | p = 1.000 |
| Pregnancy rate (% per ET cycle) | | | |
| Positive βhCG/ET | 4/23 (17.4%) | 6/19 (31.6%) | p = 0.477 |
| Ectopic pregnancy | None | 1/19 (5.3%) | p = 0.452 |
| Ongoing pregnancy | 2/23 (8.7%) | 4/19 (21.1%) | p = 0.384 |

ET: embryo transfer

βhCG: beta human chorionic gonadotrophin

Embryo transfer and pregnancy rates are also described in Table III. Normally cleaving embryos were available for transfer in all cycles in both groups. While the mean number of embryos transferred in both groups was similar, all measured

outcome parameters were more favorable in group B, although this was not statistically significant. The rate of positive βhCG in group B was 32% compared with 17% in group A (p = 0.477); furthermore, both ectopic and ongoing pregnancy rates were not significantly different between the two groups.

**Second phase of study:** Retrospective study

The study included 300 patients who underwent 374 oocyte retrieval cycles. Patient demographic variables and characteristics were essentially similar (Table IV). In group B the patients were significantly older (p < 0.0001) with a mean age of 34.0 ± 3.1 years compared with 32.0 ± 3.4 years for group A. In both the groups the indication for fertility treatment included tubal factor, male factor, idiopathic, anovulation, endometriosis and multiple factors.

Table IV. Indication for fertility treatment in the retrospective study for the control (group A; Sydney IVF Medium) and trial (group B; Quinn's Advantage Sequential Culture Medium) groups

| | Group A | Group B | p-value |
|---|---|---|---|
| Cycles | 179 | 195 | |
| Repeat cycles | 30 | 31 | p = 0.822 |
| Age (mean ± SD) | 32 ± 3.4 | 34 ± 3.1 | p = 0.000 |
| Number of previously failed IVF cycles (mean ± SD) | 2.0 ± 1.5 | 2.2 ± 1.7 | p = 0.282 |
| Indication for IVF | | | |
| Tubal factor | 17/146 (11.6%) | 10/154 (6.5%) | p = 0.180 |
| Male factor | 68/146 (46.6%) | 65/154 (42.2%) | p = 0.515 |
| Idiopathic | 12/146 (8.3%) | 17/154 (11.0%) | p = 0.532 |
| Anovulation | 7/146 (4.8%) | 18/154 (11.7%) | p = 0.051 |
| Endometriosis | 4/146 (2.7%) | 8/154 (5.2%) | p = 0.430 |
| Multiple factors | 38/146 (26.0%) | 36/154 (23.4%) | p = 0.698 |

IVF: *in vitro* fertilization

Data on fertilisation, embryo quality and cleavage rates are presented in Table V. Fertilisation did not differ significantly between the two groups (group A 786/1 201 (66%); group B 919/1 454 (63%); p = 0.231). However, there was a statistically significant increase ($p < 0.05$) for all embryo developmental parameters (early dividing embryos, good quality (day 2 and 3) and blastulation rate) in group B.

Table V. Retrospective study: Fertilisation, embryo development, embryo transfer and pregnancy rate for the control group (group A; Sydney IVF Medium) and the trial group (group B; Quinn's Advantage Sequential Culture Medium)

| | Group A | Group B | p-value |
|---|---|---|---|
| Cycles | 179 | 195 | |
| Retrieved oocytes | 1 201 | 1 454 | |
| Number of oocytes (mean ± SD) | 6.7 ± 3.6 | 7.5 ± 3.8 | |
| Fertilisation | 786/1201 (65.5%) | 919/1454 (63.2%) | p = 0.231 |
| Early dividing embryos | 156/786 (19.8%) | 263/919 (28.6%) | p < 0.05 |
| Embryo quality | | | |
| Day 2 good quality | 275/786 (35.0%) | 416/909 (45.3%) | p < 0.05 |
| Day 3 good quality | 234/639 (36.6%) | 378/795 (47.5%) | p < 0.05 |
| Blastulation rate | 74/267 (27.7%) | 175/384 (45.6%) | p < 0.05 |
| Embryo transfer (ET) | | | |
| Embryos transferred/cycle | 2.8 ± 0.7 | 2.7 ± 0.6 | p = 1.000 |
| Pregnancy rate (% per ET cycle) | | | |
| Positive βhCG/ET | 46/179 (25.7%) | 70/195 (35.9%) | p < 0.05 |
| Ectopic pregnancy | 1/179 (1.1%) | none | |
| Ongoing pregnancy | 31/179 (17.3%) | 59/195 (30.3%) | p < 0.05 |

