THE ROLE OF THE CUMULUS OOPHORUS COMPLEX DURING SPERMATOZOA CAPACITATIONAL EVENTS

MICHELLE RIJSDIJK

Thesis presented in partial fulfilment of the requirements for the degree of Masters in Medical Science (Reproductive Biology) at the University of Stellenbosch

Promoter: Prof. Daniel R. Franken

December 2005

Declaration

I, the undersigned, hereby declare that the work in this thesis is my own original work and that I have not previously or in part submitted it at any university for a degree

Signature:.....

Date: 4/8/2005

Summary

Chapter 1 contains a review dealing with nuclear and morphological changes during spermatogenesis and spermatozoa transport with emphasis on the maturation of spermatozoa, capacitation, acrosome reaction and the interaction with the cumulus oophorus complex (COC). The oocyte and cumulus oophorus complex is also discussed particularly on the topic of maturity (oocyte and cumulus maturity). Also presented is a review of the fluorescent binding agents, namely Fluorescein Isothiocyanate labeled with Pisum sativum (FITC-PSA), Chlorotetracycline test (CTC) and Chromomycin A_3 (CMA_3). **Chapter II** describes all the materials and methods used during this study. Routine semen analysis is described with emphasis on normal spermatozoon morphology according to strict criteria. The evaluation of capacitation and acrosome reaction (AR) using the CTC and PSA-FITC staining methods as well as the evaluation of spermatozoon nuclear chromatin packaging using the CMA_3 staining method is described. **Chapter III** represents the results recorded in this study. Compared with those spermatozoa cultured in medium alone, spermatozoa exposed to the cumulus mass were more likely to be capacitated and acrosome reacted, with a distinct increase in chromatin packaging quality. A general discussion of the results and future applications are discussed in **Chapter IV.** In short An *in vitro* model for spermatozoa penetration through the cumulus oophorus was established. The model can be applied to investigate the effect of the cumulus oophorus on sperm functions and to assist in the selection of functional sperm for intracytoplasmic sperm injection therapy. All relevant references are presented in **Chapter V** .

Opsomming

Hoofstuk I bevat ‘n oorsig wat handel oor die nukluêre en morphologiese veranderinge wat plaasvind tydens spermatogenese en spermatozoa migrasie, met klem op maturasie van die spermatozoa, kapasitasie, akrosoom reaksie en die interaksie met die cumulus oophorus kompleks (COK). Die oösiet en die cumulus oöphurus kompleks word ook bespreek in besonderhede op die maturasie van die oösiet en cumulus kompleks. Daar word ook ‘n oorsig aangebied oor die fluoreserende bindende agente naamlik, Fluorescein Isothiosianaat gekoppelde Pisum sativum (FITC-PSA), Chlorotetrasiklien toets (CTC) en Chromomycin A_3 (CMA_3) **Hoofstuk II** beskryf die metodes en materiale wat gebruik is gedurende hierdie studie. Roetine semen analise word beskryf met die klem op normale spermatozoa morfhologie soos beoordeel volgens die Tygerberg se streng kriteria. Die evaluasie van spermsel kapasitasie en akrosoom reaksie (AR), met die gebruik van CTC en FITC-PSA kleuring metodes, asook die evaluasie van spematazoa nukluêre chromatien verpakking soos gesien met die CMA_3-kleuringsmetode word ook beskryf. **Hoofstuk III** verwys na die resultate wat verkry word tydens hierdie studie. Opsommend toon die resultate dat spermselle wat deur die cumulus selmassa migreer het, fisiologies verder gematureer het as die selle in die kontroel populasie. n Algemene bespreking van die resultate en toekomstige toepassings word bespreek in **Hoofstuk IV**. Kortliks word die *in vitro* model, waartydens spermselle deur ’n cumulus selmassa migreer, as ‘n moontlike voorbereidingstegniek vir intra-sitoplasmatiese inspuitings behandelling gesien. Al die relevante verwysings is uiteengesit in **Hoofstuk V.**

Acknowledgements

I wish to extend my most sincere gratitude and appreciation to the following people for their contribution to the sucessful completion of this study:

Professor Danie Franken, for your unwavering support and guidance,

Professor TF Kruger, for making this course a reality, and for the fountain of knowledge that you made available to me,

To **Dr R Menkveld**, allowing me to publish Figure III.

Dr Marie-Lena Windt, for the unwavering support and well intended criticism.

To all those working at the IVF Lab namely **Drs JP van der Merwe** and **I Siebert**, **Dr R Menkveld, Frik Stander, Karin Smith, Evelyn Erasmus, Christiaan Hoogendijk, Greg Tinney, Lou-Marie Kellerman and Zulaigha Williams** (my partner in crime), thanks for all the support and many laughs in the tea room during the stressful times,

To all my friends especially **Leigh-Ann Wellington** and **Steven De Becker** for supporting me at the best and worst of times,

To my parents**, Rudy and Nell Braam,** and my sister**, Maryke Braam**, for the love and support throughout my studies.

Table of Contents:

CHAPTER I

Literature Review and Background Information

Infertility affects about 15% of couples in their reproductive age and has a major impact on public health (Liu and Baker 2004). Since the birth of the first intracytoplasmic sperm injection (ICSI) baby in 1992 (Palermo *et al.,* 1992) the clinical importance of semen analysis and sperm preparation techniques has come under scrutiny (Mc Donough, 1997). The question has often been asked "is sperm quality still important for ART (Assisted Reproductive Techniques) outcome?" Eventually it was thought that all infertile couples could be helped with the intracytoplasmic sperm injection (ICSI) technique, since sperm concentration, motility, morphology and sperm antibodies did not seem to play a role during this fertilization process (Nagy *et al*., 1998). It was believed that only a few spermatozoa are necessary to obtain fertilization, good embryo development and a healthy pregnancy (Nagy *et al*., 1998).

Today we know that in the clinical management of infertility, allocation of patients between standard *in vitro* fertilization (IVF) and ICSI is mainly decided on the basis of assessment of sperm characteristics (Kruger *et al.,*1986; Liu *et al*., 2004). Although ICSI could be used for all classes of male infertility, irrespective of sperm quality, the cost of the procedures involved, compel clinics to reserve ICSI treatment for patients with severe sperm anomalies (Bahattacharya *et a*l, 2001). Patients with subtle sperm defects that influence sperm-oocyte interaction are more efficiently treated with ICSI (Liu *et al* 2004, Esterhuizen *et al* 2000). Reliable sperm functional tests, to assist in the diagnosis of infertility and offer a reasonable selection of treatment, are valuable tools in an infertility program.

Due to the relatively high costs and risks involved with ART procedures, a fundamental need developed, to carry out research directed to understand the underlying pathophysiology, as well as to be able to prevent those conditions resulting in infertility (i.e., sexually transmitted diseases, reproductive bio-hazards and others).

Today it is recognized that sperm dysfunction is one of the most common single causes of infertility yet, remarkably, our knowledge of the cellular and biochemical basis for this condition is very limited. Indeed, our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoon, is elementary. The aim of basic research programs is often aimed to investigate the fundamental mechanisms of calcium signaling in fertile and sub-fertile human spermatozoa following binding of zona pellucida (ZP) and progesterone (the two recognized, endogenous agonists of acrosome reaction [AR]) to the sperm plasma membrane. Although defects in these pathways account for approximately 25% of cases of sperm dysfunction, our current knowledge of signaling in spermatozoa is minimal. A detailed and comprehensive understanding of the signaling process will allow rational and effective treatment to be developed.

1.1. Cumulus Oophorus Interaction with Spermatozoa.

The cumulus oophorus, unique to the egg of eutherian mammals, has been suggested to function either in oocyte transport to the oviduct ampulla, avoidance of polyspermy (Yanagimachi, 1994), enhancement of sperm fertilizing ability, or sperm guidance to the egg (Bedford, 1993). Sperm ascending from the lower oviduct encounter an oocyte which is surrounded by at least two layers (zona pellucida and the cumulus oophorus) that has a protective role as far as the oocyte is concerned. The inner layer is the acellular zona

pellucida, while the outside layer is known as the cumulus oophorus. The cumulus (explained in depth in 1.1.1.) is made up of granulosa cells and their extracellular matrix (ECM). The granulosa cell layer which is closest to the zona has different biochemical and structural features than the remaining cumulus and it is termed the corona radiata (Talbot 1985; Cherr *et a*l., 1990).

The efficiency of the fertilization process may be enhanced by gradients within the cumulus which induce both chemotaxis (attraction) and chemokinesis (motility stimulation) of the fertilizing sperm cells (Ralt *et al*., 1994, Cohen-Dayag *et al*, 1994). The cumulus ECM is produced by granulosa cells (Eppig 1982); it is formed primarily of hyaluronic acid, but also contains some proteins (Cherr *et al*., 1990 Drahorad *et al.,* 1991). The structure of the cumulus matrix is a network of openings that are smaller than the sperm head (Cherr *et al*., 1994, Yudin *et al*., 1988). Spermatozoa penetrating the cumulus display hyperactivated motility (explained in section 1.5.1) (Cummins and Yanagimaqchi 1982), and these flagellar movements may physically tear the structure of the cumulus (Yudin *et al*., 1988) and thus facilitate sperm entry.

Sperm motility may also be modulated by interaction with the cumulus and may respond to chemical stimuli, as well as physical stimuli during cumulus penetration. The chemical signals which are involved may be related to the chemotactic/chemokinetic factors demonstrated in human follicular fluid (Ralt *et al*., 1994). The speed of sperm penetration through the cumulus *in vivo* is not known, but penetration through the cumulus to the zona surface requires 3-20 min *in vitro* (Cummins and Yanagimachi 1982, Cherr *et al*., 1994).

The characteristics of the sperm surface are important for penetration of the cumulus ECM. For example, non-capacitated sperm (Cummins and Yanagimachi 1982, Cummins and Yanagimachi 1986) and acrosome reacted sperm (Cherr *et al*., 1986, Cummins and Yanagimachi 1986, Suarez *et al*., 1986) cannot enter the cumulus ECM. Some vigorously motile sperm adhere irreversibly to granulosa cells but cumulus penetration is prevented (Cherr *et a*l., 1986, Corselli and Talbot 1987, Suarez *et al*., 1986). Other spermatozoa, including sperm with intact acrosomes, are phagocytosed by the granulosa cells (Bedford 1972). The cellular mechanisms and biological significance of these sperm-granulosa cell interactions are unknown.

Although it is controversial, the view of most reproductive biologists is that the acrosome reaction of the fertilizing sperm is induced by contact with the zona pellucida, and that the fertilizing sperm has an intact acrosome during penetration of the cumulus (Bedford and Kim 1993; Esterhuizen et al., 2002;) . Experiments done in mice have demonstrated that only sperm with intact acrosomes can bind to the zona (Wassarman 1999). Experiments with human gametes suggest that the sequence of events prior to fertilization, also includes sperm zona binding followed by the acrosome reaction. Moreover there is evidence that biochemical changes in the sperm's enzymes may occur during interaction with the cumulus (Drahorad *et al*., 1994) and one result may be a priming (sensitization) of the fertilizing sperm to ensure that the acrosome reaction will take place once bound to the zona pellucida (Yanagimachi 1994, Roldan 1998).

1.1.1. Clinical Importance of the Cumulus Oophorus

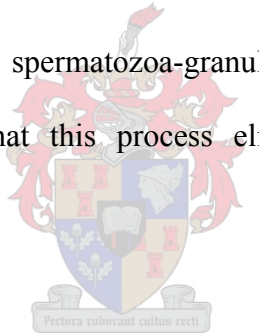
It has been shown that cumulus-free cattle oocytes are not fertilizable (Chen & Sathanathan, 1986) and that there is a lower IVF fertilization rate of naked hamster oocytes than that of cumulus-intact oocytes when transferred to the oviducts of hamsters (Moore *et al*, 1978). Despite these observations, the physiological roles of the human cumulus oophorus have been thus far unclear due to limited availability of oocyte-cumulus complexes due to ethical considerations. The cumulus oophorus complex (COC) of humans does, however, play a very important role during fertilization especially with regard to sperm selection (Chen *et al*, 1986; Overstreet, 1996).

The COC (Figure I) consists of two components; the outer stratum is the cumulus oophorus which consists of granules that are trypsin-sensitive, embedded in a viscoelastic matrix comprised mainly of hyaluronic acid (Cooper & Yeung, 2000; Fatchi *et al*, 2002). The inner stratum is the acellular zona pellucida of the oocyte which consists of a meshwork of glycoproteins. Cumulus cells do have the ability to affect the oocyte maturity especially with respect to nuclear and cytoplasmic maturity (Hong *et al.,* 2004; Tesarik *et al.,* 1997). Certain factors are responsible for this control, among them oestradiol (E_2) is the most important. E_2 acts on the oocyte surface changing the reactivity of Ca^{2+} release systems during cytoplasmic maturation. It has been reported by Tesarik *et al* (1997) that oocytes need this priming by E_2 to develop Ca^{2+} oscillation capacity during maturation.

Overstreet (1996) shows that the cumulus oophorus is responsible for the discontinuation of meiosis after the 2nd meiotic division (Appendix I and II) by secreting inhibiting factors such as cyclic adenosine monophosphate (cAMP). These act via gap junctions therefore

nurturing oocyte growth. The cumulus cells do retract from the zona pellucida (radiated ZP), thereby cutting off communication to the oocyte near the time of ovulation (figure II) (Menkveld & Coetzee, 1995; Mortimer, 1995; Overstreet, 1996; Fatchi *et al*, 2002). The increase in steroidal activity at time of ovulation has been linked to an increase in cell-plating density of the cumulus cells as seen in the study done by Bar-Ami *et al*., 1994.

Spermatozoa have been seen to display hyperactivated motility (explained in section 1.5.1) whilst penetrating the cumulus (Fatchi et al., 2002). The flagellar movements physically tear the structure of the cumulus, thus facilitating spermatozoa entry. Non-capacitated spermatozoa cannot enter the cumulus matrix, since spermatozoa have been seen to irreversibly adhere to the granulosa cells and cumulus penetration is prevented. The cellular and biological significance of the spermatozoa-granulosa cell interaction is unknown, but Fatchi *et al.,* 2002, theorize that this process eliminates morphologically abnormal spermatozoa.



The COC appears to activate the capacitation/acrosome reactions of the spermatozoa. Both steroids (estrogen and progesterone) and human follicular fluid, present in and around the cumulus matrix, have been seen to stimulate the acrosome reaction in spermatozoa (Foresta *et al.*, 1992). Spermatozoa taken from within the cumulus matrix were intact or partially acrosome reacted. As said previously the spermatozoa penetrating the cumulus appeared to reach the ZP within one hour after insemination and these were then seen to be engulfed in psuedolike processes extended by the cumulus cells (Cooper & Yeung, 1995). Present results indicate that during fertilization, cumulus cells play an important role in inducing the acrosome reaction and promoting a high fertilization rate, cleavage and development

into blastocysts *in vitro* (Fukui, 1990). Most importantly, the cumulus cells protect the oocyte from adverse environmental effects (Magier *et al*, 1990).

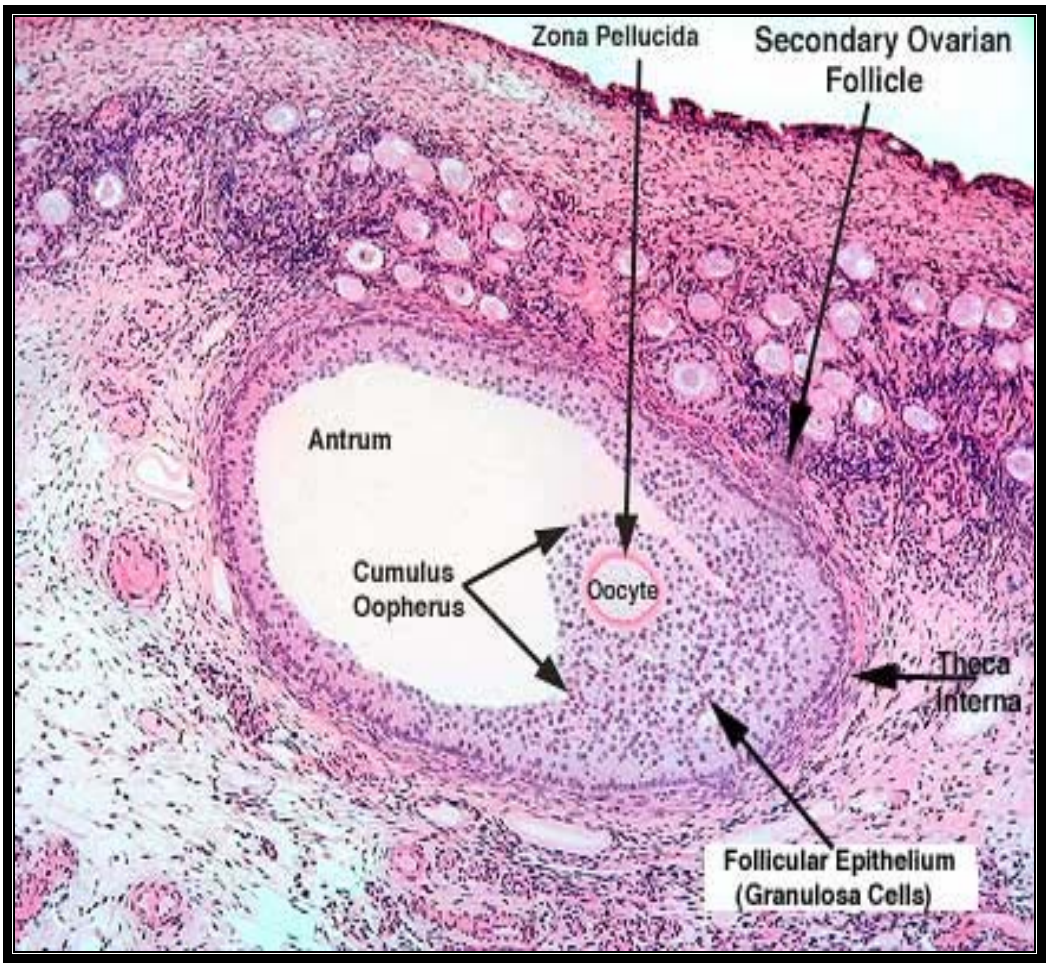


Figure 1: An illustration of the metaphase II oocyte within the mature follicle. Take note of the cumulus distribution.

