THE IMPACT OF OOCYTE MEIOTIC SPINDLE AND AUTOMATED ZONA PELLUCIDA SCORE AND SPERM CHROMATIN PACKAGING ON FERTILIZATION AND PREGNANCY FOR ASSISTED REPRODUCTION TECHNIQUES

by

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Declaration

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Abstract

Non-invasive selection of developmentally competent human oocytes may provide information on the true fertilization potential of the spermatozoon in the absence of oocyte limitations. The objective was to measure 856 oocyte's competence by assessing oocyte metaphase 2 (M2) maturity, zona pellucida score (ZS) and presence of the meiotic spindle (SPp) using birefringent imaging software. ICSI was performed and fertilization (n=90 patients) and pregnancy rates (n=89) were measured and compared to the oocyte competence and sperm DNA chromatin via Chromomycin A_3 (CMA_3) (n=89). Fisher's exact and odds ratio's (OR) were used to determine effect. In total, 856 oocytes were harvested of which 568 (66%) were M2 stage of development. SPp oocytes were (384/730) 52.60%, OR was performed on the SP to determine its relevance to fertilization, its presence in the oocyte prior to ICSI, resulted in fertilization 1.5:1 times more than when it was absent, p=0.01. SPp embryos selected for embryo transfer resulted in a 65% expanded blastocyst rate, full blastocyst rate of 58% and early blastocyst rate of 54%. A negative development competence on day 5 was also correlated to absence of meiotic spindle (SPa) prior to ICSI with 56% of day 5 embryos transferred reaching only the compacted morula stage; while 50% of SPa embryos reached the morula stage at time of embryo transfer on day 5. Although there were no statistical differences between the pregnancy rates of SPp and SPa embryos, there were slight tendencies for better embryo quality. The SPp had a pregnancy rate (PR) of 40.91% (36/88). Random effects logistic regression OR performed on 768 oocytes from 90 patients indicated pregnancy to succeed 1.4:1 when SPp (p=0.89). The mean average automated ZS was 18.96 µm (95% CI: 15.75; 22.16; n=625 positive ZS from 90 patients). The ZS revealed a GLS linear regression with p=0.04 to fertilize when the ZS was 19.20 µm (16.60; 21.79). There was no statistical difference between ZS of the pregnant and non-pregnant groups. The main objective was to prove that when oocyte quality is optimized, that fertilization rates and by implication, pregnancy rates would be improved. If not, failure to fertilize or implant would most probably be due to decreased spermatozoa capacity to fertilize possibly due to damaged chromatin packaging. The chromatin packaging (CMA_3) of the study population was 74% semen samples with >40% immature DNA. The OR for CMA_3 underlines the hypothesis, that when oocyte competence for fertilization is controlled to a degree, the success or failure of treatment can be indicated by the CMA_3 value. In this instance, the OR is highly predictive for success with pregnancy when ICSI is performed on CMA_3 values that are immature (≥41%). Logistic regression calculated the OR for immature DNA (CMA_3≥41%) to predict pregnancy to be likely with odds of 1.6:1, p=0.39. Hypothesis OR: If ICSI is performed on an oocyte with: M2 and SPp, and this embryo develops to day 5 for embryo transfer, the odds of pregnancy, if working with a semen sample with >40% immature DNA, would be 1.9: 1.

Key Words: Meiotic spindle, Zona Score, CMA_3, ICSI, Infertility

Opsomming

Nie indringende seleksie van oösiete se potensiële ontwikkelingsbevoegtheid mag inligting voorsien met betrekking tot sperm DNA chromatien. Die doelwit was om 856 oösiete se doeltreffendheid te meet deur oösiet metafase 2 (M2) volwassenheid te asseseer, zona pellucida telling (ZS) en die teenwoordigheid van die meiotiese spool (SPp) deur die gebruik van lig deurdringbare sagteware. Deur middel van intrasitoplasmiese sperm inspuiting (ISSI) is bevrugting (n=90 pasiënte) en swangerskap syfers (n=89) gemeet en vergelyk met die oösiet doeltreffendheid en sperm DNA chromatien via Chromomycin A_3 (CMA_3) (n=89). Fisher se 'exact and odds ratio's' (OR) is gebruik om die effek te bepaal. In totaal was 856 ge-oes waarvan 568 (66%) op M2 vlak van ontwikkeling was. SPp oösiete was (384/730) 52.60%. OR was toegepas op die SP om die relevansie hiervan te bepaal met bevrugting. SPp teenwoordigheid in die oösiete voor ISSI het, 1.5:1 kere meer bevrugting tot gevolg gehad, as wanneer dit nie teenwoordig was nie, p=0.01. SPp geselekteerde embrios vir embrio oorplasing het 65% uitgesette blastosiste tot gevolg gehad, volledig ontwikkelde blastosiste van 58% en vroeë blastosiste van 54%. 'n Negatiewe ontwikkelings doeltreffendheid op dag 5 is ook gekorreleer met die gebrek aan meiotiese spoel (SPa) voor ISSI met 65% van dag 5 embrios oorgeplaas wat slegs op die kompakte morula fase was terwyl 50% van SPa embrios die morula fase bereik het teen embrio oorplasing op dag 5. Hoewel daar geen statistiese verskille tussen die SPp en Spa gevind was nie, was daar klein tendense vir beter embrio kwaliteit in die SPp met 'n swangerskap syfer (PR) van 40.91% (36/88). Steekproewe van logistiese regressie van OR uitgevoer op 768 oösiete van 90 pasiënte het aangedui dat swangerskap sal slaag 1.4:1 wanneer SPp(p=0.89). Die gemene gemiddelde automatiese ZS was 18.96 µm (95% CI:15.75;22.16;n=625 positief ZS van 90 pasiënte). Uit die ZS blyk dit dat GLS linieêre regressie om te bevrug ZS 19.20µm (16.60;21.79) was, p=0.04. Daar was geen statistiese verskil tussen die ZS van die swanger en die nie swanger groepe. Die hoofdoel was om te bewys dat wanneer oösiet kwaliteit optimaal is dat bevrugting syfers en by implikasie swangerskap syfers verbeter kan word. Indien nie, sal onvermoë om te bevrug of te implanteer heel waarskynlik veroorsaak word deur die verminderde spermatozoa kapasiteit of vermoë om te bevrug as gevolg van beskadigde chromatien integriteit. Die chromatien integriteit (CMA_3) van die studie populasie was 74% semen monsters met >40% onvolwasse DNA. Die OR vir CMA_3 bevestig die hipotese dat wanneer oösiet doeltreffendheid vir bevrugting beheer word tot 'n mate, die sukses of mislukking van behandeling aangewys kan word deur die CMA_3 waarde. In hierdie geval, is die OR

hoogs voorspelbaar vir sukses met swangerskap wanneer ISSI uitgevoer word op CMA_3 waardes wat onvolwasse is (≥41%). Logistieke regressie bereken dat die OR vir immature DNA (CMA_3 ≥41%) om swangerskap te voorspel met die waarskynlikheid van 1.6:1,p=0.39. Hipotese OR: wanneer ISSI uitgevoer word op ‘n oösiet met: M2 en SPp, en die embrio ontwikkel tot dag 5 vir embrio terugplasing, sal die waarskynlikheid van swangerskap, wanneer die semen monster ‘n onvolwasse DNA CMA_3 waarde van >40% het, 1.9:1 wees.

Sleutelwoorde: Meiotiese spole, Zona Telling, CMA_3, ISSI, Infertiliteit

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"The Sound Of Silence"

Hello darkness, my old friend,
I've come to talk with you again,
Because a vision softly creeping,
Left its seeds while I was sleeping,
And the vision that was planted in my brain
Still remains
Within the sound of silence. Chapter 1

In restless dreams I walked alone
Narrow streets of cobblestone,
'Neath the halo of a street lamp,
I turned my collar to the cold and damp
When my eyes were stabbed by the flash of a neon light
That split the night
And touched the sound of silence. Chapter 2

And in the naked light I saw
Ten thousand people, maybe more.
People talking without speaking,
People hearing without listening,
People writing songs that voices never share
And no one dared
Disturb the sound of silence. Chapter 3

"Fools," said I, "You do not know.
Silence like a cancer grows.
Hear my words that I might teach you.
Take my arms that I might reach you."
But my words like silent raindrops fell
And echoed in the wells of silence Chapter 4

And the people bowed and prayed
To the neon god they made.
And the sign flashed out its warning
In the words that it was forming. Chapter 5
And the sign said, "The words of the prophets are written on the subway walls
And tenement halls
And whispered in the sounds of silence." Chapter 6

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List of Abbreviations

AMH	Anti Mullerian hormone
AO	Acridine Orange
AB	Aniline Blue
AR	Acrosome Reaction
ART	Assisted reproductive technology
BL	Blastocyst
CI	Confidence intervals
CMA_3	Chromomycin A_3
CM	Compacted morula
CO_2	Carbon Dioxide
COC	Cumulus-oocyte complex
COS	Controlled ovarian stimulation
CV	Coeffience of variance
E_2	Oestrogen/ oestradoil
EBL	Early blastocyst
ET	Embryo transfer
ExBL	Expanded blastocyst
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin releasing hormone
GnRHa	Gonadotropin releasing hormone agonist
GV	Germinal vesicle
hCG	Human chorionic gonadotropin
HZB	High zona birefringence
IUI	Intrauterine insemination
IVF	In-vitro fertilization
IVM	In-vitro maturation
ICSI	Intracytoplasmic sperm injection

LZB	Low zona birefringence
LH	Luteinizing Hormone
M	Morula
M1	Metaphase one
M2	Metaphase two
MI	Meiosis I
MII	Meiosis II
N	Nitrogen
O_2	Oxygen
OR	Odds ratio
PB	Polar body
PVS	Perivitelline space
ROS	Reactive oxygen species
SD	Standard deviation
SP	Meiotic spindle
SPa	Meiotic spindle absent
SPp	Meiotic spindle present
TVOH	Transvaginal oocyte harvest
WHO	World Health Organization
ZP	Zona pellucida
ZS	Zona score

Chapter 1: Introduction

1.1 Background

Assisted reproductive technology (ART) have evolved and improved dramatically since its inception in 1978 with the birth of Louise Brown, the first baby born to in vitro fertilization (IVF) due to the research of Edwards and Steptoe (Elder & Dale, 2012).

Many of the more recent techniques, such as intracytoplasmic sperm injection (ICSI), have greatly improved probability of pregnancy for couples suffering from severe male factor infertility, recurrent fertilization failure and where advanced age is a factor. ICSI improves success of fertilization by directly injecting a single spermatozoon into the cytoplasm of the oocyte, bypassing several natural barriers that may prevent delivery of the male nuclear content and thus ensures optimal facilitation of the fertilization process (Balaban et al., 1998; Cohen et al., 2004).

The requirements for the ICSI procedure would be a mature oocyte and a single, viable morphologically normal selected spermatozoon for injection. The success of the procedure, however, rests equally on the nuclear and cytoplasmic maturity of the oocyte as well as the morphology, viability and DNA competency of the spermatozoon together with the proficiency of the embryologist performing the microinjection procedure (Cohen et al., 2004).

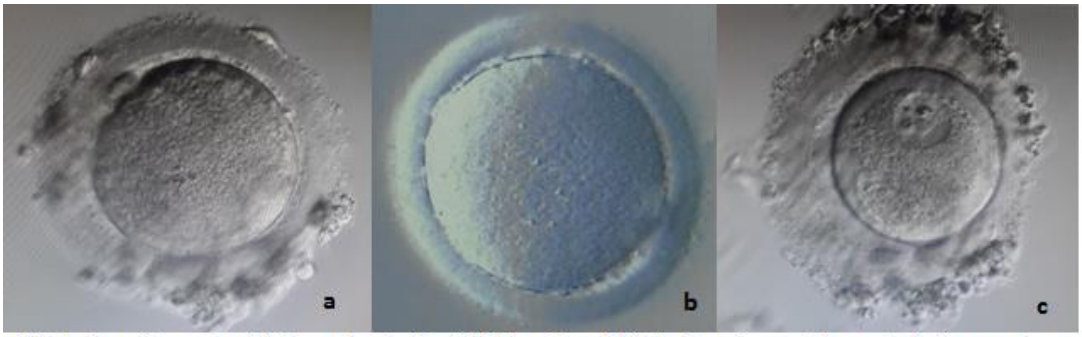
Presently in the conventional IVF setting, the criteria set for oocyte grading is only based on external appearance. Following oocyte retrieval, maturity is determined based on the expansion of the cumulus cells and corona radiata density surrounding the zona pellucida. Visualisation of maturity is achieved by conventional light microscopy using a stereomicroscope (Balaban et al., 1998; Rienzi et al., 2011).

A metaphase 2 (M2) oocyte is characterized by the well-expanded cumulus-corona radiata complex, which surrounds the aspirated oocyte, as observed by stereomicroscopy. A lesser-expanded cumulus-corona radiata complex is generally linked to immature

maturation of the oocyte during Prophase I of maturation. This method of oocyte nuclear assessment still remains subjective as it depends on the ability of the embryologist and his/her precision in identifying oocyte maturity (Balaban et al., 1998; Veeck L, 1999).

Preparation for ICSI results in the decumelation of the oocyte by use of hyaluronidase enzymes to rid the oocyte of the excess cumulus cells that surround it, enabling the embryologist to do a proper nuclear maturity assessment (method described in chapter 4).

Optimal nuclear maturity is defined as an oocyte in metaphase 2 (M2) of meiosis 2 and is characterised by the presence of the first polar body (PB) in the perivitelline space of the oocyte (figure 1.1 **a**) (Sopelak, 1997; Balaban et al., 1998; Cohen et al., 2004; Veeck, 1999). Following conventional ICSI methods, this mature oocyte can be inseminated with a normal spermatozoon from 1-5 hours post transvaginal oocyte harvest (TVOH) (Veeck, 1999). The metaphase 1 (M1) oocyte is usually characterised by the absence of both a first PB or the nucleus of the germinal vesicle (GV) as can be seen in figure 1.1 **b** (Sopelak, 1997; Veeck, 1999;Cohen et al., 2004). ICSI of the M1 oocyte commences 1-5 hours after it has reached the nuclear maturity state of M2 (PB extruded) (Veeck, 1999). The oocyte is in Prophase I if a prominent nucleus, the GV is present (figure 1.1 **c**), and is usually inseminated by microinjection 26-29 hours after TVOH if it has matured to M2 (Veeck L., 1999).



a) Metaphase 2 oocyte exhibiting polar body in 11h00 position, **b)** Metaphase 1 oocyte (no polar body or nucleus present), **c)** Germinal vesicle with prominent nucleus in the top centre half of the immature oocyte. (Images **a** & **c** courtesy of Wilgers Infertility Clinic Octax PolarAide imaging; Image **b** accessed from: http://www.advancedfertility.com/images/metaphase-1-oocyte.jpg; on 27-11-2015.

Figure 1.1 Assessment of oocyte nuclear maturity by stereomicroscope and light microscopy.

In the absence of concrete oocyte morphological indicators, other than M2 developmental maturity, all oocytes are presently subjected to insemination by ICSI, regardless of actual nuclear maturity (Rienzi et al., 2011).

In a recent meta-analysis, the risk of spontaneous abortion rates after IVF was compared to sperm DNA damage (Zini et al., 2008). Results showed that infertile men had higher levels of sperm DNA damage compared to fertile men, thereby increasing the incidence of spontaneous abortions during ART. This is of clinical relevance as nearly forty percent (40%) of men who seek assisted reproductive treatment suffer from male infertility (Agarwal & Allamaneni, 2004; Zini, et al., 2008).

Many studies conducted in recent years have focused on the basic semen analysis as a marker for male fertility (Jeyendran, 2000). The limitations of these studies are that semen quality as measured by macroscopical and microscopical parameters are not necessarily the only parameters associated with male fertility (Agarwal et al., 2009).

Many studies showed a correlation between fertilization failures and poorly packaged or damaged sperm chromatin as well as single or double stranded DNA damage (Zini & Libman, 2006). In fact, Agarwal & Allamaneni (2004) argues that spermatozoa DNA and chromatin integrity analysis has a greater prognostic and diagnostic value to infertility testing than conventional semen analysis (Zini & Libman, 2006). Spano et al. (2000) and Zini et al. (2001) have both reported that men with higher DNA damage have a low potential for natural fertility. Based on these findings, many scientists now emphasize the necessity of including sperm DNA analysis as a marker for DNA integrity as a part of the routine semen analysis.

1.2 Research problem

A common problem encountered during ART is the inability to predict the probability of success for patients enrolled in various fertility treatment cycles. Although certain techniques such as IVF and ICSI has increased chances by facilitating fertilization in the laboratory above simple sperm wash and intrauterine insemination (IUI), much still needs to

be learned on gamete failure, that is when fertilization/ and or pregnancy does not occur despite efforts made in the laboratory.

Scientists have raised much concern since the inception of regular use of ICSI, that DNA damaged spermatozoa may possibly be introduced to the ooplasm of the oocyte by sperm microinjection, since this procedure bypasses the natural selection process (Zini & Libman, 2006). In the setting of natural selection, or conventional IVF, the DNA damaged spermatozoon has been demonstrated to have much lower zona pellucida binding capacity than spermatozoa with low DNA damage (Ellington, et al., 1998; Esterhuizen, et al., 2000). In natural conception, it has also been reported that the spermatozoa with affinity to bind to the fallopian tube cells also have much lower DNA damage than the spermatozoa found not to bind (Ellington, et al., 1998; Zini & Libman, 2006;).

1.2.1 Hypothesis

If oocyte criteria* for fertilization are met, fertilization failure following ICSI of a morphologically normal spermatozoon, is due to lack of sperm DNA decondensation in the ooplasm of the oocyte.

*Criteria for successful fertilization of an oocyte was interpreted as nuclear development of a M2 oocyte exhibiting a polar body and a meiotic spindle (SP), before receiving a single-morphological-normal sperm through ICSI.

1.3 Research objectives

- Determine oocyte viability for fertilization: Maturity (M2), SP present, and zona score (ZS).
- Determine if a correlation exists between immature DNA of the spermatozoa in a semen sample and fertilization, when all other factors concerning the oocyte and sperm morphology are controlled for.

Determine if a correlation exists between immature DNA of the spermatozoon and pregnancy rate (when all factors regarding oocyte competency and sperm morphology is controlled).

1.4 Research feasibility, impact, and potential outputs

The feasibility of the research and its impact rests largely on controlling the factors concerning the oocyte. When the oocyte factors are controlled for, it will provide a better indication of fertilization potential of spermatozoa from a semen sample based on the immature DNA value since only morphologically normal sperm are selected for the ICSI procedure.

This is a much more reliable method of determining gamete failure since most studies have not been able to control for female factor other than age and M2 development stage. The incorporation of the presence of a meiotic spindle together with ZS sets this study apart from previous studies relating to fertilization potential of the spermatozoon. In this setting, the oocyte, which is a huge contributor towards fertilization potential, is controlled for, therefore should fertilization still fail; it is most possibly due to a sperm factor.

Potential outputs of this study will most certainly be a better predictive tool for successful ICSI based on a test for sperm immaturity.

1.5 Brief chapter overview

Chapter 2 – The Oocyte as Gamete

This chapter will review the current knowledge and information available on oocytes in the ART setting; from mitosis and meiosis to the data and success rates of critical structures present in the oocyte implicated for fertilization, embryo development, and implantation.

Chapter 3 – The Spermatozoon as Gamete

Chapter 3 will discuss the spermatozoon as a specialized structure with fertilization of an oocyte as its only mission. The importance of chromatin packaging and specific fertilisation potential of the spermatozoon will be highlighted with reference to successful fertilization of the oocyte with ICSI, embryo development, and implantation of the embryo.

Chapter 4 – Research Method

This chapter will as briefly as possible, whilst being as complete as possible, describe the methods and statistical interventions that will take place during the study.

Chapter 5 – Results

Chapter 5 will comprise of all the results achieved during the study pertaining to the research problem, questions and aims and objectives.

Chapter 6 – Discussion, Limitations and Future Research

The results obtained from chapter 5 will be discussed with specific relevance to the research hypothesis, aims and objectives in conjunction to the literature review and current research papers available on the topics. The possible shortfalls of the study method will be discussed and opportunities for future research of the subject will be highlighted.

Chapter 2: The Oocyte as Gamete

"*Embryogenesis begins during oogenesis"* – E.B. Wilson

2.1 Cell Biology

The biogenic law; "*Omnis cellulae e celula";* all living cells arise from pre-existing cells by Rudolf Virchow, 1855; has been a great inspiration to the "cell theory" known to biologists and scientists today (Elder & Dale, 2012).

The first principles emphasize that all living things consists of cells which in turn, is the organizational and operating component of all living things. Secondly, all cells are derived from mitosis and meiosis (only gametes) of pre-existent cells; during which process hereditary information is transferred from cell to cell (Sopelak, 1997; Elder & Dale, 2011).

2.1.1 Mitosis and Meiosis

The oocyte, with a diameter of 120µm, is the single, largest cell in the body. All living organisms rely on duplication of individual cells for reproduction and growth (Elder & Dale, 2011). Mitosis and meiosis are the two methods by which cells divide. Mitosis is the exact genetic replication of a cell (2n) and occurs in all somatic cells. Meiosis is the process of cell DNA reduction, from diploid (2n) to haploid (n) to facilitate the process of fertilization when genetic material from each parent is introduced to produce a diploid zygote (Sopelak, 1997; Saladin, 2007).

During week 10 of embryonic development, the oogonium is formed by means of germ cell mitosis and maturation. The continued mitosis of the germ cells persists until week twenty to twenty-five and a number six to seven million before they enter a state of arrested development during prophase I of the first meiotic division just before birth (Sopelak, 1997; Veeck, 1999). The characteristic of an immature oocyte, an oocyte that has not yet completed meiosis I, is a prominent nucleus, also known as the GV (Veeck, 1999).

Meiosis begins during fetal life and is characterized by chromosome conjugation and two consecutive cell divisions in which the diploid chromosome number (2n) is decreased to the haploid state (n). Meiosis mixes the parent homologous chromosomes to create new gene

combinations through a process called crossing over or the Holliday junction (named after Robin Holliday who first introduced the idea).

Meiosis consists of two phases, Meiosis I (MI) and Meiosis II (MII) and in the case of the female results in one haploid oocyte and two or three polar bodies (Veeck, 1999; Saladin, 2007; Elder & Dale, 2011).

At the time of birth, all germ cells are oocytes (GV's) and the maturation of the follicles remain in the arrested prophase I state until puberty and the increased secretions of gonadotropins from the hypothalamus prior to ovulation causes meiosis to resume (Sopelak, 1997; Veeck, 1999). MII is finally completed at the time of fertilization. Table 2.1 gives a brief summary of the processes occurring during MI & MII.

It is important to note that although the male's germ cells can continue to divide mitotically throughout his adult life, the female is born with a fixed number of oocytes which from puberty declines until the commence of menopause which signals the end of female fertility (Saladin, 2007).

Table 2.1. Phases and Process of Meiosis (Sopelak, 1997; Veeck, 1999).

MEIOSIS I	
Prophase I	
1. Leptotene	Condensation of the nuclear chromatin, the ends of both the chromosomes is attached to the nuclear envelope.
2. Zygotene	Synapse of side-by-side aligned homologous chromosomes for the exchange of genetic material; stops when all homologs have been paired.
3. Pachytene	Bivalents (chromosome threads) shorten and intertwine to form a tetrad. The Holliday juncture (crossing over) occurs with genetic exchange between non-sister chromatids of paired homologous chromosomes.
4. Diplotene	Paired bivalent chromosomes begin to repel each other except on the areas where Holliday junction has occurred. MEIOSIS I is halted before diakinesis until puberty/ovulation. The primary oocyte is characterized by the morphological appearance of a GV.
5. Diakinesis	Commences 36-48 hours before ovulation and entails the disappearing of the nucleus and the nuclear envelope as well as the formation of the spindle fibers. The primary oocyte undergoes dramatic growth due to increased GnRH secretion from the hypothalamus resulting in a sharp increase in FSH and LH secretions.
Metaphase I	Formation of the spindle followed by the chromosomes lining up on the equatorial plane.
Anaphase I	The spindle rotates perpendicular to the oocyte surface thus the entire chromosomes, with their centromeres intact, is pulled to opposite poles of the cell.
Telophase I	Cell division occurs resulting in the separation of the haploid set of chromosomes and yields one secondary oocyte and one polar body. The nuclear envelope is restored and the chromosome remains attached to it until MII.
MEIOSIS II	
Prophase II	No replication of DNA, thus there are no synapses. The chromosomes remain in a condensed form and reassemble on the equatorial plane.
Metaphase II	The nuclear envelope disappears and the spindle is reformed. Spindle fibers attach to the centromeres of each of the two chromatids of the 23 haploid chromosomes. Meiosis is again suspended until after fertilization or environmental activation.
Anaphase II	Only commences after stimulation in the form of sperm penetration (fertilization), chemical/environmental exposure or electrical trauma and results in the splitting of the chromatids at the centromeres and pulling to the opposite poles.
Telophase II	Cell division occurs yielding an activated oocyte (due to fertilization) that keeps the greater part of the cytoplasm and a second polar body.

2.1.2. Structural Development of the Primordial Follicle

The primordial follicle is located in the cortex of the ovary and connects the oolemma of the primary oocyte with cytoplasmic protrusions to a surrounding layer of granulosa cells and separated only by a basement membrane from the surrounding stroma. This is called the cumulus-oocyte complex (COC) and allows for chemical signaling between cells (Sopelak, 1997; Saladin, 2007).

The follicular phase of the ovarian cycle can be divided into two phases, the preantral (before=primary) and antral phases (after the follicle forms the cavity=secondary). The preantral phase of a follicle initiates with the primary oocyte in the primordial follicle during the first 12 – 16 weeks of gestation (Sopelak, 1997). Figure 2.1 effectively illustrates the development of the oocyte, from its location in the cortex of the ovary, through each step of maturation (mitosis & meiosis).

2.1.3. The Primary Follicle

Maturation of the primary follicle occurs as early as week 21 of gestation when the granulosa cells differentiate into cuboidal cells (Sopelak, 1997; Saladin, 2007). The primary follicle consists of multiple layers of cells due to proliferation of the granulosa cells that cause the enlargement of the follicle. This growth also sees the enlargement of the oocyte's cytoplasm from 25 to 80 µm, formation of microvilli on the oocyte surface and differentiation and dispersal of cytoplasmic organelles. This growth occurs during the Diplotene phase of Prophase I of Meiosis I.

The end of the prenatal follicular development is marked by the condensed connective tissue surrounding the granulosa cells forming the theca folliculi. Before puberty commences, some of the primordial follicles develop into secondary (antral) follicles (fig 2.2), while the rest undergo atresia (cell death) (Saladin, 2007). The preceding phases occur without pituitary gonadotropin stimulation, but further development depends upon sexual maturity and requires the maturation of the hypothalamic-pituitary-ovarian axis and stimulation of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (Sopelak, 1997).

