A Comparison of Three Spermatozoa Selection Techniques for Intracytoplasmic Sperm Injection (ICSI) using Swim-up, Cumulus Oophorus Model and PICSI ® Dish.

by

Ms Michelle Rijsdijk

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Department of Obstetrics and Gynaecology at
Stellenbosch University

Supervisor: Prof DR Franken

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Abstract

Introduction: Spermatozoa selection for Intracytoplasmic Sperm Injection (ICSI) is a paramount factor in the outcome of a fertility treatment cycle. Nature has perfected the selection process by using the cumulus matrix to select spermatozoa that are morphologically and genetically normal.

Aim: To determine which method of semen preparation delivers the best results in terms of spermatozoa selection for ICSI.

Methods: Patients were randomized into 3 groups of spermatozoa selection techniques namely the routine swim-up or density gradient, the Cumulus Model or the PICSI® dish (hyaluronic acid). The prepared collected spermatozoa were used to make slides to record the percentage of normal spermatozoa (morphological staining), the capacitational status (chlorotetracycline test), chromatin packaging quality (chromomycin A_3 (CMA_3) staining) and the DNA quality (acridine orange staining). These results were then compared to the fertilization, cleavage, pregnancy and implantation rates of the patients used in the study.

Results: All three groups displayed improvements in morphology, capacitational ability, chromatin packaging quality and DNA quality (or fragmentation). There was no significant difference in pregnancy rates between the groups and no difference in implantation rates. The PICSI® group did however show a significant improvement in the chromatin packaging quality, only if the baseline values were low.

Conclusions: All 3 groups of spermatozoa selection techniques showed improvements in spermatozoa quality. The swim-up/gradient group showed a statistical improvement in the fertilization rate when compared to the cumulus and PICSI® groups. PICSI® showed a greater improvement in selected spermatozoa parameters when baseline values for CMA_3 were low.

Keywords: ICSI, cumulus oophorus, PICSI®, morphology, capacitation, acrosome reaction, DNA fragmentation.

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Opsomming

Inleiding: Die seleksie van spermatosoa vir Intrasitoplasmatiese Sperm Inspuiting (ICSI) is 'n deurslaggewende faktor in die uitkoms van 'n fertiliteitsbehandeling-siklus. Die natuurlike seleksieproses van morfologies en geneties normale sperme, word bepaal deur die cumulus oophorus matriks.

Doel: Om die beste metode van semen voorbereiding te bepaal, waardeur optimale resultate in terme van seleksie van spermatosoë kan plaasvind vir ICSI.

Metodes: Pasiënte is ewekansig in drie groepe van spermseleksie-tegnieke verdeel, naamlik die opswemtegniek, die Cumulus Model of die PICSI® Dish (Hialuroon suur). Die voorbereide spermatosoa is gebruik om mikroskoopskyfies te maak vir die bepaling van die persentasie, normale morfologiese spermatosoë, die kapasitasie status (Chlorotetrasikline toets), chromatien verpakking kwalitiet (Chromomycin A3 kleuring) en die DNA-integriteit (Akridien Oranje kleuring). Die resultate is vergelyk tov die bevrugting-, verdeling-, swangerskap- en inplantasie syfers van die pasiënte in die studie.

Resultate: Al drie groepe het verbeterings getoon ten opsigte van morfologie, kapasitasie, chromatien verpakking kwaliteit en DNA fragmentasie. Daar was geen beduidende verskil in swangerskap of inplantasie syfers tussen die groepe nie. Die PICSI® groep het 'n statisties betekenisvolle verbetering getoon as die CMA_3 waarde laag was voor die seleksie metode.

Gevolgtrekkings: Al drie spermatosoë seleksie tegnieke het verbeterings getoon in spermatozoa kwaliteit. Die opswemtegniek-groep het 'n statistiese betekenisvolle verbetering in die bevrugtingsyfer getoon in vergelyking met die PICSI en cumulus metodes. Die PICSI® tegniek het ‘n betekenisvolle verbetering getoon as waardes vir CMA3 laag.