1.1.2. Cumulus Oophorus Models

Many models have been used in the past to try to explain the effect of the cumulus oophorus on spermatozoa. The following models have been published:

I. Cumulus oophorus complex co-incubation (Suarez *et al*, 1983; Stock *et al*, 1989; Carrell *et al*, 1993; Chen & Santhananthan, 1986; Cherr *et al*, 1983; Cummins & Yanagimachi, 1986; Corselli & Talbot, 1987)

II. Cumulus oophorus co-incubation with spermatozoa (Magier *et al*, 1990; Tesarik *et al*, 1990; White *et al*, 1990)

III. Cumulus cell monolayer co-culture (Mansour *et al*, 1995)

IV. Culture in cumulus cell-conditioned medium (Sullivan *et al*, 1990; Fetterolf *et al*, 1994)

All these models are flawed with respect to *in vivo* conditions and studying the effect of cumulus (by itself) on spermatozoa. Model I (Suarez *et al*, 1983; Stock *et al*, 1989; Carrell *et al*, 1993; Chen & Santhananthan, 1986; Cherr *et al*, 1983; Cummins & Yanagimachi, 1986; Corselli & Talbot, 1987) cannot differentiate between the cumulus or the oocyte's effect on the spermatozoa. Models II (Magier *et al*, 1990; Tesarik *et al*, 1990; White *et al*, 1990) and III (Mansour *et al*, 1995) are single layered therefore disrupting the 3-dimensional structure (matrix) of the cumulus and hence don't give the complete picture of cumulus cell physiology (Bar-Ami *et al*, 1989). Model IV does not illustrate the effect of the cell matrix upon spermatozoa function, only the hormonal/steroidal impact (Sullivan *et al*,1990; Fetterolf *et al*, 1994).

1.2. Oocytes

1.2.1. Maturation

During early *in utero* development, the primitive germ cells, or oogonia, undergo four mitotic divisions. Around the 3rd month after conception, the oogonia stop dividing and from this point on, no new germ cells are made. The oogonia in the fetus are known as the primary oocytes and then start with the first meiotic division by replicating their DNA. They do not, however, finish this division and with their 46 chromosomes, each with a sister chromatid, remain in a state of meiotic arrest during the prophase (Monesi *et al*, 1964; Pedersen *et al*, 1975; Veeck, 1986; Mortimer, 1995; Overstreet, 1996).

This meiotic arrest continues until puberty is reached, when the ovaries are activated by hormones released by the anterior pituitary gland (FSH – Folicle Stimulating Hormone and LH – Leutinising Hormone). The primary oocytes that are destined for ovulation are the only ones that will complete the first division, where each daughter cell receives 23 chromosomes, each with two chromatids. The big difference between the division of somatic cells and the male gamete to that of the oocyte is that one of the daughter cells will retain most of the ooplasm and become known as the secondary oocyte. The other is then known as the first polar body and is very small and non-functional (Figure II) (Monesi *et al*, 1964; Pedersen *et al*, 1975; Veeck, 1986; Mortimer, 1995).

The second meiotic division will only take place *after* ovulation, but only if the oocyte is fertilized. As a result of the second division, each daughter cell again receives 23 chromosomes. Once again, one daughter cell gets most of the nutrient rich ooplasm, and is now being known as the ovum while the other is the second polar body (Nagae *et al*, 1986; Veeck, 1986; Overstreet, 1996; Fatchi *et al*, 2002;).

The metaphase II oocyte is characterised as follows (Figure I and II) (Nagae *et al*, 1986; Veeck, 1986; Overstreet, 1996):

i) Nuclear status – extrusion of the first polar body

ii) Ooplasm – ooplasm is symmetrical, homogenous in colour with a smooth granular appearance (Veeck, 1986).

iii) Peripheral Cells – the cumulus (cellular material surrounding the oocyte) is expanded with a compact coronal layer ("halo" appearance).

iv) Expansion of the cumulus – lower density of the cumulus mass

v) Appearance of granulosa cells – cells are loosely aggregated within smooth masses

The maturation of the metaphase II oocyte (Figure I and II) is not just regulated by nuclear maturity but also by the vestments surrounding it (Veeck, 1986; Mortimer, 1995; Overstreet, 1996). The cumulus oophorus complex also needs to reach a certain level of maturity before fertilization can take place. This maturity is brought about by hormonal stimuli especially estrogen and progesterone (Overstreet, 1996).

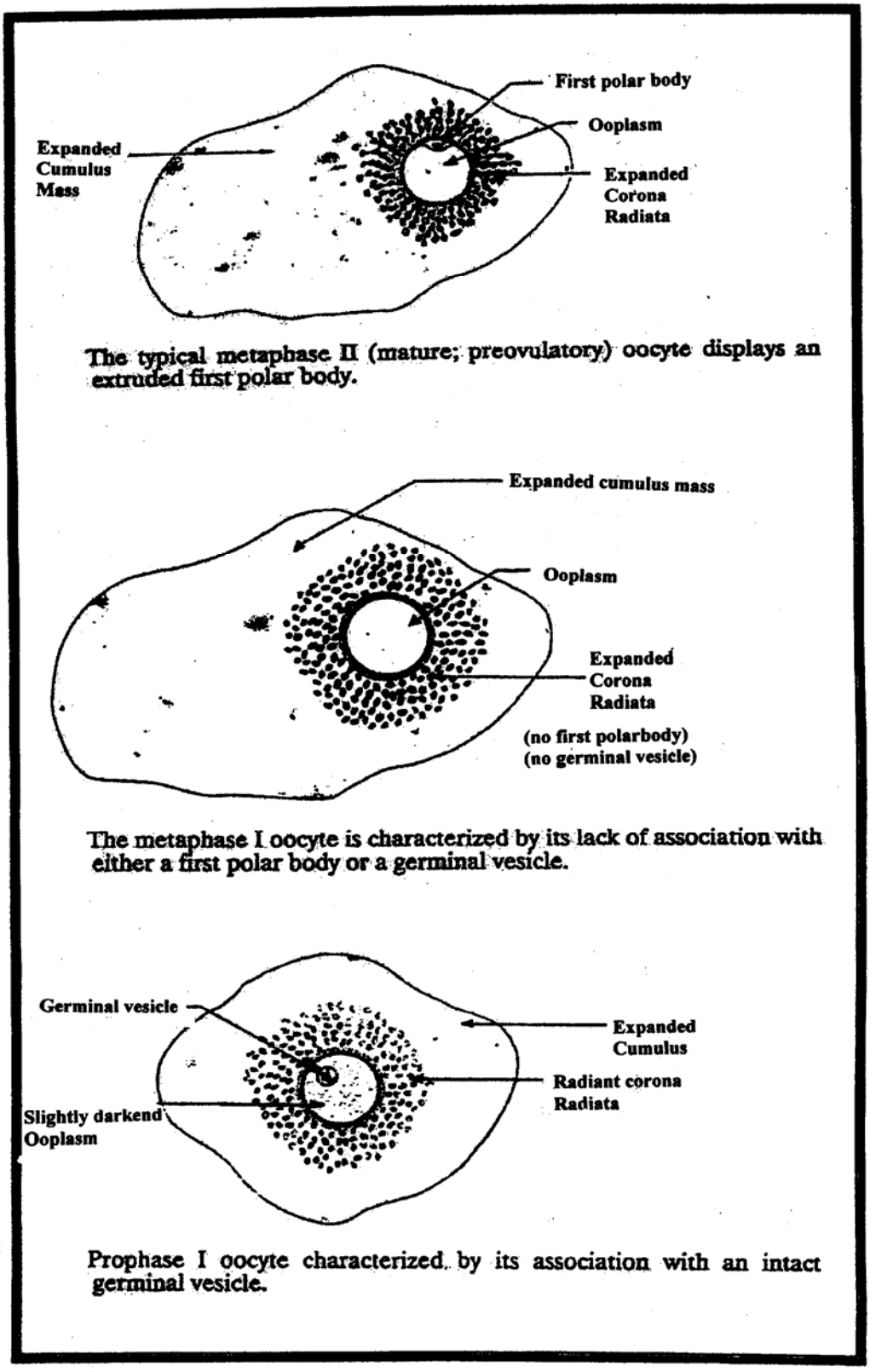


Figure II: The properties of prophase 1, metaphase 1 and metaphase 2 oocytes (Veeck *et al.*, 1986)

1.3. Spermatozoa Maturation

Spermatogenesis in the adult male is a continuous process and therefore requires a constant proliferation of differentiating germ cells from a source of stem cells which takes place in the seminiferous tubules within the testes. The spermatogonia (undifferentiated germ cells) begin to divide at puberty and the cells that result from the final mitotic division (Appendix I and II) and differentiation series are known as the primary spermatocytes (Figure III) and are analogous to the primary oogonia in the female (Acosta, 1996). It should be noted that not all spermatogonia are converted to spermatocytes but that some drop out of the cycle and revert to spermatogonia.

Each primary spermatocyte will increase in size, which is then followed by the first meiotic division (Appendix II) to form two secondary spermatocytes, each of which contains 23 two-chromatid chromosomes. Each of these in turn meiotically divides again to produce spermatids, each containing 23 chromosomes. These spermatids undergo further differentiation to finally produce mature spermatozoa (Figure III (p14) and IV (p18) Spermatogenesis is then followed by spermiogenesis (or maturation of spermatids) with both processes combined taking approximately 74 days (Acosta, 1996). Spermiogenesis is fully discussed in the next section.

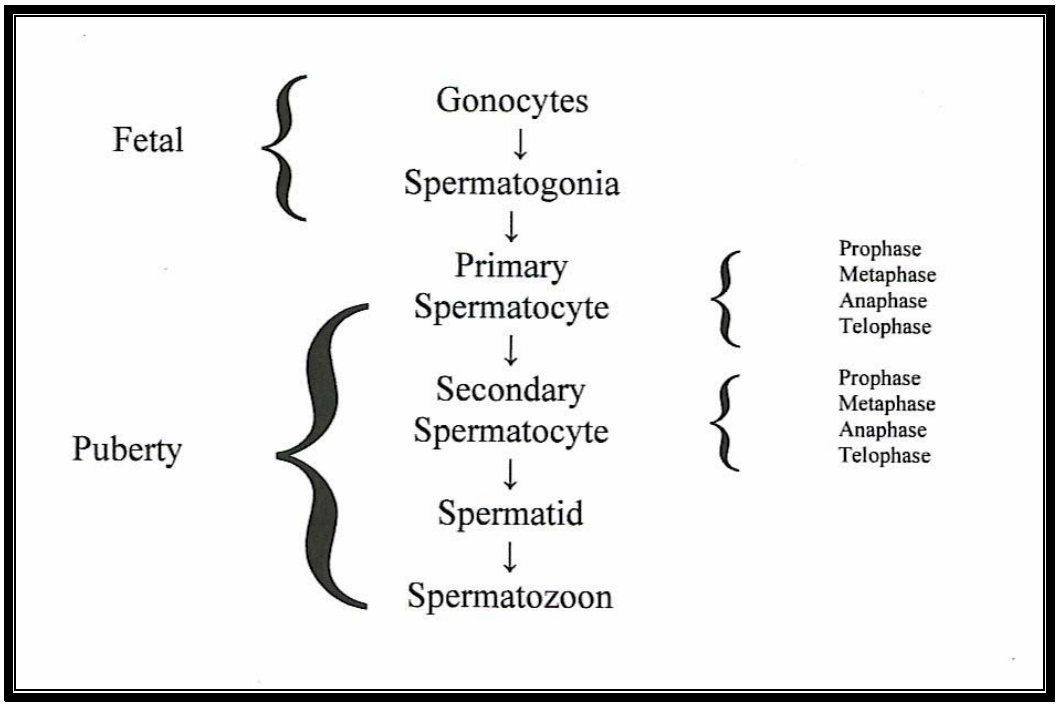


Figure III: Germ cell line

1.3.1. Nuclear shaping and Chromatin Packaging

The chromatin contained in the nuclei of mature mammalian spermatozoa is an extremely compact and stable structure. To achieve this unique condensed state, sperm DNA must be organized in a specific manner which differs substantially to that of somatic cells (Poccia 1986, Ward and Colley 1991). This DNA organization not only permits transfer of the very tightly packaged genetic information to the egg, but insures that the information is delivered in a physically and chemically organized form so that the genetic information cna be properly accessed by the developing embryo.

The very high packaging value of chromatin, is principally due to dramatic modifications of the nucleoprotein components, the most essential consisting of the replacement of histones by protamines, occurring during spermiogenesis (Ward and Coffey, 1991, Kumaroo *et al*.,

1975; Bouvier, 1977; Courtens and Loir, 1981; Balhorn, 1989). Protamines are small, very basic, arginine- rich proteins whose principal roles include DNA charge neutralization and chromatin compaction (Mali *et al*., 1988). In addition to the role of protamines the chromatin is further stabilized by the formation of intra- and inter-molecular disulphide cross-links among the cysteine residues of the protamine molecule.

The structure of this insoluble, inactive chromatin is believed to have a looped or lamellar organization as distinct from the more open nucleosomal organization observed in somatic cells (Ward and Coffey 1991). Indeed, sperm DNA has been found to be organized into loop domains that are anchored by specific sequences to the nuclear matrix in both hamster and human sperm (Ward and Coffey, 1991; Balhorn, 1982; Barone *et al*., 1994).

The most important characteristic of the gametes is that they are haploid meaning that they only have 23 chromosomes compared to the usual 46 chromosomes of other human cells (Acosta, 1996). The DNA in the spermatozoon head is complexed with proteins and is known as chromatin (De Lange *et al*, 1971). In a wide variety of species, transformation of the spermatid chromatin from a nucleohistone to a nucleoprotamine occurs during spermiogenesis (Claasens, 1991). Formation of these nucleoprotamines is responsible for the suppression of gene transcription and for the extreme condensation of the chromatin (Monesi, 1964). The density of this chromatin hinders the assessment of chromosomal order because the individual chromosomes cannot be seen.

The basic proteins within the nucleus are retained during spermatogenesis and the remainder of spermatozoon maturation. Once the spermatozoa reach the epididymis, protamine synthesis occurs (terminal stages of spermatogenesis) (Monesi, 1964) and all the

protamines are completely dephosphorylated (Claasens, 1991). There are two known protamine species and they are characteristically rich in arginine and cysteine but also contain serine and tyrosine (Claasens, 1991).

One may assume that the spermatozoa's head structure may influence the DNA-protein assembly, but according to Pedersen *et al* (1975), no differences in chromatin appearance has been seen in abnormal spermatozoon heads. It has however been found that chromatin distortion can occur when there are large numbers of vacuoles present. This could be due to distortion of the normal array of chromosomes (Pederson *et al*, 1975).

1.4. The Spermatozoon

The morphology of the spermatozoon is of importance due to its role not only during normal fertilization but also *in vitro* fertilization (Kruger *et al*, 1986; Ombelet *et al*, 1994; Eggert-Kruse *et* al, 1995). The following criteria, for morphology, is according to Tygerberg Strict Criteria (Kruger *et al*, 1986; Menkveld *et al*, 1990; Kruger *et al*, 1996) and has shown good predictive values for IVF fertilization and pregnancy rates.

1.4.1. The Spermatozoon Head and Acrosome

The spermatozoon consists of a head (containing paternal DNA), neck and tail region (provides motility) (Figure IV). Remembering that stained human spermatozoa are slightly smaller than live spermatozoa; the head is oval-shaped with a length of approx. 4 – 5.5µm and a width of approx. 2.5 – 3.5µm (Kruger *et al*, 1996). The length to width ratio of most spermatozoa falls between 1.5 and 1.75 (Kruger *et al*, 1996).

Trivial variations are seen and are considered normal but morphological anomalies are classified as follows: very large heads (macrocephalic), very small heads (pinheads or microcephalic), tapering, pyriform, amorphous, vacuolated and double headed (Menkveld & Coetzee, 1995) also fully explained in section 2.3.2. The nucleus takes up 65% of the head where the DNA is linked with other proteins to form a chromatin string that is compact and where the chromosomes are not visible (Menkveld & Kruger, 1996; Kruger *et al.*, 1996; Weinbauer *et al.*, 2000). The acrosome region covers the anterior two thirds of the head and it arises from the golgi apparatus between the spermatid and spermatozoon stages (Kruger *et al.,* 1986; Claasens, 1991; Menkveld & Coetzee, 1995; Menkveld & Kruger, 1996; Kruger *et al.*, 1996). Depending on the staining method used, the normal acrosomal size will cover 40-70% of the spermatozoon head (Kruger *et al.*, 1996). The acrosome contains hyaluronidase and proacrosin enzymes that participate in the acrosome reaction of the spermatozoa during penetration of the cumulus/oocyte complex.

1.4.2. The Mid-Piece

The mid-piece (Figure IV) contains mitochondria that are responsible for providing energy required for sperm motility. The central axis contains 11 fibrils and the outer ring has nine coarser fibrils (Claasens, 1991; Kruger *et al.*, 1996). The mitochondria are situated around the outer fibrils. The mid-piece has approximately a similar length to that of the spermatozoon head and is separated from the tail by a ring called the annulus (Kruger *et al.*, 1996; Weinbauer *et al.*, 2000).