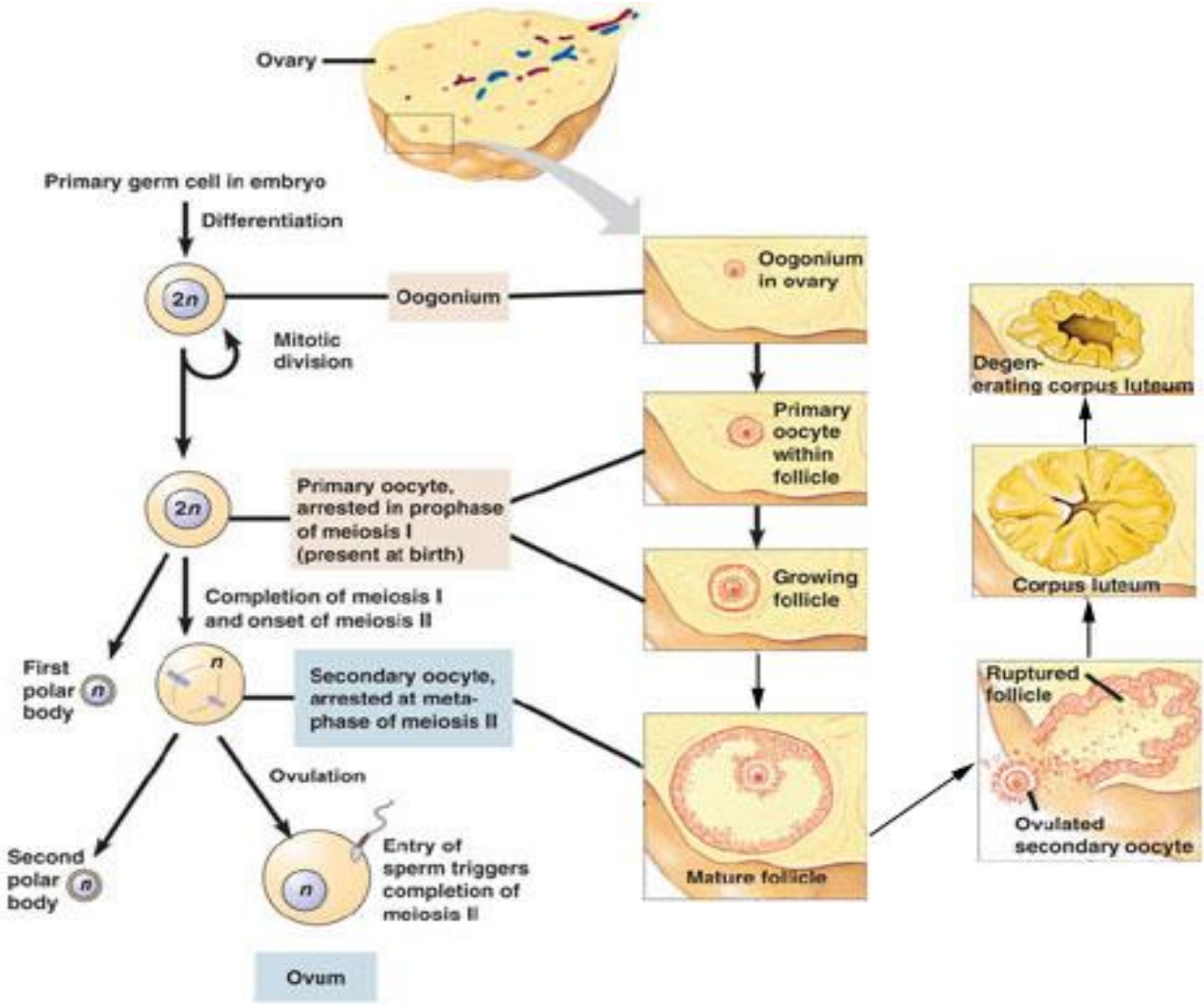


Figure 2.1 Schematic representation of oocyte maturation involving meiosis.

Available from: www.bio.miami.edu/.../c7.46.11.oogenesis.jpg.

2.1.4. The Secondary Follicle

The function of the granulosa cells of the secondary follicle is to secrete follicular fluid in pools between cells. These unite to form a fluid-filled antrum (cavity). The structure of the oocyte is surrounded by a double layer of granulosa cells. From figure 2.2, we can clearly define the structures and changes that the secondary follicle and the secondary oocyte must undergo in the development cycle. The outermost, called the cumulus oophorus, covers the oocyte, and embeds it on the one side to the follicle wall, while the innermost, called the corona radiata surrounds the zona pellucida (ZP). The theca folliculi also continues to multiply

and forms a theca externa and a theca interna. The theca externa is the fibrous outer capsule while the theca interna consists of hormone-secreting cells, which provide the granulosa cells with androgens, which they convert to oestradiol (Sopelak, 1997; Saladin, 2007).

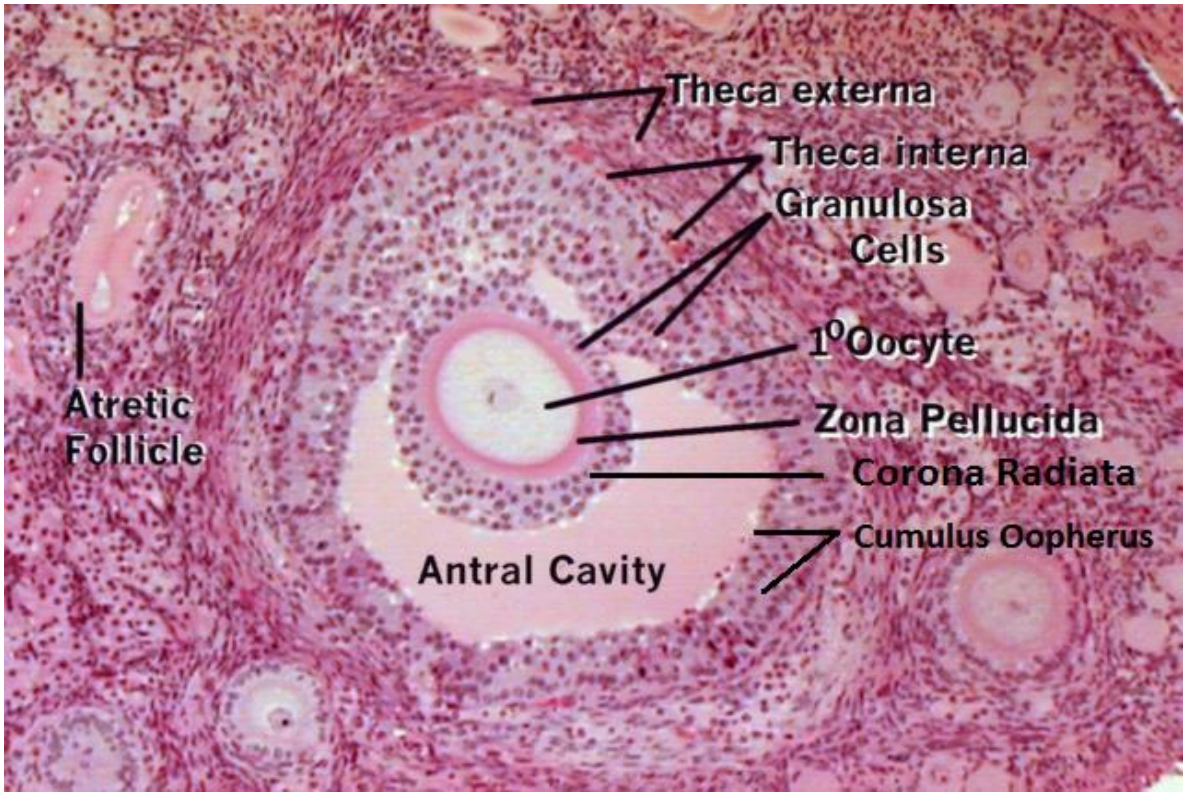


Figure 2.2 Histological illustration of the secondary oocyte, displaying the various granulosa layers and a follicle in atresia. Illustration accessed from www.stlawu.edu/~mtem/devbiol/atlas/ANTRAL1.JPG. Accessed on: 20/08/2009. Image altered 24/01/2016 (added corona radiata and cumulus oopherus).

Various neurotransmitters, e.g. dopamine, stimulate the hypothalamus to produce and release Gonadotropin Releasing Hormone (GnRH). The GnRH stimulates the anterior pituitary to release FSH and LH into the bloodstream which reaches the ovaries via the hypothalamic-pituitary-ovarian axis where it stimulates the development of the secondary follicle into the Graafian follicle (Steinmann, 2008).LH specifically stimulates the theca interna cells to produce androstenedione, which is converted to oestradiol (E_2) in the granulosa cells (the enzymes needed for this reaction is activated by FSH). E_2 is released into the follicular fluid and the bloodstream. The gradual increase of E_2 in the blood plasma leads to decreased FSH secretion via negative feedback on the higher brain center (Steinmann, 2008).

The granulosa cells of the Graafian follicle also produce and secrete the polypeptide hormone, Inhibin, which inhibits the release of GnRH and FSH. The ever-increasing oestradiol, however, reaches a critical concentration, thus again stimulating GnRH release (positive feedback) which stimulates the anterior pituitary to secrete LH (LH surge figure 2.3) and FSH (Sopelak, 1997; Saladin, 2007; Steinmann, 2008). At the beginning of each cycle ± 10-20 follicles are recruited and develop under the influence of FSH, but only one of these follicles becomes dominant (possibly due to its large number of FSH receptors and rich blood supply). E_2 stimulates FSH receptor synthesis, thus FSH tends to bind to the follicle that synthesis the most E_2. This follicle develops further while the rest undergo atresia. The primary oocyte now completes MI and begins MII, halting the maturation process in M2 shortly before ovulation. MII will only be completed once fertilization has occurred (Steinmann, 2008).

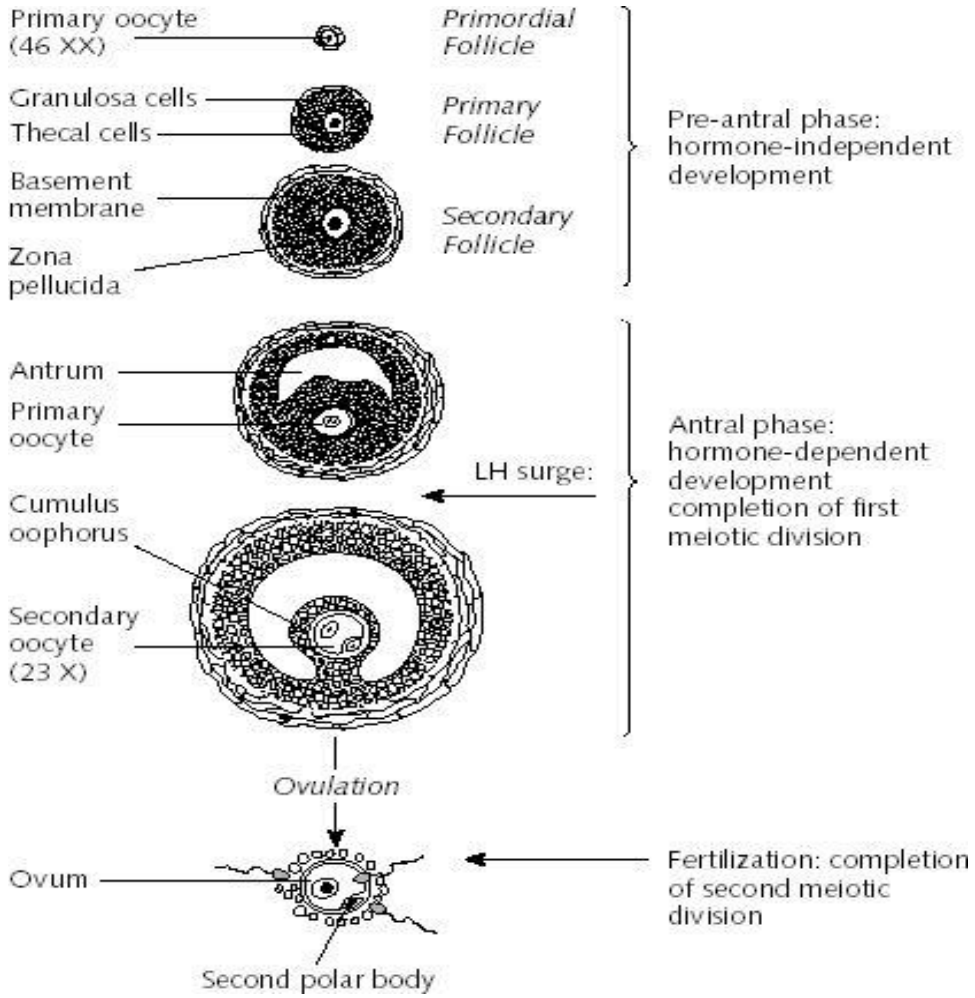


Figure 2.3 Schematic summaries of the cellular and hormonal changes during ovarian development. Available from: content.answers.com/.../019852403x.ova.1.jpg

2.1.5 Ovulatory Phase

Ovulation occurs ± 12 hours after the LH peak and 1-2 days after the E_2 secretion peaks. The follicle has a distinct fluid-filled antrum surrounded by numerous layers of granulosa cells (Saladin, 2007; Steinmann, 2008). The precise mechanism by which follicles rupture is unknown, but it is speculated that the increase in the follicular fluid volume within the follicle together with the increase in the prostaglandin concentration (Rondell, 1974) in the follicular fluid, and a possible role by a proteolytic enzyme, plasmin, may result in the disintegration of the follicle wall. Prostaglandins also stimulate the smooth muscle cells in the ovaries to increase progesterone secretions, promoting oocyte release (Rondell, 1974; Steinmann, 2008).

2.2 Critical structures and oocyte morphology implicated in fertilization, embryo development, and implantation.

2.2.1 Cumulus-oocyte complex

The granulosa cells surrounding the oocyte make it difficult to assess oocyte maturity but it is often the only method of oocyte morphology assessment when IVF is performed (Balaban et al., 1998; Veeck, 1999; Rienzi et al., 2011). Traditionally, oocyte maturation of MII is characterized by the well-expanded COC, which surrounds the aspirated oocyte, and can be observed under the microscope. A lesser-expanded COC is generally linked to immature maturation of the oocyte during Prophase I of maturation (Veeck, 1999; Ebner et al., 2003;). This method is subjective to the embryologist's precision in identifying oocyte maturity (Veeck, 1999; Ebner et al., 2003; Assidi, et al., 2011).

Oocytes are chemically and mechanically prepared for ICSI, using hydrolytic enzymes, such as bovine hyularonidase and glass drawn pipettes to remove the surrounding cumulus cells (Ebner et al., 2003). Decumelation of the oocyte, allows the embryologist to determine the presence or absence of a polar body or GV to determine the nuclear maturity of the oocyte, as can be seen in figure 2.4 (Veeck, 1999).

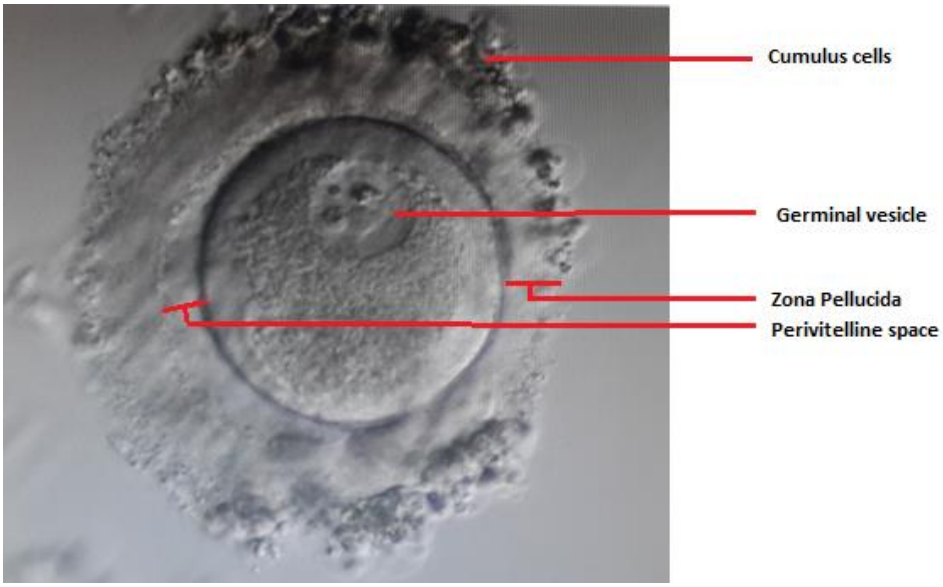


Figure 2.4 A germinal vesicle, after hydrolytic enzymatic removal of most cumulus cells. The large nucleus is clearly visible, as well as the structure of the zona pellucida and some cumulus cells left on the outside rim. (Image courtesy of Wilgers Infertility Clinic, Octax PolarAide imaging).

The COC are the immediate cells surrounding the maturing oocyte and are implicated in processes concerning the oocyte maturation, competence acquisition, ovulation, and fertilization of the oocyte (Assidi et al., 2011). The follicular environment in which the oocyte is recruited is very important to its ability to give rise to a potential viable embryo for implantation (Assidi et al., 2011).

Studies conducted on the potential of COC grading with relation to fertilization rates, embryo development and implantation rates seemed inconsistent and varying in scoring criteria (Ebner et al., 2003; Assidi, et al., 2011; Rienzi et al., 2011). The results seem to point to COC scoring as insignificant to the viability of the embryo (Assidi, et al., 2011; Rienzi et al., 2011). COC scoring is therefore deemed helpful in assessing oocyte maturity only. However, the effect of controlled ovarian hyper stimulation and exogenous administration of human chorionic gonadotropin hormone (hCG), often matures cumulus cells inconsistently to oocyte nuclear maturity in patients where E_2 levels drop or plateau (Ebner, Moser, M, Sommergruber, M, & Tews, G, 2003) resulting in well dispersed cumulus expansion with an immature oocyte.

2.2.2 Zona pellucida

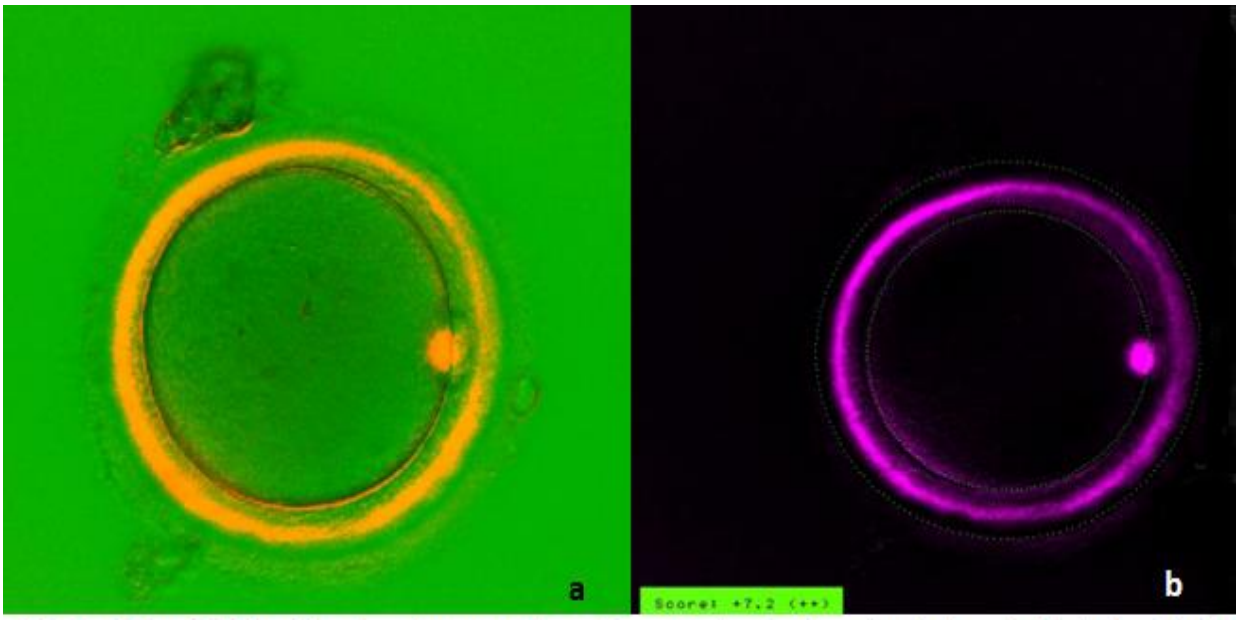
The ZP is a multilaminar glycoprotein gel that encapsulates the oocyte. The ZP consists of up to four zona proteins (Hughes & Barratt, 1999; Lefievre, et al., 2004; Ebner, et al., 2010), a dynamic, three-dimensional microfilament matrix arranged in diverging directions (Liu, et al., 2003; Braga, et al., 2010). The organizational arrangement of the ZP is a network of zona units 2 and 3 in repetitive sequence intertwined with zona protein 1 (Wassarman, 1988; Ebner, et al., 2010). The zona and granulosa cells are in continuous contact, via gap junctions, to provide nutrients to the developing oocyte (Sopelak, 1997; Liu, et al., 2003; Elder & Dale, 2011).

Novel techniques in microscopy have in recent years allowed for visualization of both the ZP as well as the presence or absence of the meiotic spindle without damaging oocytes by fixing and staining them for immunofluorescence or electron microscopy (Wang & Keefe, 2002; Madaschi, et al., 2008). This new light technology allows polarized light in association with specialized software (OCTAX PolarAIDE; OCTAX Microscience GmbH, Germany) to help improve oocyte-grading criteria with non-invasive techniques. This technology works on the basis of birefringence which in principle reveals critical structures in the oocyte by illuminating the structures very brightly through unique contrast imaging microscopy (Montag, et al., 2006; Madaschi, et al., 2008)

Complex molecules such as cell membranes, microfilaments and –tubules or structures of the cytoskeleton are highly ordered and therefore birefringence imaging can be applied as these structures alter the state of polarized light as it passes through the oocyte (Montag, Schimming, & Van Der Ven, 2006). Specific birefringent structures, such as the meiotic spindle and ZP can be seen clearly with these optics (fig 2.5) in contrast to the normal Hoffman Modulation Contrast (HMC) imaging where meiotic spindles, for example cannot be seen (Kilani et al., 2010).

The birefringence of the structures revealed can also be quantified due to the ability of the software to compute the degree of birefringence per pixel as measurements, yielding information directly relating to the level of molecular organization of these structures (Keefe *et al*., 2003; Heindryckx, et al., 2011).

The meta-analysis performed by Rienzi et al. (2011) supported the use of ZP thickness to assess embryo viability. Thinner ZP's were linked to increased fertilization rates and low birefringence of the ZP to higher miscarriage rates. A thicker ZP with high birefringence resulted in higher number of blastocysts, better embryo development and increased pregnancy rates. ZP's that had a variation in birefringence also tended to have greater embryo development (Balaban et al., 1998; Rienzi et al., 2008; Rienzi et al., 2011).



a) Octax PolarAide imaging showing metaphase 2 oocyte in telophase (meiotic spindle is the bright orange structure inside the polar body), **b)** Metaphase 2 oocyte with bright purple automatic zona score indicated in green box (Octax PolarAide software generated)

Figure 2.5 Oocyte illustrating birefringence of the meiotic spindle (in telophase I) and zona pellucida. (Available from: Wilgers Infertility Clinic, South Africa, using OCTAX polarAIDE Imaging, Germany).

2.2.3 Perivitelline space

The term perivitelline space (PVS) is designated to the space between the trilaminar layer of the ZP and the oolemma (cytoplasm) of the oocyte. Normal morphology of the PVS would include an equal spacing between the ZP and the circular oocyte without any debris or other foreign materials (Veeck, 1999).

The review compiled by Rienzi et al. (2011) compared eight publications on the single and / or combined effect of the size of, and debris present in the PVS. Mostly, no correlation was found between size of the PVS and fertilization or embryo development (De Sutter et al., 1996; Xia, 1997; Balaban et al., 2008; Valeri, et al., 2011) whilst Rienzi, et al. (2008) found that an increased PVS was associated with lower fertilization, however, overall embryo quality and pregnancy could not be found to be affected by PVS (Chamayou et al., 2006).

2.2.4 Morphology of the first PB

During telophase I of MI, cell division occurs that results in the separation of the haploid set of chromosomes yielding one secondary oocyte and one PB (Sopelak, 1997; Veeck, 1999).

Higher fertilization rates and improved embryo quality was reported by one study when oocytes for ICSI were selected according to the first PB morphology (Xia, 1997). PB morphology assessment was based on the size, or irregular shape, and/or fragmentation of the first PB (Xia, 1997; Ebner, et al., 2000). Other studies reported the PB to be a poor indicator of fertilization, embryo development and implantation/ pregnancy rates (Balaban, et al., 1998; De Santis, et al., 2005; Khalili, et al., 2005; Chamayou, et al., 2006).

2.2.5 Shape of the oocyte

Physiological factors, such as age, and the immediate vicinity of the oocyte have been reported to have a distinct effect on the shape of the oocyte (Valeri, Pappalardo, De Felici, & Manna, 2011). Authors have also associated diminished oocyte quality to apoptosis and a decrease in oocyte size (Andrux & Ellis, 2008). The specific shape of the oocyte did not prove a predictor of fertilization, embryo development, and pregnancy in any of the 9 studies part of the systematic review conducted by Rienzi et al. (2011).

2.2.6 Oocyte ooplasm (cytoplasm) appearance

Oocyte granulation (figure 2.6), homogenous and heterogeneous appearance, presence of vacuolation, cytoplasmic inclusions and / or location of granulation were found to have some effect, singular and as combination, on fertilization; embryo development; pregnancy rates and possibly miscarriage rates, however, it was not significant (Rienzi, et al., 2008; Rienzi, et al., 2011).

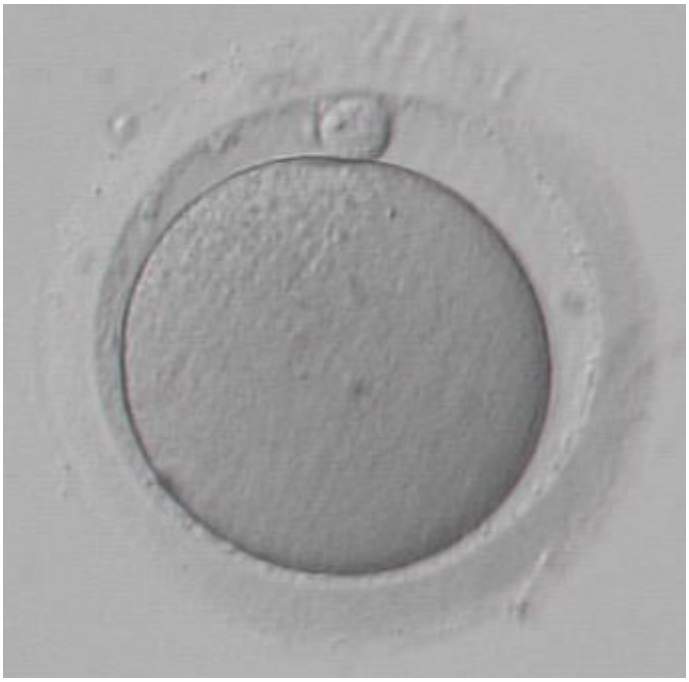


Figure 2.6 A metaphase 2 oocyte with a slightly granular ooplasm. (Image courtesy of, OCTAX polarAIDE Imaging, test images; Germany).

2.2.7 Presence and morphology of meiotic spindle

The meiotic spindle (SP) is a highly dynamic structure, formed during diakinesis of MI, consisting of barrel-shaped microtubules associated with chromosomal movement during meiosis (Cohen, et al., 2004; Montag, et al., 2006; Madaschi, et al., 2008).

The most prominent role of the SP would be its role in fertilization: chromosome separation and yielding a fertilized, activated oocyte with a second polar body (Sopelak, 1997; Cohen, et al., 2004; Braga, et al., 2008; Madaschi, et al., 2008). The SP is also influential in the early embryogenesis (Madaschi, et al., 2008; Assidi, et al., 2011) which emphasises its importance as a possible marker for fertilization and implantation potential (Cohen, et al., 2004; Montag, et al., 2006).

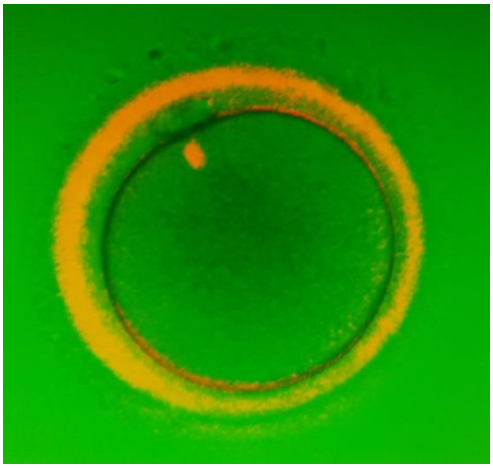


Figure 2.7 M2 oocyte illustrating the meiotic spindle perpendicular to the polar body. The ZP has a good birefringnece, as can be seen. (Image courtesy of: Wilgers Infertility Clinic, utelizing OCTAX polarAIDE Imaging, Germany).

Rienzi et al. (2011) reviewed fifteen papers on the effect of the absence, presence and morphology of the meiotic spindle on fertilisation (see figure 2.7), embryo development and pregnancy. Only one paper did not find proof of the importance of the presence of the meiotic spindle (Chamayou, et al., 2006). Several studies compared the presence of the spindle to its morphology and location vs. the location of the first polar body. Conflicting results were reported in this regard. In most cases, the presence of the meiotic spindle was associated with higher fertilization and cleavage rates; some reported that the presence of the spindle alone improved fertilization rates and pregnancy outcomes while others did not note any significant changes.