Sleutelwoorde: ICSI, cumulus oophorus, PICSI®, morfologie, kapasitasie, akrosoomreaksie, DNA fragmentasie

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Table of Contents:

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List of Figures and Tables

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Chapter 1: Literature Review

Since the introduction of intracytoplasmic sperm injection (ICSI) (Palermo *et al.* 1992) to the assisted reproductive technology (ART) arena, couples exhibiting a severe male factor have been able to accomplish their reproductive goal. ICSI has managed to bypass a number of key factors relating to the fertilization process concerning the spermatozoa. Unfortunately, the pregnancy rate is not optimal in terms of professional expectations, thus prompting continued investigation into improvement of success rates. The recruitment of follicles and ovarian stimulation cannot be altered to improve embryo quality at the stage of laboratory handling; spermatozoa selection is the major factor that can be manipulated. This study looked at the selection of spermatozoa for ICSI using three different selection techniques. All have been extensively researched but never compared to each other in terms of quality, namely, acrosome reaction (AR), morphology, DNA packaging quality and DNA quality, and fertilization, pregnancy and implantation rates.

The aim of this study was two-fold:

Primary aim: - to determine which of the three relatively cost-effective techniques delivered the best results in terms of spermatozoa quality.

Secondary aim: To compare the fertilization, embryo quality and pregnancy rates of the three different spermatozoa selection groups.

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1.1. The Spermatozoon

Spermatogenesis in the adult male requires many processes resulting in primary spermatocytes after a series of cellular differentiations (see Figure 1). After the first meiotic division, the primary spermatocytes will form two secondary spermatocytes, dividing for a second time into secondary spermatocytes which are haploid in nature (Youssef *et al.* 1996). Spermiogenesis (maturation of the spermatids) then follows the spermatogenesis, and both processes are completed in approximately 74 days (*Acosta et al. 1990*a; *Acosta et al. 1990*b). Not all spermatogonia are destined to become spermatocytes, as some fail to complete the entire process.