ET: embryo transfer

βhCG: beta human chorionic gonadotrophin

Embryo transfer and pregnancy rates are also described in Table V. There was no difference in the mean number of embryos transferred in both groups. The rate of positive βhCG in group B (70/195 (36%)) was significantly increased ($p < 0.05$) compared with that in group A (46/179 (26%)). This significance was reflected in the

ongoing pregnancy rate, which was significantly increased ($p < 0.05$) in group B (59/195 (30%)) compared with that in group A (31/179 (17%)). Only 1 ectopic pregnancy was noted in this study; it occurred in group A.

## DISCUSSION

Sydney IVF Medium has previously been compared with Ménézo B2 Medium (De Clerk *et al.*, 2001), G1.2 Medium (Van Lagendockt *et al.*, 2001) and P1 Medium (De Clerk *et al.*, 2001). All these studies found a delay in early embryonic development when culture took place in Sydney IVF Medium compared with another medium in question (De Clerk *et al.*, 2001; Veeck, 2001; Ben-Yosef *et al.*, 2004). Slow cleaving embryos play an important role in the success of the IVF cycle, as it is well known that early dividing (2-cell stage at 25 - 27 hours post insemination/injection) embryos significantly improve pregnancy rates when they are selected and transferred (Shoukir *et al.*, 1997; Windt *et al.*, 2004). Furthermore, fast cleaving human embryos have a greater potential to develop to blastocysts *in vitro* (Muggleton-Harris *et al.*, 1995). In the prospective randomized study we found a higher, although not significant, blastulation and pregnancy rate in group B. The study cohort in the prospective randomized study is small (39 patients); this is because an interim analysis 3 months into the study showed that there was a significant increase in day 3 embryo quality for embryos cultured in Quinn's Advantage Sequential Culture Medium. Furthermore, the pregnancy rate was better for embryos cultured in this medium (31.6% (group B) v. 17.4% (group A)). Although it was not significant a trend was observed, and these observations prompted an immediate change-over in our culture system.

The retrospective study reflected the observations of the prospective trial. In the retrospective study patients in group B (34.0 ± 3.1) were significantly older ($p < 0.0001$) than those in group A (32 ± 3.4). It is therefore of considerable interest that we not only found a higher incidence of early dividing embryos (EDE) in group B, but that all other embryo development parameters as well as pregnancy rates were significantly increased. In an attempt to explain this one should be aware that *in vitro* embryo development and survival is affected by various factors. These can

collectively be categorized as firstly, the immediate environment of the embryo (the culture system) and secondly, the DNA composition of the embryo.

The culture systems employed in this study differed only in the sequential culture medium; all other culture conditions were kept identical. The two culture media used in this study differ in various ways; we can only speculate that these differences have a cumulative beneficial effect for embryo development in the one medium. Differences (Table I) include the absence of labile glutamine and free alanine in Quinn's Advantage Medium, and conversely, the presence of stable alanyl-glutamine. The absence of alanine in this medium promotes pyruvate transamination. In turn, the process of pyruvate transamination inhibits the build-up of toxic ammonia ($NH_3$) in the medium. Furthermore, it is possible that the absence of inorganic phosphate in the Quinn's Advantage Cleavage Medium is beneficial for early embryo development, as glycolysis is then not promoted and the co-factors and other metabolites involved in glycolysis may be utilized for more energy-productive processes, such as oxidative phosphorylation. An additional difference between these media occurs between the blastocyst media, where there is a higher $Mg^{2+}$ concentration in the Sage product, which lowers the uptake of $Ca^{2+}$ from the medium, which has, in turn, a beneficial effect on maintaining mitochondrial activity (Beavis and Powers, 2003).

The specific sequential growth medium used, as previously mentioned, can influence embryo quality and development. However, it is well known that the DNA composition of the embryo also plays a major role (Pellestor, *et al.*, 2005). The DNA composition of the embryo is influenced by an array of factors, including chromosomal abnormalities of the spermatozoon and oocyte (Harper *et al.*, 1995; Delhanty *et al.*, 1997; Munne, 2002; Pellestor, *et al.*, 2005;), spermatozoon DNA fragmentation, endogenous production of reactive oxygen species (ROS) by the spermatozoa and apoptosis of the spermatozoa (Lopes *et al.*, 1998; Irvine *et al.*, 2000; Munne, 2002; Henkel *et al.*, 2004).