Chapter 3: The Spermatozoon as Gamete

3.1 Semen Analysis

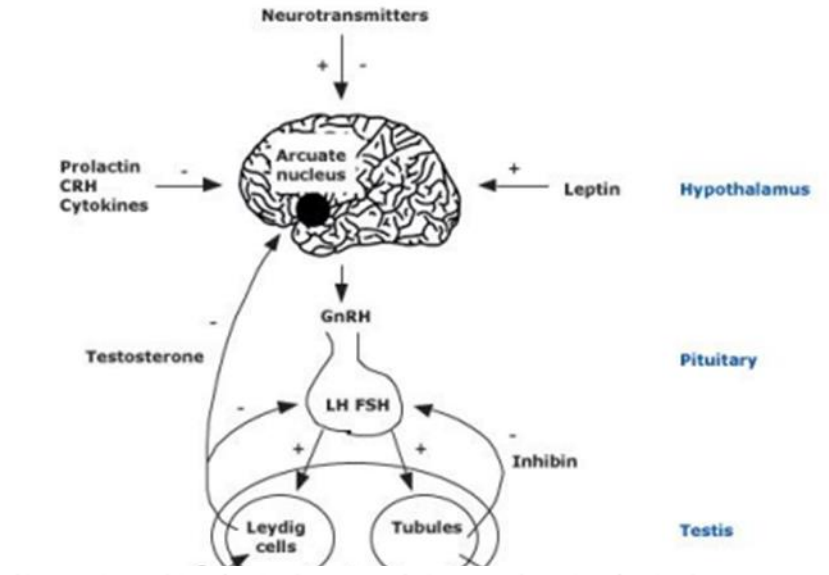
Male fertility is usually measured according to the number of spermatozoa (per milliliter), as well as sperm motility, sperm morphology, semen pH and semen volume (Jeyendran, 2000; WHO, 2010). Interpretative semen analysis has at its goal to assess the specific fertilization potential of a male's semen sample with the option of adding other specialized tests, such as DNA fragmentation, reactive oxygen species (ROS) investigations and acrosome reaction (AR), if necessary (Jeyendran, 2000; Zini & Libman, 2006; du Plessis, et al., 2015). Many studies conducted in past have focused on basic semen analysis as a marker for male fertility. The limitations of these studies are that semen quality is not necessarily associated with male fertility. There is a difference in semen quality and specific fertility potential of spermatozoa, as can be measured with sperm chromatin packaging (Agarwal et al., 2009). Tests of sperm nuclear chromatin or DNA structure are specialized tests that may provide additional information to semen analysis in the assessment of male infertility and could be more appropriate to possibly predict success of ART (Bungum, et al., 2004; Alvarez, 2005; Spano, et al., 2005; O'Brien & Zini, 2005; Benchaib et al., 2007; Griffin et al., 2015).

The World Health Organization (WHO) amended its Laboratory manual for human semen and sperm-cervical mucous interaction (1999, 4th edition) with a brand new manual in 2010 titled: Laboratory Manual for the Examination and Processing of Human Semen. The 5th edition described more concise methods for worldwide standardization of human sperm diagnostics and processing in laboratories. With this new edition, came new and much lower reference values regarding sperm parameters (table 3.1). The new values stemmed from large studies conducted in laboratories across the world, and included 1900 men whose partners were pregnant within 12 months of unprotected intercourse (Cooper et al., 2010).

Table 3.1 Revised World Health Organization lower reference values for semen and sperm analysis.

Parameters	Units	95% Confidence Intervals
Volume	1.5ml	1.4 -1.7ml
Sperm concentration	15 x10^6/ml	12 – 16x10^6/ml
Total number of sperm	39x10^6/ejaculate	33 – 46x10^6/ejaculate
Morphology	4% normal forms	3 – 4% normal forms Tygerberg Strict Criteria
Vitality	58% live	55 – 63 % live sperm
Progressive motility	32% motile	31 – 34% motile sperm
Total motility (progressive + non progressive)	40%	38 – 42%

3.2 The hypothalamic-pituitary-testicular axis



CRH: corticotrophin-releasing hormone; GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone.

Figure 3.1 Schematic representation of the hypothalamic-pituitary-testicular axis. (Image taken from: Griffin JE, Wilson JD. In: Williams Textbook of Endocrinology, 7th ed, Wilson, JD, Foster DW (Eds), WB Saunders, Philadelphia, 1985, p. 802).

Hormones, neurotransmitters, cytokines, and Kisspeptin regulate the hypothalamic secretion of gonadotropin-releasing hormone (GnRH) in a pulsatile fashion to stimulate the anterior pituitary release of LH and FSH which in turn act on the testes (figure 3.1) (Gnessi et al., 1997; Krsmanovic et al., 2009; Griffin et al., 2015).

In the testes, spermatogenesis is controlled by two hormones, FSH (FSH receptors are located in Sertoli cells and spermatogonia in the seminiferous tubules) and testosterone (produced in the Leydig cells in the interstitium, under influence of LH). Androgen receptors are located in the Sertoli, Leydig, and Myoid cells (figure 3.2) (DeKrestser et al., 1996; Griffin et al., 2015).

FSH stimulates the Sertoli cells to increase secretions of androgen-binding protein, transferrin, inhibin B, aromatase enzyme (CYP19), and plasminogen activator; as well as transporting glucose to the Sertoli cells and converting glucose to lactate. LH influences the Leydig cells to produce testosterone, 100 times more than the circulatory concentration (DeKrestser et al., 1996; Lie et al., 2009; Griffin et al., 2015).

As the serum testosterone levels rise above physiological levels necessary for spermatogenesis, the serum testosterone triggers negative feedback to the anterior pituitary as well as the hypothalamus (CYP19 converts testosterone to estradoil; estradoil is the mechanism which inhibits the hypothalamus secretions of GnRH), to decrease LH secretions. In the same manner, the increase in testosterone also triggers negative feedback in the seminiferous tubules by increasing Inhibin B secretion which can act on the anterior pituitary to decrease FSH secretion (Santen, 1975; Means et al., 1976; Wahlstrom et al., 1983; Griffin & Wilson, 1985; Morishima et al., 1995; DeKrestser et al., 1996; Hayes et al., 2000; Hayes et al., 2001; Krsmanovic et al., 2009;).

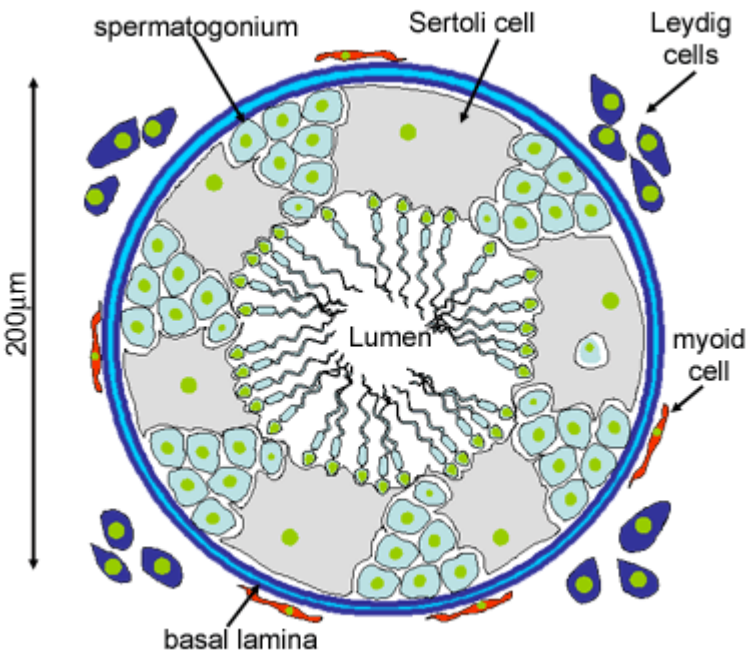


Figure 3.2 Representation of the Sertoli, Leydig, and Myoid cells involved in spermatogenesis in the seminiferous tubules. (Image taken from: http://www.histology.leeds.ac.uk/male/assets/tubule.gif, Accessed on: 26/11/2015).

3.3 Spermatogenesis

Spermatogenesis is defined as the process by which a spermatogonial stem cell gives rise to a spermatozoon. Spermatogenesis can be divided into 3 phases: mitosis, meiosis (1 and 2) and maturation.

During fetal and post-natal development, the primordial germ cells migrate to the urogenital ridge, the testes differentiate, and germ cells become distributed within the sex cords (Sopelak 1997; Veeck 1999). Following limited mitotic proliferation, primitive germ cells arrange in pairs near the periphery, interspersed among Sertoli and Leydig cells (figure 3.2), and are now called prespermatogonia (figure 3.3). The prespermatogonia resemble adult stem cells.

Prior to the onset of puberty and spermatogenesis, prespermatogonia undergo nuclear condensation, nucleolar enlargement, and cytoplasmic volume reduction yielding formation

of the pale (type A), or dark (type A) and type B spermatogonia (Sopelak, 1997; Veeck, 1999; Peckham, online). These spermatogonia are arranged along the basal lamina of the seminiferous tubules (Veeck, 1999; Peckham, Online). At puberty, the number of spermatogonia per testis is 6 x 10^8.

During the mitotic phase, multiplication of the spermatogonia occurs which leads to the formation of spermatocytes (diploid=2n) while maintaining their number by renewal (every spermatogonium undergoing differentiation gives rise to 16 primary spermatocytes) (Johnson et al., 1983). Differentiation of the spermatogonia occurs in the seminiferous tubules in an organized fashion.

Each dark spermatogonium (type A) differentiates into two additional dark type A spermatogonia, each of which differentiates into two pale type A spermatogonia. The pale type A spermatogonia divides further to form two type B spermatogonia, which are located either at the tubular periphery or adjacent to the type A cells. Each type B spermatogonium undergoes differentiation yielding two primary spermatocytes (see figure 3.3). Each day, approximately 1.5-3 million spermatogonia begin this differentiation process (Griffin et al., 2015).

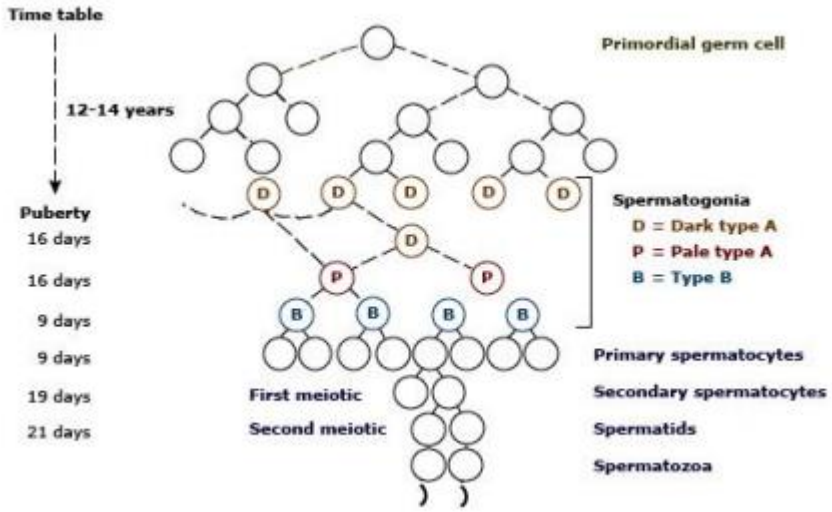


Figure 3.3 Time-line schematic representation of spermatogenesis. (Image taken from: Griffin JE, Wilson JD. In: Williams Textbook of Endocrinology, 7th ed, Wilson, JD, Foster DW (Eds), WB Saunders, Philadelphia, 1985, p. 810).

After mitosis, each primary spermatocyte undergoes two meiotic divisions resulting in the formation of four haploid (n) spermatids; two with 22 autosomes and a ‘x’ sex chromosome, and two with 22 autosomes and a ‘y’ sex chromosome.

Meiosis 1

The duration of meiosis 1 is 19 days. The process of prophase 1 of meiosis 1 involves the primary spermatocyte to increase in cellular volume (enlarge). The synthesis of DNA and RNA becomes apparent as unique membrane proteins appear. Finally, the Golgi apparatus expands as the nucleus completes the metaphase, anaphase, and telophase of meiosis 1 to yield 2 secondary spermatocytes. (Johnson et al., 1983; Sopelak, 1997; Veeck, 1999).

Meiosis 2

The secondary spermatocyte enters the 2nd meiotic division and results in the formation of 2 haploid spermatids as can be seen in figure 3.4 (Johnson et al., 1983; Sopelak, 1997; Veeck, 1999).

When meiosis 1 and 2 are complete, the maturation phase, spermiogenesis, can commence. During this phase, the spermatid undergoes a series of metamorphic changes resulting in a highly differentiated spermatozoon. Changes that the spermatid undergoes during spermiogenesis include the expansion of the acrosome, and the condensation of chromatin and eccentric nucleus, (*histone replacement by protamine) (Griffin et al., 2015; Lie et al., 2009). The sperm cell elongates and the axial filament is formed, during which process the mitochondria (necessary for movement) becomes more associated with the proximal portion of the tail, and all excess cytoplasm is separated from the cell (Johnson et al., 1983;; Sopelak, 1997; Veeck, 1999; Lie et al., 2009; Griffin et al., 2015).

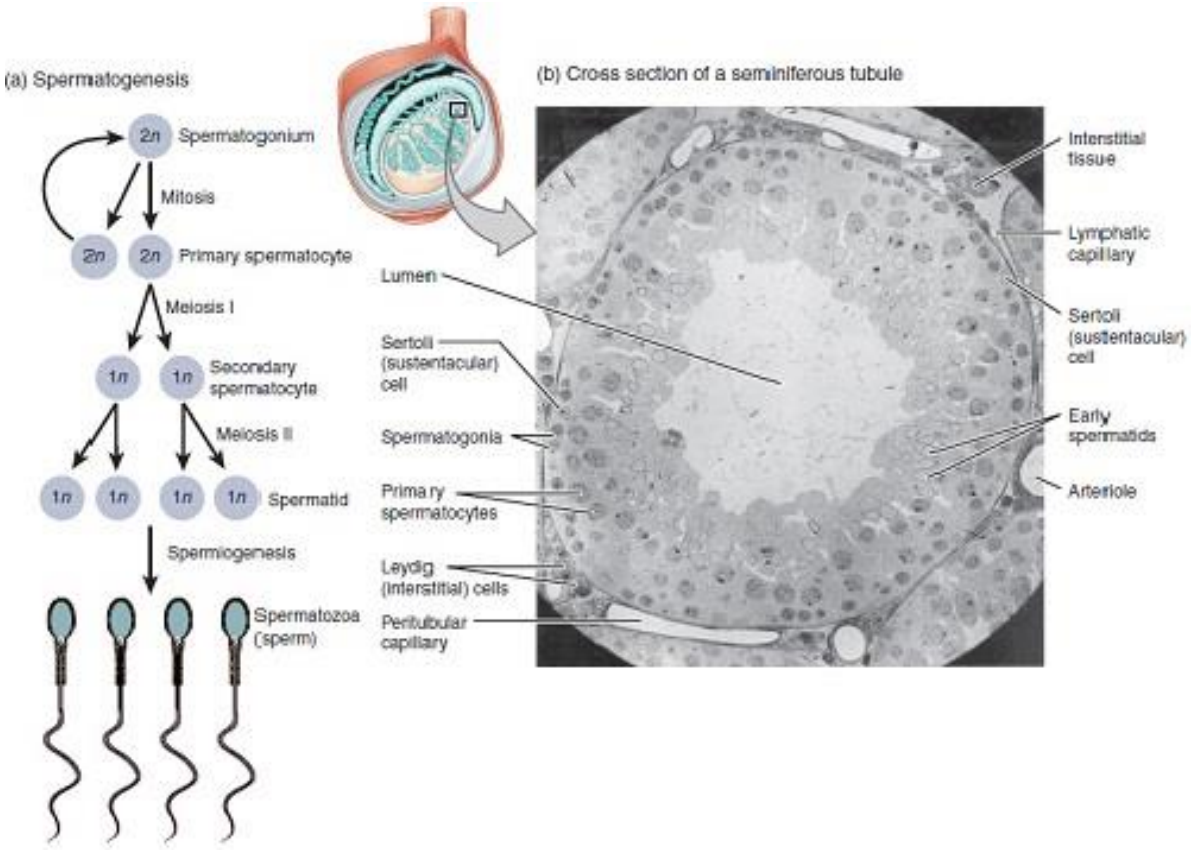


Figure 3.4 Schematic illustration of the process of spermatogenesis. (Image accessed from: Anatomy & Physiology, Connexions Web site http://cnx.org/content/col11496/1.6/; Accessed on: 27-11-2015).

*During spermiogenesis the spermatid's nucleus becomes condensed due to transition proteins displacing the histones, which in turn, is replaced by protamines. The spermatid's chromatin is restructured for tight protamine-DNA compaction by the DNA strands that form tigthly coiled loops around the protamine molecules (Ward & Coffey, 1991; Brewer et al., 1999; Steger et al., 2000; Zini & Libman, 2006;). This highy superstructured DNA-protamine association's function is to stabilize the sperm nucleus and to protect the spermatozoon's nuclear material from external influensors, such as temperature and ROS (Kosower et al., 1992; Zini & Libman, 2006; Du Plessis et al., 2015).

Spermiation follows spermatogenesis and is the process where the mature spermatid becomes motile by freeing itself from its association with the Sertoli cells and enters the lumen of the seminiferous tubule as a spermatozoon. From the beginning of differentiation until the formation of a motile spermatozoon takes 70-75 days. The spermatozoa stay ± 10

days in the epididymis, where the final maturation takes place, and is only transported to the ejaculatory duct once ejaculation occurs (Johnson et al., 1983; Griffin et al., 2015).

3.4 Factors causing anomalies in sperm chromatin status

The ‘modern age’ has afforded both men and women the opportunity to build successful careers and combined with the increased use of contraceptives, the age of the couple wishing to conceive has increased dramatically. A study conducted in Germany by Kühnert & Nieschlag (2004) reported on the increase in time to marriage for men as the average age to get married rose from 26.6 years in 1985 to 31.8 years in 2002. Similarly, the average time to fathering a first child increased during this period from 31.3 years to 33.1 years.

Spermatogenesis continues without cessation in the male and results in the formation of uniform spermatozoa in the ejaculate. Increasing time to fatherhood has become a global phenomenon and researchers have investigated the effects of male aging on the semen parameters and its relationship with semen quality, infertility, and possible chromosomal abnormalities (Lambert et al. 2006). It has been shown that in the aging male there is an age-associated decrease in the hypothalamic-pituitary axis control as reflected in lower serum-steroid-androgen secretion and circulation to the testes. Age, testicular trauma (varicocele), diseases (such as orchitic mumps), and physiological apoptosis of spermatozoa are all causal agents of increased production of reactive oxygen species (ROS) in the testes due to increases of the amount of leukocytes present (Gnessi et al., 1997; Maya et al. 2009; Dain et al. 2011; Du Plessis et al., 2015; Griffin et al., 2015). This continued replication of spermatozoa through spermatogenesis possibly causes spontaneous mutations within the male germline. Apoptosis (cell death) of spermatozoa with compromised DNA is an integral part of spermatogenesis that guarantees natural selection of spermatozoa with normal DNA (Lambert et al. 2006; Agarwal et al. 2009; Nijs et al. 2011; Du Plessis et al., 2015). The extent of DNA compromised spermatozoa can be determined amongst others, by performing Chromomycin A_3analysis (CMA_3).

3.5 Sperm chromatin and DNA assays

The usefulness of tests of DNA integrity for prediction of fertility is still debated (Collins et al., 2008; Du Plessis, et al. 2015; Griffin et al., 2015). Collins et al., (2008) suggested that sperm chromatin investigations would be beneficial to subfertile men although this sperm parameter was found to be non-predictive in a meta-analysis with pregnancy outcome and ART programs (Griffin et al., 2015).

A recent meta-analysis, comparing the risk of spontaneous abortion in IVF with sperm DNA damage, reported that infertile men had higher levels of sperm DNA damage compared to fertile men, thereby increasing the likelihood of spontaneous abortions following ART (Zini et al., 2008). This is of clinical relevance as nearly 40% of men who seek assisted reproductive treatment suffer from male infertility.

Many scientists now emphasize the necessity of including sperm DNA analysis as part of routine semen analysis based on many studies providing associations between fertilization failure and poorly packaged or damaged sperm chromatin or other DNA damage. In fact, Argawal & Allamaneni (2004) argue that spermatozoa DNA and chromatin integrity analysis has a greater prognostic and diagnostic value to infertility testing than conventional semen analysis (Franken et al., 1999; Zini & Libman, 2006; Kazerooni et al., 2009). Infertile men are believed to retain a higer histone: protamine ratio compared to fertile men (Steger et al., 2000; Zini & Libman, 2006). Although 15% of DNA-histone binding is normal (protamin-DNA binding is thought to be 85%), when the histone-DNA binding is greater than 15%, the chromatin structure is less tightly compacted and more exposed to external influencers (Kosower et al., 1992; Zini & Libman, 2006).

There are ample tests to assess the chromatin structure of sperm. Flow cytometry through low pH denaturation of sperm chromatin evaluates chromatin integrity and sperm function as well as chromosome status, cellular fragments, and immature germ cells (Hacker-Klom et al., 1999; Evenson et al., 2000; Larson et al., 2000; Griffin et al., 2015). Chromosome status refers to the number of chromosome sets present in the mature spermatozoon, and flow cytometry has been applied to distinguish between haploid (n; normal) and diploid (2n;

abnormal) spermatozoa. DNA fragmentation has also been used effectively to determine the nuclear integrity of a spermatozoon (Donnelly et al., 2000; Chohan et al., 2006; Griffin et al., 2015). Flourochrome dyes have been proven to compete for protamine binding sites and loosely packaged sperm chromatin. These flourochrome dyes include aniline blue (AB), acridine orange (AO) and chromomycin A_3 (Franken et al., 1999; Kazerooni et al., 2009).

Fertilization failure, and even recurrent miscarriage, can be greatly attributed to the gamete failure caused by a protamine defiency or incorrect replacement of histones by protamines in the spermatozoon (Manicardi et al., 1995; Nasr-Esfahani et al., 2007; Kazerooni et al., 2009). The protamine constituent of a spermatozoan can be measured indirectly through flourochromes such as CMA_3. CMA_3 has been reported to compete with protamine successfully, enabling a quick, cost-effective and efficient method for observing nicked, deficient protamine and partially denatured DNA (Manicardi et al., 1995; Bizzaro et al., 1998; Nasr-Esfahani et al., 2007). CMA_3 staining has been reported to be increased in men of infertile populations (Nasr-Esfahani et al., 2007; Kazerooni et al., 2009) and the stain has been inversely correlated to the state of protamination of the spermatozoon (Bizzaro et al., 1998), correlated to anomalies in chromatin ultrastructure (Iranpour et al., 2000) and compared to assisted reproduction outcomes (Nasr-Esfahani et al., 2007).

The preferred CMA3 reference is ≤40% immature DNA according to Esterhuizen et al. (2000), while Sakkas et al. (1996) and Nasr-Esfahani et al., (2007) established CMA3 cut-off values to be as low as 30%. This implies that ≥31% immature DNA could be regarded as high CMA3 positivity.

This study is the first to the author's knowledge to investigate the effect of chromatin packaging on assisted reproduction outcomes when special care is taken to control possible oocyte factors (maturity, presence of meiotic spindle and automated ZS) and sperm morphology (through ICSI) that influence fertilisation and pregnancy. Forthcoming chapters will describe the methods, results and discussion to follow.

Chapter 4: Materials and Methods

4.1 Research Design

This study employed a pure hypothetical deduction research approach consisting of an observational design, gathering of quantitative data and statistical analysis. A diagrammatic representation of the study design is shown in figure 4.1.

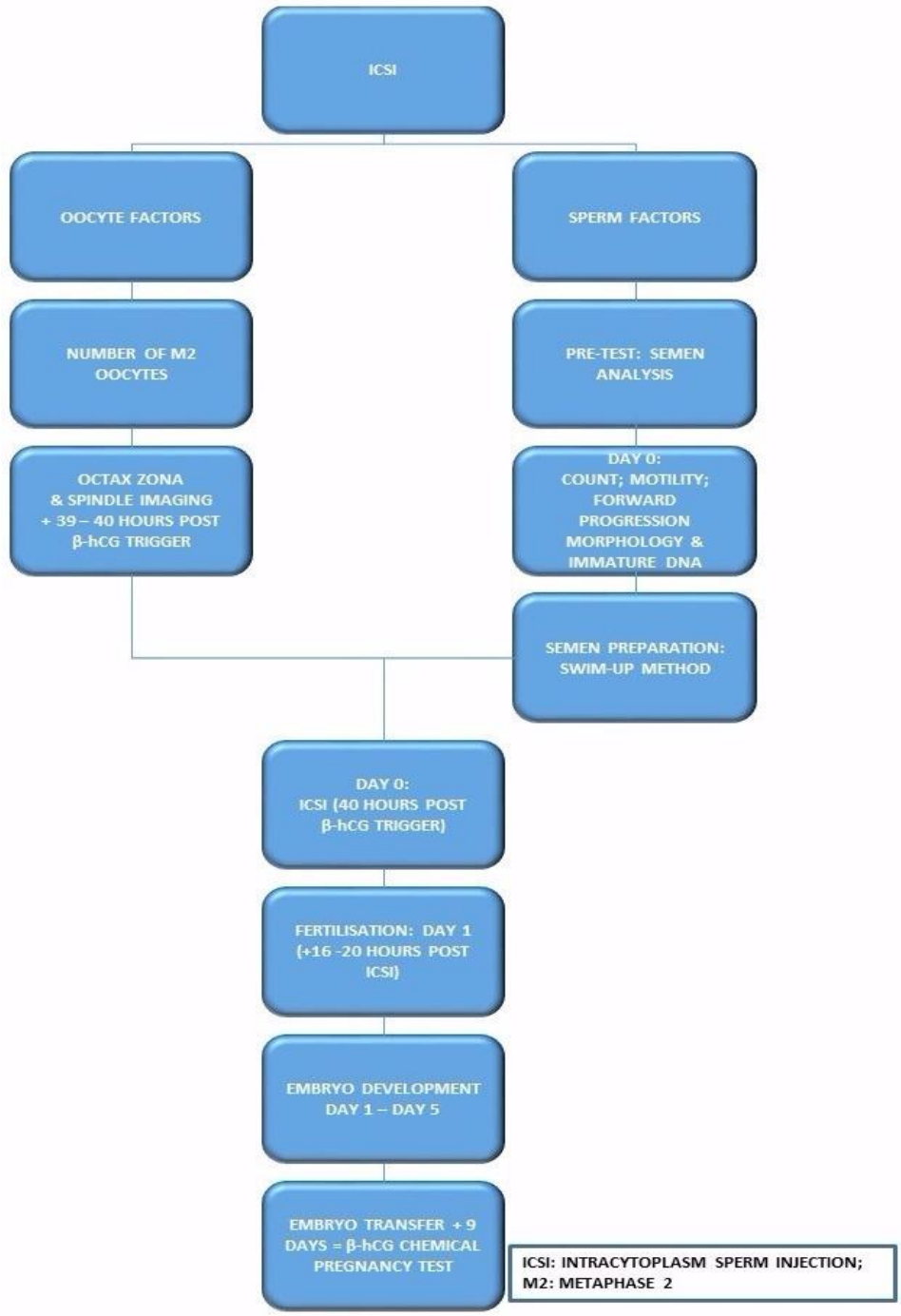


Figure 4.1 Layout of the study parameters measured.

4.2 Sampling (Study population)

4.2.1 The number of subjects

In total, a number of 95 couples were recruited as research participants through convenient sampling as part of their scheduled ICSI treatment during August to December of 2013 at Wilgers Infertility Clinic, a private fertility laboratory at Life Wilgers Hospital, Pretoria, South Africa. It is the standard procedure for gynaecologists at Wilgers Infertility Clinic to provide every couple that enrols for ART with ICSI as it has been established that the vast majority of patients seeking treatment fall within the ICSI category. The reason for this is believed to be the fact that the embryologists and gynaecologists from Wilgers Infertility Clinic are well known for their expertise and proficiency in performing ICSI.

4.2.2 Research participant identification

The study involved 856 oocytes and 95 semen samples from the 95 couples participating in the assisted reproduction program at the Wilgers Infertility Clinic. The oocytes included in this study were collected after controlled ovarian stimulation (COS) from patients undergoing oocyte retrieval for ICSI by means of convenient sampling according to the relevant inclusion and exclusion criteria set out below.