1.4.3. The Spermatozoon Tail

The tail of the spermatozoon (Figure IV) arises in the spermatid stage (Figure III). In human spermatozoa the flagellum (tail) reveals the same basic pattern of microtubles and matrix components that can be seen in the cilia and flagella throughout the animal kingdom (Pederson *et al.*, 1975). This pattern consists of nine evenly spaced, pheripheral doubled microtubles and a central pair of single microtubles. The tail has two parts namely the principal piece and the end piece. The principal piece of the tail is the longest with the entire tail being 4-10μm long and less than 1μm in diameter (Kruger *et al.*, 1996).

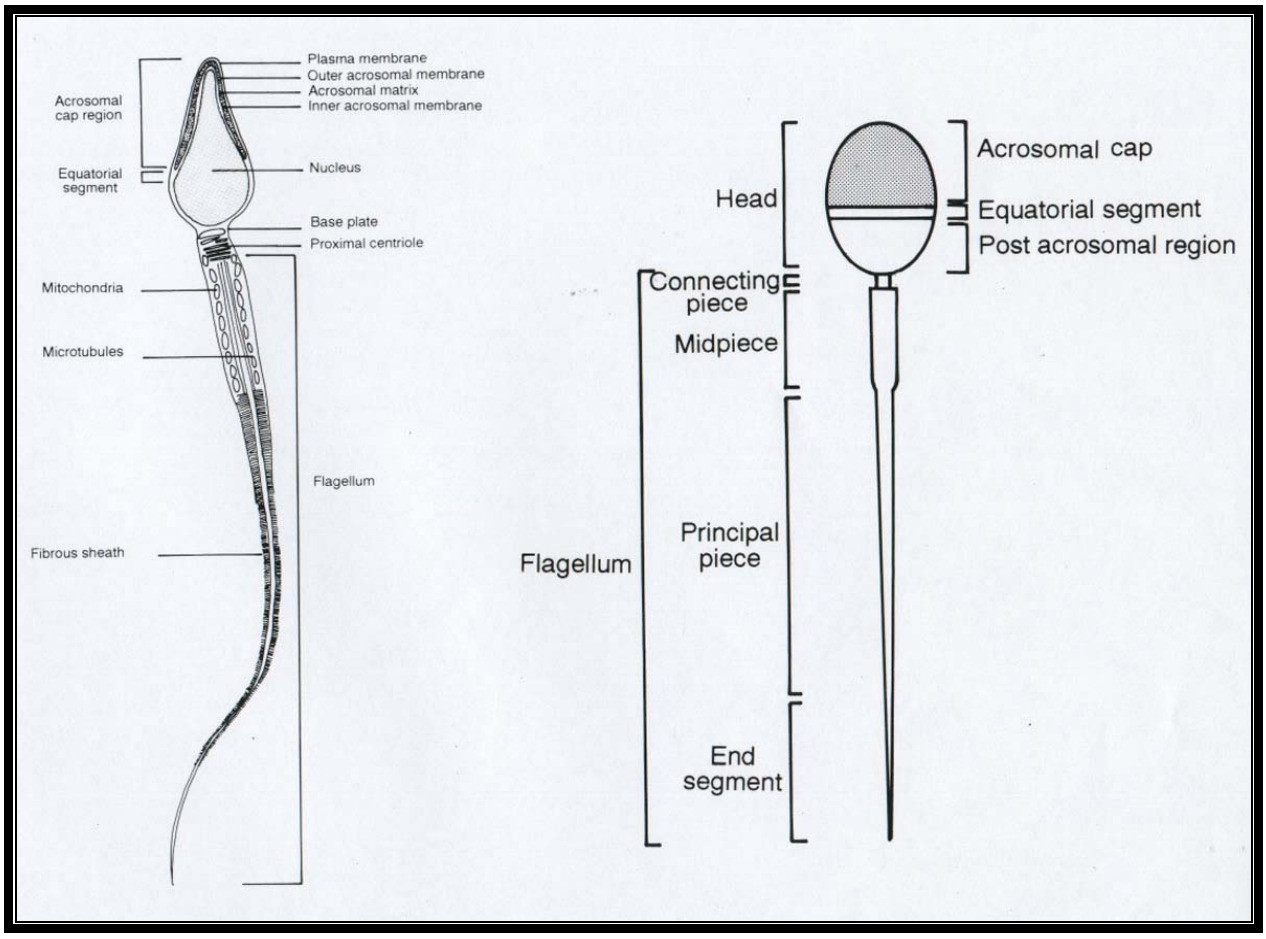
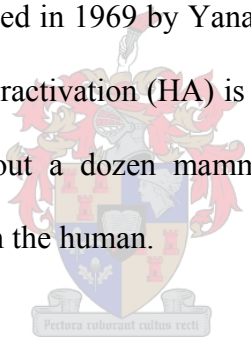


Figure IV: A longitudinal section and schematic drawing of a normal spermatozoa (Menkveld *et al*, 1995)

1.5. Processes Spermatozoa Undergo before Fertilization

1.5.1. Hyperactivation

Mature spermatozoa of all mammalian species studied must complete a series of membrane and metabolic changes before they can fertilize an intact ovum (Yanagimachi 1988). This process has been termed capacitation and is functionally associated with the sperm acrosome reaction and acquisition of a distinctive type of motility. The acrosome reaction (AR) is no longer viewed as the sole phenomenon which signals the completion of capacitation. Increasingly, hyperactivated motility of spermatozoa has been included as a second functional marker (Yanagimachi 1988, Bedford 1983, Chang 1984, Fraser 1984, Yanagimachi 1969). First identified in 1969 by Yanagimachi (1969) and also by Gwatkin and Anderson, (1969) sperm hyperactivation (HA) is a distinctive, vigorous motility which has now been described for about a dozen mammalian species, (Yanagimachi, 1969) including two primates and now in the human.



Hyperactivation is a movement pattern seen in sperm at the site and time of fertilization in mammals. It may be critical to the success of fertilization, because it enhances the ability of sperm to detach from the wall of the oviduct, to move around in the labyrinthine lumen of the oviduct, to penetrate mucous substances and, finally, to penetrate the zona pellucida of the oocyte. The movement of hyperactivated sperm appears different under varying physical conditions and in different species, but basically it involves an increase in flagellar bend amplitude and, usually, beat asymmetry. Presumably, a stimulus exists in the oviduct ,initiating hyperactivation at the appropriate time; however, none has yet been identified with certainty. While the signal transduction cascade regulating hyperactivation remains to be completely described, it is clear that calcium ions interact with the axoneme of the

flagellum to switch on hyperactivation. Although hyperactivation often occurs during the process of capacitation, the two events are regulated by somewhat different pathways (Van Ahlen *et al* 2000).

1.5.2. Capacitation

Unlike their invertebrate counterparts, mammalian sperm are not immediately fertile upon release from the male reproductive tract, despite their ability to exhibit vigorous motility. They require a species-dependent period of time during which they undergo a series of changes, collectively referred to as 'capacitation' (Loeb 1915, Yanagimachi 1994), that are needed for cells to become fully competent in order to fertilize an oocyte. When capacitated, mammalian sperm can express hyperactivated motility (Loeb 1915), the very vigorous, thrusting pattern of motility which is needed for penetration of the oocyte investments (Bedford 1983, Yanagimachi 1994) and interact with oocytes (including cumulus cells, follicular fluid and zona pellucida) to undergo the acrosome reaction. The latter is an exocytotic event that promotes interaction with and penetration through the zona pellucida and confers fusogenic properties on the remaining plasma membrane in the sperm head (Bedford 1983). It has been suggested (Caswell and Hutchison, 1974) that the importance of capacitation may actually be to prevent sperm from becoming fertile too quickly, given that cells are deposited into the lower regions of the female reproductive tract and then migrate some considerable distance to reach the fertilization site.

It has long been known that successful fertilization is dependent on the extracellular ionic environment, in large because this can modify the intracellular ionic composition of the gametes. The first observations on this as published 90 years ago, when Loeb

noted that fertilization in the sea urchin did not occur in the absence of extracellular Ca^{2+} (Loeb, 1915). Later studies revealed that this was due to defective sperm function, namely failure of the acrosome reaction to occur. After the development of successful culture systems for mammalian gametes it was possible to demonstrate that mammalian sperm fertilizing ability, like that of invertebrate sperm, can be modulated by alterations in the extracellular ionic composition.

The following events occur during the capacitation process (Overstreet, 1996):

- There is removal or modification of the sperm surface proteins
- Efflux of cholesterol from the spermatozoon membrane (albumin and high density proteins are responsible for the removal of cholesterol)
- Changes in oxidative metabolism
- Hyperactivity of spermatozoa occurs (more whip-like movement of the tail)
- Increase in phosphotyrosine phosphorylation of certain proteins
- Decrease in the calmodulin binding proteins
- Increase in Ca^{2+} uptake (utilising Ca^{2+}-ATPase pumps) (DasGupta *et al*, 1994)
- Increase in intracellular pH
- Increase in cAMP levels

All of the above bring about changes in the membranes of the spermatozoa head which allow for the onset of the acrosome reaction (DasGupta *et al*, 1994). A time dependant decrease in endogenous enzyme activity would allow the intracellular concentration of Ca^{2+} to rise to a critical value necessary for the initiation of acrosomal exocytosis and subsequent succesfull fertilization (DasGupta *et al*, 1994). Some spermatozoa will 'over

capacitate' and undergo spontaneous acrosome loss (Fraser *et al*, 2003). This is undesirable since acrosome-reacted spermatozoa are non-fertilizing (Fraser, 1984; Fraser *et al*, 2003). To test whether or not the spermatozoa do in fact undergo capacitation, spermatozoa can be exposed to human follicular fluid, disaggregated zona pellucida or progesterone and be tested for acrosome reacted spermatozoa (Overstreet, 1996). Only spermatozoa which were already capacitated would be able to undergo the acrosome reaction (Chen *et al*, 1986; Nagae *et al*, 1986; Overstreet, 1996).

1.5.3. Acrosome Reaction

For successful fertilization of oocytes by spermatozoa, a set of functionally normal parameters with regard to oocyte and spermatozoal maturity are of paramount importance. In the spermatozoon, besides motility and zona binding, the occurrence of the acrosome reaction is of primary importance in the development of functional capability (Chen *et al*, 1986; Nagae *et al*, 1986; Kruger *et al*, 1995;Overstreet, 1996; Fatchi *et al*, 2002).

The acrosome is a membrane-bound, secretory organelle which appears during spermatogenesis as a product of the Golgi complex (Kruger *et al*, 1995; Overstreet, 1996). Acrosomal membranes underlying the plasma membrane are referred to as outer acrosomal membranes, those overlying the nuclear membrane as inner acrosomal membranes (Nagae *et al*, 1986). The acrosome reaction is an exocytotic process involving fusion of the spermatozoon's plasma membrane and outer acrosomal membrane that must take place before the spermatozoon can penetrate the oocyte vestments and fertilize the oocyte. Only

acrosome-reacted spermatozoa can penetrate the zona pellucida (Nagae *et al*, 1986; Kruger *et al*, 1995; Fatchi *et al*, 2002).

Calcium influx is believed to be an initiating event in the normal acrosome reaction. Multiple steroids help to induce the Ca^{2+}-influx with one of them being identified by Osman *et al*, 1986, as 4-pregnen-3,20-dione (progesterone) and 4-pregnen-17alpha-ol-3,20-dione (17-alpha-hydroxyprogesterone) which are present at the fertilization site *in vivo*. The assessment of acrosomal status after induction of the acrosome reaction by calcium ionophore identifies acrosome reaction dysfunction (Chen *et al*, 1986; Nagae *et al*, 1986). Various staining methods are available for assessing the acrosomal status of human spermatozoa using light or fluorescence microscopy (these are briefly discussed in section 1.6).

The acrosome reaction *in vitro* can be divided into five stages namely:

Stage 1 (Fig V A) is defined by an intact acrosome. Both the acrosomal membranes are intact and the acrosomal matrix is homogenous and compact (Nagae *et al*, 1986; Claasens, 1991).

Stage 2 (Fig V B) shows a swollen or decondensed matrix while the plasma and outer acrosomal membranes remain intact (Nagae *et al*, 1986; Claasens, 1991).

Stage 3 (Fig V C) is characterised by the presence of many vesicles within the acrosomal cap. Derangement of plasma and outer acrosomal membranes are commonly seen. The acrosomal matrix remains intact (Nagae *et al*, 1986; Claasens, 1991).

Stage 4 (Fig V D) is seen as fusion of the plasma and outer acrosomal membranes to each other in the cap/anterior region of the equatorial segment. The matrix is seen to be largely missing (Nagae *et al*, 1986; Claasens, 1991).

During **Stage 5** (Fig V E, F) the inner acrosomal membrane is exposed. The plasma and outer acrosomal membranes as well as the matrix are missing. The equatorial segment of the acrosome at this stage is usually intact but vesiculated or absent in some spermatozoa (Nagae *et al*, 1986; Claasens, 1991).

Spermatozoa of men with proven fertility showed that after 24 hours, a mean of 15.4% spermatozoa had initiated the acrosome reaction with 9.7% of this population having completed the acrosome reaction (Stock *et al*, 1987). Stock *et al*, 1987, therefore claimed that initiation of the acrosome reaction occurs at a fairly constant rate.

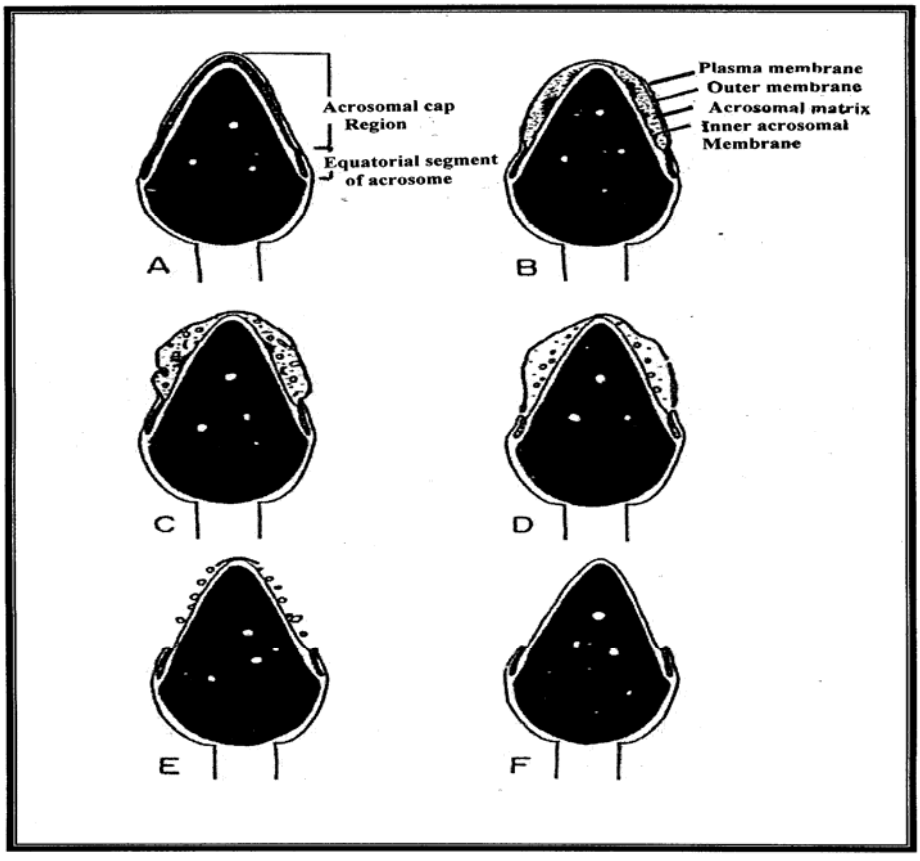


Figure V: The probable processes of the acrosome reaction in human Spermatozoa (Nagae *et al.*, 1986)

1.6 Techniques to determine Sperm Acrosomal and Capacitational Status

1.6.1 Microscopic evaluations

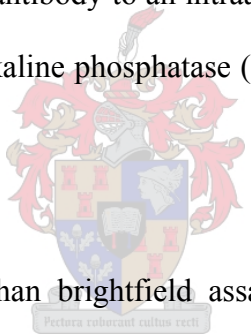
Assays that reveal the loss of the acrosome material

Acrosomal exocytosis is a critical event in the fertilization process, and the ability to discern whether or not sperm acrosome reaction is an essential tool in the study of sperm function. The human acrosome has so far defied attempts to visualize it with phase contrast or differential contrast optics due to its small size and optical properties and for many years research was limited by the need to use electron microscopy to determine if sperm were acrosome reacted. In the past decade, however many simpler techniques have been developed, and now the major problem is deciding which assay to use.

Assays that detect the presence or absence of acrosomal material require the sperm to be permeable to the probe. When sperm are treated with stains for brightfield microscopy, the harsh conditions of the fixative and staining solutions disrupt the sperm membrane. When a lectin or an antibody is to be used, the sperm are usually permeabilized with ethanol or methanol and then exposed to the probe. All of these assays show acrosome-intact sperm as labeled over the entire anterior head. Acrosome-reacted sperm lose much or all of the component to which the labeling attaches during staining, so that these reacted cells have no label over the anterior head. With most probes an equatorial band of label persists in reacted sperm, but a few probes do not react with the equatorial segment so the head of a reacted sperm is unlabeled.

Talbot and Chacon (Talbot and Chacon 1980, 1981) developed the first light microscope methods for assessing human acrosomal status. Their triple-stain procedure, in which trypan blue serves as a viability stain, and Bismark brown Y and Rose Bengal reveal acrosomal status, is often criticized for producing too- subtle distinctions between intact and reacted sperm. It is still in use, however, sometimes varied by omitting the trypan blue (De Jonge 1989).