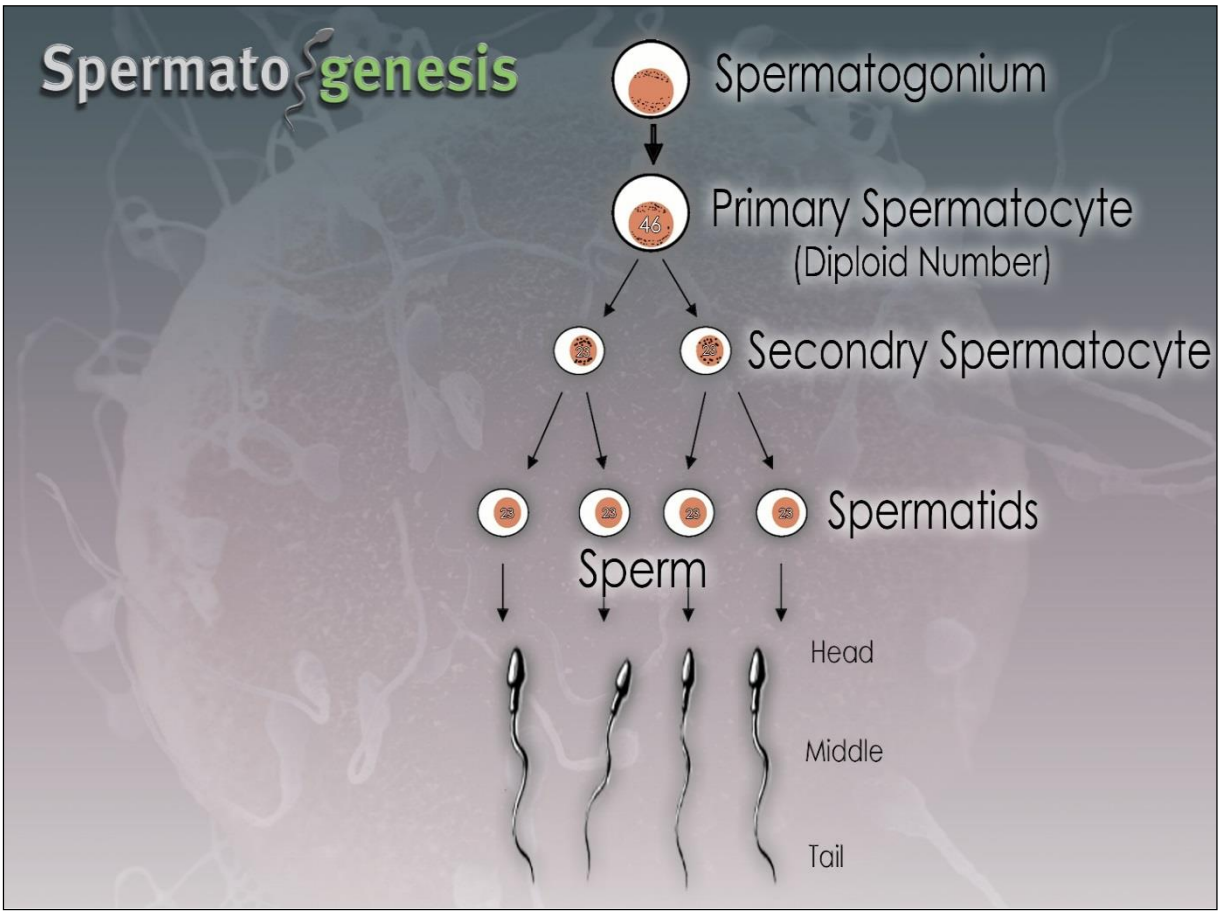

Figure 1: A representation of spermatogenesis **Picture by J Wilde**

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Spermatozoon chromatin is incredibly stable and compact. To achieve this condensed format, the spermatozoa DNA is specifically organized to allow transfer of the densely packaged genetic material to the oocyte. This format of packaging is due to significant modifications of the nucleoprotein compartments and replacement of histones by protamines (Ward & Coffey, 1991; Bouvier, 1977; Balhourn, 1989). Protamines are arginine-rich proteins that primarily neutralize the DNA charge and compact the chromatin (Mali *et al.* 1988) and this is complemented by the formation of intra- and inter-molecular disulphide cross-links between the cysteine residues. Once the spermatozoa reaches the epididymis, protamine synthesis occurs and all protamines have been dephosphorylated (Claasens, 1991).

The mature spermatozoon morphologically consists of a head with acrosome, the mid-piece and the tail regions **(**Figure 1). These regions play important roles in normal fertilization and their criteria have been extensively explained according to the Tygerberg Strict Criteria (Kruger *et al.* 1986; Kruger *et al.* 1996; Menkveld *et al.* 1990).

The **head** is oval shaped with a length of 4 – 5.5µm and width of 2.5 – 3.5µm (Menkveld *et al.* 1990). Numerous variations are considered normal but anomalies such as large heads, small heads (pinheads), tapering, amorphous and vacuolated heads (Menkveld & Coetzee, 1995) have also been identified. The nucleus constitutes 65% of the head where the DNA is linked with other proteins to form a chromatin string so that the chromosomes are no longer visible (Kruger *et al.* 1996; Menkveld & Kruger, 1996; Weinbauer *et al.* 2000). The acrosomal region covers the anterior two-thirds of the head (arising originally from the golgi complex) (Claasens, 1991; Kruger *et al.* 1986; Kruger *et al.* 1996; Menkveld & Coetzee, 1995; Menkveld & Kruger, 1996).

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The **mid-piece** contains mitochondria providing the energy needed for the spermatozoa motility and is approximately the same length as the head, separating the head and tail regions (Kruger *et al.* 1996; Weinbauer *et al.* 2000).

The **tail**, or flagellum, has 9 evenly spaced, peripheral, doubled microtubules and a central pair of single microtubules. The tail is the longest region with a length of 4 – 10μm and a diameter of 1μm (Kruger *et al.* 1996)

Human spermatozoa, unlike those of other species, have to undergo a series of physiological and morphological changes once ejaculated, called capacitation, before fertilization can take place (Loeb, 1915; Yanagimachi, 1994). These changes are mandatory along with hyperactivation (a unique, vigorous motility) of the spermatozoa, to enter the oocyte and its vestments (Yanagimachi, 1988**;** Yanagimachi, 1994).