4.2.3 Inclusion and Exclusion criteria

Inclusion Criteria

Couples undergoing ICSI were selected to participate in the study according to the following criteria:

- Signed informed consent form.
- Semen parameters measured according to WHO 2010 standards:

    1) Macroscopic semen analysis;
    2) Morphology Assessment (Tygerberg Strict Criteria);
    3) CMA_3 evaluation to evaluate DNA chromatin packaging.

Exclusion Criteria

- Cycles where no oocytes were retrieved;
- Cycles where semen samples were Azoospermic and couples did not want the use of a donor.

4.3 Data Collection, Analysis, and Bias

4.3.1 Semen preparation and processing

On the day of each couple's scheduled oocyte retrieval, the male partner collected a semen sample by means of masturbation or *coitus interruptus* in a patient-unique marked container prior to the start of the ultrasound-guided transvaginal aspiration.

Samples were evaluated approximately 20-40 minutes after collection to allow for liquefaction and according to standard protocols for spermatozoa concentration, motility and forward progression (WHO, 2010).

4.3.1.1 Semen volume and sperm concentration, motility, and forward progression

The ejaculate volume was measured with a sterile 10ml pipette and the sperm concentration, motility and forward progression was assessed manually by the same technician, using a Makler counting chamber and bright-field light microscopy under 100 x magnifications (Nikon Alphaphot with 10x objective lens). The Makler counting chamber has a depth of 10 µm and the base contains a grid area of $1mm^2$ subdivided into 100 small squares, each of which covers $0.01mm^2$. 10µl of semen was pipetted onto the grid and covered with the cover slip.

The number of spermatozoa in 10 small squares was counted; this value was the sperm concentration per millilitre expressed as millions of sperm per millilitre (_$x10^6$/ml). Motility was assessed by counting the number of motile sperm in the row of 10 squares and dividing it by the total number of sperm counted in the same row, and was expressed as a percentage. Forward progression was determined by the forward motion of the sperm and its speed indicated as 1+/-; 2, 2+ and 3 with 3 being excellent.

Two smears were made before processing of each semen sample. One smear was made for morphology assessment (Tygerberg Strict Criteria) and one smear was made for chromatin packaging assessment (CMA_3 evaluation).

Methanol/glacial acetic acid in a ratio of 3:1 was used for fixing both semen smears (morphology & CMA_3) at 4°C for 20 minutes after which the slides were allowed to air dry for another 20 minutes at room temperature.

4.3.1.2 Semen preparation

The aim of semen processing is to rid the semen sample of immotile, morphologically abnormal spermatozoa and other semen constituents such as prostaglandin, thereby retaining the motile spermatozoa with normal morphology for use in ART. The final sample will still contain abnormal and immotile spermatozoa, however semen preparation yields a much better sample compared to the initial neat sample.

The liquefied neat semen was washed in Ham's F10 medium (Sigma Chemicals, St Louis, USA) with a 1:1 ratio, in a 15ml Falcon conical tube (AEC Amersham, South Africa) and centrifuged for 10min. at 300g. The supernatant was discarded and the pellet was layered with 0.5ml of gamete buffer (COOK Medical, Sydney, Australia). Spermatozoa were allowed to swim up for 20 min post layering at room temperature. The upper 0.5ml fraction was aspirated from the top of the meniscus with a sterile, glass drawn Pasteur pipette and transferred into a 5ml Falcon conical tube. The final sperm concentration was determined by placing a 10µl droplet onto the Makler counting chamber (WHO, 1999; WHO, 2010), as described in 4.3.1.1.

4.3.1.3 Morphology Analysis

The spermatozoa morphology assessment was performed according to criteria described in the WHO 2010 manual and based on Tygerberg Strict Criteria (Kruger et al., 1986).

For the morphology assessment, the spermatozoon was divided into its head (and neck) and tail (midpiece and principle piece) portions and when both portions were considered normal the spermatozoon was considered normal. According to this method, all borderline forms were considered abnormal.

Spermatozoa morphology was assessed using a phase contrast Nikon microscope with brightfield optics at x1000 magnification with oil immersion. With the aid of a laboratory

counter, ten spermatozoa per field were examined in twenty fields until two hundred spermatozoa per semen sample were counted.

Explained in brief, the Tygerberg Strict Criteria, judged the spermatozoon's head to be morphologically normal when it was smooth, oval in shape and with a clear acrosome covering between 40 and 70% of the head. No more than 2 small vacuoles were allowed to be present in the acrosome and no vacuoles in the post acrosomal region as this is where the paternal DNA is located (Menkveld et al., 2001). The sperm head's dimensions should be 4-5µm in length and ±3µm in width. The midpiece was recorded as normal when it was similar in length to the head, 4µm and < 1µm in width. When the cytoplasmic droplets were > 30% of the sperm head size the midpiece was recorded as abnormal (Mortimer & Menkveld, 2001). The tail was regarded as normal when it was ±45µm in length (10x the length of the head) and was allowed to be looped back on itself but any sharp, angular bents were recorded as abnormal (WHO, 2010).

4.3.1.4 Chromatin Packaging Quality Assessment

Two hundred spermatozoa per semen sample were evaluated according to the following criteria:

a. No staining / No fluorescence of the sperm head;

b. Fluorescent band at the equatorial region of the sperm head;

c. Faint yellow fluorescent staining of the entire sperm head;

d. Bright yellow fluorescent staining of the entire sperm head.

Reporting results:

1) Classes a) and b) are added and reported as mature DNA;

2) Classes c) and d) are added and reported as immature DNA.

The CMA_3 cut-off values of 40% immature DNA, as suggested by Esterhuizen et al. (2000) and also applied by Ampath Laboratories, were used. Mature DNA was regarded as ≤40% CMA3 fluorescent staining of the sperm head; and ≥41% fluorescent staining was regarded as immature DNA.

4.3.2 Ovarian Stimulation

The COS protocols varied and were adapted according to the diagnosis of fertility for the couple. Mainly two COS protocols were followed; the “long” and “short” protocols.

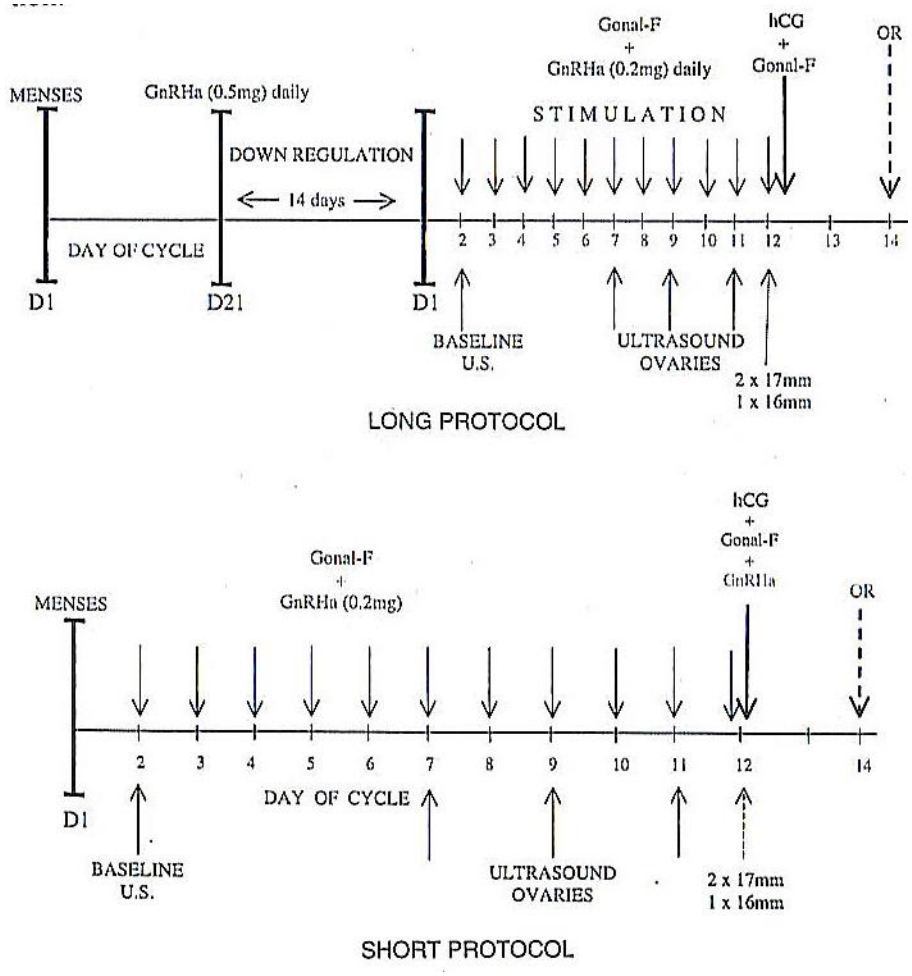


Figure 4.2 Schematic representation of comparison between the long and short controlled ovarian stimulation protocols. (Image captured from Merck Serono Infertility-care-patient brochure).

The short COS protocol was achieved by administering exogenous follicle stimulating hormone (FSH; Gonal-F; Serono, Johannesburg, South Africa) and/or recombinant FSH & luteinizing hormone (LH) injections (Menopur, Ferring, Johannesburg, South Africa). It was supplemented with or without either clomiphene citrate (Fertomid / Clomid to suppress Estrogen secretion) or the gonadotropin releasing hormone antagonist (GnRHa), cetrorelix

acetate (Cetrotide, Serono, Johannesburg, South Africa) to prevent premature LH surge and untimely ovulation. Serum estrogen (E_2) levels were monitored and once the leading follicles reached 18-20mm, ovulation was triggered with a 10 000IU human chorionic gonadotrophin (hCG) injection (Ovitrelle; Serono Johannesburg, South Africa) followed 36h later by ultrasound guided transvaginal oocyte retrieval.

The long COS pituitary down-regulation was achieved using the GnRH agonist Leuprolene acetate (Lucrin; AbbVie Limited, Johannesburg, South Africa) during the mid-luteal or late luteal phase for 14 days prior to day 1 of the menstrual cycle in order to down regulate oestrogen secretion. When desensitization was achieved, recombinant exogenous FSH (Gonal-F; Serono, Johannesburg, South Africa) was administered in the early follicular phase. Ovulation was triggered with 10 000IU recombinant hCG (Ovitrelle; Serono, Johannesburg, South Africa) once leading follicles measured 18-20mm and serum E_2 were at satisfactory levels.

Ultrasound scans were performed from day 2 of controlled ovarian stimulation until the day of transvaginal oocyte retrieval to monitor follicular development i.e. the number of follicles and their measurements. On day 3 of stimulation, the E2, FSH, and LH hormone levels were determined and the stimulation protocols were adjusted according to these levels. The endometrial thickness was also monitored, since too thin endometrial width (<8mm on day of hCG trigger) has been associated with failure to implant, and pregnancy loss.

4.3.3 Oocyte retrieval, denudation, and maturation assessment

Transvaginal oocyte retrieval

Ultrasound guided transvaginal oocyte retrieval was the method of choice and was performed 36hrs after hCG-trigger, according to techniques commonly applied by gynecologists.

After retrieval, oocytes were incubated in culture medium (COOK medical, Sydney, Australia) for 60-120 minutes at 37°C and 5% CO_2 prior to denudation of oocytes. All incubation steps were performed in a water-jacketed incubator (FORMA® Water-jacketed CO_2 Incubator; LABOTEC, Johannesburg, South Africa) and according to COOK culture medium protocol.

Denuding of Oocytes

On day 0 of embryo culture, cumulus cells were stripped from the oocytes 60-120 minutes after retrieval. Denuding was performed in a sterile laboratory in a laminar flow cabinet (AFM, Johannesburg, South Africa) using a stereo microscope (Nikon, IMP, Johannesburg, South Africa) and a hand-drawn glass pipette (Labretoria, Pretoria, South Africa).

Granulosa cells were removed by repeatedly pipetting the oocyte-cumulus-complex for approximately 30 seconds to one minute in 0.1% Hyularonidase (Sigma Chemicals, St Louis, MO, USA) in gamete buffer (COOK medical, Sydney, Australia). Hyularonidase is a hydrolytic enzyme, commonly used to remove most of the granulosa cells surrounding the oocyte. Denuded oocytes were loaded in individual droplets of medium (Cleavage medium, COOK medical, Sydney, Australia) under oil in a glass bottom dish (Fluorodish, Delfran, Pretoria, South Africa).

Nuclear maturity of the individual oocytes was briefly evaluated and they were cultured for another 60 – 120 min prior to oocyte imaging using OCTAX polarAIDE™ software (MTG, OCTAX Microscience GmbH, Germany).

In cases where the oocyte retrieval yielded more than 10 oocytes, only 10 oocytes were randomly selected for OCTAX polarAIDE™ imaging.

Zona and Spindle Birefringence Analysis

Oocytes were individually imaged using the OCTAX PolarAIDE (MTG, OCTAX Microscience GmbH, Germany) on an inverted microscope (Nikon TE200, IMP, Johannesburg, South Africa). The microscope was equipped with a motorized stage (MTG, OCTAX Microscience GmbH, Germany) containing fully heated aluminium insert. The entire stage was calibrated and heated to 37°C ±0.5°C and the temperature was adjusted with an external calibrated sensor to maintain 37.0°C±0.5°C in the medium droplet during calibration.

The oocyte images were captured and saved for analysis of presence of the meiotic spindle and zona birefringence score on the OCTAX database. Unique patient codes were used to identify specific oocytes and reports were generated from the software provided by OCTAX PolarAIDE.

The presence of birefringent meiotic SP was analysed. To prevent improper SP alignment in the event of spindle absent (SPa) oocytes, the oocyte was rotated whilst secured to the holding pipette so ensure the accurate visualization of the presence or absence of the SP. The oocytes were classified as having a visible SPp or SPa.

The zona birefringence of M2 oocytes were classified as having a high zona pellucida birefringence (HZB) or a low zona pellucida birefringence (LZB). (Method adapted from: Montag, et al., 2006; Braga et al., 2008; Madaschi, et al., 2008; Ebner, et al., 2010; Valeri et al., 2011). For the purpose of this study and in order to determine in-house threshold values for our laboratory, 5 categories were compiled, namely ZS<10; 10>ZS<20; 20>ZS<30; 30>ZS<40; 40>ZS<60; ZS>60.

4.4 Reliability, Validity & Objectivity

4.4.1 Standardization of results for Morphology and CMA_3 staining

To eliminate bias, technician and sample coefficients of variation (CV) was recorded for both morphology and CMA_3 assessments. Intra-assay variation was determined by evaluating 1000 cells on different microscopic fields from the same stained slide for both morphology and CMA_3. Inter-assay variation was measured by counting 200 cells on five different slides from the same semen sample. Coefficient of variation (Mortimer, 1994) for both intra- and inter assay values were calculated using the following formula:

$$CV\ (\%) = \frac{SD}{mean} \times \frac{100}{1}$$

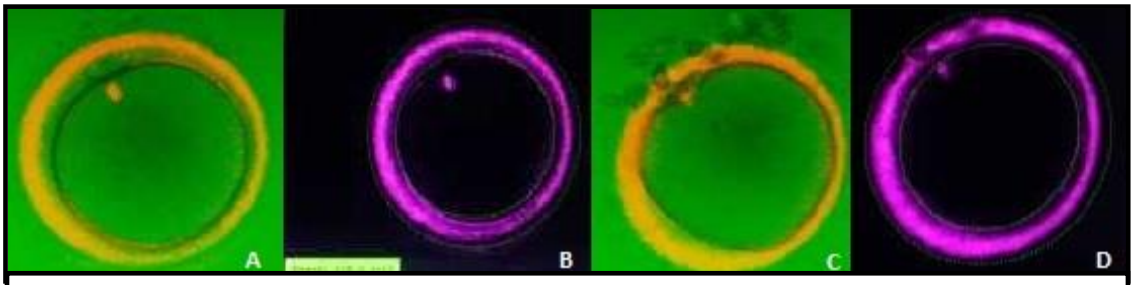
The CV during the standardization of results was < 15% in all instances.

(Mortimer, 1994; Esterhuizen et al., 2000; WHO, 2010, acceptable difference table).

4.4.2 Oocyte Zona and Spindle Imaging

Zona and Spindle Imaging

The birefringence analysis, including auto-calibration, was a process that was fully controlled by a polarization imaging software module (OCTAX PolarAIDE™, Altdorf, Germany) implemented with an imaging software system. No bias was present in calculations, reliability, and objectivity. Validity was ensured for all oocyte images. The software calculated a score based on intensity and uniformity of the zona pellucida around the entire oocyte. Oocytes with a birefringence score ≥10 were considered as HZB (fig 4.4) and oocytes with a score ≤10 as LZB. The SP was bright flourecent orange or purple, depending on software mode, when present (fig 4.4).



A) M2 oocyte with meiotic spindle; B) The same M2 oocyte with automated zona score of 18.1µm; C) M2 oocyte in telophase 1, with the SP still perpendicular to the polar body; D) Telophase M2 oocyte in purple and black contrast image to illustrate the meiotic spindle and zona pellucida birefringence.
(Images courtesy from Wilgers Infertility Clinic using OCTAX polarAIDE imaging software).

Figure 4.3 Birefringent imaging of two metaphase 2 oocytes with green & orange and purple & black birefringent contrast.

The OCTAX PolarAIDE images in figure 4.4 reveals a M2 oocyte with a SP perpendicular to the polar body (A) and an automated HZB score of 18.10µm (B) indicated by the black and purple image. The second oocyte exhibits a M2 oocyte in telophase 1 (C & D), with the SP still perpendicular to, and exchanging genetic material with the polar body. In this case, ICSI was delayed for 90 min so that spindle can complete exchange and reform again in M2 oocyte.

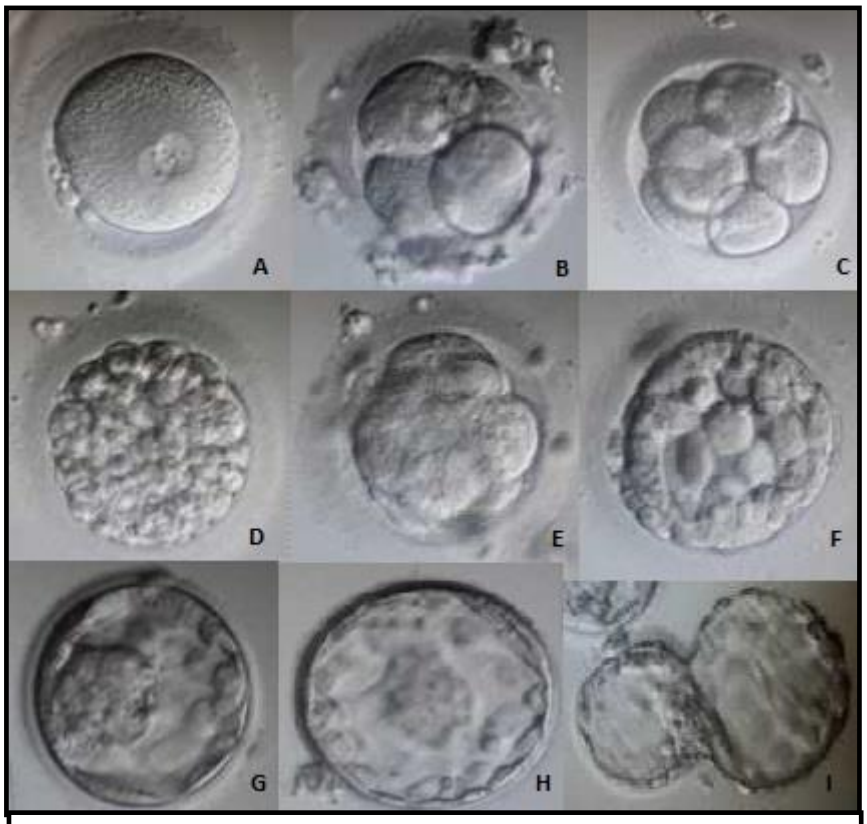
4.5 ICSI Procedure and Embryo Culturing

After completion of oocyte zona and spindle imaging, the oocytes were transferred into an ICSI dish with droplets of HEPES-buffered media (Gamete buffer, Cook medical, Sydney, Australia) and PVP (Cook Medical, Sydney, Australia) under oil (LifeGuard oil, LifeGlobal, Delfran, South Africa). The standard protocols for performing ICSI were adhered to. ICSI commenced 40 hours post β-hCG triggered oocyte retrieval. Very briefly, the selection criteria for a 'morphologically normal spermatozoon' was based on the morphology of the sperm as well as its motility. The single, morphologically normal spermatozoon selected for microinjection into the ooplasm of the oocyte, was immobilized by striking its tail perpendicular to the ICSI dish in the 10% polyvinyl pyrrolidone (PVP, COOK medical, Sydney, Australia). The spermatozoon was aspirated into the injection pipette (The Pipette Company, Delfran, Pretoria, South Africa) tail-first and injected into the oocyte at the 3 o'clock position once the oocyte was secured at the 9 o'clock position by the holding pipette (The Pipette Company, Delfran, Pretoria, South Africa) with the polar body in the 12 o'clock or 6 o'clock positions. Upon the initial contact of the injection pipette through the zona pellucida and into the ooplasm, the ooplasm of the oocyte was aspirated in order to ensure the mixing of the spermatozoon and the ooplasm of the oocyte. The spermatozoon was deposited into the oocyte and the injection pipette was gently withdrawn.

After completion of the ICSI procedure, each oocyte was transferred into a culture dish (Day 0) prepared with a cleavage medium droplet per oocyte harvested (Cleavage medium, Cook medical, Sydney, Australia) and covered under oil (LifeGuard, LifeGlobal, Delfran, Pretoria, South Africa).

Oocytes were checked for fertilization 16-18 hours post ICSI (Day 1) on an inverted microscope (Nikon TE200, IMP, Johannesburg, South Africa) by taking images of the oocytes on the OCTAX system (OCTAX PolarAIDE™, OCTAX Microscience GmbH, Altdorf, Germany). Fertilization was confirmed when two pronuclei with nucleolar precursor bodies inside were visualized. All embryo development checks were performed early in the morning. On day 2 of embryo culture (40-42 hours post ICSI), the early cleavage embryos were imaged again and graded according to the number of blastomeres, homogeneity of the blastomere sizes and amount of fragmentation present in the embryo. The development curve for day 2 embryos suggests that the embryos should have had two to four cells each. On day 3 of embryo culture (64-68 hours post ICSI), the embryos were expected to have

between six and eight cells each and were transferred into a culture dish prepared with blastocyst medium. Depending on the age of the female, and the amount of fertilized and developing embryos, either they were transferred to the uterus of the female of the commissioning couple on day 3, or culture was extended up until day 5 of development. Every day, except for day 4 of culturing, images were taken of every embryo with the OCTAX system and recorded on the database (OCTAX PolarAIDE™, OCTAX Microscience GmbH, Altdorf, Germany). According to the Cook Culture System Manuel, the embryos were best not disturbed on day 4 for evaluation, but rather after the last media changeover on day 3, remained untouched in the incubator for 48 hours until the day 5 embryo transfer (± 110-116 hours post ICSI).



Images captured at 200x magnification

A) Fertilized oocyte(zygote); B**)** 4 cell embryo; **C)** 8 cell embryo; **D)** Morula(M)
E**)** Compacted morula (CM); **F)** Early Blastocyst (EBL); G**)** Full blastocyst (BL);
H) Expanded blastocyst (ExBL); **I)** fully hatched Expanded Blastocyst

Figure 4.4 Microscopic images illustrating the lifecycle of the fertilised oocyte from day 1 to day 5 development. (Images courtesy of Wilgers Infertility Clinic using OCTAX PolarAIDE software).

The embryo transfer (ET) was performed by the gynaecologist using his/her catheter of choice (K-SOFT-5002; K-JETS-6019-SIVF, COOK Medical, Sydney, Australia). The two top-quality embryos were selected, firstly according the development: Hatching blastocyst (BL), Expanded BL, full BL, BL and Early BL and then secondly, the automated zona score and the presence or absence of the meiotic spindle was also taken into account before making the final decision on the two embryos to be transferred.

The ET catheter was primed for the embryos prior to the transfer by flushing it with Blastocyst culture media (Cook Medical, Sydney, Australia) before drawing up the embryos for transfer. All media preparations and culturing were performed using the COOK Culture System and according to its specific recommendations described in the Cook Culture System Manuel (Catt & Sydney IVF team, 2002).

A biochemical pregnancy test was performed 7 to 9 days after the embryo transfer, with hCG values above 5.0 mIU/ml indicating a biochemical pregnancy towards the unlikely to low β-hCH value, whilst a β-hCG value above 25 mIU/ml was considered a good pregnancy prognosis. Foetal heartbeat confirmed clinical pregnancy at 6 weeks.

4.6 Ethical considerations

4.6.1 Ethical clearance

The principles of Beneficence, Autonomy, non-Maleficence, and Justice were adhered to.

A written informed consent was obtained (Appendix A), in which patients agreed to share the outcomes of their own cycles for research purposes. Ethical clearance was obtained from the Ethics Committee of the University of Stellenbosch (and Tshwane University of Technology).

4.6.2 Anticipated risks

All procedures performed during the study forms part of the normal treatment that the research participant would have received. No additional specimens were required from the participants, the imaging of oocytes are completely non-invasive; the semen utilized for ICSI were prepared according to the unit's standard protocol, with the exception that two smears were made for diagnostic evaluation purposes. Considering all aspects, the participant only benefits from this study. There are no risks involved in this study. None of

the factors under evaluation is experimental or harmful in any way to the research participant or the success of their treatment.

4.6.3 Statistics

The statistics was performed by Prof P Becker from the Faculty of Science, University of Pretoria and Prof M Kidd from Stellenbosch University, Biostatistics Division.

STATA 13, Statistics Data Analysis was employed to test & determine: 1) intra-category variances or associations between inter-categories with Fisher's exact test and chi-squared tests; 2) Generalized least squares (GLS) models were used to determine variance within, between and overall, in a patient specific, as well as in a population setting; 3) Random-effects logistic regression group variables were used to determine probability of conception in the form of likelihood-ratio testing; and odds ratio calculations. Statistical results were regarded as significant when 'p'-value was <0.05 or when clinical significance is highlighted.

4.6.4 Records

All records of the study will be kept at Wilgers Infertility Clinic for four years. Patient data sheets are kept in files (hardcopy and electronic) in the locked laboratory facility of the Wilgers Infertility Clinic with limited access.

4.6.5 Publications

The final dissertation and parts thereof may be published in the appropriate peer-reviewed journals nationally and internationally upon its acceptance for a Master's degree in Medical Physiology.

Chapter 5: Results

5.1 Study population results

The study involved 856 oocytes from 95 couples included in the assisted reproduction program at the Wilgers Infertility Clinic. Results concerning the nominal ('n') value differed due to the variance in the data available per couple. All patients gave written informed consent; however, the researcher could not access all patient information from files since one of the physicians' rooms was located in a rural part of South Africa. The specialist biostatisticians involved considered all these factors in the statistical analysis. Standard deviations are indicated with '±' in brackets, and the 95% Confidence intervals are indicated as (95% CI) in brackets.

5.1.1 Age distribution

The mean maternal age was 34.5 (±4.5) years with the median age at 35 years, ranging from 24 – 45 years. In comparison to their female counterparts, the mean paternal age was relatively higher (not significantly) at a mean of 38.5 (±6.2) years, median age of 37 and ranging from a minimum age of 29 to a maximum age of 59 years (fig 5.1). The study involved couples undergoing ICSI at Wilgers Infertility Clinic as part of their routine fertility treatment.

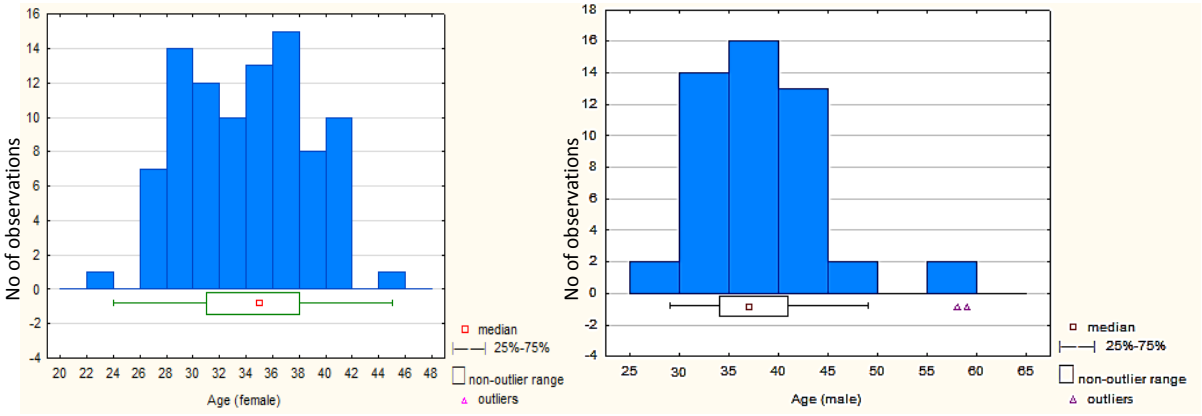


Fig 5.1 The ages of females and males attending Wilgers Infertility Clinic for assisted conception.