There are other stains that can be observed by brightfield microscopy. Coomassie blue (Aarons *et al*., 1993) and silver staining (Gosalvez *et al*., 1986, Anderson *et al*., 1992) have been directly or indirectly compared to transmission electron microscopy, with satisfactory results. Binding of a monoclonal antibody to an intraacrosomal antigen has been visualized using alkaline phosphatase-antialkaline phosphatase (Braun *et al*., 1991) but the labeling is of low intensity.



Fluorescence assays are better than brightfield assays because the labeling has higher contrast, and the difference between reacted and intact patterns is much more obvious. Lectin-based assays are very fast, requiring as little as five minutes to label the sperm.

Assays that reveal externalized acrosome material

The acrosome reaction exposes to the extracellular space a new set of sperm components, including the inner acrosomal membrane and the acrosomal contents. Some assays are designed to detect the emergence of these components. In these assays the sperm are not permeabilized so the probe will only have access to the target if the sperm is acrosome-reacted.

The heads of acrosome-intact sperm are essentially unlabeled; the acrosomal region of reacted sperm are labeled. The probes include Con A, soybean trypsin inhibitor and monoclonal antibodies GB24 and MH61 (Fenichel *et al*., 1989, Holden *et al*., 1990, Arts *et al.*, 1994, Okabe *et al.,* 1990). They are visualized by fluorescence microscopy.

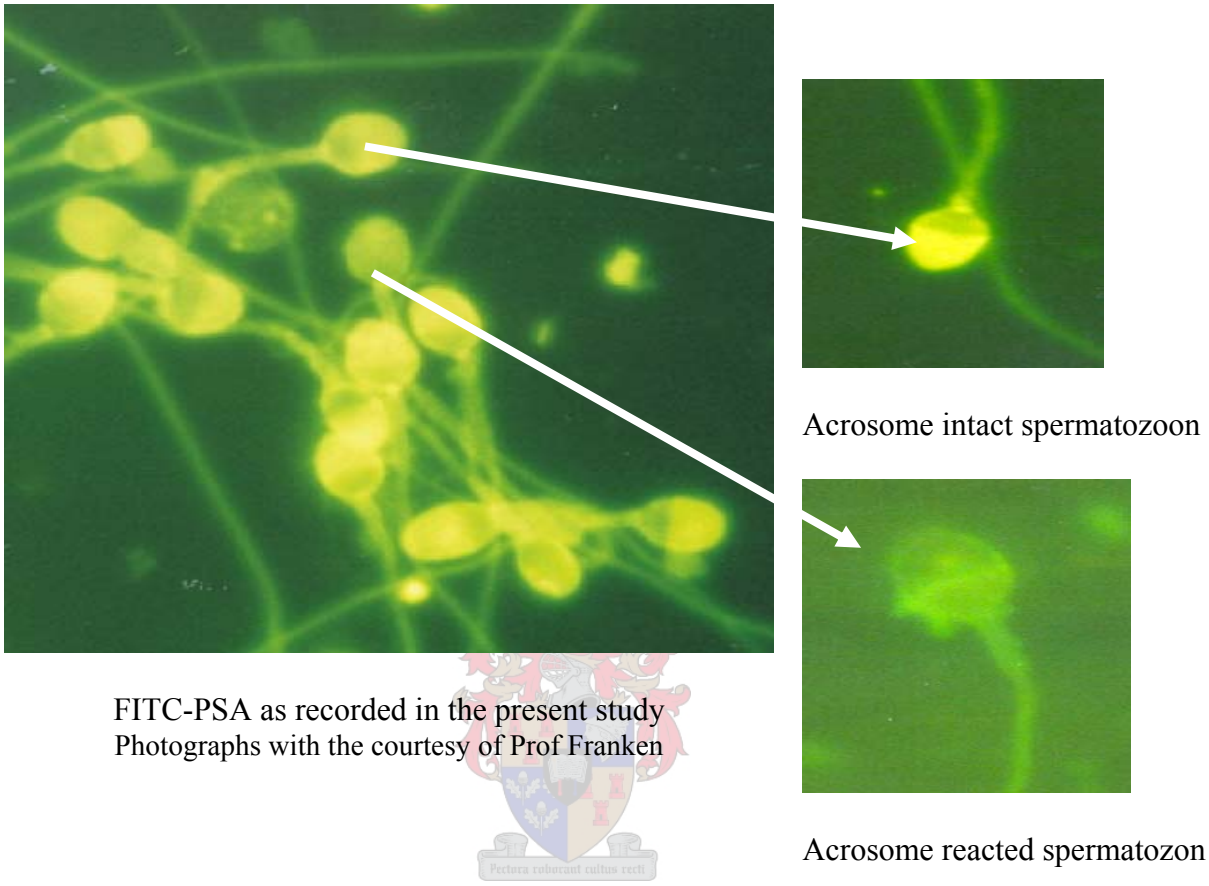
In a different approach Ohashi *et al.* (1992) devised a semi- quantitative assay that employs beads coated with a monoclonal antibody that binds to the anterior head of acrosome-reacted sperm. The formation of mixed aggregates of beads and sperm is a function of the number of acrosome-reacted sperm.

These assays have some advantages over assays in which the sperm must be permeabilized. It may be possible to carry out the assay on living sperm, so the acrosomal status of motile sperm can be determined. Alternatively, the first step of the assay can be fixation with formaldehyde (Holden *et al*., 1990), so that the experimenter has precise control of the time at which sperm are assayed This is a useful feature if the population is changing rapidly. Sperm can be formaldehyde-fixed before permeabilizing them to label with a probe for an extracellular marker, but the fixation conditions may have to be carefully controlled (Morales and Cross 1989).

1.6.1.1 Fluorescein Isothiocyanate labeled Pisum sativum

The use of fluorescein isothiocynate (FITC) conjugated with Pisum sativum agglutinin (PSA) was used for its ability to distinguish acrosome-intact from acrosome-reacted human spermatozoa (Figure VI). This specific type of FITC (PSA-FITC) was used for its ability to

target the acrosomal content for detection of both partial and complete AR (Franken *et al*, 2000).



FITC-PSA as recorded in the present study
Photographs with the courtesy of Prof Franken

Figure VI: Photos of FITC-PSA Stained Slides

Pisum sativum agglutinin (Cross *et al*., 1986) binds to acrosomal contents while peanut agglutinin (Mortimer *et al*., 1987) binds to the outer acrosomal membrane. Ricinus communis agglutinin produces a labeling pattern similar to the other two lectins, although its site of binding has not been identified (Talbot and Chacon 1980). Many modified versions of these assays have been described (Mendoza *et al*., 1992, Morales and Cross 1989, Tesarik *et al*., 1993, Aitken and Brindle 1993)

1.6.1.2 Chlortetracycline test (CTC)

The chlortetracycline (CTC) assay works on the principle that a complex of CTC and calcium binds to membranes which thus showing as highly fluorescent (Veeck, 1986; Agarwal & Damer, 2004). This complex binds to the surface membranes of the spermatozoa when changes occur to these membrane surfaces (these changes are explained under the heading Capacitation).

Chlortetracycline produces patterns that reveal acrosomal status, although the mechanism by which it works is unknown. Treating human sperm with chlortetracycline produces three or four patterns of head fluorescence (DasGupta *et al*., 1993, Lee *et al*., 1987). The relative abundance of these patterns changes with the length of incubation in vitro and following treatment with agents that induce acrosome reactions. A pattern representing acrosome-reacted sperm has been identified, as well as one believed to be characteristic of capacitated sperm.

This assay is rapid and simple to conduct; sample processing is limited to 5 mins, and presents fine resolution (Veeck, 1986; Franken *et al*, 2000). The pattern classification used offers a more precise description of human spermatozoa capacitation *in vitro* (Perry *et al*, 1996).

1.6.2 Flow cytometry

Flow cytometry provides an objective measure of cell fluorescence and it can provide data in real time on a large number of cells thereby improving the sensitivity of the assay. It is

also considerably less fatiguing for the operator than microscope- based assays. Flow cytometry has been applied to sperm labeled with monoclonal antibodies (Fenichel *et al*., 1989, Okabe *et al*., 1990, Tao *et al*., 1993), peanut agglutinin (Purvis *et al*., 1990), and Pisum sativum agglutinin (Henley *et al*., 1994). The monoclonals that recognize antigens that are externalized by the acrosome reaction are particularly useful for flow cytometry because they avoid the need to permeabilize the cells, a treatment which often causes the sperm to aggregate. Supravital dyes have been incorporated into some of these procedures (Henley *et al*., 1994, Tao *et al*., 1993).

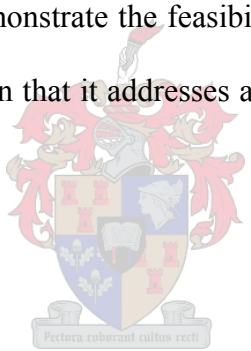
1.7. Techniques to determine Spermatozoa Chromatin Packaging Quality: Chromomycin A_3 (CMA_3).

Conventional semen parameters sometimes fail to predict fertilization outcome suggesting other hidden abnormalities, lying at sperm membrane level or at a chromatin level Chromomycin A_3 can detect these anomalies, being a guanine-cytosine-specific fluorochrome that reveals chromatin that is poorly packaged in human spermatozoa (Claasens, 1991). The chromomysin A_3 and protamines compete for the same binding sites in the DNA which is also one limiting factor of CMA_3 because protamine levels vary according to spermatozoa maturity therefore limiting CMA_3 accesability to the DNA (Bianchi *et al*, 1996). A high CMA_3 fluorescence is a strong indicator of low protamination of the spermatozoal DNA (Franken *et al*, 1999). A number of studies have shown that abnormal chromatin packaging relates to infertility in men (Evenson *et al.*, 1980; Monaco & Rasch, 1982; Foresta *et al.*, 1992). The bright yellow spermatozoa (CMA_3 positive) are easily distinguished from the dull yellow spermatozoa (CMA_3 negative). Odds ratio analysis as shown by Esterhuizen *et al.*, (2002), indicated that being in the >60% CMA_3 staining group resulted in a 15.6 fold increase in the risk of decondensation failure, relative

to CMA_3 staining <44% group. CMA_3 staining has a sensitivity of 73% and a specificity of 75% (Franken *et al.*, 1999). Distinction between fertilization failure or success can therefore be made although it has been seen in some ICSI cases that CMA_3 positivity (very low protamination) does not indicate fertilization failure, but rather the failure of decondensation (although chromatin packaging is also responsible for the decondensation process) (Franken *et al.*, 1999).

1.8 Aim of the Study

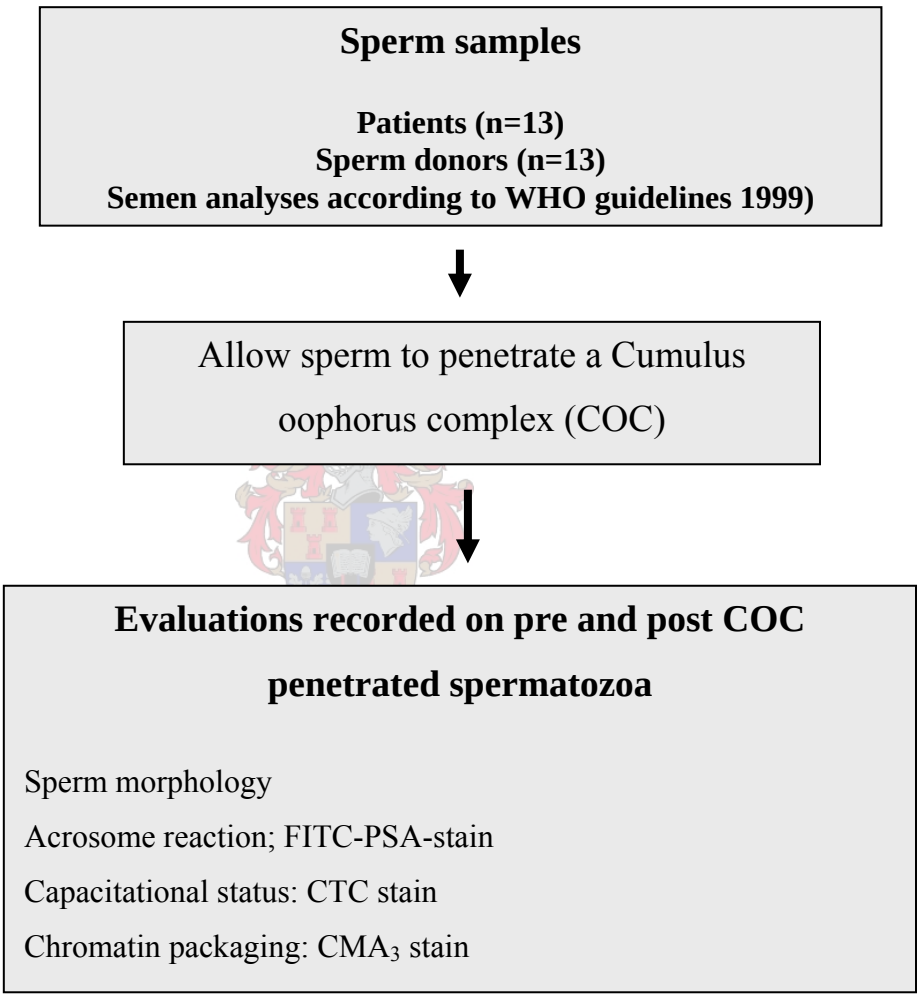
The study aimed to record the (i) capacitational status (ii) acrosomal status and chromatin packaging quality of spermatozoa before and after cumulus oophorus complex penetration. In this study we have tried to demonstrate the feasibility of this model, also used by Hong *et al*, 2004. This study is unique in that it addresses all the deficiencies of precious models (see section 1.1.2 page 8).



CHAPTER II

Materials and Methods

2.1 Experimental design



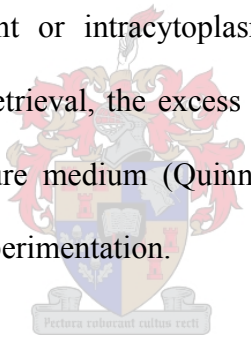
2.2. Spermatozoa Handling and Preparation

A total of 26 semen samples were obtained from (i) 13 men attending the fertility program at Tygerberg Infertility Clinic (patients) and (ii) 13 individuals from our sperm donor program (donors). After complete liquefaction at room temperature, a basic semen analysis was performed according to the WHO manual for semen examination (WHO 1999). Hence,

recordings were made of the semen volume, concentration, spermatozoa motility and forward progression. Separate duplicate slides were prepared to evaluate the percentage normal cells, with more duplicate slides made after cumulus penetration to evaluate the quality of chromatin packaging (CMA_3) , acrosome reaction (FITC-PSA) and capacitational status (CTC) of the sperm samples. All samples were then introduced to the *in vitro* cumulus model, making duplicate slides for the FITC-PSA, CTC and CMA_3 stainings.

2.3. Cumulus Oophorus Retrieval

The cumulus-oocyte complex was obtained from stimulated patients who underwent *in vitro* fertilization (IVF) treatment or intracytoplasmic sperm injection (ICSI). After follicular aspiration and oocyte retrieval, the excess cumulus oophorus was collected and pooled in HEPES buffered culture medium (Quinns, USA) supplemented with Human Serum Albumin (HSA) before experimentation.

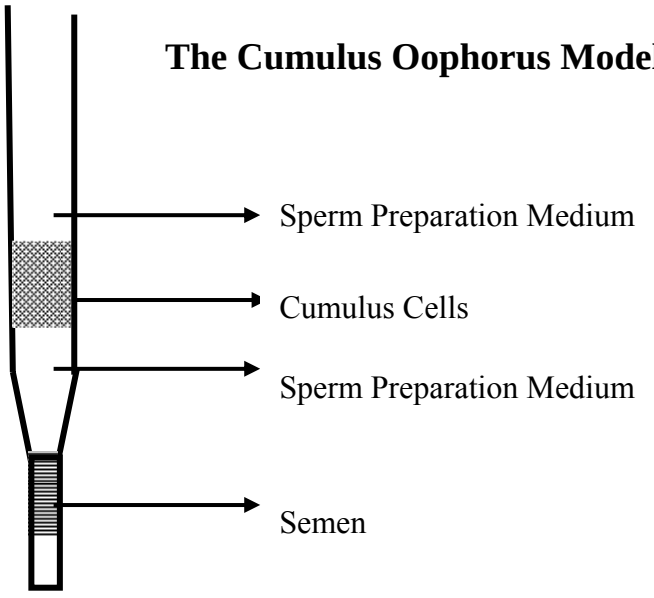


2.3.1. The *In vitro* Cumulus Oophorus Model

Sterile Pasteur pipettes were pre-warmed to 37^0C. Culture medium (Sperm Preparation Medium, Quinns, USA) was aspirated to a length of 10mm from the end of the pipette. This was followed by aspiration of the cumulus oophorus and again sperm preparation medium, thus forming a column the length of 12mm. A pipette containing only sperm preparation medium served as the control (Figure VII).

Four pipettes containing the cumulus oophorus were used for each of the thirteen patients with one of these being the control. The pipettes were then kept at 37^0C for 1 hour in a heated test tube block. After incubation, the pipettes were cut using a diamond-tipped pen at the interface between the cumulus oophorus column and the sperm preparation medium (Quinns, USA) column above the cumulus layer. This medium column contained spermatozoa that have passed through the cumulus oophorus column (therefore penetrated spermatozoa). These spermatozoa were then expelled into an eppindorf tube, with the spermatozoa from several pipettes being pooled. The control pipette was also cut at the same distance from the end of the pipette as those containing cumulus, with the spermatozoa having swum through the medium also being pooled. These samples were then analysed for motility, concentration and forward progression (CASA) and morphology. Slides were made and fixed in a 1:1 ether/alcohol solution for 24 hours and then left to air dry for 4 hours. All the subsequent tests were then performed on these slides.