Hyperactivation is a pattern of movement seen in the spermatozoa which is a distinctive, vigorous motility as first described by Yanagimachi in 1969 (Yanagimachi *et al*; 1969). This movement involves increased flagellar bend amplitude and beat symmetry. Capacitation is imperative to prevent spermatozoa from becoming fertile too quickly, given that during coitus, spermatozoa are deposited in the vagina and the distance the spermatozoa covers, to get to the oocyte, is extensive. Once capacitation has occurred, it allows for commencement of the AR (DasGupta *et al.* 1993).

Capacitation is a series of the following processes:

1. removal of the spermatozoa surface proteins (as seen by the green bars surrounding the spermatozoa in Figure 2), allowing better for better binding of the spermatozoa to the oocyte

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2. efflux of cholesterol from the spermatozoon membrane, allowing for improved permeability of Ca^{2+}
3. changes in the oxidative metabolism
4. hyperactivity (more whip-like movement of the tail)
5. increases in phosphotyrosine phosphorylation of certain proteins
6. decreases in calmodulin binding proteins
7. increases in Ca^{2+} uptake (using the Ca^{2+}-ATPase pumps)
8. increases in intracellular pH and lastly increases in cyclic adenosine monophosphate (cAMP) levels (DasGupta *et al.* 1993; Overstreet, 1996).