5.1.2 Racial Distribution

The racial distribution recorded for 74 couples were evenly distributed between the Caucasian (48.65%) and the African groups (51.35%) (table 5.1).

Table 5.1 Racial distribution of the study population (n=74)

Race	Percentage
Caucasian	48.65% (36/74)
African	51.35% (38/74)

5.1.3 Infertility status

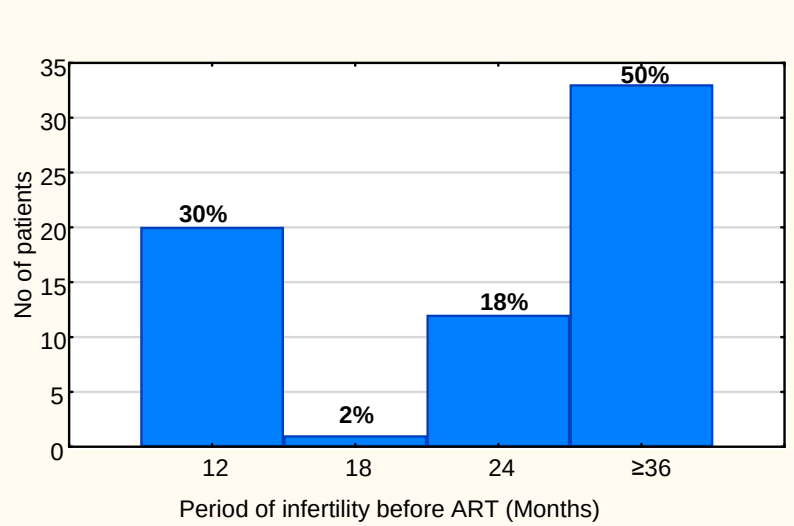


Fig. 5.2 The period of infertility for couples attending Wilgers Infertility Clinic prior to seeking ART.

The WHO, in their most recent report update for sexual reproductive health, defines primary infertility as the inability to either become pregnant or carry a pregnancy to a live birth; inclusive of pregnancy with recurrent miscarriages (Zegers-Hochschild, et al., 2009). Secondary infertility is described much the same as primary infertility, with the exception of a prior ability to carry a conception to a live birth (Zegers-Hochschild, et al., 2009). Data for primary vs. secondary infertility was only available for 74 couples.

Table 5.2 Diagnosis of infertility (n=74)

Diagnosis	Percentage
Secondary Infertility	20.27% (15/74)
Primary Infertility	79.73% (59/74)

Diagnosis of primary infertility (table 5.2) was responsible for 80% of all infertility cases with only 20% of couples struggling with a second/ third conception.

Table 5.3 Subfertility categories in the study population (n=75)

Subfertility	Frequency in population
Idiopathic (Unexplained) Infertility	9.33% (7/75)
Male Infertility	25.33% (19/75)
Female Infertility	20% (15/75)
Combined Infertility	45.33% (34/75)

The diagnosis of infertility was further expressed in the various categories of male, female, combined, and idiopathic (unexplained) infertility, as listed in table 5.3.

5.2 Semen characteristics

5.2.1 Volume

The neat semen was measured with a calibrated wide-mouth pipette, as described in section 4.3.1.1. 95 sperm samples were collected; 5 samples were collected through testicular sperm aspiration (TESA) because of obstructive Azoospermia, 10 samples were collected through masturbation, but with culture media in the collection container due to poor motility and / or known presence of antibodies (spermiogram during work-up revealed antibodies). The volume for each semen sample was measured, except for these 15 samples.

The mean semen volume recorded for the remaining 80 samples were 3.1 ml; standard deviation (SD). 1.99 ml.

5.2.2 Sperm concentration

Sperm concentration was measured as described in section 4.3.1.1. The mean sperm concentration was 29.53x10^6/ml, the median value was 20x10^6/ml with a maximum sperm concentration of 100x10^6/ml and a minimum sperm concentration of 0.1x10^6/ml, SD 28.84x10^6/ml (n=80; concentration was not determined for 5x TESA samples and 10x samples ejaculated in culture media).

5.2.3 Motility and forward progression

Motility was assessed as described in section 4.3.1.1. The mean motility measured for 80 semen samples ('n' value: 95-15=80 samples) was 45.96% with a median motility of 50% and within a maximum range of 75% motile sperm and a minimum of 5% motile sperm; SD 13.8.

Forward progression was indicated with a score of 1+ if the sperm had non-progressive motility. Sperm was scored as 2- 3 when the spermatozoa were moving actively, linearly or in a large circle, irrespective of speed. The mean forward progression was scored at 1.91 with a St. Dev of 0.33 and a maximum forward progression of 2, a median forward progression of 2, and a minimum forward progression of 1.

5.3 Spermatozoa morphology and chromatin packaging

5.3.1 Morphology

The overall spermatozoa morphology had a mean value of 6.39% normal forms (the current WHO lower reference limit is ≥ 4%; WHO, 2010) with a median percentage of 6% and standard deviation of 5.35% normal forms, the minimum value was one percent normal morphology and the maximum was twenty-one percent according to Tygerberg Strict Criteria (fig 5.3).

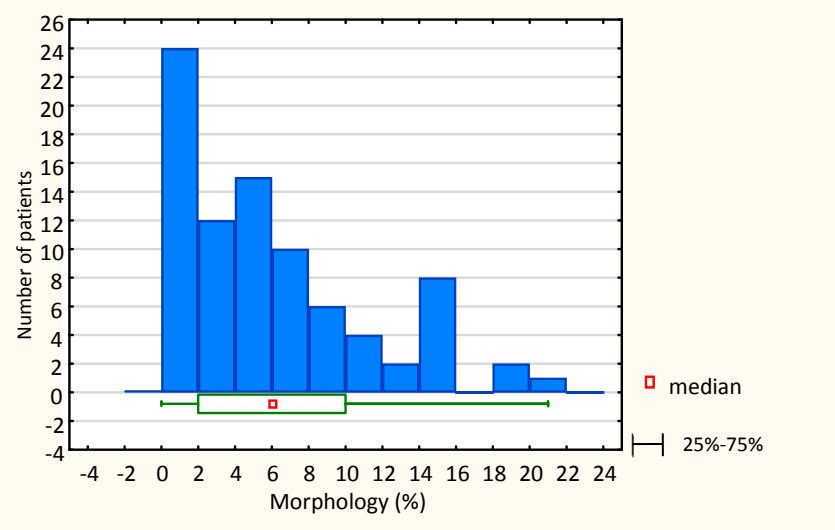


Fig.5.3 Illustration of the spermatozoa population's morphology (n=84 samples)

When the study population was defined according to recommendations made by the WHO (2010) into two main categories; normal spermatozoa morphology <4% or ≥4%; the study population presented with ± 63% of males that were classified with ≥4% normal forms and 37% of males presented with semen samples <4% normal forms (table 5.4).

Table 5.4 Spermatozoa categories based on morphology (n=93).

Morphology	Percentage
≥4% Normal	63.44% (59/93)
<4% Subfertile	36.56% (34/93)

5.3.2 Chromatin packaging

The majority (74%) of spermatozoa from males that participated in the ICSI cycles at Wilgers Infertility Clinic, presented with immature DNA ≥41% as shown in table 5.5.

Table 5.5 Illustration of the study population's CMA_3 categories (n=93).

CMA3 Categories	Percentage
≤40%	25.81% (24/93)
≥41%	74.19% (69/93)

The chromatin packaging of the spermatozoa sample showed a mean percentage of 53.98 (±21.66) with a median of fifty-five percent immature DNA, minimum value was one percent and maximum CMA_3 value was ninety-six percent (fig. 5.4).

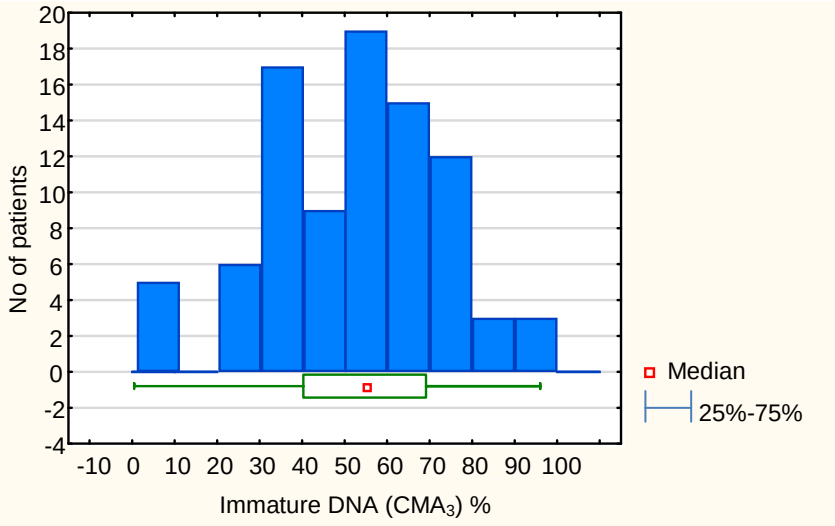


Fig. 5.4 Representation of the chromatin packaging of the spermatozoa of male participants prior to processing for intracytoplasmic sperm injection.

This places the overall spermatozoa population in the group where ICSI is recommended, as damage to chromatin packaging would prevent a spermatozoon's ability to fertilise an oocyte. ICSI overrides the issue with fertilization by directly delivering a morphological normal spermatozoon into the cytoplasm of the oocyte. The second potential problem associated with increased immature DNA is the delayed embryo development that could be caused by the 'late paternal effect'.

5.4 Oocyte retrieval

The short COS protocol was followed in the majority of cases (protocols described in chapter 4). Patients' hormone levels were monitored on day 3 to ensure that the patient population would be capable of recruiting 'good' quality oocytes. The mean hormone levels were as follows: FSH 8.34 ± 4.6 IU/ml; LH 8.84 ± 9.6 IU/ml; AMH 4.22 ± 3.18 IU/ml and Estradiol 166.85 ± 95.73 IU/ml; all within normal ranges. Figure 5.5 illustrates the median endometrium thickness before β-hCG trigger (timed-ovulation) as 8.3 mm with a mean

endometrial thickness of 8.41 mm (±1.6) a minimum thickness of 6 mm and maximum thickness of 13 mm.

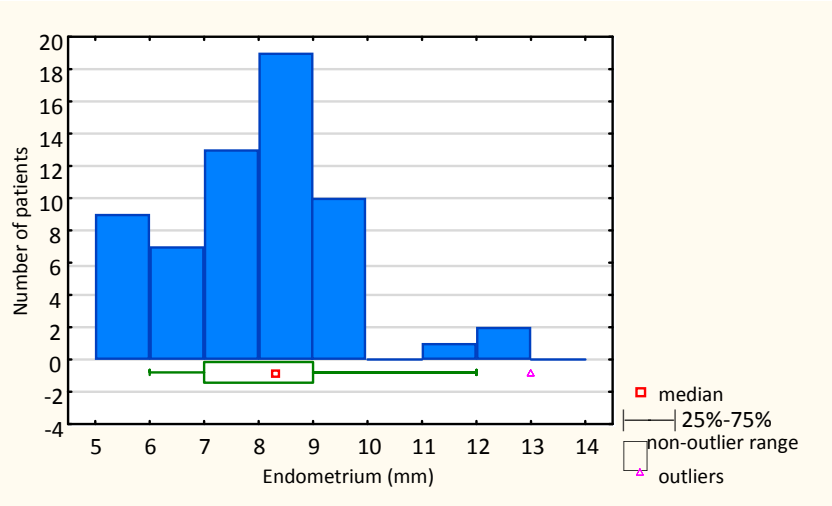


Fig. 5.5 The mean endometrium thicknesses (mm) before transvaginal oocyte harvest.

Oocytes were retrieved by ultrasound guided transvaginal oocyte harvest (TVOH) 36 hours after β-hCG trigger. Because of COS, a mean number of 11.32 (±6.71) follicles were recruited per patient with a median of 10 follicles per patient and a minimum of one follicle and maximum of thirty-one follicles (fig 5.3). The actual number of oocytes harvested resulted in a mean number of 9.07 oocytes (±5.14) per patient per ultrasound guided oocyte retrieval with a median of nine oocytes. The minimum number of oocytes harvested was one and the maximum number of oocytes harvested during the study was twenty-three oocytes (fig 5.6).

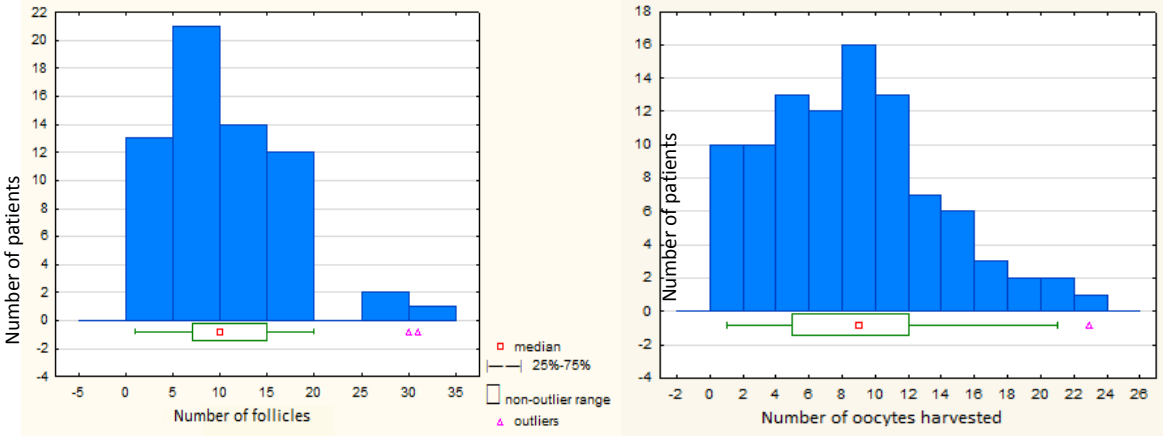


Fig 5.6 The mean number of follicles achieved after controlled ovarian stimulation vs. the actual amount of oocytes harvested after transvaginal oocyte harvest.

5.5 Oocyte maturity and Zona score

5.5.1 Maturity status

In total, 856 oocytes were harvested of which 568 (66%) were M2, 162 (19%) were M1 and 126 (15%) were in the GV stage of development (fig 5.4).

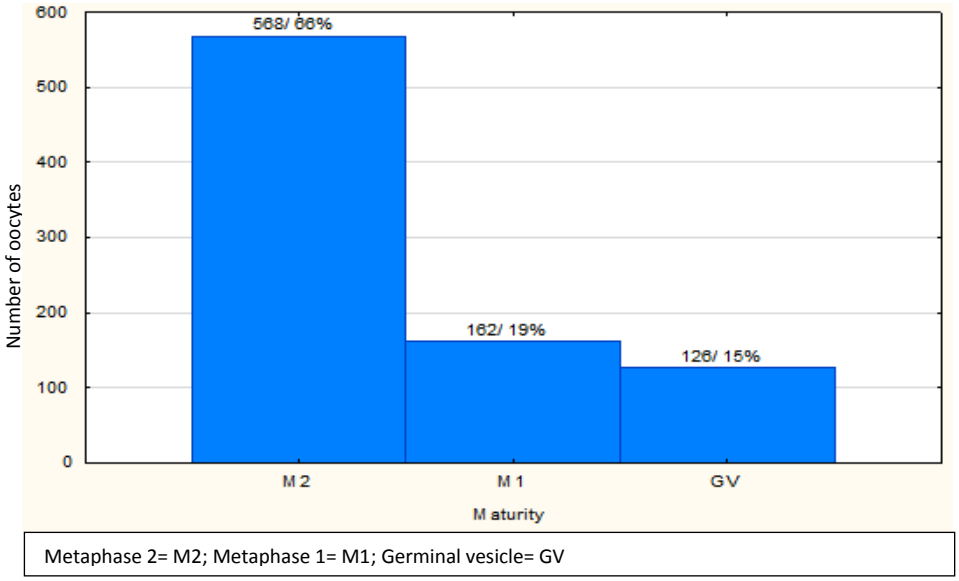


Fig. 5.7 Maturity of oocytes after transvaginal oocyte harvest.

5.5.2 Automated Zona score

From the present study it is evident that 40% (304) of oocytes had a LZB of <10 (fig 5.8). The results of the 60% (464) HZB oocytes were: 25% (194) had zona scores of 10-20; 15% (114) of oocytes were in the zona score category of 20-30, 8% (63) of oocytes had zona scores of 30-40; 10% (78) of oocytes' scores ranged between 40-60 and 2% (15) oocytes had zona scores of >60.

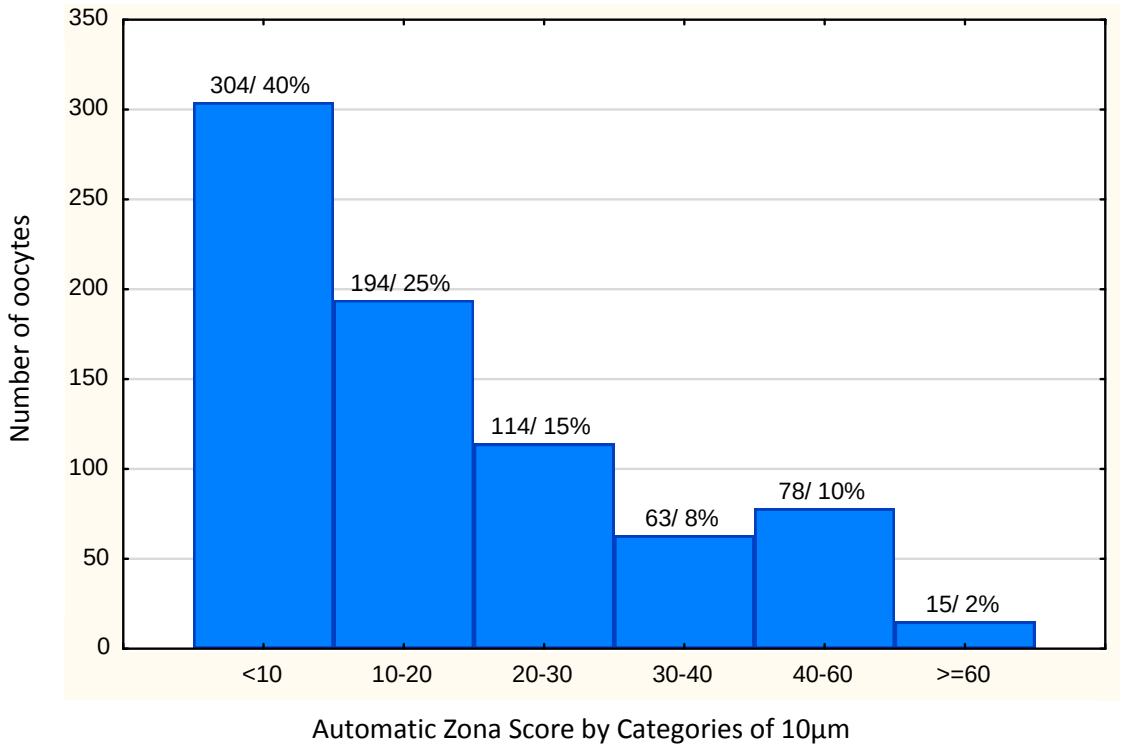


Fig. 5.8 Automatic zona scores for oocytes measured by the OCTAX polarization software.

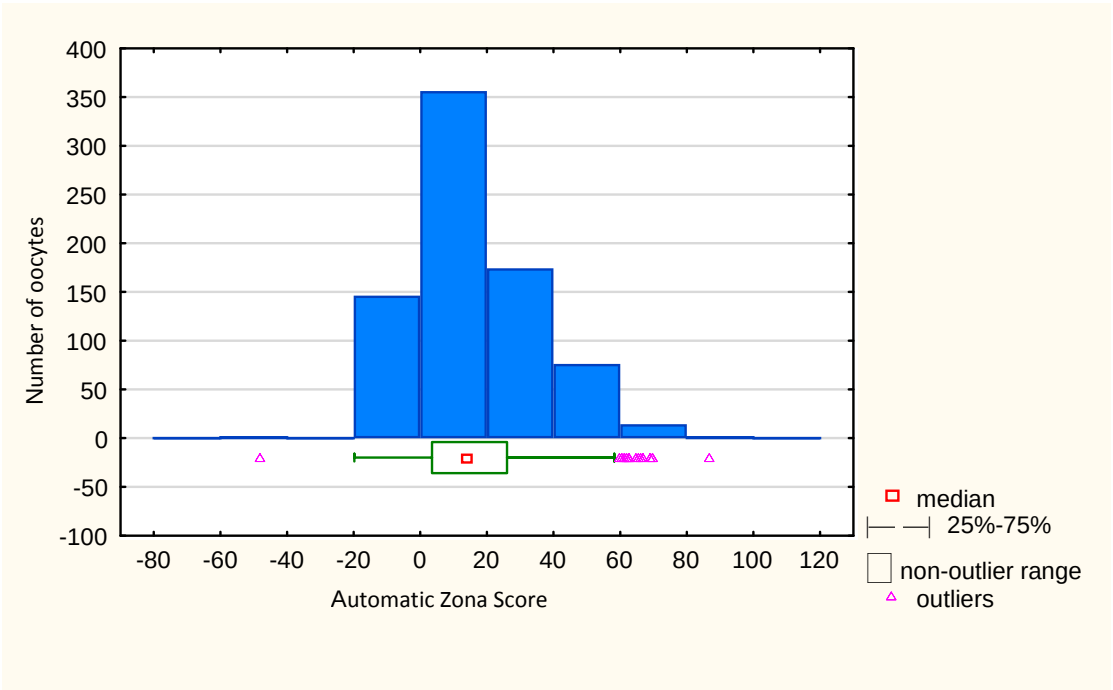


Fig 5.9 Representation of the median and range of the oocytes' automated zona scores.

The median zona score measured by the automated OCTAX software imaging was 13.3µm, with a mean of 15.99 µm, SD±18.12 µm and a maximum measurement of 86.7 µm and the minimum was -48.4 µm (fig. 5.9).

5.6 Meiotic spindle

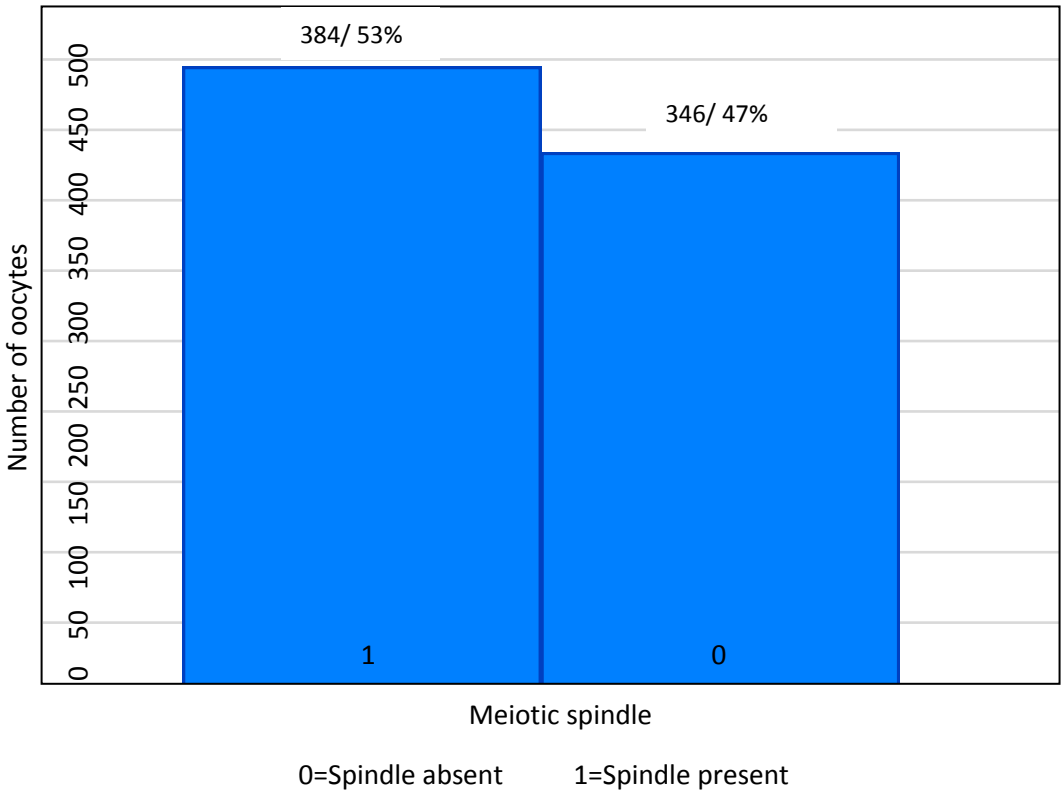


Fig. 5.10 Histogram to illustrate the presence or absence of the meiotic spindle in the metaphase 1 and metaphase 2 oocytes (n=730).

5.7 Fertilization results

5.7.1 Fertilization results of study population (n=856)

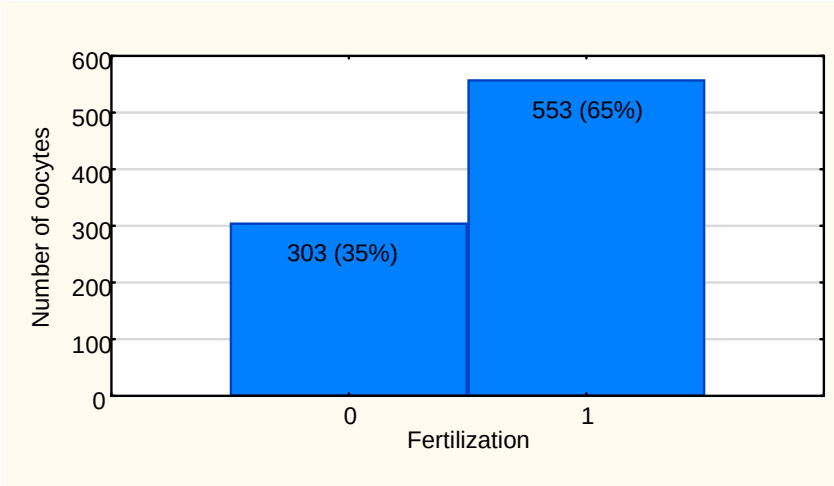


Fig. 5.11 Histogram of fertilization.

The overall fertilization rate for the study was 65% (fig 5.11). All M2 oocytes were inseminated on the day of oocyte retrieval by means of ICSI (method discussed in chapter 4). Immature oocytes (M1 & GV) were in vitro maturated and inseminated the following day. Fertilization was established by the extrusion of the second PB with both the male and female pronuclei present in the zygote (2PN).

5.7.2 Fertilization results for sperm morphology

Table 5.6 Fertilization results compared to morphology cagtegories ≥4%; <4% normal forms.

Level of factor	N	Proportion of fertilization	Effect: 95% CI
Morphology <4%	31	0.72	(0.62 ; 0.82)
Morphology ≥4%	58	0.67	(0.62 ; 0.73)

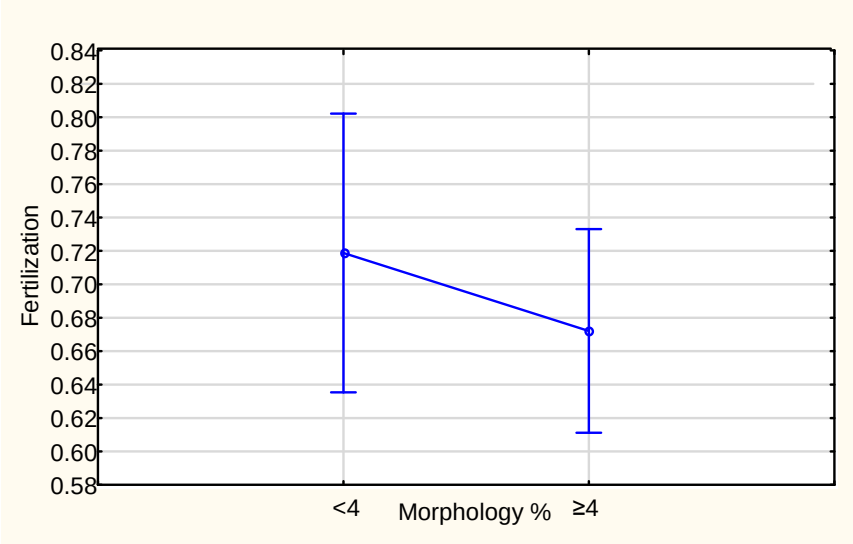


Figure 5.12 Comparison between fertilization of the oocyte according to sperm morphology categories.