The Cumulus Oophorus Model Used in This Study



2.4. Semen Samples:

Semen samples were collected from the IVF patients and tsperm donors with normal semen parameters according to World Health Organization criteria. After liquefaction and routine semen analysis, the semen was used as explained in 2.2.1. (Appendix III)

2.4.1. Semen Analysis:

Slide Preparation

One drop of semen was placed on a clean microscope slide and covered with a coverslip. The drop should fill the area under the coverslip, with no empty edges and no floating of the specimen. The regular microscopic (400x ph. magnification) examination of this wet preparation can then be used not only to estimate motile quality, but also spermine phosphate crystal formation, aggregation/agglutination and the presence of microorganisms.

Motility

In determining quantitative motility (manually) one distinguishes the percentage of motile spermatozoa from the percentage of immotile spermatozoa. The estimation of the percent motile is made to the nearest 10 percent (Menkveld & Kruger, 1996; Menkveld & Coetzee, 1995)

Forward progression

In determining qualitative motility in our laboratory the nature of motility is evaluated on a scale of 0 to 4.

0	no movement
1	movement - none forward
1+	movement - a few now and then
2	movement - undirected, slow
2+	movement - slowly but directly forward
3-	movement - fast but undirected
3	movement - fast and directly forward
3+	movement - very fast and directly forward
4	movement - extremely fast and directly forward (wave-like)

Concentration

Dilutions were made according to the number of spermatozoa per high field magnification observed on the wet preparation (e.g. 1/10; 1/20; 1/100). Samples were diluted with distilled water or with a mixture of NaHCO3 (16 gm) and phenol (4 gm) in 400 mL of distilled water, mixed well and transferd to a Neubauer counting chamber (haemocytometer), a slide with engraved squares. Counting chambers were place in a humidified petri dish for 10 minutes and the spermatozoa were counted in two 1 mm^2 areas which are divided into 16 squares (Menkveld & Kruger, 1996; Menkveld & Coetzee, 1995). (Appendix IV)

MAR-Test

Spermatozoa contain antigenic components and this factor may be of significance in the fertility of the male. The MAR-test is a simple and reliable routine screening test for the

presence of IgG antibodies, the most commonly found spermatozoa antibody (Menkveld & Kruger, 1996; Menkveld & Coetzee, 1995). (Appendix V)

Computer Assisted Sperm Analysis (CASA)

The Hamilton-Thorne (HTM-IVOS) analyzer operates as a computerized cell motion analyzer. Integrated components include internal phase-contrast optics with stroboscopic illumination, an internal automated heated specimen stage, image digitizer and a 80486 computer. Samples are loaded and the Integrated Visual Optic System (IVOS) can either evaluate the sample automatically, selecting random fields (maximum 16 fields), or the user can manually select the fields. The random method was used in this study. Various parameters were evaluated by the HTM-IVOS system in this study; spermatozoa concentration, motile and progressively motile concentration, percentage motile and progressively motile, average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat frequency, straightness and linearity (Figure VIII and Appendix VI) (Menkveld & Kruger, 1996).

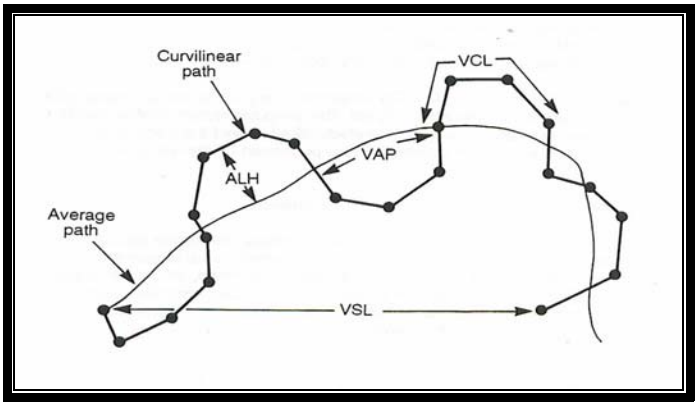


Figure VIII: Spermatozoon head motion parameters typically determined by CASA analysis.

2.4.2. Sperm Morphology – Strict Criteria

Standard sperm morphology evaluations as described by the Tygerberg Strict Criteria (Kruger *et al*, 1986; Menkveld *et al*, 1990)were applied to determine the percentage normal spermatozoon cells (Figure IX) of the raw samples (both IVF patient and donors) as well as the penetrated samples (both IVF patients and donors) (stained with Papanicolaou staining method, Appendix VII). The whole spermatozoon (head, neck, midpiece and tail) was taken into consideration as well as the presence of other cells. The head is required to have a smooth oval configuration with a well defined acrosome, comprising 40-70% of the spermatozoon head (Kruger *et al.*,, 1986; Menkveld *et al.*,, 1990; Menkveld & Coetzee, 1995; Kruger *et al.*,, 1996).

The somatic cells (leukocytes, histocytes and epithelium) were observed on the "albumin" slide and expressed as follows:

+ = A few on the slide
+ = 1-5 cells per high power field (400X magnification)
++ = 6-10 cells per high power field
+++ = >10 cells per high power field

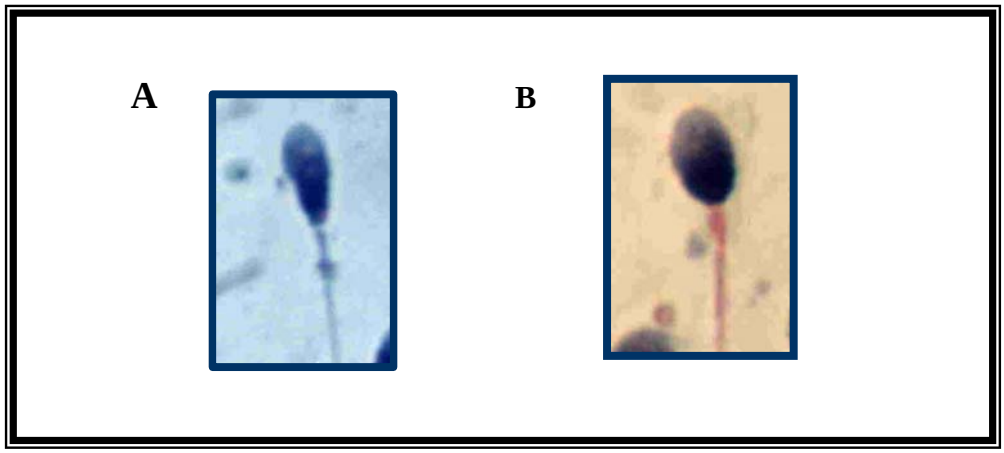


Figure IX: Morphology of Human Spermatozoa (A=Abnormal, B=Normal Sperm) (Menkveld *et al.,* 1995)

2.5 Chlorotetracycline Test (CTC test) for Sperm Capacitation Evaluation

Chlortetracycline fluorescence is used to determine the capacitational status as well as the acrosomal status of a sperm population. Temporal changes in relative proportions of the staining patterns reflect changes in the fertilizing ability of the entire sperm population. Changes in chlortetracycline fluorescence associated with the time sequence of capacitation and the acrosome reaction have been described in both mouse and human spermatozoa (Lee *et al.,* 1987). By allowing assessment of the relative proportions of uncapacitated and capacitated sperm within the acrosome-intact group of cells, CTC analysis provides unique information relating to functional potential (Fraser 1995). The capacitational status of spermatozoa was determined by chlortetracycline staining (Yao *et al*, 2000)

2.5.1. Staining solution

CTC solution comprises a mixture of 5 mM CTC, 20 mM Tris, 5 mM cysteine, and 130 mM NaCl. It is prepared by dissolving 10 mg Tris, 3 mg cysteine, and 30 mg NaCI in reagent water, adding 40µl of a 500 mM aqueous stock solution of CTC, and bringing to a total volume of 4.0 ml. The pH is adjusted to 7.8 ±0.5 using 1 N HCI, and the solution is kept at 4^{o}C. The final CTC solution should be used only on the day of preparation.

2.5.2. Fixative

Glutaraldehyde fixative is a 3% (v/v) solution of glutaraldehyde in 20 mM Tris buffer pH 8.0. The Tris buffer is prepared by dissolving NaCI 7.889 g/l (135 mM), KCI 0.373 g/l (5 mM), and Tris 4.481 g/l (37 mM) in reagent water and adjusting to pH 8.0 with 5 N HC1. The fixative is prepared by mixing 125 µl stock (25% v/v) glutaraldehyde and 875 µl Tris

buffer. The final pH should be 7.8 ± 0.5. Fixative is stored at 4^0C and use only on the day of preparation. Fixative should be discarded when it develops a slight yellow tinge and prepare a fresh solution.

2.5.3. Method

Specimen: Capacitating sperm populations of 5 x 10^6 to 10 x 10^6/ml were used.

1. Place 2.5µl of the sperm suspension on a clean microscope slide at 37°C and quickly add 2.5µl CTC solution. Multiple slides (at least three) should be pre-prepared in case of problems with labeling or reading.
2. Within 10 seconds, 0.25µl glutaraldehyde fixative was added and mixed using the tip of the microsyringe dispenser.
3. Keep the slides at ambient temperature in a light-shielded humid chamber until ready to score.
4. Two slides were scored for each experiment. Slides were randomized and coded, under epifluorescence, counting 250 spermatozoa per slide using an Olympus B40 fluorescent microscope fitted with a 395nm excitation filter (UV plus violet) with 520 nm emission filter and a barrier filter with 455 nm. Each spermatozoon is classified according to the distribution of fluorescence.

2.5.4. Evaluation of Fluorescence Patterns Observed in Human Sperm Cells

CTC staining patterns of the sperm head will be identified according to the method of Perry *et al* (1995):

- Uncapacitated patterns: CTC1, a fluorescent band in the postacrosomal region;
  CTC2, a bright fluorescent head with a nonflourescent postacrosomal region;
  CTC3, a bright fluorescent head with a nonflourescent thin band in the postacrosomal region;
- Capacitated pattern: CTC4, uniform head fluorescence;
- Acrosome Reacted pattern: CTC5, a decrease in or loss of uniform fluorescence over the head;

2.6 Fluorescein isothiocyanate labeled *Pisum sativum* (PSA-FITC) for Acrosomal Status Evaluation

Fluorescein isothiocyanate labeled with pea (*Pisum sativum*) agglutinin (FITC-PSA; Sigma) was used to evaluate the acrosomal status of the sperm. Pisum Sativum agglutinin (PSA) has a specificity for alpha–methylmannose residues and labels the acrosomal content (Cross *et al.,* 1986).

2.6.1. Preparation and Staining

The sperm was placed as a 5µl droplet on a slide and left to dry. Subsequently the slides were fixed in cold ethanol and left to dry after which the area was flooded with the FITC-PSA for 30mins in darkness. Slides were then rinsed in distelled water and mounted with glycerol with a cover slip.

2.6.2. Evaluation of Staining Patterns

The fluorescence pattern of 200 spermatozoa in randomly selected fields was determined under a microscope (Zeiss, Oberkochen, Germany) with 1000x magnification. The acrosomal status of spermatozoa was classified according to the three typical lectin staining patterns: (i) intact acrosome, complete staining of acrosome; (ii) reacting acrosome, partial or patchy staining of acrosome; and (iii) reacted acrosome, complete staining of the equatorial segment only or no staining of the whole sperm head. The proportions of intact, reacting and reacted acrosome were expressed as percentages of the respective patterns in the total number of spermatozoa counted (Perry *et al*, 1995).

2.7. Chromomycin A_3 (CMA_3 Test) for Sperm Chromatin Packaging Quality Evaluation

The quality chromatin packaging was determined using CMA_3 staining (Sigma Chemicals, St Louis, MO USA Cat 2659), techniques (Manicardi *et al.,*., 1995).

2.7.1. Preparation and Staining

In short, for CMA_3 staining thin smears were prepared, air dried and fixed in methanol/acetic acid 3:1 at room temperature for 20 minutes. The slides were air dried and stained with 60-100ul CMA_3 in a dark chamber for 20 minutes. Slides were then washed in McIlvaine buffer (Sakkas *et al.,* 1996) and mounted using Dabco (Aldrich Chemicals Co, Milwaukee, USA cat No. 29,073-4). Two hundred spermatozoa were evaluated under a Olympus B40 fluorescent microscope (Wirsam Scientific Cape Town, South Africa Filter Fx 465-495).

2.7.2. Evaluation of Staining Patterns

Fluorescence was performed using a FITC-filter and an Eplan 100X objective. A total of 200 spermatozoa were randomly evaluated on each slide (Franken *et al.,* 1999). The evaluation of the CMA_3 staining is easily done with the bright yellow spermatozoa (CMA_3 positive) being distinguished from the dull yellow spermatozoa (CMA_3 negative). CMA_3 positive cells have decondensed chromatin were as the CMA_3 negative cells have normal chromatin packaging.

2.8 Incorporation of Subfertile Men (IVF Patient) into the Cumulus Oophorus Model

Couples undergoing *in vitro* Fertilization (IVF) were included in the study. The patients were undergoing IVF treatment due to abnormal semen parameters in the male. Female patients were superovulated and the oocytes recovered. The functional tests were then compared to fertilization rates of the patients in the present study, attending the IVF program.

2.8.1. Ovulation Induction

Ovarian stimulation was carried out by the administration of gonadotrophin-releasing hormone (GnRHa) (Synarel®; Montosano South Africa, Searle) in a long protocol, followed by human menopausal gonadotrophins (HMG) (Pergonal®Serono, South Africa (Pty) Ltd) and/or pure follicle-stimulating hormone (FSH) (Metrodin®; Serono, South Africa, Pty., Ltd.) from day 3 of the cycle. Patients were followed up by doing estradiol

determinations as well as serial ultrasonographical measurement of the Graafian follicle. Ovulation was induced by the administration of human chorionic gonadotrophin (HCG) (Profasi®; Serono, South Africa (Pty) Ltd) as soon as the leading follicle reaches 18mm in diameter.

2.8.2. Oocyte retrieval and preparation during IVF

Females underwent transvaginal or laparoscopic follicle aspiration. Oocytes were recovered from the aspirated fluid within 20 to 120 seconds. The resultant embryos were then incubated (37^{o}C, 6% CO_2in air) sequentially with fertilization, cleavage and blastocyst media (Quinns Advantage^{TM} Sequential Embryo Media (Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA).

Oocytes were incubated with an intact cumulus until insemination. Metaphase II oocytes were inseminated with 100 000 to 500 000 motile spermatozoa each, according to percentage of morphologically normal spermatozoa. Fertilized oocytes were then placed in sequential media until transfer.

2.8.3. Fertilization and embryo culture

Fertilisation was verified by the presence of two pronuclei as well as 2 polar bodies, at 16-18 hours after insemination and again at 26 hours. On day 2, the embryos were evaluated for cleavage and quality (morphology) to the 4-cell stage and all those showing division before 26 hours were classified as being early dividers. On day 3, embryos destined for

culture to the 8-cell stage, were transferred to blastocyst medium and evaluated for embryo quality. Embryos not transferred were cultured until blastocyst stage.

2.8.4. Embryo transfer

Two, three or four embryos (4 to 8 cell stage) were transferred into the fallopian tubes (laparoscopy) or the uterus with an Embryon thin wall catheter (Rocket Medical). In cases where early cleavage embryo(s) of good quality were available, they were selected and preferably transferred. In cases where no early cleavage took place, the best quality embryo(s) were transferred.

2.9. Statistical analysis

All statistical analyses were performed with MedCalc 8.0.1.0 for Windows (MedCalc Software, Mariakerke, Belgium). A repeated measure of variance was done on CTC, FITC-PSA and CMA_3 data. Results were then analysed using the Students t-test to calculate the mean difference, confidence interval and p-values of the control and cumulus test model for all of the following between the pre- and post-penetration of both the donors and the patients: CTC, FITC-PSA, CMA_3, and morphology, VAP, VSL, VCL, ALH, BCF, STR and LIN. P-values <0.05 was accepted to be significant.

CHAPTER III

Results

3.1. Semen Analysis Results for Sperm Donors and IVF Patients.

The semen analysis results for both the donors and the IVF patients are tabulated in Table I below.

Table I

The mean (±SD) semen analysis parameters for the donors and IVF patients

Samples	Motility % Progresssive	Forward Progression Rated 1 - 4	Concentration	Morphology % Normal Cells
Donors (n=13)	50±12%	3^{-}	64±14 x 10^{6}/ml	7±2%
IVF Patients (n=13)	55±9%	3	90±16 x 10^{6}/ml	6±3%

3.2. Effect of cumulus oophorus on selecting motility of the sperm donors.

The effect of the cumulus oophorus on sperm motility parameters (only done on the donor samples) are shown in Table II.

Table II

Effect of cumulus oophorus on sperm motility

Group	Pre-cumulus penetration	Post cumulus penetration	p-Values
VCL (µm/s)	58.2	55.3	0.630
VAP (µm/s)	39.6	36.0	0.351
VSL (µm/s)	31.5	31.7	0.951
BCF (Hz)	16.0	27.2	<0.0001
ALH (µmol/l/s)	3.8	2.7	<0.027
LIN (%)	54	54.5	1.000
STR (%)	79	85	<0.0001*

3.3. Effect of Cumulus oophorus in selecting morphologically normal spermatozoa.

The semen samples (donors and IVF patients) used, were p- and g-morphologic patterns. The mean morphology collected from the control capillary was 6.8%. The spermatozoa that transversed the cumulus oophorus had no significantly higher morphology (p = 0.92 and p=0.80 for the donor and IVF patient groups, respectively) compared to the controls for both the donors and IVF patients.

3.4. Results of FITC-PSA, CTC and CMA_3 staining of semen samples before and after Cumulus penetration

3.4.1. CTC results

Sperm donor and IVF patient's results to indicate changes in the capacitational status of the spermatozoa as determined by CTC staining are represented in Table III.