However, because randomization of patients occurred in the prospective study and patients were matched in the retrospective study, the types of patients studied in the two groups are essentially similar. Furthermore, it should also be noted that during

the course of this study variables such as embryologist, embryo transfer techniques and physicians were all constant. Five embryologists work on a rotational basis that was implemented before the onset of the study. Every rotation includes two embryologists in the IVF laboratory. The embryo transfer technique used did not change over the period of this study (January 2002 - May 2004). We can therefore speculate that the impact on embryo quality and pregnancy rate that we noted in both groups is mainly due to the sequential culture media used.

In conclusion, in the randomized prospective trial we noted a significant increase in day 3 embryo quality in group B (Quinn's Advantage Sequential Medium). Retrospectively, we found a significant increase of early dividing embryos, good quality embryos (day 2 and 3), blastulation rate and pregnancy rate in Quinn's Advantage Medium compared with Sydney IVF Medium. Although the randomized controlled trial is a small study, the trends observed are reflected by the results from the retrospective study. However, to prove unequivocally that one medium is superior to the other, a larger prospective randomized control trial would have to be undertaken. At present, taking all results into account, the embryo culture medium of choice in our laboratory is Quinn's Advantage Sequential Culture Medium. This study highlights the importance to have a holistic view on implantation and embryo quality. It is important to note that there are multiple factors (e.g. the state of spermatozoa, the genetic composition of the oocyte as well as laboratory conditions) affecting embryo quality and implantation.

**REFERENCES**

Alves da Motta EL, Alegretti JR, Baracat EC, Olive D and Serafini PC (1998) High implantation and pregnancy rates with transfer of human blastocysts developed in preimplantation stage one and blastocyst media. Fertil Steril 70, 659-663.

Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C and Trounson AO (1995) Blastocyst development and pregnancy after *in vitro* maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod 10, 3243-3247.

Beavis AD and Powers M (2003) Temparature dependance of the mitochondrial inner membrane anion channel. J Biol Chem 279, 4045-4050.

Behr B, Pool TB, Milki AA, Moore D, Gebhardt J and Dasig D (1999) Preliminary clinical experience with human blastocyst development *in vitro* without co-culture. Hum Reprod 14, 454-457.

Ben-Yosef D, Amit A, Azem F, Schwartz T, Cohen T, Mei-Raz N, Carmon A, Lessing JB and Yaron Y (2004) Prospective randomized comparison of two embryo culture systems: P1 Medium by Irvine Scientific and the Cook IVF Medium. J Assist Reprod Genet 21, 291-295.

Bolton VN, Wren ME and Parsons JH (1991) Pregnancies after *in vitro* fertilization and transfer of human blastocysts. Fertil Steril 55, 830-832.

Cooke S, Quinn P, Kime L, Ayres C, Tyler JPP and Driscoll GL (2002) Improvement in the early human embryo development using new formulation sequential stage-specific culture media. Fertil Steril 78, 1254-1260.

De Clerk E, Janssens R, Joris H, Verheyen G and Van Streiteghem A (2000) Prospective, controlled, randomized comparison of two embryo culture systems: Ménézo B2 medium and Cook IVF medium. ESHRE Annual Meeting, Bologna, Italy, 25th to 28th June, 2000, 801-900.

Delhanty JDA, Harper J, Ao A, Handyside AH and Winston RML (1997) Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. Hum Genet 99, 755-760.

Ebrahim A, Rienhardt G, Morris S, Kruger TF and Lombard CJ (1993) Follicle stimulating hormone levels on cycle day 3 predict ovulation stimulation response. J Assist Reprod Genet 2, 130-136.

Fong C-Y and Bongso A (1998) Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture. Hum Reprod 14, 774-781.

Gardner DK and Lane M (1997) Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum Reprod Update 3, 367-382.

Gardner DK, Vella P, Lane M, Wagley V, Schlenker T and Schoolcraft WB (1998a) Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. Fertil Steril 69, 84-88.

Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J and Hesla J (1998b) A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Hum Reprod 13, 3434-3440.

Gardner DK, Lane M, Stevens J, Schlenker T and Schoolcraft WB (2000) Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 73, 1155-1158.

Harper JC, Coonen E, Handyside AH, Winston RML, Hopman AHN and Delhanty JDA (1995) Mosaicism of autosomes and sex chromosomes in morphologically normal, monospermic preimplantation human embryos. Prenat Diagn 15, 41-49.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB and Kruger TF (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 18, 965-972.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA and Aitken RJ (2000) DNA integrity in human spermatozoa: relationships with semen quality. J Androl 21, 33-44.