Fertilization was compared to the two categories of sperm morphology. The current effect was: F (1, 87) =0.80590, p=0.37 Mann-Whitney U p=0.29, not significant. This is due to the intervention of intracytoplasmic sperm injection which was applied for every patient, regardless of diagnosis of male fertility (fig 5.12, table 5.6).

From a clinical point of view, morphology seems to corresponded to diagnosis of infertility as can be seen in fig 5.13. The primary infertility category presented with the highest percentage (84%) of abnormal sperm (<4% normal forms).

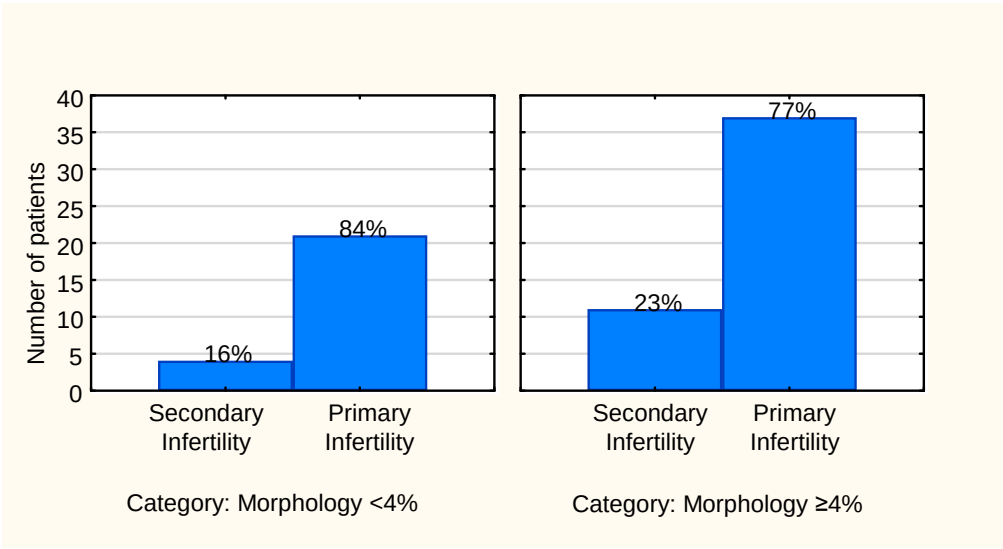


Fig 5.13 Diagnosis of infertility compared to the categories of male fertility (n=89).

5.7.3 Fertilization results for sperm chromatin packaging

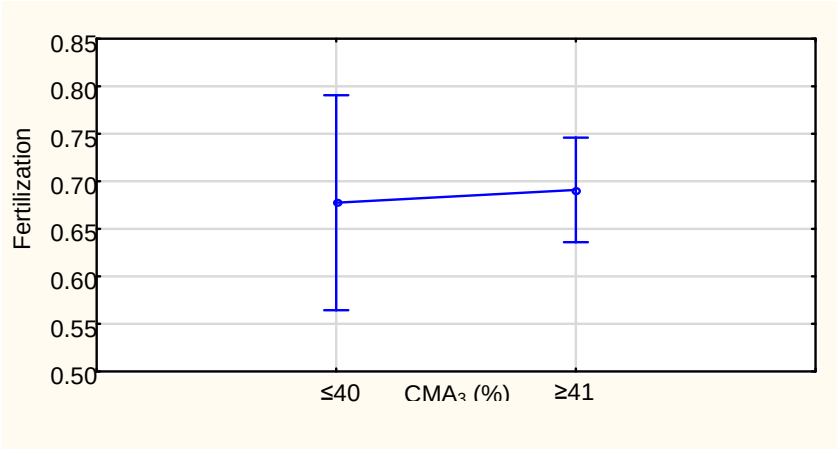


Fig 5.14 Fertilization rates between spermatozoa in the groups of CMA_3 ≥41% and ≤40%.

There was no significant difference in fertilization rates between CMA3 categories with ≥41% immature DNA and ≤40% immature DNA (current effect: F (1, 87) = 0.04524, p=0.83 Mann-Whitney U p=0.74). This could confirm suggestions to perform ICSI on spermatozoa populations with higher than acceptable CMA3 values (exceeding 40% immature DNA and

chromatin packaging problems) (Esterhuizen et al., 2000; Zini et al., 2001; Nasr-Esfahani et al., 2007).

5.7.4 Fertilization results for zona score

Table 5.7 Comparison of fertilization factor categories with respect to zona score.

Factor	**Category**	**Effect**	Linear prediction ***Mean (95% CI)**	**p-Value**
Fertilization	No		21.21 (18.37 ; 24.05)	**0.04**
	Yes	-2.01	19.20 (16.60 ; 21.79)	
CMA_3	≤40%		20.02 (17.48 ; 22.55)	0.29
	≥41%	0.08	20.04 (17.52 ; 22.57)	
Morphology	<4% Normal		20.06 (17.52 ; 22.61)	0.75
	≥4% Normal	0.08	20.13 (17.58; 22.70)	
*** Random effects General Least Squares (GLS) regression**				

Table 5.6 provides a brief overview of the main factors involved in fertilization when compared to ZS. Firstly, random effects GLS regression linear predictions were perfomed on the effect of the ZS, the sperm morphology and the immature DNA on fertilization (table 5.7). The ZS revealed a GLS linear regression with p=0.04 to fertilise when the ZS was 19.1972 (±95% CI: 16.60 ; 21.79). The GLS linear regression also confirmed that neither sperm morphology or immature DNA was indicative of fertilization when ICSI is the intervention of choice.

5.7.5 Fertilization rates for meiotic spindle

Random effects: logistic regression was employed to measure the effect on the presence or absence of the meiotic spindle in fertilization, OR was used to illustrate the effect. When the SPp, there was an OR of 1.4: 1 to fertilize (95% CI ± 1.011 ; 1.95), standard error 0.24 and p=0.04, which was significant.

Table 5.8 Comparison of fertilization factors by odds ratio's.

Fertilization	**Odds Ratio**	p-Value	[95% Conf. Interval]
Zona Score	0.99	0.37	(0.98 ; 1.01)
Meiotic Spindle	**1.50**	**0.01**	**(1.10 ; 2.00)**
Immature DNA (CMA3≥41%)	0.85	0.61	(0.45 ; 1.61)
Morphology <4% normal forms	1.46	0.20	(0.82 ; 2.62)

Random effects logistic regression was performed on fertilization and was expressed in odds ratios (OR) results favoured the meiotic spindle as a good indicator for fertilization when it was present (table 5.8).

Zona score was not a reliable factor for OR since the data is based on specific but varying values, p=0.37. OR was performed on the SP to determine its relevance to fertilization, its presence in the oocyte prior to ICSI, will likely result in fertilization 1.5:1 times more than when it is absent, p=0.01. Random effects logistic regression would imply that the 'subfertile' fraction of the spermatozoa (morphology <4% normal forms) had a 1.5:1 times more likelihood to fertilise than the normal group, but the p=0.2 is insignificant. Linear predictions for the ZS revealed that a ZS of 19.2 µm (95% CI± 16.603; 21.791) with p=0.04 was significant in predicting fertilization, however, the logistic regression OR found it to be insignificant with 0.99:1 likelihood to predict fertilization; p=0.37. Neither did immature DNA have a positive predictive effect on fertilization, with OR = 0.85:1, p=0.61. If Immature DNA is compared to fertilisation, and expressed as an OR, it seems to have an inhibiting effect on fertilisation, as suggested by authors (Esterhuizen, et al., 2000).

5.7.6 Embryo development according to meiotic spindle (Day 1- 5)

The results on embryo development and pregnancy rates did not include all of the embryos that were in culture or frozen-stored for future use, only the embryos that were utilized in embryo transfers were used for statistical analysis. Nominal values differ in accordance to relevant information available per patient couple.

Embryo development according to presence (1) or absence (0) of meiotic spindle

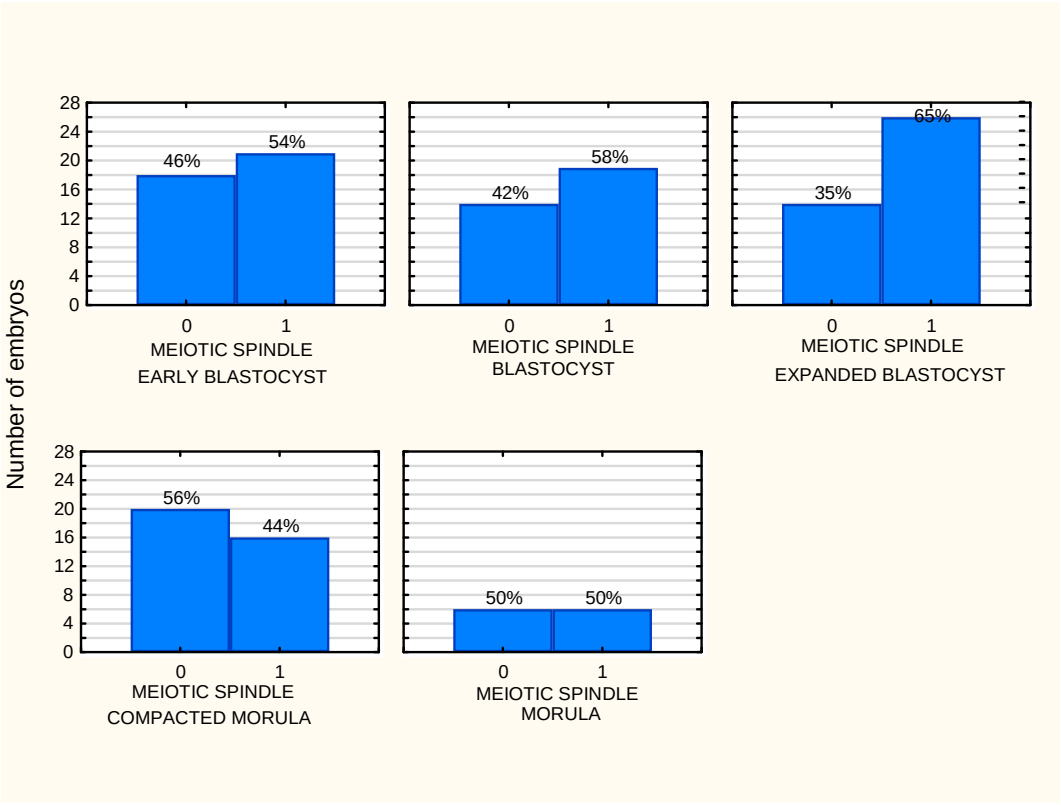


Fig. 5.15 Categorized histogram based on presence of meiotic spindle and day 5 embryo development.

Both fig 5.15 and fig. 5.16 show positive effects with embryo development on day 5 and the presence of a meiotic spindle; the automated zona scores also correlated with more good quality embryos. The data on embryo development only includes the embryos that were selected for embryo transfer, and not all of the embryos that were cultured, therefore due to the small sample size of embryos eventually selected for embryo transfer the ‘p’-values were not found to be significant in this regard.

5.7.7 Embryo development according to zona scores (Day 1- 5)

Embryo development according to software automated zona scores

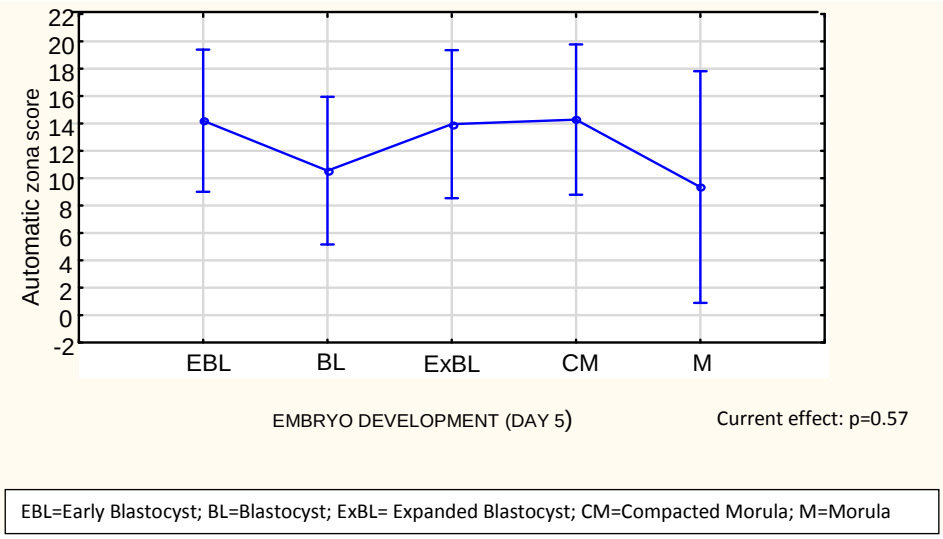


Fig. 5.16 Quality of embryos transferred on day 5 with corresponding zona scores.

The mean zona scores (95% confidence intervals) is represented over the categories of embryo quality on day 5 for embryo transfer in fig 5.12. Statistically, the groups do not differ from each other, clinically, it is relevant to note that the lowest quality of embryo for ET derived from the lowest mean ZS.

5.8 Pregnancy results

5.8.1 Overall pregnancy rates for study population

Table 5.9 Pregnancy and biochemical pregnancy rates of the study.

Pregnant	Percentage
No	60.67% (54/89)
Yes	39.33% (35/89)

One hundred and fifty eight (158) embryos were transferred on day 5 with an average of 1.76 embryos transferred per couple. Eighty nine (89) couples received day 5 embryo transfers (ET); whilst four couples (4.21%) received day 3 ET's and two couples (2.11%)

did not receive embryo transfers due to hyper stimulation & uterine lining too thin on the day of oocyte harvest. In the latter cases, all of the viable day 5 embryos were frozen for future use in natural frozen-thawed embryo transfer cycles. The pregnancy rate for the study was 39.33% (table 5.9).

5.8.2 Pregnancy results for race

Table 5.10 Pregnancy rates by racial groups in the study population (n=70).

Race	**Pregnancy rate**	**Fisher's exact & p-Value**
Caucasian	40%	
African	37.14%	1.000

Pregnancy rates did not differ between the racial groups (70 couples that were defined according to race, received embryo transfers, table 5.9) with 40% (14/35) of the Caucasian group falling pregnant, and 37.14% (13/35) of the African group.

5.8.3 Pregnancy results for infertility status

Table 5.11 Pregnancy rates of primary and secondary infertility based on column and row percentages.

Diagnosis	Frequency	**Pregnancy rate by diagnosis**	**Fisher's exact & p-Value**
Primary Infertility	79.73% (59/74)	35.59% (21/59)	
Secondary Infertility	20.27% (15/74)	53.33% (8/15)	0.17

The overall pregnancy rate was 39% (table 5.8) and is well within world standards, especially when patient population and intervention (ICSI) is taken into account. The secondary infertility group was expected to have less 'subfertility issues' than the primary group due to previous conception and birth of a child, and played a role with pregnancy rates of 53% and 35% respectively (Table 5.11) p=0.17, not statistically significant

Pregnancy rates based on diagnosis of infertility

Table 5.10 demonstrates the subfertility categories in the diagnosis of infertility with their individual pregnancy rates. No statistical significance was detected, p=0.22.

Table 5.12 Intra- and inter subfertility pregnancy rates (n=75).

Subfertility	Frequency by population	Pregnancy rate per category	Fisher's exact & p-Value
Idiopathic (Unexplained) Infertility	9.33% (7/75)	71.43% (5/7)	
Male Infertility	25.33% (19/75)	31.58% (6/19)	
Female Infertility	20% (15/75)	26.67% (4/15)	
Combined Infertility	45.33% (34/75)	41.18% (14/34)	0.22

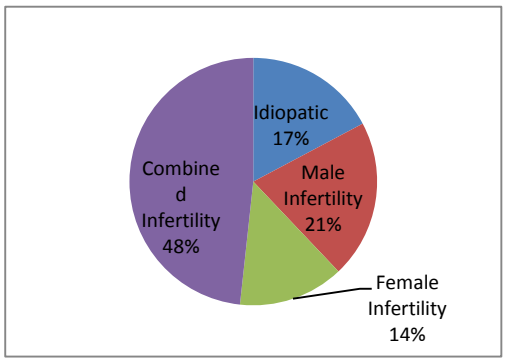


Fig 5.17 Pie chart to illustrate how subfertility groups contributed towards the overall pregnancy rate (n=29 pregnant couples from 75 couples whose infertility status is known).

5.8.4 Pregnancy results for sperm morphology

Table 5.13 Pregnancy rates according to spermatozoa morphology categories (n=93; n=87).

Morphology	Frequency	*Pregnancy rate by morphology category	Fisher's exact & p-Value
≥4% normal forms	63.44% (59/93)	38.89% (21/54)	
<4% normal forms	36.56% (34/93)	36.36% (12/33)	1.00
*** 'n' value differ because 89 couples received embryo transfers, for which**			
87 couples' morphology information and pregnancy test was concurrently available.			

Table 5.13 indicates the presentation of, and distinction of the WHO (2010) recommended morphology groups: <4% normal forms yielding a pregnancy rate of 36.36% (representing 36.56 % of the entire study population; n=34/93); and ≥4% normal morphology group had a similar pregnancy rate of 38.89% and represented 63.44% of the population; n=59/93).

OR for morphology (<4% normal forms) when logistic regression was employed to measure its effects on pregnancy, was insignificant (p=0.81) to predict likelihood of pregnancy with 0.8:1. The intervention was ICSI; therefore, poor morphology could not correlate with pregnancy prognosis since morphological normal spermatozoa were selected for injection into the oocyte's cytoplasm.

5.8.5 Pregnancy results for sperm chromatin packaging

Table 5.14 CMA_3 categories compared to pregnant and non-pregnant groups (n=93; n=84).

CMA_3	Frequency	***Pregnancy rate by CMA_3 category**	**Fisher's exact & p-Value**
≤40% Immature DNA	25.81% (24/93)	31.82% (7/22)	
≥41% Immature DNA	74.19% (69/93)	41.94% (26/62)	0.46
*** 'n' value differ because 89 couples received embryo transfers, for which**			
84 couples' CMA_3 information and pregnancy test was concurrently available.			

CMA_3 values were categorized according to recommendations given by Esterhuizen et al. (2000) as groups with ≤40% immature DNA (represented 26% of the population; n=22/84) with a pregnancy rate of 31.82%; and groups with ≥ 41% immature DNA (representing 74 % of the entire study population; n=62/84) yielded a pregnancy rate of 41.94% (table 5.14). Clinically it is significant to note that 78.79% of the pregnant couples' semen derived from the >41% immature DNA populations. Although CMA_3 results were recorded for 95 couples, only 84 couples' CMA_3 values could be compared to pregnancy results. The variance in nominal value was statistically compensated for.

The results highlight that ICSI is a powerful method to be utilised in male factor infertility since no statistical difference was noted in pregnancy when distinction was made between spermatozoa with immature DNA of more than 41% or less than 40% (p=0.46). It is significant to note, that 74% of males in the study population had CMA_3 values in the ≥41% immature DNA chromatin packaging groups.

Logistic regression compiled the OR for immature DNA (CMA_3≥41%) to predict pregnancy to be likely with odds of 1.6:1, p=0.39.

5.8.6 Pregnancy results for oocyte maturity

Table 5.15 Pregnancy rates and maturity of the oocytes per embryo transfer (n=89 ET's).

Maturity	Frequency (proportion)	Pregnancy rate per category	Maturity contribution to overall pregnancy rate	Fisher's exact & p-Value
Germinal Vesicle (GV)	14.61% (13/89)	15.38% (2/13)	5.71% (2/35)	
Metaphase 1 (M1)	12.36% (11/89)	27.27% (3/11)	8.57% (3/35)	
Metaphase 2 (M2)	73.03% (65/89)	46.15% (30/65)	85.71% (30/35)	0.095

Table 5.15 reflects the maturity of the oocytes on the day of TVOH and the embryos transferred on day 5 for all of the 89 couples that received ET's. From this breakdown, it is

clear that from the 35 couples that were pregnant in the entire study, M2 oocytes represented 73% of the embryos transferred on day 5 and led to pregnancy in 46% of cases. The M1 and GV oocytes that were initially immature were in vitro matured and ICSI commenced on day 1 of cell culture. The pregnancy data for M1 and GV was derived from these embryos that resulted after initial immaturity and after prolonged embryo culture (day 6).Although marginal, it is significant to note that the immature oocytes (M1 & GV) resulted in much lower pregnancy rates (27% & 15%) after initial in vitro maturation and ICSI on the following day (p=0.095).

5.8.7 Pregnancy results for zona score

Automated zona scores of 768 oocytes were evaluated. Some 18.62% or 143 oocytes exhibited negative scores (ranging from -0.1µm to -11.8µm) and were deemed undetectable by the system. This was less than the third of oocytes that Ebner et al. (2010) reported undetectable to the OCTAX software. Random effects generalized least squares (GLS) regression was employed to model variation within a patient or group on the remaining 625 (positive scores) ZS from 90 patients (n=90). Results tabulated in table 5.16.

Table 5.16 Average overall automated zona scores for all oocytes with positive zona scores.

Population	Number of oocytes (N)	Number of couples (n)	Zona score (µm)	95% Confidence Interval	Fisher's exact & 'p'-Value
Overall	625	90	18.96	(15.75 ; 22.16)	
Pregnant			21.27	(17.24 ; 25.31)	
Non-pregnant			18.96	(15.75 ; 22.16)	0.38

When random effects GLS regression was employed once more, on only the particular oocytes that gave rise to the embryos that were selected for embryo transfer on day 5 of embryo culture, the following results were recorded in table 5.16.

Table 5.17 Zona scores for transferred embryos only (day 5 development).

Population	Number of oocytes (N)	Number of couples (n)	Zona score (µm)	95% Confidence Interval	Fisher's exact & 'p'-Value
Overall	158	76	18.10	(14.46 ; 21.73)	
Pregnant			17.44	(12.82 ; 22.07)	
Non-pregnant			18.10	(14.46 ; 21.73)	0.83

5.8.8 Pregnancy rates for meiotic spindle

OCTAX PolarAIDE software detected 384 meiotic spindles (SP) present in 730 oocytes (568 x M2 oocytes + 162 x M1 oocytes excluding GV data (126 oocytes)) ≈ 52.60 % SP. GV's do not yet possess the ability for the SP fibres to form since it is suspended in the diplotene phase of meiosis I.

Table 5.18 Pregnancy rates between meiotic spindle present and meiotic spindle absent oocytes following day 5-embryo culture and transfer.

Diagnosis	Frequency	Pregnancy rate by meiotic spindle	Fisher's exact & p-Value
Meiotic spindle absent	44.30% (70/158)	37.14% (26/70)	
Meiotic spindle present	55.70% (88/158)	40.91% (36/88)	0.38

Of the 158 embryos transferred on day 5, embryos derived from 36 oocytes with SP present on day 0 resulted in pregnancies with a pregnancy rate of 41%, not significantly higher, however, to the SPa group with a similar PR of 37%, p=0.38 (table 5.18).

Random effects logistic regression was performed on 158 embryos that were transferred and found that SP present was not a prognostic marker (p=0.84) for pregnancy with an OR of 0.6:1 to fall pregnant. When random effects logistic regression was performed on all oocytes (n=768) from 95 patients (N=95), SP present was an indicator of pregnancy with OR 1.4:1 (p=0.87), however, the 'p' value is not significant.

5.9 Hypothesis

Hypothesis odds ratio

Logistic regression was employed to test the hypothesis, should all the factors controlling oocyte quality be controlled (i.e. maturity, SP present & automatic ZS) as well as spermatozoa morphology (since intervention is ICSI), failure to fertilise and subsequently, to fall pregnant, would be due to immature DNA. A last OR was performed, considering the immature DNA as predictor of pregnancy, in the event of mature oocytes that were injected, those exhibited SP and were the actual embryos transferred, had an OR of 1.9:1 to predict pregnancy (p=0.81). However, the ‘p’ value is not significant.

Chapter 6: Discussion, Limitations, and Future Research

6.1 Introduction

For most couples of reproductive age (15-49yrs), having a baby seems to be the easiest and most natural thing to do. For the approximately 15% of couples who suffer from some form of subfertility, it can become the most difficult and emotionally taxing 'journey' that they might ever embark on (Rutstein & Shah, 2004; Huyser & Boyd, 2012). The inability to become pregnant can lead to a variety of emotions, including anxiety, depression, anger, shame, and guilt (Chen, et al., 2004; Rutstein & Shah, 2004; Huyser & Boyd, 2012). Anxiety disorder (23% prevalence) and major depressive disorder (17% prevalence) were two of the main psychiatric diagnoses made in some 40% of infertility patients suffering from psychiatric disorders from a study conducted by Chen, et al. (2004). Huyser & Boyd (2012) puts the emotional trauma experienced by the childless infertile couple presenting at infertility clinics into context as "…a condition whereby a couple cannot contribute to the future of a community within a social structure where children signify security and continuance of life." In modern society both men and women are expected to excel in their careers while balancing it socially, however, the same cultural expectancies of childbearing and -raising still remains as much a focus as in pre-modern times (Rutstein & Shah, 2004).

The South African landscape of infertility treatments and techniques is extremely dynamic, and interestingly, predominantly in the hands of private practice. Patients are responsible to pay for services offered (Medical insurance companies do not pay for ART, except for one medical aid, CAMAF; Government offers ART treatment to qualifying couples at subsidized prices, however the opportunities are limited and only available at a small number of tertiary institutions) (Huyser & Boyd, 2012). South Africa recently experienced an increase in 'reproductive tourism' with a substantial influx of couples from neighbouring African countries (e.g. Mozambique, Angola, DRC), and the rest of the world (the list is virtually endless). The 'tourism' can be attributed to a number of factors including (i) a lack of adequate and reliable health services in their own countries, (ii) certain foreign governments (via their embassies) paying for ART; (iii) favourable exchange rates, and (iv) less strict human tissue legislation in South Africa. The current study involved 95 couples undergoing ICSI at Wilgers Infertility Clinic, a private infertility clinic located in Pretoria, Gauteng, South Africa, as part of their routine fertility treatment. The mean pregnant maternal age was 34.16 years (SD±4.6) and the non-pregnant group was aged 34.6 years (SD±4.7). This was not statistically relevant. In this study, maternal age was demonstrated not to be a factor to hinder fertilization or pregnancy; however, maternal age still is and in

all probability will remain one of the biggest factors in the success of ART (Rienzi, et al., 2011; Valeri, et al., 2011).

The racial distribution for this study was distributed evenly, with equal presentation of African (48.65%) and Caucasian couples (51.35%). The PR for both racial groups were similar in this study, African women had a PR of 37.14% and 40% of Caucasian women were pregnant after ICSI was performed. A recent press release from the office of The American Society for Reproductive Medicine (ASRM) indicated that in the United States (US) a large cohort study of 4000 women were undertaken to determine pregnancy rates between both major racial groups of African(-American) and Caucasian women. The study conducted from 2010-2012 astounded Chicago fertility specialists with 30.7% PR for Caucasian women after ART and only 16.9% PR for women of African heritage with ART (ASRM Office of Public Affairs, 2014). Researchers investigated sexual health patterns and knowledge between the two main target groups, and despite much research regarding sexual transmitted infections-history and neglected urinary tract infections, could not explain the lowered pregnancy rates for African women. This specific racial PR trend has been observed at our laboratory as well in the past. Factors influencing the results of this study could possibly be attributed to, the random convenient sampling nature of the study, which somehow resulted in the unexpected equal racial group pregnancy results, or the excellent patient-treatment diagnosis combined with ICSI intervention could have been a causative factor.

6.2 Study population profile

Keeping track of the patient population of an IVF clinic is important in establishing the specific ‘niche’ market and population the clinic caters for. Equally as important as the presentation of race at IVF clinics, is the diagnosis of primary or secondary infertility and distinction of specific type of subfertility that the couple experience. In order to ‘tailor’ the specific assisted reproduction treatment for the couple, the infertility workup must be thorough and all information recorded, and evaluated prior to commencement of the ART cycle.