3.4.2. FITC-PSA results

Table III shows the acrosomal status as determined by FITC - PSA staining of the transversed versus control spermatozoa (AR in Table III) for both the donors and IVF patients. Significantly more acrosome-reacted spermatozoa were seen in the transversed spermatozoa (p=0.0001) than in the control for both donors and IVF patients. Also, significantly more un-reacted (intact acrosome) sperm were found in the control rather than the transversed samples for both groups (Donors = 13.0% vs. 46.8%; IVF Patients = 13.9% vs. 27.0%).

3.4.3. CMA_3 results

The effect of cumulus oophorus on chromatin packaging are shown in Table III as determined by CMA_3 staining of the spermatozoa. No significant differences were seen between the pre- and transversed samples for the CMA_3 positive staining (33.5% vs. 32.69%).

The results for the CMA_3 staining (Table III) for the IVF patient group showed a significant increase in the CMA_3 positive population of the transeversed spermatozoa population (43.6% vs. 58.3%; p=0.00001) vs. the control population.

Table III

Sperm test results of the donors and IVF patients control and penetrated groups

Sperm population	FITC-PSA Acrosome reacted sperm	CTC 1,2,3	CTC 4	CTC 5	CMA_3
Donor Pre-COC penetration	13.0%	44.6%	39.4%	16.0%	33.5%
Donor Post-COC penetration	46.8%	30.5%	41.2%	28.3%	32.69%
p-Values	0.0001*	0.0001*	0.42	0.0001*	0.51
Patient Pre-COC penetration	13.9%	46.4%	38.4%	15.2%	43.6%
Patient Post-COC penetration	27.0%	31.4%	40.1%	28.5%	58.3%
p-Values	0.0001*	0.0001*	0.55	0.0001*	0.0001*

3.5. Sperm donors vs. IVF Patients

3.5.1. Pre-cumulus penetration

The FITC-PSA, CTC and CMA_3 results recorded for pre-penetration sperm populations, were compared between the donors and the IVF patients. The results are tabulated in Tables IV and also graphically represented in Graphs III. Only the CMA_3 results showed a significant difference (p=0.0001) between the donors and IVF patients

Table IV

Sperm acrosome, capacitatational status and chromatin packaging quality of sperm obtained from donors vs. IVF patients <u>before</u> Cumulus penetration.

	FITC-PSA Acrosome reacted sperm	CTC 1, 2, 3	CTC 4	CTC5	CMA_3
Donors	13.0%	44.6%	39.4%	16.0%	33.5%
Patients	13.9%	46.6%	38.4%	15.2%	43.6%
p-values	0.600	0.580	0.720	0.560	0.0001*

3.5.2. Post Penetration Results

The post penetration results of the donors vs. the IVF patients were compared for post-penetration reading and are depicted in Table V.

Table V

Sperm acrosome, capacitatational status and chromatin packaging quality of sperm obtained from donors vs. IVF patients after Cumulus penetration.

	FITC-PSA Acrosome reacted sperm	CTC 1, 2, 3	CTC 4	CTC5	CMA_3
Donors	46.8%	30.5%	41.2%	28.3%	32.69%
Patients	27.0%	40.1%	38.4%	28.5%	58.3%
p-values	0.0001	0.760	0.710	0.870	0.0001

If we consider the pre-penetration recordings as baseline values, the post–COC penetration scenario can be expressed by subtracting the baseline values form the post penetration values (Table VI).

Table VI

Results of mean changes (increase/decrease) in values recorded for percentage acrosome reacted sperm, capacitational status and percent CMA_3 positive sperm

	FITC-PSA Acrosome reacted sperm	CTC 1, 2, 3	CTC 4	CTC5	CMA_3
Donors	33.8%	-14.1%	1.8%	12.3%	0.81%
Patients	13.1%	-15.0%	1.7%	13.3%	14.7%
p-value	0.001	0.341	0.560	0.07	0.0001

3.6 Fertilization rates and post penetration values for acrosome reacted spermatozoa, capacitational status and CMA_3 positive sperm cells.

As seen in Table VII, there was no significant difference recorded between the fertilization rate of the IVF patients and the post penetration acrosome reaction (FITC-PSA and CTC 5), uncapacitated spermatozoa (CTC 1, 2, 3), and the capacitated spermatozoa (CTC 4). There was a significant difference seen with the CMA_3 positive sperm and the fertilization rate (p-value = 3×10^{-4}).

Table VII

Fertilization rates reported for the IVF patients compared to the percent acrosome reacted spermatozoa, capacitational status and percent CMA_3 positive sperm cells.

	p-Values
% Fertilization rate vs AR (FITC-PSA)	0.655
% Fertilization rate vs. CTC 1, 2, 3	-0.03
% Fertilization rate vs. CTC 4	0.135
% Fertilization rate vs. CTC 5	-0.05
% Fertilization rate vs. CMA_3	0.3×10^{-5}

CHAPTER IV

Discussion

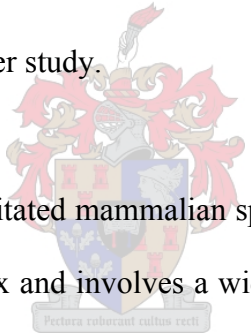
In order to achieve its objective of fertilizing the ovum, the male gamete has to display a wide variety of diverse properties involving differential patterns of movement, membrane restructuring during capacitation, zona recognition, signal transduction, acrosomal exocytosis, the concomitant processing of recognition and fusion of molecules on the sperm head and, ultimately, fusion with the vitelline membrane of the oocyte itself (Veeck, 1986). In view of this complexity, it follows that the functional assessment of human spermatozoa cannot be achieved on the basis of any single global test. Instead, a range of bioassays have been developed, each one of which focuses on a different aspect of sperm function (Menkveld *et al*, 1996). In this study the percentage of morphologically normal sperm was not significantly (controls donors p=0.92 and IVF patients p=0.80) higher in the cumulus penetrated population than that of the control. This is in contrast with the findings of Hong et al., (2004) who described a significant increase in the percentage of normal spermatozoa increase after cumulus penetration. This discrepancy can be because they used sperm populations of extreme high quality especially as far as motility and morphology is concerned. In that study the percentage normal cells in the ejaculates were recorded as 18% and in the post cumulus penetrated population 36%. Similar results were reported in a study by Menkveld et al., (1991); using human zona pellucida as sperm morphology selector (Menkvld et al., 1991). During the latter study it was concluded that sperm samples with morphology value <10% and motility of <60%, did not show the same increase in percent normal cells after zona binding, However, sperm samples with >14% normal sperm and motility >60% also revealed a significant increase in the percentage normal sperm that were zona bound. Both the donors and IVF patients in the present study

had <10% motile sperm with motility values of 50±12% (donors) and 55±9% (IVF patients).

The effect of human cumulus oophorus on movement characteristics of human spermatozoa previously incubated *in vitro* under capacitating conditions was studied using automated digital image analysis. When spermatozoa were incubated for a short time with whole cumuli, most of those that penetrated the cumulus intercellular matrix were characterized by a linear movement with small amplitudes of lateral head displacement, but with elevated values of beat cross frequency (Hong et al., 2004). Short (5 min) incubation with solubilized cumulus intercellular matrix of spermatozoa preincubated in capacitating conditions (6 h) significantly reduced the percentage of spermatozoa showing the 'hyperactivated' type of motility characterized by high curvilinear velocity, low progressive velocity and elevated values of lateral head displacement which was also seen by Hong *et al.* (2004). Moreover, a subpopulation of spermatozoa with very high values of progressive velocity and beat cross frequency and with reduced amplitudes of lateral head displacement appeared in these conditions. This cumulus-related motility pattern was not seen in fresh spermatozoa or in those incubated in the absence of cumulus material. Changes in the sperm movement characteristics similar to those observed in the presence of the solubilized cumulus matrix could also be induced by some of its high pressure liquid chromatography (H.P.L.C.) fractions. These results show that the intercellular matrix of the human cumulus oophorus exerts a specific effect on human sperm motility, probably acting preferentially on the 'hyperactivated' sperm subpopulation

The motility of penetrated spermatozoa in this study was also seen to be more rapid, linear and have a better progressive fashion. The BCF and STR (VAP:VSL) was significantly

higher in the cumulus penetrated population ($p < 0.001$). The ALH was significantly lower ($p = 0.027$) in the penetrated versus the control populations. These findings are not in correlation with Hong *et al* (2004), who found that VAP, VSL and BCF were significantly higher. The present results of the ALH was, however, the same, namely, significantly lower ($p = 0.027$) (Hong *et al*, 2004). These findings imply that the hyperactivated motion of sperm, namely high VCL, ALH and low VSL is lower in the penetrated population versus the control. Hong *et al* (2004), also obtained these results which is contrary to other scientific research done by Tesarik *et al.,* (1990). It has however been suggested by Drobnis *et al* (1988) that the mechanical resistance of the cumulus matrix influences the motility patterns of spermatozoa. These findings are then supportive of the suggestion that there are unknown components within the matrix. The area of the exact composition and physiology of the cumulus matrix needs further study.



The transformation of an uncapacitated mammalian sperm cell into a acrosome reacted cell (Table III) is clearly very complex and involves a wide array of interacting systems which control fluxes of ions and hence their intracellular concentrations. The capacitational state of the sperm populations as evaluated by the CTC-test provided interesting information as far as the kinetics of capacitation is concerned. At present, these are only beginning to be understood and we currently have more information about mechanisms that may help to regulate events occurring primarily in the sperm head.

Sperm capacitation is a prerequisite of fertilization. Many previous studies (Bastias *et al.,* 1993; Chen *et al.,* 1986; Yeo *et al.,* 2000; Osman *et al*, 1989; Fukui, 1990) show that cumulus cells help induce capacitation (in addition to follicular fluid, progesterone, etc). In this study there is not a significantly ($p = 0.42$) higher percentage of capacitated

spermatozoa found in the penetrated population versus the control for both the donor (p=0.42) and patient (p=0.55) groups.

On the other hand (Table III) the number of uncapacitated sperm before penetration is significantly (p=0.0001) higher than after penetration for both the donor and IVF patient groups. This indicates that although the capacitated sperm is not significantly different pre- and post-penetration, the uncapacitated populations has possibly undergone spontaneous acrosome loss as shown by the significant (donors: p=0.0001 and IVF patients: p=0.0001) increase in the amount of acrosome reacted sperm. These observations can be explained, firstly, the cumulus oophorus stimulates capacitation and subsequently the acrosome reaction, of the spermatozoa passing through it due to the presence of progesterone secreted by the cells, and secondly, that capacitated spermatozoa pass through the cumulus faster than uncapacitated spermatozoa (Cherr *et al*, 1986). Capacitation, however, is very difficult to define and therefore the data presented here cannot differentiate between the two above possibilities and the start of the acrosome reaction. The sperm reaching the zona pellucida should be initiating the acrosome reaction, not fully reacted, because it has been shown that the zona does not allow acrosome reacted sperm to pass through it.

Comparison between sperm capacitational kinetics of donor and IVF patient populations (Tables III and IV) indicated a clear difference between pre –and post COC penetration values. Furthermore, the dramatic differences (p=0.0001) in both donor and IVF patient populations between uncapacitated versus capacitated (and acrosome reacted) populations before COC penetration, is indicative of the dynamics of the capacitation kinetics. It is also an indication of the heterogeneity of the sperm populations as far fertilization potential is concerned. Direct comparison between pre-& post COC penetration further illustrates the

dynamics between capacitated and uncapacitated sperm A gradual decrease in uncapacitated sperm (illustrated by the open columns in Graphs III and IV) and the consistent elevated levels of capacitated sperm (illustrated by black columns in Graphs III and IV) is recorded.

If we recognize capacitation as a time dependant physiological regulator of the fertilization capacity of spermatozoa, then we will also comprehend the heterogeneity concept of spermatozoa. Furthermore, it is known that capacitation can be visualized by two endpoints, namely, the occurrence of the acrosome reaction and specific changes in the motion characteristics of sperm population viz. hyperactivation. This is possibly nature's way to ensure that there are always capacitated sperm ready to acrosome react after binding to the zona pellucida to eventually achieve fertilization.

The penetrated spermatozoa showed significantly higher levels of acrosome reaction (CTC5 for donors: p = 0.0001; CTC5 for IVF patients: p=0.0001; FITC-PSA for donors p=0.0001; FITC-PSA for IVF patients: p=0.0001) compared to the control samples for both the donors and the IVF patients. These findings are consistent with other studies; Stock *et al* (1989) and Fukui *et al* (1990) showed that human COC increases the percentage of acrosome reacted spermatozoa when sperm are co-incubated for 14 to 18 hours post insemination. Tesarik *et al* (1985) and Carrell *et al* (1993) also reached similar conclusions. The exact mechanism responsible for this activation of the acrosome reaction cannot be fully explained. It can, however, be attributed to the hormones (progesterone) and other activation components (example hyaluronic acid) found to be present within the cumulus matrix. Interstingly, Adeoya-Osiguwa and Fraser (2005) found that addition of cathine and norephedrine stimulated capacitation but inhibited the acrosome reation (the acrosome

reaction can then be stimulated with progesterone (Foresta *et al*., 1992) at a later point). This can also be used during IVF to ensure that the sperm population, during the washing process, does not undergo premature acrosome loss (this acrosome loss is described by green *et al*., 1999).

These observations might be of clinical relevance since the COC model can be used as a physiological selector of sperm designated to be injected in an ICSI program. By using the present model, sperm cells that have successfully completed an important physiological step in the fertilization cascade, namely capacitation and acrosome reaction (Fukui *et al.,* 1990) can be selected without risk factors involved during cytoplasmic injection. One of the anomalies present in spermatozoa that lead to an adverse paternal influence on the developing embryo may be related to the sperm nuclear structure (Sakkas et al., 2002)

There was a significant improvement ($p = 0.0001$) of chromatin packaging quality between the penetrated and non-penetrated populations for both the donor and patient groups. Poor chromatin packaging may contribute to decreased fertilization rate (FR) or fertilization failure during IVF as well as sperm decondensation failure after intra-cytoplasmic sperm injection (ICSI) and subsequently fertilization failure (Sakkas *et al*, 1996) therefore this model can be used to select CMA_3 positive (good packaging quality) sperm. As reported by Agarwal *et al* (2004), semen samples with high CMA3 positivity (>30%) may have significantly lower fertilization rates if used for ICSI (Tesarik *et al*, 1997) but severe p-pattern morphologies were not used in this study and therefore no conclusive decision can be made as to whether CMA_3 can be used as a complimentary assay for ICSI procedures to already existing diagnostic approaches for these patients.

Perspectives

The question is often asked whether evaluation of sperm quality still plays a role in the outcome of ART. In light of the present evidence it is clear that the evaluation and adequate processing of human semen samples has an important role in the male fertility workup. Analyzing the quality of a sperm sample will define the type of ART proposed to the patient. Clinically relevant cut off values have been validated especially for normal morphology, sperm zona binding and zona induced acrosome reactions. These cut off values can be used to identify those samples to be used in a first line infertility treatment regime such as IUI (intrauterine insemination) before introducing the more costly and more intense IVF or ICSI procedures.

Furthermore, the study of the individual spermatozoon injected during ICSI procedure should become a priority research effort. This is especially important since we are still not certain that ICSI therapy is without any risk factor. For example, different morphological-ICSI studies revealed a relationship between morphology of the injected spermatozoon and its genetic constitution.

Biochemical markers such as acrosin, aniline blue reactive oxygen species, creatine kinase, hypo-osmotic swelling test and others can also be used in clinical practice to further evaluate sperm fertilizing potential. Moreover, sperm nuclear DNA stability and chromatin packaging and apoptotic events are equally important clinical issues that need to be addressed to fully understand the fertilizing capacity of the ICSI injected spermatozoon and to address the important question of paternal contributions to embryogenesis.

Chapter V

References

Aarons D, Boettger-Tong H, Biegler B, George G, Poirier GR. The acrosomal status of human sperm evaluated by Coomassie Blue staining and electron microscopy. Mol Androl 1993; 5: 31-37.

Acosta AA. Chapter 1: Spermatogenesis and Intratesticular Sperm Maturation. Human Spermatozoa in Assisted Reproduction. 2nd Edition, The Parthenon Publishing Group 1996; 3-11.

Acosta AA. Chapter 4: The Fertilization Process in the Human. Human Spermatozoa in Assisted Reproduction. 2nd Edition, The Parthenon Publishing Group 1996; 33-43.

Adeoya-Osiguwa SA, Fraser LR. Cathine and norephedrine, both phenylpanolamines, accelerates capacitation then inhibits acrosome loss. Human Reprod. 2005; 20 (1): 198-207.

Agarwal A, Damer MS. Sperm Chromatin Assessment. Textbook of Assisted Reproduction Techniques. 2nd Edition, Taylor and Francis Group, London 2004; chapter 7: 93-160.

Aitken RJ, Brindle JP. Analysis of the ability of three probes targeting the outer acrosomal membrane or acrosomal contents to detect the acrosome reaction in human spermatozoa. Hum Reprod 1993; 8: 1663-1669

Anderson RA, Feathergill KA, De Jonge CJ, Mack SR, Zaneveld I. Facilitative effect of pulsed addition of dibutyryl cAMP on the acrosome reaction of non capacitated human sperm. J Androl. 1992; 13: 398-408

Arts EG, Kuiken J, Jager S. A new method to detect acrosome reacted spermatozoa using biotinylated soybean trypsin inhibitor. Fertil Steril 1994; 62: 1044-1055.

Austin CR. The Capacitation of the mammalian sperm. Nature, London 1952; 170: 326.

Bahattacharya S, Hamilton MP, Shaaban M, Khalaf Y, Seddler M, Ghobara E. Conventional *in-vitro* fertilization versus intracytoplasmic sperm injection for the treatment of non-male factor infertility: a randomized controlled trial . Lancet 2001; 357: 2075-9.