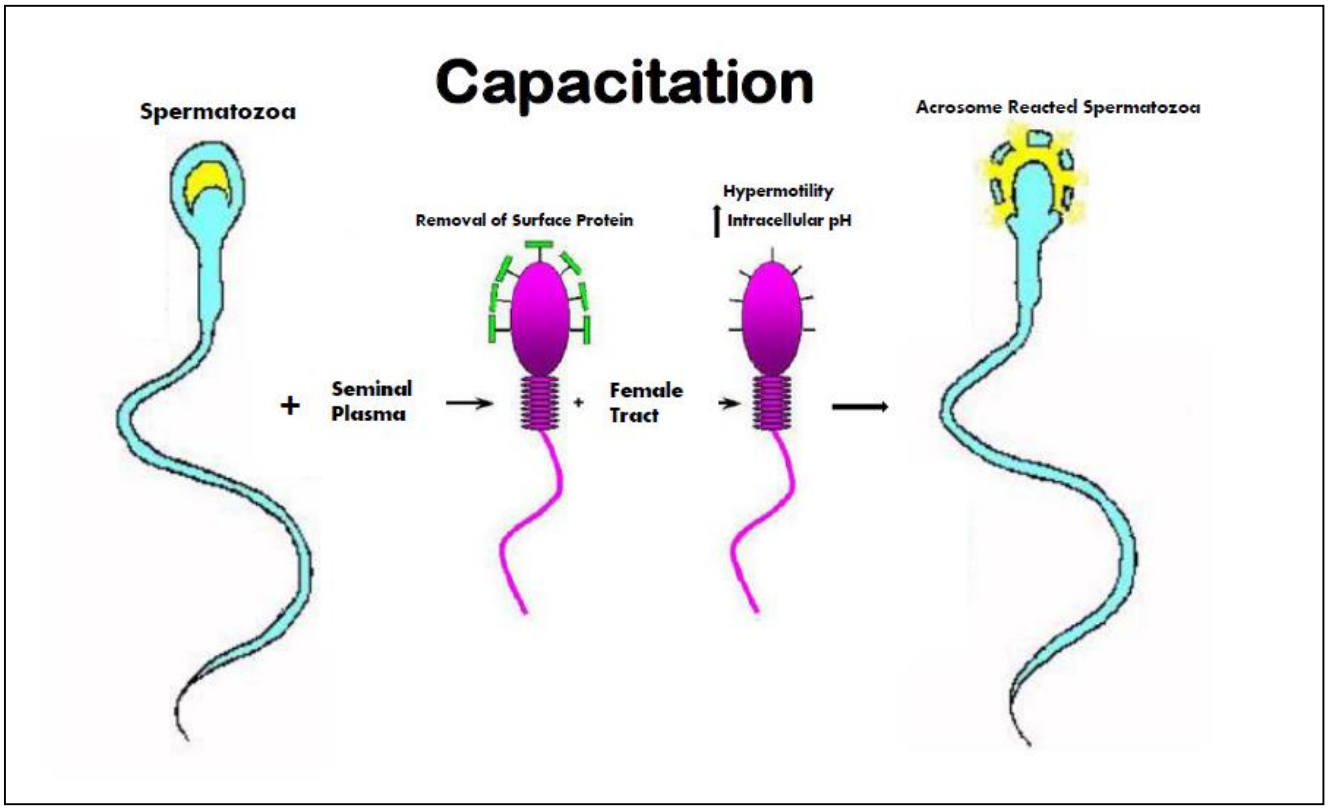

Figure 2: A representation of the mechanisms following ejaculation from capacitation to the acrosome reaction

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Multiple steroids help to induce the Ca^{2+}-influx with one of them being identified by Osman *et al.* (1989), 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 AR is an enzymatic activity resulting in increases in intracellular Ca^{2+} allowing acrosomal exocytosis and therefore leading to successful fertilization (DasGupta *et al.* 1993).

There are 5 stages of the AR:

Stage 1: Intact acrosome: Acrosomal membranes are intact and the acrosomal matrix is homogenous.

Stage2: Decondensed matrix: sealing of the matrix while the outer acrosomal membranes remain intact.

Stage 3: Vesicles and matrix: Vesicles are present within the acrosomal cap and rearrangement of the plasma and outer acrosomal membranes take place (as seen by the last spermatozoa in Figure 2)

Stage 4: Fusion: The outer acrosomal and plasma membranes fuse to each other in the cap region of the equatorial segment. Disappearance of the matrix is also evident.

Stage 5: Inner acrosomal membrane exposed: The plasma and outer acrosomal membranes have vanished; the equatorial segment is still usually intact.(Claassens, 1991; Nagae *et al.* 1986).

Capacitation and AR are therefore the two fundamental steps that the spermatozoon needs to complete before fertilization can occur. Cumulus cells have been shown to stimulate the AR (Hong *et al.* 2009) as spermatozoa that have been co-incubated with cumulus have lower numbers with intact acrosomes.

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1.1.1. The Molecular Selection of Mature Spermatozoa using Hyaluronic Acid

Spermatozoa have been known to suffer from physiological and environmental stressors which lead to morphological aberrations, gene mutations and chromosomal abnormalities (Evenson *et al.* 2002) which ultimately lead to a disruption in the biochemical events leading up to and including fertilization as well as embryogenesis. Spermatozoa chromatin abnormalities induce chromatin structural problems and could lead to apoptosis and necrosis (Darzynkiewicz *et al.* 1997) which arise during spermiogenesis if the DNA nicking and ligating activities are abnormal (Evenson *et al.* 2002).

Other biochemical processes and thus possible markers that can be used to determine the maturity of the spermatozoon have been carefully researched. Creatine kinase (CK) is one of the markers that has been found to be higher in men with lower fertility potential (oligozoospermic males) (Huszar *et al.* 1988). Autoradiographic and CK immunostaining (Cayli *et al.* 2003; Huszar and Vigue, 1993) have provided an explanation that increased CK and other protein concentrations suggest that a developmental defect has occurred in the terminal stages of spermatogenesis. Increases in CK, as well as other cytoplasmic protein concentrates, cause process malfunctions in the extrusion of the cytoplasm (see Figure 2); 'Residual bodies', otherwise known as cytoplasmic droplets, are left in the adluminal area. These cytoplasmic droplets are therefore a visual indication of immaturity of the spermatozoon. Huszar *et al*. (1992) showed in their in vitro fertilization (IVF) study that all normozoospermic men with high levels of CK did not achieve successful pregnancy results vs. other patients in the normozoospermic group. This was in agreement with their earlier study (Huszar and Vigue; 1990) which looked at the correlation of intrauterine insemination (IUI) pregnancy rates with spermatozoa

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parameters; they found that the spermatozoa CK activity was a significant predictor of outcome in comparison to other parameters such as count and motility.