The ‘infertility jargon’ and vocabulary have confused many patients, and even healthcare providers, to such an extent that the European Society for Human Reproduction and Embryology (ESHRE) called a special taskforce to life, the European Classification of

Infertility Taskforce (ECIT), in order to simplify existing terminology (Jenkins, et al., 2004). Certain misconceptions exist regarding the definition of 'infertility', and it is often erroneously referred to as sterility (Rutstein & Shah, 2004). Rutstein & Shah (2004) describes the common definition of 'infertility' to be the "inability to carry a pregnancy to a live birth," a statement that does not clearly distinguish between a permanent fertility issue (sterility) or a temporary fertility issue (subfertility). Zegers-Hochschild et al. (2009) authored a revised glossary on ART terminology as yet another taskforce, The International Committee for Monitoring Assisted Reproductive Technology (ICMART), this time for the WHO, was formed in 2009. The authors expressed 'infertility' as "…a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse", thereby including a time-frame as part of the explanation (Zegers-Hochschild, et al., 2009). ECIT (Jenkins, et al., 2004), however, eloquently clarifies the misapprehension by likening 'infertility' to any other physical symptom such as abdominal pain or bleeding which is associated with a temporal element that can be of short (i.e. acute) or long (i.e. chronic) duration. In this context it is considered to represent a 'relative' condition rather than 'sterility' which is an 'absolute' condition." Jenkins, et al. (2004) suggests investigation of the 'absolute' or 'relative' cause of infertility to be emphasized in the patient workup.

Diagnosis of infertility, primary or secondary, played a role with primary representing 80% (59/74 couples) and secondary 20% (15/74 couples) of the study population. The PR for primary infertility was 35% and 53% for secondary infertility, with the secondary infertility group-performing better. This higher PR to the secondary infertility is attributed to the group having less serious subfertility issues compared to the primary group, since these couples have already had a child born previously.

According to the latest report issued by the National Institute for Health and Care Excellence (NICE) in February 2013, the proportions of subfertility in the UK were found to consist of 25% idiopathic (unexplained) infertility, 30% male infertility and 45% female infertility, which was split between ovulatory disorders (25%), tubal infertility (20%), and uterine or peritoneal disorders (10%). Subfertility was diagnosed as combined infertility in at least 40% of couples (NICE, 2013). The results from this study was compared to the study conducted in the UK (NICE, 2013) and presentation of subfertility proved to be quite similar. In the present study, male infertility presented in 25% of couples vs. NICE's 30% in the UK. Female infertility was found to be present in 20% of couples vs. 25% (NICE, 2013).

Idiopathic infertility was diagnosed in only 10% of couples visiting Wilgers Infertility Clinic vs. 25% in the UK study. Idiopathic infertility is commonly referred to as failure to determine actual cause for infertility. Combined infertility was present in 45% of couples in the present study vs. 40% in the NICE (2013) study. Both studies had similar male infertility of 70 %(25% male infertility + 45% combined infertility in the present study; NICE had 30% male infertility + 40% combined infertility). Similarly, both groups also found 65% female infertility.

The PR harmonized with the presentation of the type of subfertility. Idiopathic infertility presented as 9.33% of the study population (7/75 couples) with the highest individual subfertility PR of 71.43% (5/7 couples were pregnant) and an overall subfertility category PR of 17% (5/29 pregnant couples). Nevertheless, the PR for this group is remarkable at 71.43%. The low overall subfertility PR of 17% is attributed to the low numbers of couples assigned to this category, only 7 from 75 couples presented with idiopathic infertility.

Male infertility consisted of 25.33% of the population with a subfertility category PR of 31.58% (6/19 couples where the male was sub-fertile) and a category PR of 20.69% (6/29 couples in the overall PR). Female infertility presented as 20% of the population (15/75 couples) with a PR of 26.67% (4/15 couples presenting with female fertility only, became pregnant) and the overall category PR was a mere 13.79% (4/29 pregnant couples).

Both male and female infertility groups performed poorly in relation to the overall PR. The poor PR of female infertility at 13.79% highlights the belief that the oocyte's competency is crucial for success in ART therapies, more so than the competency of the spermatozoon. The obvious impact of oocyte competency vs. the spermatozoon's integrity is highlighted especially since this laboratory performs ICSI as standard intervention for all patients. Duly noted, however, was the presentation of 74.19% of spermatozoa populations in this study with $CMA_3 \geq 41\%$, necessitating ICSI as only treatment option.

Finally, combined infertility proved to be the most successful group comprising 45.33% of the entire population (34/75 couples) with a PR of 41.18% (14/34 couples presenting with combined infertility were pregnant) and contributing to 48.28% of all pregnancies (14/29 of all pregnant couples). This subfertility group performed surprisingly well, and suggests that

in cases of combined infertility, where COS with ICSI is the only method of treatment in most cases, it is an effective technology to apply.

The overall PR of the study was 39.19% (β-hCG quantitative >25 mIU/ml and confirmation of heartbeat after 6 weeks) and excluded all biochemical pregnancies (11% had a 5> β-hCG quantitative <25 mIU/ml value). Overall PR competes well with international clinics; Ebner, et al. (2010) had a PR of 39.1 % in Linz; Austria and Madaschi, et al. (2008) achieved an overall PR of 31% in Sao Paulo; Brazil.

6.3 Oocyte profile

6.3.1 Maturity

Gynaecologists practicing in South Africa, under a specific super-specialization Reproductive Medicine Specialist qualification at the Health Professionals Council of South Africa (HPCSA) use COS protocols to manipulate the ovaries through hormonal supplementation in order to produce supernumerary oocytes simultaneously. COS is said to produce oocytes of varying quantity and maturity (Keefe, et al., 1997; Khalili & Mojibian, 2005). Patients' hormone levels were monitored on day 3 to ensure that the patient population was capable of recruiting 'good' quality oocytes. The mean hormone levels were as follows: FSH 8.34 ± 4.6 IU/ml; LH 8.84 ± 9.6 IU/ml; AMH 4.22 ± 3.18 IU/ml and Estradiol 166.85 ± 95.73. La Marca, et al. (2011) suggested that AMH was a marker to predict the number of oocytes retrieved and therefore advocated its measurement prior to COS, as a baseline prognostic indicator of success. The normal AMH references ranges are between 1.5 – 4.0 ng/ml. The AMH hormone levels placed the patient population, despite their individual diagnosis, in the good responders' category with COS.

Harvested oocytes generally exhibit different stages of maturation, prophase 1 (GV), metaphase 1 (M1) and metaphase 2 (M2) and is due to a-synchronization in the maturation process occurring in the ovaries (Cha & Chian, 1998; Trounson, et al., 2001; Magli, et al., 2006; Braga, et al., 2010). COS resulted in a mean number of 11.32 (SD = 6.71) follicles that were recruited per patient and resulted in a mean number of 9.07 (SD = 5.14) oocytes harvested per patient, per ultrasound guided oocyte retrieval. La Marca, et al. (2011) suggested that the number of oocytes retrieved and fertilised have been reported to be a reliable prognostic tool for IVF success. In total, 856 oocytes were harvested of which 568 (66%) were M2, 162 (19%) were M1 and 126 (15%) were in the GV stage of development.

The first brief to determine the oocyte's competency for fertilization was the maturity. In this study, only 66% of oocytes harvested complied with the very first criteria for fertilization potential. The couple's sense of emergency for pursuing treatment (Chen, et al., 2004) resulted in mainly the use of short COS stimulation protocols and could be a major contributing factor for the quality of oocyte stimulation and maturity. Half of the population that presented at Wilgers Infertility Clinic had ≥36 months of unprotected intercourse with an inability to fall pregnant before seeking assisted conception.

Various authors all reported decreased pregnancy rates when immature oocytes were in vitro matured, with rates between 22% and 35% (Lanzendorf, 2006; Li et al., 2006; Son et al., 2007). These studies correspond to results gathered from the present study after initial IVM (day 0, the day of TVOH) and ICSI (on day 1 of embryo culture) of immature oocytes: M1 & GV's, the day after TVOH and following IVM culturing, resulting in pregnancy rates of 27% & 15% respectively (p=0.095). Although the "p" value seems to be statistically only slightly significant, it has a definite significant clinical value of interpretation. IVM therefore is a 'need-to-do' in the case of the 'poor-responder' with very few oocytes of compromisable maturity, and in the case of M1, it could have a pregnancy rate of 27% and in the case of prophase 1, it still has a 15% possibility of pregnancy.

M2 oocytes from the present study resulted in 85% of the overall pregnancies and a PR per maturity category of 46%, this is also in agreement with other studies that majority of pregnancies resulted from M2 in vivo matured oocytes (Li, et al., 2006; Braga, et al., 2008).

6.3.2 Birefringent analysis of oocytes

The protocol for oocyte imaging was simple, for each patient, should the TVOH yield >10 oocytes, the 10 most mature (M2) oocytes were imaged. For the patients with <10 oocytes, all oocytes were imaged. For the most part of the PolarAIDE imaging, the recommendations of authors such as (Keefe, et al., 1997; Cohen, et al., 2004; Chamayou, et al., 2006; Montag, et al., 2006; Braga, et al., 2008; Rienzi, et al., 2008; Braga, et al., 2010; Ebner, et al., 2010; Valeri, et al., 2011) was adhered to.

6.3.3 Meiotic spindle

Of the total amount of oocytes harvested (856), only 384 (46%) exhibited meiotic spindles. Realistically, only M2 and M1 oocytes have the ability to exhibit the meiotic spindle (SP) and therefore the accurate number of SP present oocytes was (384/730) 52.60%. Our SP present oocytes 53%, was lower compared to other studies (Madaschi, et al., 2008) that had SP present of 62.8% and 61% SP present for Valeri, et al., (2011). The lowered SP present rate could not be attributed to 'timing' of SP imaging, as this was done at 40-42 hours post β-hCG trigger and SP should have been formed by then. Nor could it have been attributed to the SP microtubules lying in a position not visible to the OCTAX software since imaging was done with a holding pipette so that the oocyte could be rotated if the SP was not obvious in the ooplasm of the oocyte. The author suggests that the lowered SP present rate might be due to differences in COS, our unit predominantly utilizing the short stimulation model and Madaschi et al. (2008) utilizing the long stimulation protocol.

The study of Chamayou, et al. (2006) was the only one highlighted in a large systematic review by Rienzi, et al. (2011) that did not find statistical differences in fertilisation or embryo development due to SP absence or SP presence. In the present study, OR was performed on the SP to determine its relevance to fertilization, its presence in the oocyte prior to ICSI, resulted in fertilization 1.5:1 times more than when it was absent, p=0.01. The remaining 14 papers evaluated by Rienzi, et al. (2011) found that there was a positive effect concerning cleavage of the embryos, as supported by the outcome of this study.

Multiple researchers have investigated the relevance of the presence of the meiotic spindle for fertilisation and embryo development (Rienzi, et al., 2003; Cohen, et al., 2004; Montag, et al., 2006; Braga, et al., 2008; Madaschi, et al., 2008; Kilani, et al., 2010; Kilani, et al., 2011; Rienzi, et al., 2011). Day 3 embryo transfers were excluded from this study since we focused on day 5 transfers and blastocyst formation. In addition, very limited literature on embryo culture beyond 3 day was available since a number of countries have legal restrictions regarding the amount of oocytes to be fertilised and days allowed for in vitro culture (Madaschi, et al., 2008; Montag, et al., 2006; Valeri, et al., 2011). This study clearly demonstrated that when the meiotic spindle was present, the embryos selected for embryo transfer at our clinic on day 5 resulted in a 65% expanded blastocyst rate, with an equally impressive blastocyst rate of 58% and early blastocyst rate of 54%. A negative development competence on day 5 was also correlated to absence of meiotic spindle prior

to ICSI with 56% of day 5 embryos transferred reaching only the compacted morula stage; while 50% of meiotic spindle absent embryos reached the morula stage at time of embryo transfer on day 5. Although there were no statistical differences between the SP present and absent groups, there was slight tendencies for better embryo quality in this study (Rienzi, et al., 2003; Montag, et al., 2006; Braga, et al., 2008; Madaschi, et al., 2008; Ebner, et al., 2010; Kilani, et al., 2011;).

The presence of the meiotic spindle had a slight effect on pregnancy with a PR of 40.91% (36/88, p=0.87) similar to results reported by Madaschi, et al. (2008) and also correlated with the same study that there was only ever so slight a tendency in the presence or absence of SP in the pregnant and non-pregnant groups.

Random effects logistic regression OR performed on 768 oocytes from 95 patients indicated pregnancy to succeed 1.4:1 should the SP be present (p=0.87). The ‘p’ value is not significant due to the large variance in oocyte-to-oocyte and patient-to-patient data, and more research with greater amounts of oocytes are needed to explore the options.

6.3.4 Automated zona pellucida score

The third factor for determining oocyte competence for fertilization also includes the detection and automated scoring of the zona pellucida (ZP) by the OCTAX PolarAIDE software. Several studies classified the automated zona score (measured in micrometres µm) into two categories; i.e. ZP scores <10 as low zona birefringent (LZB) or ZP>10 as high zona birefringent (Valeri, et al., 2011). Madaschi, et al. (2008) evaluated the ZS for 100 oocytes to establish a median value of 5.5 which they used as a base-line reference for HZB (ZS>5) and LZB (ZS <5). Valeri et al. (2011) reported a LZB score of 43% and HZB score of 57%, much like the 40% LZB score and 60% HZB scores obtained in the present study.

According to the literature, oocytes with a HZB would be the ones most likely to fertilize and develop into the quality embryos that produce pregnancies based purely on scores of the ZP (Assidi, et al., 2011; Ebner, et al., 2009; Montag, et al., 2008). Ebner, et al. (2010) found a difference in ZS due to differences in ovarian stimulation protocols. They reported HZB to

be more prevalent in the long stimulation protocol, with the short stimulation protocol achieving lower ZS (Ebner, et al., 2010). When the average automated zona score was calculated for all the oocytes imaged with the OCTAX PolarAIDE software (n=625 positive ZS from 90 patients), the mean average automatic zona score was 18.96µm (15.75; 22.16).

Random effects GLS regression linear predictions were performed on the effect of ZS, morphology and immature DNA on fertilization. The ZS revealed a GLS linear regression with p=0.04 to fertilise when the ZS was 19.20 µm (16.60; 21.79). No linear predictions could be made for the impact of neither spermatozoa morphology nor immature DNA by CMA_3 with ZS and fertilisation as impact. ICSI is the insemination method of choice since the oocytes are denuded completely from all surrounding cumulus and granulosa cells to enable zona and SP imaging. When ICSI is performed, the embryologist gives the selected spermatozoon an unfair advantage, by injecting a morphologically suitable spermatozoon into the oocyte's ooplasm, bypassing the normal cumulus interactions and zona binding route that spermatozoa would have had to configure. The surrounding corona radiata and cumulus oopherus cells together with the structure of the ZP are nature's natural barriers to prevent unsuitable spermatozoa access to fertilise an oocyte (Zini & Libman, 2006).

Several studies also assessed the prognostic value of the structure of the ZP in fertilization, embryo development, and pregnancy (Keefe, et al., 1997; Madaschi, et al., 2008; Braga, et al., 2010; Ebner, et al., 2010; Rienzi, et al., 2011). Automatic ZS were not found to significantly influence embryo development during this study either, what was notable however, was that embryos that did not develop past morula stage on the day of embryo transfer (day 5) had low birefringent ZP scores of < 10. Ebner, et al. (2010) could prove a correlation between blastulation and ZS but not between ZS and fertilisation, embryo quality or pregnancy. The present study proved a statistical association between ZS and fertilisation (p=0.04) when the ZS was HZB with a mean of 19.20 µm (16.60; 21.79). The present study could also not confirm a statistical difference between ZS of the pregnant and non-pregnant groups, much the same as was found by Ebner, et al. (2010).

Factors summarizing oocyte competence for fertilization prior to intervention: ICSI

1) Oocyte maturity = 66% M2 oocytes with polar bodies extruded;
2) Presence of Meiotic spindle = 53%;
3) Oocytes in the 'preferred' HZB range= 60%

According to criteria, research objective 1 answers the question of the competence of the oocytes harvested to be able to be fertilized as 53% [three factors divided by three and multiplied by 100 = [(66+53+60/3*100=59.67% ≈60%)]. This discovery of competency of an oocyte according to its morphologic structures, including nuclear maturity, presence of meiotic spindle and the soundness of the structure of the ZP only yielding a 60% chance of fertilization competency, leaves the researcher at a dilemma when confronting the three main criteria for this study to control for oocyte-fertilization-competency, however, similar results were found by other authors (De Sutter, et al., 1996; Balaban, et al., 1998; Chamayou, et al., 2006;). It could also be argued that the oocyte can only give 50% chance for fertilisation, since without the complement of a morphologically normal spermatozoon, no physiological fertilisation can occur, in which case, 60% would be sufficient oocyte competence? Some authors did find significant evidence for oocyte morphological factors to play a role in fertilization (Khalili, et al., 2005; Rienzi, et al., 2008; Rienzi, et al., 2011).

6.4 How does the oocyte data reflect on the hypothesis and spermatozoa profile?

6.4.1 Sperm morphology and Chromatin packaging

The percentage of normal spermatozoa morphology for the entire sperm population was recorded to have a mean of 6.39 % normal forms (SD±5.35), which would place the sperm population as a whole in a favourable category concerning morphology, since WHO (2010) guidelines amended the lower reference limit for fertile sperm as ≥4% normal forms and <4% normal forms for male subfertility. Morphology was compared to fertilization, to measure a possible effect, but as could be expected from an intervention where ICSI is applied, there was no indication that morphology could predict fertilization.

Spermatozoa with ‘normal’ (≥4%) morphology and ‘sub-fertile’ (<4%) morphology categories were also compared to pregnancy. As was expected once again, due to ICSI as intervention, there was no measurable effect of morphology (good or poor) on pregnancy, with no statistical difference in results for the two groups: ≥4% morphology (38.89%) and <4% morphology (36.36%), p=1.000.

Primary infertility indicates that neither partner in the couple has conceived a child before. What was not to be expected was that despite the mean percentage normal morphology

was >6% normal forms for the entire population, there was not a significant difference in diagnosis of infertility. Males that had ≥4% normal spermatozoa (77%) still faced primary infertility. The researcher believes that this could be due to elevated CMA_3 values (74% of the population), caused by lifestyle factors and ROS in the testis environment, aging (mean male age was 38.5 years, SD±6.2), as well as due to oocyte-, and female fertility issues, rather than sperm morphology. The data does seem to point to issues of competence of the oocyte to fertilize or immature sperm DNA, rather than spermatozoa morphology when ICSI is involved.

The amendments to the WHO (2010) guidelines included the lowering of the morphology reference value to ≥4% normal forms according to the Tygerberg Strict Criteria, and this has received much criticism. Prior to the WHO 2010 recommendations, ≥15% morphologically normal sperm as evaluated using Strict Criteria would have been considered as a normal sperm population. With this study as an example, it would have classified 87% of the present study's population as possessing lower than normal sperm morphology. Results from Esterhuizen et al., (2000) corresponds to rates achieved in the current study, with 41 males presenting with semen samples with a mean morphology of 4.0% and within range 3-5% and corresponding CMA3 mean of 80.9% with a range of 77-83% immature DNA. Instead, the 'new' lower reference values, now includes 63% of the study population to be considered to have normal sperm morphology and only 37% to be classified as morphologically abnormal (vs. 87% according to the previous classification system).

Barratt, et al. (2011) argues that by amending the reference limits according to the semen characteristics of fertile men, the WHO has made the error of comparing fertile men, with those who aren't. The researcher of this manuscript agrees with the findings of Barratt et al. (2011) and points out that due to the lowering of reference values from ≥15% normal morphology to only ≥4% normal morphology had an implication on the results of the entire study population of this study. Under the previous 4th WHO laboratory manual, 87.10% (n=81/93) of the male participants would have been classified as having male subfertility in the form of teratozoospermia. This is on par with the 74% of the male participants presenting with CMA_3 values exceeding 40% immature DNA. Increased CMA_3 values have in the past correlated with poor sperm morphology (Esterhuizen, et al., 2000). In the study conducted by Esterhuizen et al. (2000), ± 81% of men presented with a ≥81% immature DNA and the mean of these men's semen samples had 3-4% normal forms, with a mean sperm morphology of 4%. Under the current lower reference limits for morphology, the tables have turned considerably, and instead of the majority of patients falling in the group

of subfertility, 63.44% of male participants now qualify to be included in the fertile group as far as normal sperm morphology goes. It is staggering, since the best treatment option for these couples would always have been ICSI, since damage to chromatin packaging would have inhibited natural fertilisation as well as conventional IVF. These men would have been highly unlikely to succeed in getting their partners pregnant naturally. This is specifically the reason why they sought medical assisted reproductive methods out, and yet, they are classified as falling in the 'normal' sperm morphology group. These reference values need to be revisited with the focus on a clear distinction between reference values for fertile men (partners pregnant within 12 months of unprotected intercourse) and sub-fertile men (whom did not succeed in pregnancy within 12 months, in absence of a gross female subfertility).

However, several authors have in years prior to the WHO reference value update, published works of high scientific value that supports the lower morphology reference value of 3-4% normal sperm morphology using Tygerberg Strict Criteria (Ombelet et al., 1997; Guzick et al., 2001 ; Gunalp et al., 2001 ; Menkveld et al., 2001; Cooper, et al., 2010). Many of these earlier works were based on the sperm found in the endocervical tract, the uterus, and the Fallopian tubes (site of fertilisation). These sights are associated with lower amounts of spermatozoa, and supposedly higher motility and of higher normal morphology than the ones not able to scale the journey from semen deposition in the vagina (Sakkas, et al., 2015).

The author of this manuscript does not by any means discredit the valuable research presented by such reputable researchers, but only seeks to provoke thought as to why the lower reference values should be considered as 'normal' in a sub-fertile population.

The second research objective was to determine if there was a correlation between the percentages of immature DNA (chromatin packaging via CMA_3) in the sample of spermatozoa that might hinder fertilization and a possible pregnancy in a controlled sperm morphology environment such as the case with ICSI.

The study results strongly support the research hypothesis that when ICSI is applied, normal morphology spermatozoa is selected for insemination. Since the mean percentage of normal spermatozoa in the population was >4%, the researcher had a slightly less

difficult ICSI to the extent of sperm selection and provides the hypothesis assumption that fertilization failure (and subsequent pregnancy failure) would be due to incorrect chromatin packaging.

The mean average for immature DNA in the spermatozoa sample was calculated to be 54% (SD±21.66). This is considerably higher than the suggested value of ≤40% immature DNA or more correctly, damage of spermatozoa chromatin packaging. Furthermore, 74% of the study population had >41% CMA_3 values which relates to immature sperm DNA and chromatin packaging. Results did not differ between categories for chromatin packaging ≥41% or ≤40% when compared to fertilization. CMA_3 ≥41% immature DNA had a mean fertilization rate of 0.69 (SD±0.24) and CMA_3 ≤40% had a fertilization mean of 0.68 (SD±0.22).

Couples were the male has DNA damage higher than the norm, have been reported to have a decreased natural fertility and has a longer time to pregnancy (TTP) than the average population (<12 months of unprotected intercourse) (Spano, et al., 2000; Zini, et al., 2001) and this was evident from the present study's male population. The majority of couples presented with immature DNA greater than 40% and the average time before attempting fertility consultation was >36 months. The high amount of couples presenting with semen analysis with CMA_3 values ≥41% also firmly supports Wilgers Infertility Clinic's protocol of standard ICSI for all couples attending our fertility clinic, since 74% of our population presented with increased immature DNA.

The research objectives of this particular study were examined and found to support the null hypothesis and reject the hypothesis, which was that failure to fertilize, was due to immature DNA (chromatin packaging) of the spermatozoon with OR of 0.85: 1, p=0.61. Logistic regression calculated the OR for immature DNA (CMA_3≥41%) to predict pregnancy to be likely with odds of 1.6:1, p=0.39. In fact, the study proved the efficacy of ICSI regardless of normal spermatozoon morphology and especially when immature DNA, as measured by CMA_3, is ≥41%.

The initial prerogative of the study was to determine that should an oocyte, harvested during routine COS and TVOH, be prepared for intervention (ICSI) and complying to the

standard of nuclear maturity (M2; meiotic spindle present and a ZP>10); then gamete failure, or failure to fertilize would be the result of damage to the spermatozoon's chromatin packaging. ICSI involves selecting the 'most' morphologic normal spermatozoon for insemination, ruling spermatozoa morphology out as a possible factor of fertilization failure.

The results demonstrated that neither spermatozoa morphology, nor chromatin packaging damage had any measurable effect on fertilization or pregnancy outcome. The overall fertilization rate of the study was 65%. Furthermore, fertilization was no different in the categories of <4% normal morphology (0.72±0.26) or ≥4% morphology (0.68±0.22). This is in contrast to previous studies, which indicated a normal morphology ≥4% would have greater fertilization potential (Kruger, et al., 1986; Mortimer, 1994; Jeyendran, 2000; Menkveld, 2001; Mortimer & Menkveld, 2001; WHO, 2010). However, it must be remembered that the mentioned studies were based on in-vivo, and in vitro fertilisation models. The intervention of this study was ICSI, which should explain why there was no difference of significance in fertilization rates and deems as an independent quality control for this particular laboratory and its embryologists' experience in accurate spermatozoa selection for injection.

The main objective was to prove that when oocyte quality is optimized, that fertilization rates and by implication, pregnancy rates would be improved. If not, failure to fertilize or implant would most probably be due to decreased spermatozoa capacity to fertilize possibly due to damaged chromatin packaging.

The OR for CMA_3 underlines the hypothesis, that when oocyte competence for fertilization is controlled to a degree, the success or failure of treatment can be indicated by the CMA_3 value. In this instance, the OR is highly predictive for success with pregnancy when ICSI is performed on CMA_3 values that are immature (≥41%). In fact, when doing an ICSI on an oocyte that is mature (M2) and if there is a SP present, and this embryo develops to day 5 for embryo transfer, the odds of pregnancy, if working with a semen sample with >40% immature DNA, would be 1.9: 1, p=0.81. DNA The statistical 'p' value indicated insignificance to the parameters measured; clinically this is not a true reflection. The insignificant 'p' value is attributed to the small sample size available for testing due to the 'if' conditions for the OR to be met. The first condition controlled the maturity of the oocytes; only M2 oocytes were included for the OR [only 66% (568/865) of all oocytes had M2 maturity, thereby eliminating 288 of all oocytes]. Secondly, the oocytes needed to fertilize in

order to develop until day 5. Fertilization rate was 65% (553/856 oocytes). Thirdly, the oocytes needed to have a meiotic spindle present, the results excluded another 346 oocytes to only 53% (384/730 M1 & M2 oocytes with SPp). The CMA3 group breakdown was also partial since 74% of all semen samples (n=69/93) had more than 41% immature DNA.

6.5 Concluding remarks

Taking on this particular subject, has enriched the knowledge of the researcher, as well as her unit. It has been fulfilling to be able to conduct a study, of which the results have been able to compare to renowned units across the globe. Not only could we compare results, we could successfully investigate a hypothesis that to the knowledge of the researcher has not yet been presented elsewhere.

In conclusion to the study presented, the researcher would like to remind of the scientific information presented in this thesis.

Sperm DNA chromatin has been associated in past with failure to fertilize and pregnancy failure. This paper had as its aim to see what would be the outcome of sperm DNA on fertilization and pregnancy, should the oocyte competence have been proved. The oocyte competence was based on the nuclear maturity (M2), the presence of the meiotic spindle, and the relevance, if any, of a ZS >10 µm.

The results proved a distinct correlation in favour of ZS and fertilization, p=0.04 when 19.20 µm (16.60 ; 21.79). OR was performed on the SP to determine its relevance to fertilization and resulted in fertilization 1.5:1 times more than when it was absent, p=0.01.

Finally, the hypothesis was proved sound by an OR indicating 1.9:1 likelihood of fertilization, when the oocyte is competent in the event of damage to sperm DNA chromatin packaging.

6.6 Limitations

Due to the project, design and nature of the research project there were a few limitations to the study, which are listed below.