Balhorn R. A model for the structure of chromatin in mammalian sperm. J Cell Bio 1982; 93: 298-305.

Balhorn R. Mammalian protamines: Structures and molecular interactions. In: KW Adolph (ed), Molecular biology of chromosome function. New York- Berlin-Heidelberg: Springer Verlag, 1989; 366-395.

Bar-Ami S, Gitay-Goren H, Brandes JM. Different morphological and steroidgenic patterns in oocyte/cumulus-corona cell complexes aspirated at *in vitro* fertilization. Biol Reprod 1989; 41: 761-770.

Bar-Ami S, Khoury C. Effect of cell-plating density on the steroidgenic activity of human cumulus cells. J Reprod & Fertility 1994; 101: 729-735.

Barone JG, De Lara J, Cummings KB, Ward WS. DNA organization in human spermatozoa. J Androl. 994; 15: 139-144.

Bastias MC, Kamijo H, Osteen KG. Assessment of Human Sperm, functional changes after in vitro, coincubation with cells retrieved from the human reproductive tract. Human Reproduction 1993; 8: 1670-1677.

Bedford JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals, Biol. Reprod.1983; 28: 108.

Bedford JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biol Reprod 1985; 28: 108-120.

Bedford J.M., Kim HH. Cumulus oophorus as a sperm sequestering device, *in vivo*. J Exp Zool. 1993; 265(3): 321-8.

Bedford JM . An electron microscopic study of sperm penetration into the rabbit egg after natural mating. AM J Anat.1972; 133 213-54.

Bianchi PG, Manicardi GC, Urner F, Campana A, Sakkas D. Chromatin packaging and morphology in ejaculated human spermatozoa: Evidence of hidden anomalies in normal spermatozoa. Mol. Human Reprod. 1996; 2: 139-144.

Bouvier D. Chemical aspects of histone acetylation and replacement in mouse spermatids at different stages of maturation. Cytobiologie 1977; 15: 420-438.

Braun J, Hirsch T, Krause W, Ziegler A. Evaluation of the acrosome reaction using monoclonal antibodies against different acrosomal antigens -comparison with the triple stain technique. Int J Androl 1991; 14: 431-436.

Caswell AH, Hutchison JD. Visualization of membrane bound cations by a fluorescent technique. Biochem Biophys Res Commun 1971; 42: 43-49.

Chang, M C. The meaning of sperm capacitation; a historical perspective, J. Androl. 1984; 5: 45

Cherr GN, Yudin AL, Katz D. Organization of the Hamster cumulus extracellular matrix: a hyaluronidase-glycoprotein gel which modulates sperm access to the oocyte. Dev Growth Diff 32; 1990; 353-65

Cherr GN, Lambert H, Meizel S, Katz DF. *In Vitro* studies of the golden hamster sperm acrosome reaction: completion on the zona pellucida and induction by homologous soluble pellucidae. Dev. Biol. 1986; 114: 119-131.

Claasens OE. The Evaluation of Human Sperm. Chromosomal Status and the Relation with Sperm Morphology, Motility and Fertilization *in Vitro*. Stellenbosch University Press 1991; 10-16.

Coetzee K, Kruger TF, Menkveld R, Swanson RJ, Lombaard CJ, Acosta AA. Usefulness of Sperm Penetration Assay in Fertility Predictions. Arch. of Androl. 1989; 23: 207-172.

Courtens J, Loir M. A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat and stallion spermatids. J. Ultrastruct. Res. 1981; 74: 327-340.

Cohen-Dayag A, Ralt D, Tur Kaspa I, Manor M., Makler A, Dor J, Mashiach S, Eisenbach M. Sequential acquisition of chemotactic responsiveness by human spermatozoa Biol Reprod 1994; 50: 786-90.

Cooper TG, Yeung CH. Chapter 4: Physiology of Sperm Maturation and Fertilization. Andrology: Male Reproductive Health and Dysfunction. 2nd Edition, Springer, Berlin 2000; 63-82

Corselli J, Talbot P. *In vitro* penetration of hamster oocyte-cumulus complexes using physiological numbers of sperm. Dev. Biol. 1987; 122: 227-242.

Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res. 1986; 15: 213- 226.

Cummins JM and Yanagimachi R. Sperm-egg ratios and the site of the acrosome reaction during in vivo fertilization in the hamster. Gamete Res.1982; 5: 239-56

Cummins JM, Yanagimachi R. Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated *in vitro*, in relation to the timing of acrosome reaction. Gamete Res. 1986; 15: 187-212.

DasGupta S, Mills CL, Fraser LR. A possible role for Ca^{2+}- ATPase in human sperm capacitation. J Reprod Fertility 1994; 102 (1): 107-116.

De Jonge CJ, Mack SR, Zaneveld Lill. Synchronous assay for human sperm capacitation and the acrosome reaction. J. Androl. 1989; 10: 232-239.

De Lange RJ, Smith EL. Histones Structure and Function. Ann. Rev. Biochem. 1971; 40: 279-314.

Drahorad J, Tesarik J, Cechova D and Vili M. Proteins and glycosaminoglycans in the inter- cellular matrix of the human cumulus oophorus and their effect on conversion of proacrosin to acrosin.]. Reprod. Fertil.1991; 93: 253-62.

Drobnis EZ, Yudin AI, Cherr GN and Katz DF. Kinematics of hamster sperm during penetration of the cumulus cell matrix. Gamete Res. 1988; 17: 367-383.

Eggert-Kruse W, Reimann-Anderson J, Rohr G, Pohl S, Tilgen W, Runnebaum B. Clinical relevance of sperm morphology assesment using strict criteria and the relationship with sperm-mucus interaction *in vivo* and *in vitro*. Fertil Steril 1995, 63: 612-624.

Eppig JJ. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev. Biol 1982; 19 89, 268-72

Esterhuizen AD, Franken DR, Lourens JGH, van Rooyen LH. Clinical importance of zona pellucida induced acrosome reaction (ZIAR test) in cases of failed human fertilization. Hum Reprod, 2002; 16: 138-144.

Esterhuizen AD, Franken DR, Becker PJ, Lourens JGH, Muller II, Van Rooyen LH. Defective sperm decondensation: a course study for fertilization failure. Andrologia 2002; 34: 1-9.

Evenson DP, Darzynkiewiez Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980; 240: 1131-1133.

Fatchi AN, Zeinstra EC, Kooij RV, Colebrander B, Bevers MM. Effect of Cumulus Cell Removal of *in Vitro* Matured Bovine Oocytes prior to *in Vitro* Fertilization on Subsequent Cleavage Rate. Theriogenology 2002; 57: 1347-1355

Fenichel P, Hsi BL, Farahifar D, Donzeau M, Barrier-Delpech D, Yehy CJ. Evaluation of the human sperm acrosome reaction using a monoclonal antibody, GB24, and fluorescence activated cell sorter. J Reprod Fertil. 1989; 87: 699-706.

Fetterolf PM, Jurisicova A, Tyson JE, Casper RF. Conditioned medium from human cumulus oophorus cells stimulated human sperm velocity. Biol Reprod 1994; 51: 184-192.

Foresta C, Rossato M, Mioni R, Zorzi M. Progesterone induces Capacitation in Human Sperrmatozoa. Andrologia 1992; 24: 33-35.

Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: Abnormal persistence of histones in spermatozoa in infertile men. Int. J. Androl 1992; 15: 330-337.

Franken DR, Bastiaan HS, Oehninger SC. A micro-assay for sperm acrosome reaction assessment, J Assisted Reprod & Gen. 2000; 17: 156-161.

Franken DR, Franken CJ, de la Guerre H, De Villiers A. Normal sperm morphology and chromatin packaging: Comparison between Analine Blue and Chromomycin A3 staining. Andrologia 1999; 31: 361-366.

Fraser, L. R., Mechanisms controlling mammalian fertilization, Oxford Rev. Reprod. Biol. 1984; 6: 5.

Fraser L. Mechanism regulating capacitation and the acrosome reaction in Human sperm acrosome reaction Eds Fenichel P , Parinaud J. Colloque INSERM John Libbey Eurotext Ltd, Paris 1995; 236: 17-33.

Fraser LR, Adeoya-Osiguwa SA, Baxendale RW. First messenger regulation of capacitation via G-protein-coupled mechanisms: a tale of serendipity and discovery. Mol Human Reprod 2003; 9 (12): 739-748.

Fukui Y. Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine oocytes matured *in vitro.* Mol Reprod Dev 1990; 26 (1): 40-46.

Goldberg RB, Geremia R, Bruce WR. Histone synthesis and replacement during spermatogenesis in the mouse. Differentiation 1977; 7: 167-180.

Gonsalvez J, Lopez-Fernandez C, De La Torre J, Suja JA, Rufas JS. A method for visualizing the acrosome by light microscopy. Stain Technol 1986; 61: 227-230.

Green S, Fischel S, Rowe P. The incidence of spontaneous acrosome reaction in homogenous populations of hyperactivated human spermatozoa. Human Reprod. 1999; 14: 1819-1822.

Gwatkin RBL, and Anderson OF. Capacitation of hamster spermatozoa by bovine follicular fluid, Nature (London) 1969; 224: 1111.

Henley N, Baron C, Roberts KD. Flow cytometric evaluation of the acrosome reaction of human spermatozoa: a new method using a photoactivated supravital stain. lnt J Androl 1994; 17: 78-84

Holden CA, Hyne RV, Sathananthan AH, Trounson AO. Assessment of the human sperm acrosome reaction using concanavalin A lectin. Mol. Reprod Dev. 1990; 25: 247-257.

Hong SJ, Chiu PC, Lee KF, Tse JMY, Ho PC, Yeung WSB. Establishment of a capillary-cumulus model to study the selection of sperm for fertilization by the cumulus oophorus. Human Repro. 2004; 19: 1562-1569**.**

Katz DF, Drobnis EZ, and Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments, Gamete Res.1989; 22: 443.

Irvine DS. Epidemiology and etiology of male infertility. Hum Reprod. 1998; 13 (1): 33-44.

Kruger TF, Menkveld R. Reaction, Acrosine Levels and Sperm in Assisted Reproduction. Assisted Reproduction Reviews. 6: 27-36.

Kruger TF, Menkveld R, Oehninger S. Chapter 2: Anatomy of the Mature Spermatozoon. Human Spermatozoa in Assisted Reproduction. 2nd Edition, The Parthenon Publishing Group 1996; 13-17.

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

Kumaroo KK, Jahnke G, Irving JL. Changes in basic chromosomal proteins during spermatogenesis in the mature rat. Arch Biochem Biophys 1975; 168: 413-424.

Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. Fertil Steril 1987; 48: 649-658

Lee MA, Trucco GS. Bechtol KB, Wummer N, Kopf GS., Blasco L, and Storey BT. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay; Ferfil. Steril.1987; 48: 649.

Leob J. On the nature of conditions which determine or prevent the entrance of the spermatozoa into the egg. Amer Nat 1915; 49: 257-285.

Liu DY and Baker HW. Inducing the human acrosome reaction with calcium ionophore A23187 decreases sperm-zona pellucida binding with oocytes that failed to fertilize *in vitro*. J. Reprod Fetility 1990; 89: 127-134.

Liu DY, Garrett G, Baker HWG. Clinical application of sperm-oocyte interaction tests in in vitro fertilization-embryo transfer and intracytoplasmic injection programs. Fertil Sterill, 2004; 5: 1252-1263.

Magier S, Van der Ven HH, Dietrich K and Krebs D. Significance of Cumulus Oophorus in in vitro Fertilization and Oocyte viability and fertility. Human Reproduction 1990; 5: 847-852.

Mali P, Sandberg M, Vuorio E, Yelick PC, Hecht NB, Parvinen M. Localization of protamine 1 mRNA in different stages of the cycle of the rat seminiferous epithelium. J Cell Biol 1988; 107: 407-412.

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

Mansour RT, Aboulghar MA, Serour GI, Abbas AM, Elattar I. The live span of sperm motility and pattern in cumulus co-culture. Fertility and Sterility 1995; 63: 660-662.

McDonough P. Editorial comment: Has traditional sperm analysis lost its clinical relevance? Fertil Steril, 1997; 67: 585-587.

Mendoza C, Carreras A, Moos J, Tesarik J. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using Pisum satiuum agglutinin. J Reprod Fertil 1992; 95: 755-763.

Menkveld R, Stander FSH, Kotze TJdW, Kruger TF, van Zyl JH. The evaluation of morphologic characteristics of human spermatozoa according to stricter criteria. Hum Reprod. 1990; 5: 586-592

Menkveld R, Franken DR, Kruger TF, Oehninger SC, Hodgen GD. Sperm selection capacity of human zona pellucida. Mol Reprod Dev 1991; 15: 346-52.

Menkveld R, Coetzee K. Andrology Manual Tygerberg Hospital, 1995.

Menkveld R, Kruger TF. Chapter 6: Basic Semen Analysis. Human Spermatozoa in Assisted Reproduction. 2nd Edition, The Parthenon Publishing Group 1996; 53-87.

Monaco PJ, Rasch EM. Differences in staining with DNA-specific flourochromes during spermatogenesis. J. Histochem. Cytochem 1982; 30: 585.

Monesi V. Autoradiographic Evidence of Nuclear Histone Synthesis during Mouse Spermiogenesis in the absence of detectable quantities of Nuclear Ribonucleaic Acid. Expl Cell Res 1964; 36: 683-688

Moore HD, Bedford JM. An *in Vitro* Analysis of Factors influencing the Fertilization of Hamster Eggs. Biol. Reprod. 1978; 19: 879-885.

Morales P, Cross NL. A new procedure for determining acrosomal status of very small numbers of human sperm. J. Histochem. Cytochem. 1989; 37: 1291-1292.

Mortimer D, Curtis EF, Miller RG. Specific labeling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J Reprod Fertil 1987; 81: 127-135.

Mortimer D. Practical Laboratory Andrology. Oxford University Press 1995; 25-26.

Nagae T, Yanagimachi R, Srivastava PN, Yanagimachi H. Acrosome Reaction in Human Spermatozoa. Fertil. Steril. 1986; 4: 701-707

Nagy ZP, Verheyen G, Tournaye H , Van Stierteghem A. Special applications of intracytoplasmic sperm injection: the influence of sperm count, motility morphology, source and sperm antibody on the outcome of ICSI. Hum Reprod 1988;13 (1): 143-54.

0hashi K, Saji F, Dato M, Okabe M, Mimura T, Tanizawa o. Evaluation of acrosomal status using MH6l-beads test and its clinical application. Fertil Steri1. 1992; 58:803-808.

0kabe M, Nagira M, Kawai Y, Matzno S, Mimura T, Mayumi T. A human sperm antigen possibly involved in binding and/or fusion with zona free hamster eggs. Fertil Steril 1990; 54: 1121-1126.

Ombelet W, Fourie R, Vandeput H, Bosmans E, Cox A, Janssen A. Teratozoospermia and *in vitro* fertilization: a randomized prospective study. Hum Reprod 1994; 9: 1479-1484.

Osman RA, Andria ML, Jones AD, Meizel S. Steroid induced exocytosis: the human sperm acrosome reaction. Biochem Biophys Res Commun. 1989; 160 (2): 828-833.

Overstreet JW. Chapter 3: Physiological changes in Sperm during Transport through the Female Reproductive Tract. Human Spermatozoa in Assisted Reproduction. 2nd Edition, The Parthenon Publishing Group 1996; 19-31.

Palermo G, Joris H, Devroey P, Van Stierteghem A. Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte, Lancet 1992; 340: 17-18.

Perry RL, Naeeni M, Barratt CL, Warren MA, Cooke ID. A time course study of capacitation and the acrosome reaction in human spermatozoa using revised chlortetracycline pattern classification. Fertil. Steril. 1995; 64: 150-159.

Perry RL, Naeeni M, Barrat CL, Warren MA, Cooke ID. A time course study of capacitation and the reaction in human spermatozoa using a revised chlorotetracycline pattern classification. Fertil. Steril. 1996; 65 (6): 1258-1259.

Poccia D. Remodelling of nucleoproteins during gametogenesis, fertilization, and early development. Int. Rev. Cytol. 1986; 105: 1-65.

Purvis K, Rui H, Scholberg A, Hesla S, Clausen OP. Application of flow cytometry to studies on the human acrosome. J. Androl. 1990; 11: 361-366.

Ralt D, Manor M. Cohen-Dayag A, Tur-Kaspa I, Ben-Shlomo I, Makler A, Yuli I, Dor J, Blumberg S, Mashiach S, Eisenbach M. Chemotaxis and chemokines of human spermatozoa to follicular factors. Biol. Reprod. 1994; 50: 771-85.

Roldan ERS. Role of phospolipases during sperm acrosomal exocytoisis. Frontiers in Biosciences, 1998; 3: 1109-1119.

Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Human Reprod. 6; 11 (4): 837-843.

Sakkas D, Urner F, Bianchi PG, Bizarro D, Wagnere I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Hum Reprod 1996; 11: 837-843

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

Stock CE, Fraser LR. The acrosome reaction in human sperm from men of proven fertility. Human Reprod 1987; 2: 109-119.

Stock CE, Bates R, Lindsay KS, Edmonds DK, Fraser LR. Human oocyte-cumulus complexes stimulate the human acrosome reaction. Journal of Reproductive Fertility 1989; 86: 723-730.

Suarez S, Katz D, Meizel S. Changes in motility that accompany the acrosome reaction in hyperactivated hamster spermatozoa. Gamete Res. 1994; 10: 253-65.

Suarez SS, Ho HC. Hyperactivated motility in sperm. Reproduction in Domestic Animal, 2003; 28 (2): 119-124.

Suarez SS, Katz DF, Overstreet JW. Movement Characteristics and Acrosomal Status of Rabbit Spermatozoa recovered at the site and time of Fertilization. Biologic Reproduction 1983; 29: 1277-1287.