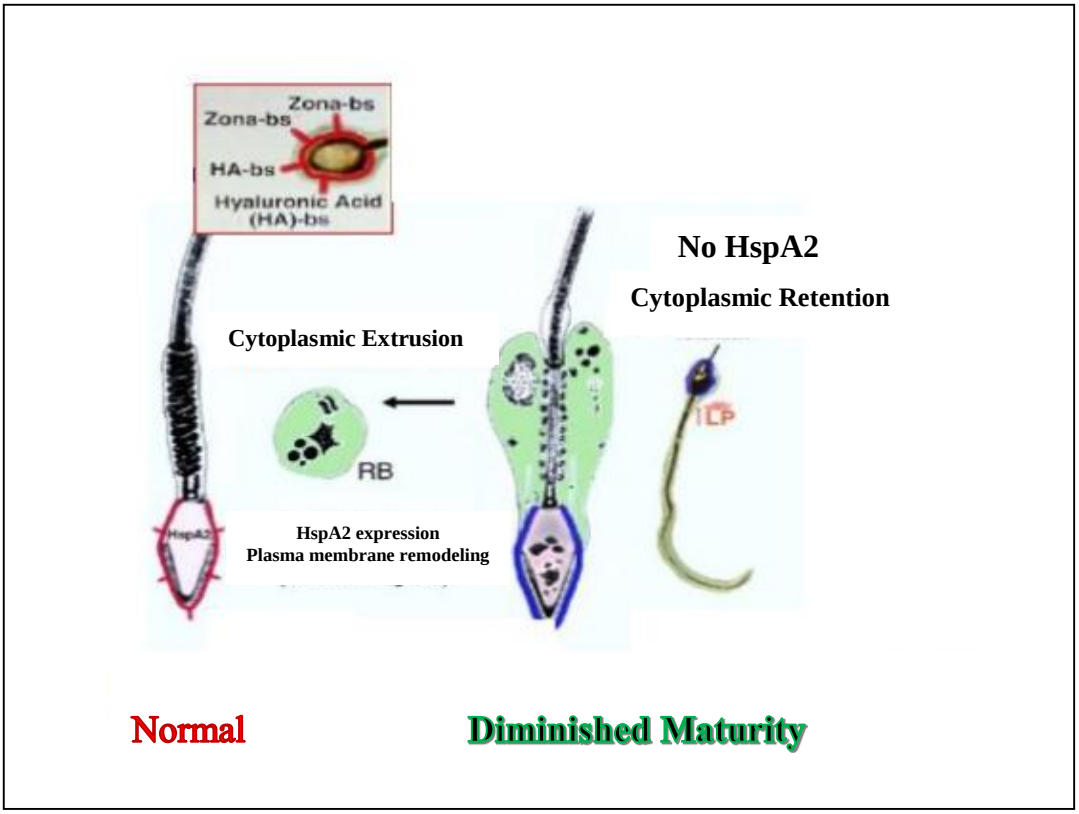

Figure 3: A diagram depicting the difference between the cytoplasmic retention of a diminished mature spermatozoon and that of a normal spermatozoon.

Through subsequent research of Huszar and Vigue (1990), another protein was identified as the 70KDa testis-expressed heat shock protein known as HspA2 (Huszar *et al.* 2000). This heat shock protein, HspA2, first appears in primary and secondary spermatocytes, and concurrently with the expression of HspA2, major protein movements occur and result in cytoplasmic extrusion and remodelling of the spermatozoa plasma membrane (Huszar *et al.* 1997*;* Huszar *et al.* 2000, Huszar *et al.* 2007*).* This supports the theory that HspA2 supports chromosomal crossing-over as it is responsible for the delivery of DNA repair enzymes as well as histone-protamine replacement in terms of the membrane remodelling (Ergur *et al.* 2002; Jakab *et al.*

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2005). Lack of this testis-specific chaperone (HspA2) can lead to meiotic errors (Nixon *et al*. 2015). Anomalies in the expression of HspA2 have been seen to be concurrent with an increase in numerical chromosomal aberration (Sakkas *et al.* 1991 (1); Sakkas *et al*. 1999 (2)), higher levels of reactive oxygen species (ROS) (Huszar *et al.* 1993) and increases in DNA chain fragmentation (Aitken *et al.* 1994; Nixon *et al*. 2015). These have all been supported by the discovery that low levels of HspA2 expression are more prevalently seen in men suffering severe oligo-astheno-teratozoospermia (OATS) (Griffin *et al.* 2003; Jakab *et al.* 2005, Twigg *et al.* 1998)