1. The overall maturity of oocytes after COS was only 66% M2 oocytes and was a limitation due to amount of oocytes that had potential to fertilise, and the viable day 5 embryos for transfer was reduced due to this. Madaschi, et al., (2008) had similar M2 maturity rates of 60% (Brazil); Braga, et al., (2008) had similar maturity of 64.3% M2 (Brazil); Braga, et al.

(2010) had a 66.2% M2 (Brazil). Montag, et al. (2006) in Germany, and Ebner, et al. (2010) in Austria, achieved the highest M2 maturity rates of 92% and 88% of M2 oocytes respectively.

It was significant to note that the immature oocytes (M1 & GV) resulted in much lower pregnancy rates (27% & 15%) after initial IVM and ICSI on the following day. In cases of IVM and ICSI, prolonged embryo culture up to the day-5 embryo development for the embryo was employed and resulted in embryos being in culture for up to 6 days. The prolonged culture could be a reason for lower pregnancy rates since a critical endometrial stage could have passed before embryos were capable of implanting.

2. Only 53% of M2 oocytes had a SP present. This is much lower than the majority of studies and is not on account of a drop or change in temperature, pH, or culture conditions. All these aspects were controlled for by an entirely heated and calibrated stage, mineral oil to cover droplets of culture media, swift imaging of oocytes (<10 min in all cases, time-stamps recorded on imaging software) and imaging oocytes in buffered culture media (COOK). The lower SP present rate could most probably be attributed to the use of the short COS protocol vs. long pituitary-down regulation protocols and the residual higher M2 rate for the long stimulation protocol. The possible advantage of using the long COS protocol was evident in the studies from Montag, et al. (2006), which had M2 oocytes with 84% SP present and Braga, et al. (2008) that had 74.3% SP present.

3. Embryos chosen for transfer were selected according to development potential, and not solely according to initial maturity, presence of meiotic spindle or the automated ZS. Ebner, et al. (2010) had similar restrictions, although due to specific review board instructions to

select the best developed embryos for transfer on day 5, in our case, it was not requested by the ethics committee to select the best quality embryos, but due to our own protocol of patient-service first. That said, when examining the data of the development of the embryos chosen for transfer, the majority of good quality blastocysts were embryos that mostly did exhibit meiotic spindles and high birefringent ZP scores.

4. During the gathering of data, the researcher could not be as critical of maturity by limiting OCTAX PolarAIDE oocyte imaging only to M2 oocytes. Wilgers Infertility Clinic offers OCTAX PolarAIDE oocyte imaging free of charge to all patients enrolled in ICSI programs, however, in South Africa, ART treatment is funded privately by the patient and therefore OCTAX PolarAIDE is performed on all oocytes, up to a maximum amount of 10 oocytes per TVOH. Imaging of 10 oocytes deemed fit to make an accurate assumption for the entire population of oocytes should the TVOH yielded more than 10 oocytes. (Kilani, et al., 2010; Kilani, et al., 2011).

5. The ‘new’ lower reference values for normal male morphology (≥4% normal forms) as recommended by the WHO (2010) are a great concern to the researcher. This very low value does not allow for distinction between normal sperm morphology, which translates to ‘male partners with a high probability of fertility’, and poor morphology, which translates, to the ‘male partner with a poor probability of being fertile’ and subsequently is sub-fertile (Barratt, et al., 2011).

6. Not all nominal (‘n’) values were consistent during the statistical evaluations. This was due to varying medical information available per patient and was corrected for statistically. Only the information available per statistical analysis available was analysed. All 95 couples represented and included, gave written informed consent and received a copy of the study information; however, for the reason that many couples’ treatment was via guest gynaecologists’ stimulating and diagnosing patients in other practices, the specific medical records of those patients’ history was unattainable, despite efforts made by the researcher.

6.7 Future research

As a follow up to the current study, it is suggested that a larger study (including thousands of oocytes) should be performed. Furthermore, the selecting of only embryos exhibiting meiotic spindles and ZP scores >10 for embryo transfer can also possibly be considered.

APPENDIX A

INFORMATION LEAFLET AND INFORMED CONSENT

PROJECT TITLE: THE IMPACT OF OOCYTE SPINDLE MORPHOLOGY AND SPERM CHROMATIN PACKAGING ON ICSI AND EMBRYO DEVELOPMENT FOR ASSISTED REPRODUCTION TECHNIQUES

Primary investigator: K. Raubenheimer, BTech (Reproductive Biology)

Study leader: Prof SS du Plessis, PhD, MBA, Medical Physiology, Faculty of Medicine, Stellenbosch University

Dear Potential research participant,

You are invited to participate in a research study that forms part of my formal Master's degree studies. This information leaflet will help you to decide if you would like to participate. Before you agree to take part, you should fully understand what is involved. You should not agree to take part unless you are completely satisfied with all aspects of the study.

WHAT IS THE STUDY ALL ABOUT?

I, Kara Raubenheimer, a qualified Clinical Technologist specializing in reproductive biology, am conducting research on gamete failure and the possible reasons for it. Research is just the process to learn the answer to a question. In this study, I want to learn if there are diagnostic tools that can better predict behaviour of gametes (oocytes and sperm) in vitro.

The main objective of this study is to determine oocyte viability (egg quality) for fertilization and if a correlation exists between immature DNA of the spermatozoa (sperm fertilization ability) in a semen sample and fertilization.

By using new technology and studying the potential of the oocyte to fertilize combined with the male's semen, specifically the spermatozoon's DNA integrity, potentially new and novel data can be gathered for use in assisted reproduction treatment for couples undergoing intra cytoplasmic sperm injection (ICSI).

WHAT WILL YOU BE REQUIRED TO DO IN THE STUDY?

If you decide to take part in this study, your participation shall not expose you / or require of you any more procedures or samples except the samples you have agreed to provide by commencing the IVF program.

Should you participate, you will be required to:

- Sign this informed consent form,
- Give permission for your medical records to be accessed,
- The overall study design will require that the male partner produce one (1) semen sample on the day of oocyte aspiration for use in ICSI. No additional specimens will be required from any partner. The sample must be produced by means of masturbation (at the laboratory) or coitus interruptus, for semen analysis including determining concentration, motility, forward progression, and determination of the integrity of the sperm DNA chromatin packaging.
- The female research participant's oocytes (collected during oocyte retrieval) will be photographed with birefringent technology to visualize meiotic spindles and zona pellucida characteristics.

THE RESEARCH PROTOCOL WILL INCLUDE THE FOLLOWING PROCEDURES:

1. Semen production – The male will be given a sterile container, and will be asked to produce in that container by means of masturbation or coitus interruptus.

2. Research procedures – the sample will be analysed according to the revised protocol for semen analysis by the World Health Organization.

Basic semen analysis

1. Determination of semen volume;
2. Evaluating spermatozoa concentration;
3. Calculate the motility of the spermatozoa;
4. Determine the percentage of normal morphology spermatozoa in the sample;
5. Determine the percentage of immature DNA (chromatin packaging by CMA_3).

3. Semen processing will follow the normal unit protocol for ICSI at Wilgers Infertility Clinic. A smear will be made of the post preparation sample for immature DNA evaluation.

4. Transvaginal oocyte aspiration – the female partner will undergo the oocyte aspiration procedure during which the super ovulated ovaries are aspirated to recover the oocytes for the ICSI procedure.

5. Mature oocytes will be prepared as usual for ICSI with the Oosight Imaging analysis to determine the viability of the oocyte to fertilize just before the ICSI procedure.

6. OCTAX PolarAide is a new technology, employed to visualize important structures in the oocyte that are necessary for fertilization. This procedure is completely safe and non-invasive.

7. All mature oocytes will be injected with the most morphologically normal sperm present in the sample, as is protocol of Wilgers Infertility Clinic.

Intervention

1. Determine the viability of the mature oocyte to be fertilized by using technology to visualize critical structures in the oocyte;
2. Determine the DNA integrity by assessing the chromatin packaging of the spermatozoa in the sample.

CAN ANY OF THE STUDY PROCEDURES RESULT IN PERSONAL RISK, DISCOMFORT, OR INCONVENIENCE?

There are no risks regarding the study as we will perform accepted basic semen analysis including the sperm concentration, motility, forward progression (movement of the sperm) and chromatin integrity of the sperm sample. The World Health Organization's protocols for semen analysis will be applied.

The research participant will not be exposed to any risks by participating in this study, other than the risks associated with the infertility treatment and ICSI for which they have voluntarily approached the Wilgers Infertility Clinic. No experimental research procedures will be performed on research participant's gametes, only non-invasive observations will be made from diagnostic evaluations.

WHAT ARE THE POTENTIAL BENEFITS THAT MAY COME FROM THE STUDY?

The benefits of participating in this study are:

- You will receive special diagnostic investigations that do not normally form part of any fertility treatment cycle - that will benefit current and future fertility treatment ;
- You will make a contribution towards establishing new data with regard to the critical structures in oocytes, sperm DNA, fertilization, and ultimately pregnancy results.

WILL YOU RECEIVE ANY FINANCIAL COMPENSATION OR INCENTIVE FOR PARTICIPATING IN THE STUDY?

Please note that you **will not** be paid to participate in the study since the technology used can only benefit your IVF cycle by providing key diagnostic information not generally included for IVF cycles at infertility centers.

Due to the expensive nature of the technology utilized for this study, the research participant benefits personally in their current IVF cycle as well as providing key diagnostic information for their future IVF treatments.

The research participant will not be liable to costs incurred for basic semen analysis and determination of spermatozoa morphology and DNA integrity of the semen sample nor for the use of the expensive birefringent technology.

WHAT ARE YOUR RIGHTS AS A PARTICIPANT IN THIS STUDY?

Your participation in this study is entirely voluntary. You have the right to withdraw at any stage without any penalty or future disadvantage whatsoever. You do not even have to provide the reason/s for your decision. Your withdrawal will in no way influence your continued care and relationship with the health care team. Note that you are not waiving any legal claims, rights, or remedies because of your participation in this research study.

HOW WILL CONFIDENTIALITY AND ANONYMITY BE ENSURED IN THE STUDY?

All the data that you provide during the study will be handled confidentially. This means that access to your data will be strictly limited to the researcher, the supervisors of the study, and members of the research ethics committee and/or the designated examiners (appointed by Stellenbosch University). In addition, your data and personal information will be kept and stored in a confidential format that will only be accessible to the researcher. Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law.

IS THE RESEARCHER QUALIFIED TO CARRY OUT THE STUDY?

The researcher is an adequately trained and qualified researcher in the study field covered by this research project, specifically in reproductive biology and embryology.

HAS THE STUDY RECEIVED ETHICAL APPROVAL?

Yes. The Faculty Higher Degrees Committee and the Research Ethics Committee of Tshwane University of Technology and Stellenbosch University have approved the formal study proposal. In addition, the partners of Wilgers Infertility Clinic have granted written approval for the study. All parts of the study will be conducted according to internationally accepted ethical principles.

WHO CAN YOU CONTACT FOR ADDITIONAL INFORMATION REGARDING THE STUDY?

The primary investigator, Ms K Raubenheimer, can be contacted during office hours at Tel (012) 807-8398, or on her cellular phone at 082 724-8671. The study leader, Prof SS du Plessis, can be contacted during office hours at

Tel +27 21 938-9388.

DECLARATION: CONFLICT OF INTEREST

The researcher has nothing to declare.

A FINAL WORD

Your co-operation and participation in the study will be greatly appreciated. Please sign the informed consent below if you agree to participate in the study. In such a case, you will receive a copy of the signed informed consent from the researcher.

CONSENT

I hereby confirm that I have been adequately informed by the researcher about the nature, conduct, benefits, and risks of the study. I have also received, read, and understood the above written information. I am aware that the results of the study will be anonymously processed into a research report. I understand that my participation is voluntary and that I may, at any stage, without prejudice, withdraw my consent and participation in the study. I had sufficient opportunity to ask questions and of my own free will declare myself prepared to participate in the study.

Research participant's name: _____ (Please print)

(Husband)

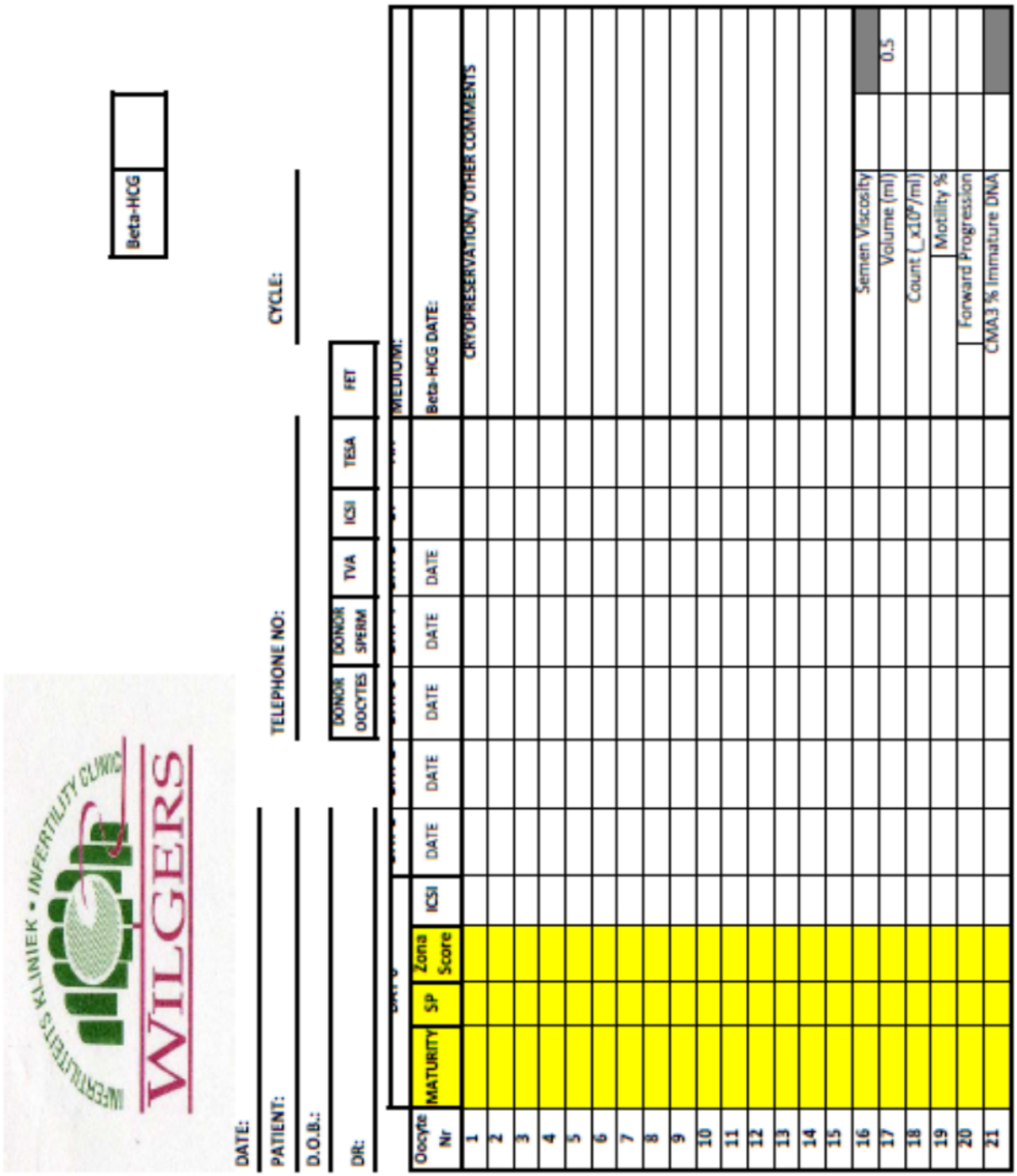
Research participant's signature: _____Date: _____

Research participant's name: _____ (Please print)

(Wife)

Research participant's signature: _____Date: _____

Appendix B



APPENDIX C

Reagents and Method of Papanicolaou morphology stain (WHO, 2010)

Papanicolaou constituent stains (Harris's haematoxylin; G-6; EA-50)

Harris's haematoxylin without acetic acid

1. Haematoxylin dark crystals colour index 75290;
2. Ethanol 95% (v/v);
3. Aluminium ammonium sulphate dodecahydrate ($AINH_4(SO_4)_2 \cdot 12H_2O$);
4. Mercuric oxide (HgO);
5. Purified water.

Aluminium ammonium sulfate dodecahydrate (160g) was dissolved in 1600ml of purified water by heating. Haematoxylin crystals (8g) was dissolved in 80ml of 95% (v/v) ethanol and added to the aluminium sulfate solution whilst the mixture was heated to 95°C. The mixture was removed from the heat and 6g of mercuric oxide was added slowly while stirring. The container was plunged into a cold water bath and the mixture filtered after cooling. The filtered solution was stored away from light (dark-brown or aluminium-foil-covered bottles) at room temperature, left to stand for 48 hours before the required amount was diluted with equal amounts of purified water, and filtered again before use.

Orange G6

1. 10g Orange G crystals colour index 16230;
2. 100ml Purified water;
3. 1000ml 95% ethanol (v/v);
4. 0.15g Phosphotungstic acid.

Stock solution 1 (orange G6, 10% solution)

10g of Orange G crystals was dissolved in 100ml purified water and mixed well. Solution 1 was stored away from light at room temperature for 7 days before use.

Stock solution 2 (orange G6, 0.5% solution)

950ml 95% ethanol (v/v) was added to 50ml of stock solution 1.

0.15g Phosphotungstic acid was added to the mixture and mixed well. Solution 2 was stored away from light, at room temperature and filtered before use.

EA-50

1. 10g Eosin Y colour index 45380;
2. 10g Bismarck brown Y colour index 21000;
3. 10g Light-green SF colour index 42095;
4. 300ml Purified water;
5. 2000ml 95% Ethanol (v/v);
6. 4g Phosphotungstic acid;
7. 0.5ml saturated aqueous lithium carbonate.

Stock solutions

10% solutions of each of the 3 stains were prepared separately:

1. 10g eosin Y was dissolved in 100ml purified water;
2. 10g Bismarck brown Y was dissolved in 100ml purified water;
3. 10g light-green SF was dissolved in 100ml purified water.

Finally, the Papanicolaou stain is prepared by mixing the following solutions together:

1. 50ml eosin Y stock solution;
2. 10ml Bismarck brown Y solution;
3. 12.5ml light-green SF stock solution;
4. 1927.5ml 95% ethanol (v/v);
5. 4g Phosphotungstic acid;
6. 0.5ml saturated lithium carbonate.

The mixture was filtered with a 0.45µm filter before use and stored in a dark cupboard, at room temperature.

Staining the fixed smear: The slides were immersed sequentially according to the following instructions:

1.	Ethanol 80% (v/v)	30 seconds
2.	Ethanol 50% (v/v)	30 seconds
3.	Purified water	30 seconds
4.	Harris's haematoxylin	4 minutes
5.	Purified water	4 minutes
6.	Acidic ethanol 50% (v/v)	4-8 dips (1dip = 1 second)

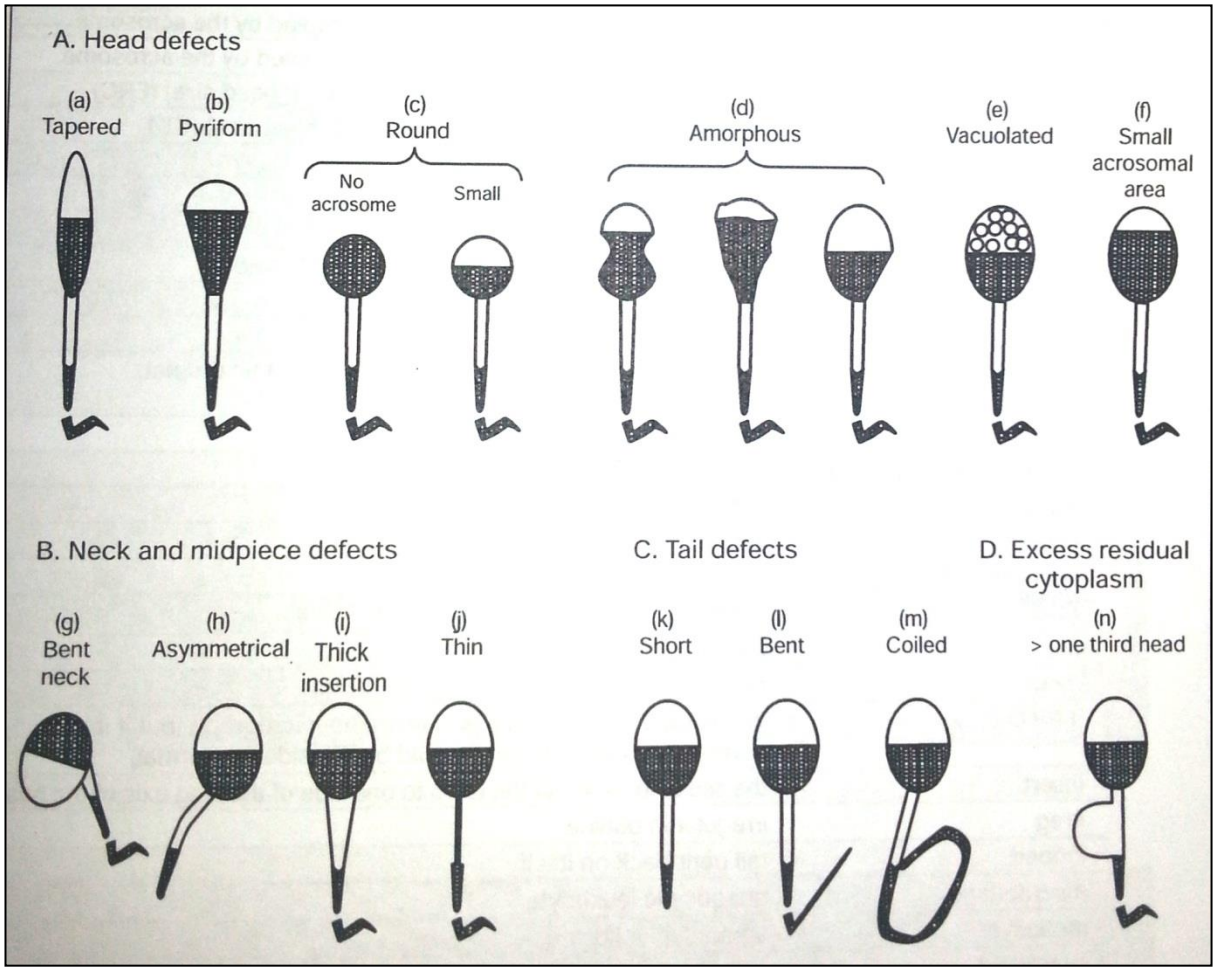
(Acidic ethanol: 1.0ml of concentrated hydrochloric acid to 200ml of 70% (v/v) ethanol)

7.	Running cold tap water	5 minutes
8.	Ethanol 50% (v/v)	30 seconds
9.	Ethanol 80% (v/v)	30 seconds
10.	Ethanol 95% (v/v)	≥15 minutes
11.	G-6 orange stain	1 minute
12.	Ethanol 95% (v/v)	30 seconds
13.	Ethanol 95% (v/v)	30 seconds
14.	Ethanol 95% (v/v)	30 seconds
15.	EA-50 green stain	1 minute
16.	Ethanol 95% (v/v)	30 seconds
17.	Ethanol 95% (v/v)	30 seconds
18.	Ethanol 100% (v/v)	15 seconds
19.	Ethanol 100% (v/v)	15 seconds

The stained semen smear was submerged in Xylene: Ethanol (1:2) for 1 minute; allowed to drain for 1-2 seconds and mounted with Dabco mounting medium (Aldrich Chemicals, Milwaukee, USA cat No. 29,073-4). The mounted smears were allowed to dry horizontally in a slide drying rack for at least 24 hours in a fume cupboard (Andrology Laboratory, Ampath, Pretoria).

APPENDIX D

Illustration of spermatozoa with head, neck and tail defects according to Tygerberg Strict Criteria (Taken from WHO (2010) and adapted from Kruger et al., 1993).



APPENDIX E

Reagents of Chromomycin A_3 (CMA_3)

20. CMA_3;
21. McIlvaines Buffer;
22. Magnesium Chloride in McIlvaines Buffer.

CMA_3

For the CMA_3 solution 2.0ml EtOH (absolute ethanol) was added to 10mg CMA_3 (Sigma Chemicals, St. Louis, MO. USA Cat C2659).

McIlvaines Buffer

The McIlvaines solution was prepared with 19ml 0.1M Citric Acid solution + 81ml 0.2M $Na_2HPO_42H_20$ (Sigma Chemicals, St. Louis, MO).

Citric acid solution: 2.101g Citric Acid and 100ml sterile water (0.1M Citric Acid) and

7.12g di-Sodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$) + 100ml sterile water

= 0.2M $Na_2{\cdot}HPO_4{\cdot}2H_20$

Magnesium Chloride in McIlvaines buffer

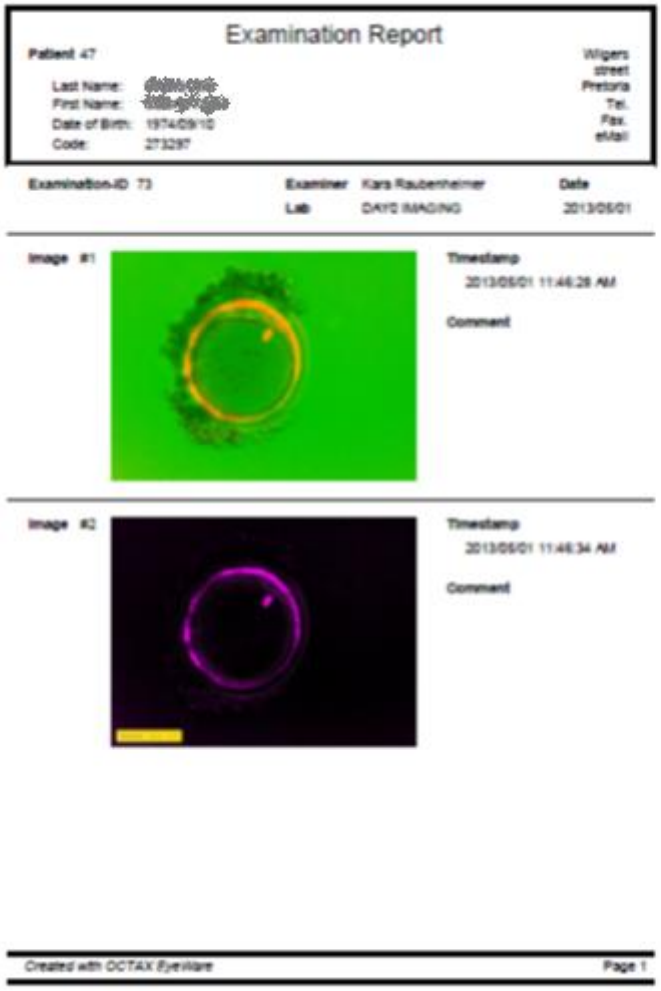
0.2033g Magnesium Chloride (Sigma Chemicals, St. Louis, MO) + 100ml McIlvaines Buffer (10mM Magnesium Chloride).

Each slide was treated with 100µl CMA_3 solution and left for 20 minutes in a dark drawer. The CMA_3 solution comprised of 0.25mg/ml CMA_3 in McIlvaines buffer (Geigy Scientific Tables 1984) supplemented with 10mM $MgCl_2$ (Esterhuizen, et al., 2000).

Slides were rinsed in McIlvaines buffer, mounted with Dabco (Aldrich Chemicals Co, Milwaukee, USA cat No. 29,073-4) and kept overnight at 4°C. Fluorescence of the spermatozoa head was evaluated the next morning using a Fluorescence microscope (Nikon TE200, IMP, Johannesburg, South Africa) fitted with a triple band filter fluorescein isothiocyanate (FITC), rhodamine, 4',6-diamidino-2-phenylindole (DAPI) and an Eplan 100x objective (Esterhuizen, et al., 2000).

APPENDIX F

Report generated from OCTAX polarAIDE software indicating automated zona birefringence score and meiotic spindle.



Examination Report

Patient 47

Last Name: [redacted]
First Name: [redacted]
Date of Birth: 1974/09/10
Code: 273297

Wigers street
Pretoria
Tel.
Fax.
eMail

Examination-ID 73 | Examiner Kara Rautenheimer | Date
Lab DAYO IMAGING | 2013/05/01

Image #1

Timestamp
2013/05/01 11:46:28 AM

Comment

Image #2

Timestamp
2013/05/01 11:46:34 AM

Comment

Created with OCTAX Eyeware Page 1

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