Sullivan R, Duchesne C, Fahmy N, Morin N, Dionne P. Protein synthesis and acrosome reaction-inducing activity of human cumulus cells. Human Reproduction 1990; 5: 830-834.

Talbot P, Chacon RS. A new procedure for rapidly scoring acrosome reactions of human sperm. Gamete Res. 1980; 3 :211-216.

Talbot P, Chacon RS. A triple-stain technique for evaluating normal acrosome reactions of human sperm. J Exp Zool. l98l; 215:201-208.

Talbot P. Sperm penetration through oocyte investments in mammals. Am J Anat. 1985; 174: 331-46.

Tao J, Du JY, Critser ES, Critser JK. Assessment of the acrosomal status and viability of human spermatozoa simultaneously using flow cytometry. Hum Reprod 1993; 8: 1879

Tesarik J, Mendoza C, Carreras A. Fast acrosome reaction measure: a highly sensitive method for evaluating stimulus-induced acrosome reaction. Fertil Steril 1993; 59: 424-430.

Tesarik J, Mendoza C. Direct Non-Genomic Effects of Follicular Steroids on Maturing Human Oocytes; Oestrogen versus Androgen Antagonism. Human Reproduction Update 1997; 3: 95-100.

Tesarik J, Mendoza OC, Testart J. Effect of human cumulus oophorus on movement characteristics on human capacitated spermatozoa. J. Reprod Fertil. 1990; 88: 665-675.

Veeck L. Atlas of the human oocyte and early conceptus. Williams & Wilkens, Baltimore USA. 1986; 1-7.

Wang WH, Abeydeera LR, Fraser LR, Niwa K. Functional analysis using chlorotetracycline fluorescence and *in vitro* fertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. J Reprod. Fertil. 1995; 104: 305-313.

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

Wassarman, P.M. (1999) Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis and fusion. Cell, 96, 175-183.

Ward WS, Coffey DS. Specific organization of genes in relation to the sperm nuclear matrix. Biochem Biophys. Res. Comm. 1990; 173:20-25.

Weinbauer GF, Gromoll M, Simoni M, Nieslag E. Chapter 3: Physiology of Testicular Function. Andrology: Male Reproductive Health and Dysfunction. 2nd Edition, Springer, Berlin 2000; 23-61

White DR, Phillips DM, Bedford JM. Factors affecting the acrosome reaction in human spermatozoa. J. Reprod. Fertil. 1990; 90:71-80.

Yao, Y.Q., Ho, P.C. and Yeung, W.S.B.. Effects of human follicular fluid on the capacitation and motility of human spermatozoa. Fertil. Steril. 2000; 73: 680-686.

Yanagimachi, R., Mechanisms of fertilization in mammals, in Fertilization and Embryonic Development *In Vitro*. Mastroianni, L. and Biggers, J. B., Eds., Plenum Press, New York, 1981.

Yanagimachi R.. *In vitro* capacitation of hamster spermatozoa by follicular fluid, J. Reprod. Fertil. 1969; 18: 275.

Yanagirnachi, R., Mammalian fertilization, in *Physiology of Reproduction,* Knobil, E., Neill, J. Raven Press, New York 1988.

Yudin, A. I., Cherr, G. N. and Katz, D. F. Structure of the cumulus matrix and zona pellucida in the golden hamster: A new view of sperm interaction with oocyte-associated extracellular matrices. Cell Tisssue Res. 1988, 251: 555-64

Chapter VI

APPENDIX I

Definition of Terms

Mitosis

Mitotic division produces two identifiable daughter cells, each containing the same number and type of chromosomes in their nuclei as the original mother cell.

Interphase

The nucleus and nucleoli is clearly visible whilst the chromatin shows no chromosome differentiation. Just before prophase the cell actively synthesizes nucleic acids and proteins for the process of chromosome duplication.

Prophase

The chromosomes become visible as long thin, thread-like structures. Due to spirilization they become shorter and thicker. Each chromosome consists of two identical chromatids that are joined by a centromere. The nucleolus becomes smaller and usually disappears. The spindle forms during prophase or at the start of metaphase.

Metaphase

Chromosomes orientate on the equatorial region of the spindle and connect onto the spindle by their centromeres.

Anaphase

Each centromere divides and moves to different poles to separate the two chromatids of each chromosome.

Telophase

A nucleus membrane encircles the daughter chromosomes at each pole. The chromosomes become invisible due to despirilization and nucleoli reappear.

APPENDIX II

MEIOSIS

Meiotic prophase is subdivided classically into four stages that are denoted as **leptontene, zygotne, pachytene, and diplotene**. These stages are each based on a progression of distinctive chromosomal events. **Leptotene**, the first stage, is defined by the gradual condensation and spiralization of the chromosomes, and by the appearance of thin unpaired axial elements. A single axial element develops lengthwise between the two sister chromatids of each homologous chromosome and is attached to each end to the nuclear envelope.

During the transition from leptotene to **zygotene** the homologous chromosomes are aligned to within 200-300 nm. The sister chromatids gradually rotate to bring the homologous axial elements into suitable positions. The onset of zytogene is denoted by a closure of the intervening space between homologous axial elements to approximately 150nm, and the accompanying appearance of a structured central region. This event precedes the initial formation of the synaptonemal complex and point-to-point manifestation of the homologous chromosomes, which continues throughout zygotene until synapsis is complete. Autosomal synapsis is usually initiated at the distal ends (telomeres) of homologus chromosomes and terminated at the kinetochores. The chromosomes initiate and complete synapsis asynchronously, particularly the XY-chromosome pair which commence synapsis during late zygotene and terminate early. The zygotene spermatocyte shows a denser, more rough and granulated chromatin.

The **pachytene** stage, the longest period of meiotic prophase, begins following complete formation of the synaptonemal complexes between all homologues. A general

condensation and shortening of the chromosomes probably results even in the XY-pair, which forms a heterochromatic body that is spatially segregated from the autosomes. The pachitene and short-lived **diploteen**-spermatocytes have the largest nucleus and the densest chromatin.

Diplotene is defined by desynapsis of the synaptonemal complex and separation of the homologous chromosomes. Disjunction of the homologous is not complete, however, instead they remain attached at chiasmata that mark exchange points for intrachromosomal recombination. At diakinesis the chiasmata undergo terminalization, the nuclear membrane disperses, and the spermatocytes enter metaphase I. At anaphase the homologous chromosomes are randomly segregated into daughter cells, the secondary spermatocytes (in stage VI). This meiotic division is followed quickly by a mitotic division in which the chromatids of each homologue are segregated at random into separate haploid cells. A round spermatid (Sa1) is formed after this second meiotic division in stage I, which contains the haploid (n) number of chromosomes.

APPENDIX III

Terminology

Since it is often to describe all deviations from normal semen variables with words and numbers the following international nomenclature was introduced. As these terms may have different values allocated to them in different laboratories it is very important that, when used for the first time in a publication, it should be specified what the numerical value of each term is:

-spermia	Refers to the ejaculate
Aspermia	No semen ejaculated
Hypospermia	<1 ml ejaculate volume
Hyperspermia	>6.5 ml ejaculate volume
-zoospermia	Refers to spermatozoa in ejaculate
Azoospermia	No spermatozoa in semen
Asthenozoospermia	Poor motility and/or forward progression (<=20% motility and/or <2 forward progression)
Globozoospermia	Round headed acrosomeless spermatozoa
Necrozoospermia	All spermatozoa are dead as confirmed by a supravital stain
Normozoospermia	All parameters are normal in the ejaculate
Oligozoospermia	Low spermatozoa concentration (<10 x 10^6 sperm/ml)
Polizoospermia	>250 x 10^6 sperm/ml
Teratozoospermia	Reduced percentage of morphological normal spermatozoa (<=14%)

APPENDIX IV

Calculate sperm concentration as follows:

$$\frac{\text{No of sperm x dilution x 10 } \boldsymbol{x\ 1000}}{\text{No. of blocks counted } \boldsymbol{(1\ mm^2)\ x\ 1000}} = \text{millions/mL}$$

Where:
10 = a compensation factor for the 0.1 mm depth of the chamber.
1000 = a conversion factor, mm^3 to cm^3 = ***mL***

Short method: for 2 blocks counted:

$$\text{1:10 dilution} = \frac{\text{total No. of sperm counted (N)}}{2 \times 10}$$

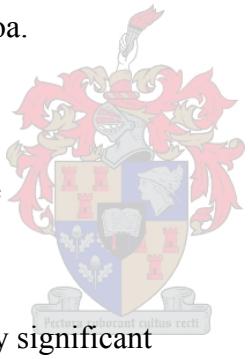
$$\text{1:20 dilution} = \frac{N}{2 \times 5}$$

$$\text{1:100 dilution} = \frac{N}{2}$$

APPENDIX V

MAR Test Method:

- Mix 1 drop of semen with 1 drop of anti-IgG and 1 drop of R1R2 sensitized red blood cells coated with IgG or IgG bound latex particles on a glass slide
- Cover with coverslip and leave for 10 minutes to allow for the agglutination of the red blood cells. No interpretation can be made without agglutination of the red blood cells or latex particles.
- If antibodies are present on spermatozoa they will bind the spermatozoa to the red blood cell agglutinates.
- Estimate the percentage of spermatozoa bound as compared to the percentage of free-swimming spermatozoa.



Interpretation: <10% = negative

>10% = positive

>80% = clinically significant

If a positive MAR-test is found, blood and seminal plasma must be obtained from the particular patient to determine whether antisperm antibodies are present in the patients blood serum and seminal plasma.

APPENDIX VI

Definition of CASA terminology:

Curvilinear velocity (VCL); is the measure of the rate of travel of the centroid of the spermatozoa head over a given time period. This is calculated from the sum of the straight lines joining the sequential positions of the spermatozoa head along the spermatozoa's track.

Straight line velocity (VSL); is the straight line distance between the first and last centroid positions for a given time period.

Average path velocity (VAP); is the spatially averaged path that eliminates the wobble of the spermatozoa head, while preserving the basic curvature of the path.

Linearity of forward progression (LIN); is reported as the ratio of VSL to VCL and expressed as a percentage. A value of 100 representing cells swimming in a perfectly straight line.

Straightness (STR); measures the straightness of the average path.

Maximum lateral head amplitude (maxALH); the centroid positions are recorded and a mean trajectory, based on each of these positions, is generated. For each point, the adjacent 2 points on either side are averaged and a least-squares curve fit applied.

Beat cross frequency (BCF); is defined as the number of times the centroid of the spermatozoa head crosses the computer generated mean trajectory for that cell in Hz (number of beats/second). This parameter provides an estimate of flagellar beat frequency based on the movement of the spermatozoa head.

APPENDIX VII

Papanicolaou Staining Procedure

This staining method is used for the analysis of semen morphology.

Preparation of Specimens:

- Morphology – an evenly spread thin smear, air dried.
- Cytology – 1 drop of egg albumin + 1 drop of semen, mixed and evenly smeared and then fixed in ether:ethanol (50:50) for at least 30 minutes

Staining Procedure:

1.	80% ethyl alcohol	10X
2.	70% ethyl alcohol	10X
3.	50% ethyl alcohol	10X
4.	Distilled water	10X
5.	Meyer's Hematoxylin	5 mins
6.	Running water	3 mins
7.	0.5% HCl	2X
8.	Running water	5 mins
9.	Scott's water	1 min
10.	Running water	3 mins
11.	50% ethyl alcohol	10X
12.	70% ethyl alcohol	10X
13.	80% ethyl alcohol	10X
14.	95% ethyl alcohol	10X
15.	Orange G6	5 mins
16.	95% ethyl alcohol	10X
17.	95% ethyl alcohol	10X
18.	EA 50	8 mins
19.	95% ethyl alcohol	10X
20.	95% ethyl alcohol	10X
21.	95% ethyl alcohol	10X
22.	Xylol	10X
23.	Xylol	10X
24.	Xylol	leave for 20 mins

Mount immediately in DPX
Note: X = number of dips

APPENDIX VIII

Frequently used abbreviations

AR: Acrosome Reaction/ Acrosome Reacted

Ca^{2+}: Calcium

cAMP: Cyclic Adenosine MonoPhosphate

CMA_3: Chromomycin A_3

COC: Cumulus Oophorus Complex

CTC: Chlorotetracycline test

HSA: Human Serum Albumin

ICSI: IntraCytoplasmic Sperm Injection

IVF: *In Vitro* Fertilization

PSA-FITC: Fluorescein Isothiocyanate labeled with *Pisum sativum*

ZP: Zona Pellucida.

APPENDIX IX

Table VIII: Pre-penetration Results of Donors and Patients

	PRE PENETRATION VALUES OF DONORS and PATIENTS											
Sample	**Morphology donors**	**Morphology patients**	**Acrosome reacted sperm-donors**	**Acrosome reacted sperm-donors**	**CTC 1 donors**	**CTC 1 patients**	**CTC 4 donors**	**CTC patients**	**CTC 5donors**	**CTC 5 patients**	**CMA3 donors**	**CMA3 patients**
1	6	12	9	10	47	47	35	35	18	18	30	50
2	10	8	11	9	27	27	58	58	15	15	31	48
3	8	8	14	18	50	50	30	30	20	20	30	50
4	9	6	10	16	48	48	38	38	14	14	28	49
5	5	5	13	20	46	46	38	38	16	16	38	45
6	4	8	12	11	39	39	39	39	22	22	38	44
7	10	7	8	17	40	40	40	40	20	20	30	51
8	11	7	14	18	38	38	44	44	18	18	30	50
9	8		16	25	58	58	32	32	10	10	38	33
10	4	6	20	10	50	50	38	38	12	12	33	44
11	5	7	11	9	37	37	49	49	14	14	45	40
12	4		15	10	52	52	33	33	15	15	34	40
13	5		16	17	48	48	38	38	14	14	30	38
		6		8		58		32		10		34
		7		10		58		32		10		38
AVERAGE	**6.8**	**7.25**	**13**	**13.87**	**44.62**	**46.4**	**39.38**	**38**	**16**	**15.2**	**33.5**	**43.6**
SD	**2.6**	**1.76**	**3.3166**	**5.139**	**8.099**	**8.854**	**7.478**	**7.4**	**3.44**	**3.82**	**4.93**	**6.1**
MIN	**4**	**5**	**8**	**8**	**27**	**27**	**30**	**30**	**10**	**10**	**28**	**33**
MAX	**11**	**12**	**20**	**25**	**58**	**58**	**58**	**58**	**22**	**22**	**45**	**51**
MEDIAN	**6**	**7**	**13**	**11**	**47**	**48**	**38**	**38**	**15**	**15**	**31**	**44**

APPENDIX X

Table IX: Post-penetration Results of Donors and Patients.

	POST PENETRATION VALUES OF DONORS and PATIENTS											
SAMPLE NO.	**Morphology donors**	**Morphology patients**	**Acrosome reacted sperm-donors**	**Acrosome reacted sperm-patients**	**CTC 1 donors**	**CTC 1 patients**	**CTC 4 donors**	**CTC 4 patients**	**CTC donors**	**CTC 5 patients**	**CMA3 donors**	**CMA3 patients**
1	7	12	45	20	25	25	45	45	30	30	35	60
2	12	8	38	29	21	21	51	51	28	28	28	60
3	9	8	42	34	28	28	45	45	27	27	31	52
4	9	6	51	35	26	26	39	39	35	35	25	62
5	7	5	50	42	37	37	34	34	29	29	35	50
6	5	8	47	22	45	45	23	23	32	32	30	51
7	11	7	49	28	20	20	46	46	34	34	28	53
8	14	7	55	30	27	27	48	48	25	25	27	54
9	8	8	48	30	37	37	33	33	30	30	37	58
10	5	6	54	28	34	34	41	41	25	25	38	55
11	5	7	50	25	27	27	49	49	24	24	40	61
12	6		27	20	40	40	39	39	21	21	36	76
13	6	4	52	25	30	30	42	42	28	28	35	65
		6		16		37		33		30		58
		7		21		37		33		30		60
Mean	**8**	**7.17**	**46.8**	**28.3**	**30.5**	**31**	**41**	**41**	**28**	**28**	**33**	**58.33**
SD	**2.89**	**1.99**	**7.56**	**6.26**	**7.52**	**7.5**	**7.7**	**7.7**	**4**	**4**	**4.8**	**6.586**
MIN	**5**	**4**	**27**	**20**	**20**	**20**	**23**	**23**	**21**	**21**	**25**	**50**
MAX	**14**	**12**	**55**	**42**	**45**	**45**	**51**	**51**	**35**	**35**	**40**	**76**
MEDIAN	**7**	**7**	**49**	**28**	**28**	**28**	**42**	**42**	**28**	**28**	**35**	**58**

APPENDIX XI

TABLE X: Results of Statistical Evaluation Using Independant Sample And Paired T-Tests

	% Normal sperm	% Acrosome reacted sperm	Uncapacitated sperm	Capacitated sperm	% Acrosome reacted	% Chromatin packaging defects
Pre-cumulus penetration recordings of donor sperm vs. sperm from sub-fertile men						
p-values	0.65	0.60	0.58	0.72	0.58	0.0001
Pre-vs. Post cumulus penetration recordings of donor sperm						
p-values	0.92	0.0001	0.0001	0.42	0.0001	0.51
Pre-vs. Post cumulus penetration recordings of sperm from subfertile men						
p-values	0.80	0.0001	0.0001	0.55	0.0001	0.0001
Pre-cumulus penetration recordings of donor sperm vs. sperm from subfertile men						
p-values	0.65	0.60	0.58	0.72	0.56	0.0001
Post-cumulus penetration recordings of donor sperm vs. sperm from subfertile men						
p-values	0.32	0.0001	0.76	0.71	0.87	0.0001

Pectora roborant cultus recti