Jones GM, Trounson AO, Gardner DK, Kausche A, Lolatgis N and Wood C (1998a) Evolution of a culture protocol for successful blastocyst development and pregnancy. Hum Reprod 13, 169-177.

Jones GM, Trounson AO, Lolatgis N and Wood C (1998b) Factors affecting the success of human blastocyst development and pregnancy following *in vitro* fertilization and embryo transfer. Fertil Steril 70, 1022-1029.

Lopes S, Jurisicova A, Sun JG and Casper RF (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 13, 896-900.

Loutradis D, Drakakis P, Kallianidis K, Sofikitis N, Kallipolitis G, Milingos S, Makris N, and Michalas S (2000) Biological factors in culture media affecting *in vitro* fertilization, preimplantation embryo development, and implantation. Ann NY Acad Sci 900, 325-335.

Marek D, Langley M, Gardner DK, Confer N, Doody KM and Doody KJ (1999) Introduction of blastocyst culture and transfer for all patients in an *in vitro* fertilization program. Fertil Steril 72, 1035-1040.

Ménézo Y, Veiga A and Benkhalifa M (1998) Improved methods for blastocyst formation and culture. Hum Reprod 13, suppl 4, 2562-2564.

Mortimer D, Henman M, Catt JW, Bowman MC and Jansen RPS (1998) Reduced embryo fragmentation leads to higher implantation and pregnancy rates using improved IVF culture systems. Fertil Steril 70, suppl 1, S18.

Muggleton-Harris AL, Glazier AM and Wall M (1995) A retrospective analysis of the in-vitro development of 'spare' human *in vitro* fertilization preimplantation embryos using 'in-house' prepared medium and 'Medi-Cult' commercial medium. Hum Reprod 10, 2976-2984.

Munne S (2002) Chromosome mosaicism in cleavage stage human embryos evidence for the maternal age effect. RBM Online 465, 223-232.

Noda Y, Goto Y, Umaoka Y, Shiotani M, Nakayama T and Mori T (1994) Culture of human embryos in alpha modification of Earle's medium under low oxygen tension and low illumination. Fertil Steril 62, 1022-1027.

Pantos K, Athanasiou V, Stefanidis K, Stavrou D, Vaxevanoglou T and Chronopoulou M (1999) Influence of advanced age on blastocyst development rate and pregnancy rate in assisted reproductive technology. Fertil Steril 71, 1144-1146.

Parinaud J, Veiga A, Vietez G, Busquets A, Milhet P, Calderon G, Richoilley G and Barri P (1998) Randomized comparison of three media used for embryo culture after intracytoplasmic sperm injection. Hum Reprod 13, suppl 4, 212-217.

Pellestor F, Anahory T and Hamamah S (2005) The chromosomal analysis of human oocytes. An overview of established procedures. Hum Reprod Update 11, 15-32.

Quinn P, Kerin JF and Warnes GM (1985) Improved pregnancy rate in human *in vitro* fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 44, 493-498.

Racowsky C, Jackson KV, Cekleniak NA, Fox JH, Hornstein MD and Ginsburg ES (2000) The number of eight-cell embryos is a key determinant for selecting day 3 or day 5 transfer. Fertil Steril 73, 5585-64.

Scholtes MCW and Zeilmakern GH (1996) A prospective randomized study of embryo transfer results after 3 or 5 days of embryo culture in *in vitro* fertilization. Fertil Steril 65, 1245-1248.

Shoukir Y, Campana A, Farley T and Sakkas D (1997) Early cleavage of *in vitro* fertilized embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum Reprod 12, 1531-1536.

Steller H (1995) Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

Van Langendockt A, Demylle D, Wyns C, Nisolle M and Donnez J (2001) Comparison of G1.2/G2.2 and Sydney IVF cleavage/blastocysts sequential media for the culture of human embryos: A prospective, randomized, comparative study. Fertil Steril 76, 1023-1031.

Veeck LL (2001) Abnormal morphology of the human oocytes and conceptus. In: Veeck LL (eds) Atlas of the Human Oocyte and Early Conceptus. Vol. 2. The Parthenon Publishing Group, Lancaster, UK pp.151-864.

Windt M-L, Kruger TF, Coetzee K and Lombard CJ (2004) Comparative analysis of pregnancy rates after the transfer of early dividing embryos versus slower dividing embryos. Hum Reprod 19, 1155-1162.