The HspA2 also acts as a hyaluronan receptor during normal fertilization (Huszar *et al.* 2003; Parmegiani *et al.* 2008). It has been shown that hyaluronan-bound spermatozoa (spermatozoa bound to the PICSI dish) have increased developmental maturity, increased chromatin integrity, increased morphology, increased functional competence (Keel *et al.* 1987), decreased aneuploidy, and decreased active caspase-3 (Cayli *et al.* 2003; Jakab *et al.* 2005; Worrilow *et al.* 2009; Worrilow *et al*. 2013). The Petersen group from Brazil (2008 and 2010) do not agree with these findings and have published several articles discrediting the efficacy of the PICSI dish.

This protein, localized in several organs, is found on the spermatozoa's surface (Ghosh *et al.* 2003). With the phosphorylation of hyaluronan-binding protein -1 (HABP-1) there is a reversal in the production of D-mannosylated albumin (which prevents the spermatozoa-egg binding) and this action therefore promotes sperm-egg binding (a type of cell signalling) (Ghosh *et al.* 2007). HABP-1 is a 34kDa glycosaminoglycan consisting of 209 amino acids (Ghosh *et al.* 2007) and is produced in the seminiferous tubules as a pro-protein (Bharadwij *et al.* 2002). The protein is located in many bodily organs and but more specifically on the spermatozoa surface and also acts as a mannose-

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binding site for zona pellucida (ZP) recognition (Ghosh *et al.* 2003; Ghosh *et al.* 2007). Samples that have <20% motility (asthenozoospermia) have been shown to be deficient in HABP-1 and therefore there is a significant decrease in the spermatozoa fertilization potential (Ghosh *et al.* 2003).Conversely, the oocyte's granulosa cells also express HABP-1 and are seen to increase during ovulation; this results in the expansion of the cumulus oophorus complex (COC) which in turn exerts an effect on the follicular fluid (Thakar *et al.* 2006).

Apoptosis is another important regulator of normal spermatogenesis; it can be explained as programmed cell death or a controlled disassembly of cells from within (Seli *et al.* 2004). Apoptosis starts with movement of negatively charged phospholipids of the plasma membrane from the inner surface to the outer surface (Seli *et al.* 2004). This is associated with the activation of caspases and the cytosolic cysteine-containing aspartate-specific proteases (De Fried & Danaday, 2010). Fas, a cell-surface protein, then binds to FasL, which is produced by the Sertoli cells, and results in apoptosis of the spermatozoon (Seli *et al.* 2004). Apoptosis then leads to the breaking of the double-stranded DNA within the spermatozoon and the process of self-demise is complete (Ahmadi & Ng, 1999).

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1.2 The Oocyte and its Vestments

Mature human oocytes are in a constant state of meiotic arrest (at prophase) (Mortimer& Swan, 1995; Overstreet 1996; Veeck 1986). This continues until puberty when the ovaries are activated by hormones.

These hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are produced by the anterior pituitary gland. Only oogonia that are ovulated will complete the first meiotic division resulting in two daughter cells, each with haploid number of chromosomes. The first is known as the oocyte as it retains most of the ooplasm and the second, the first polar body (Mortimer, 1995; Veeck, 1986). The second division takes place after ovulation of the Graafian follicle, only if the oocyte is fertilized (Fatchi *et al.* 2002; Nagae *et al.* 1986; Veeck, 1986) and the second polar body will then become visible.

The maturation characteristics of the metaphase II oocyte are as follows (Figure 4)

1. Extrusion of the first polar body
2. Symmetrical ooplasm which is homogenous in colour and smooth granularity
3. The cumulus matrix should appear as a ‘halo’ around the oocyte with an expanded coronal layer
4. Expansion of the cumulus mass
5. Granulosa cells are loosely packed within smooth masses

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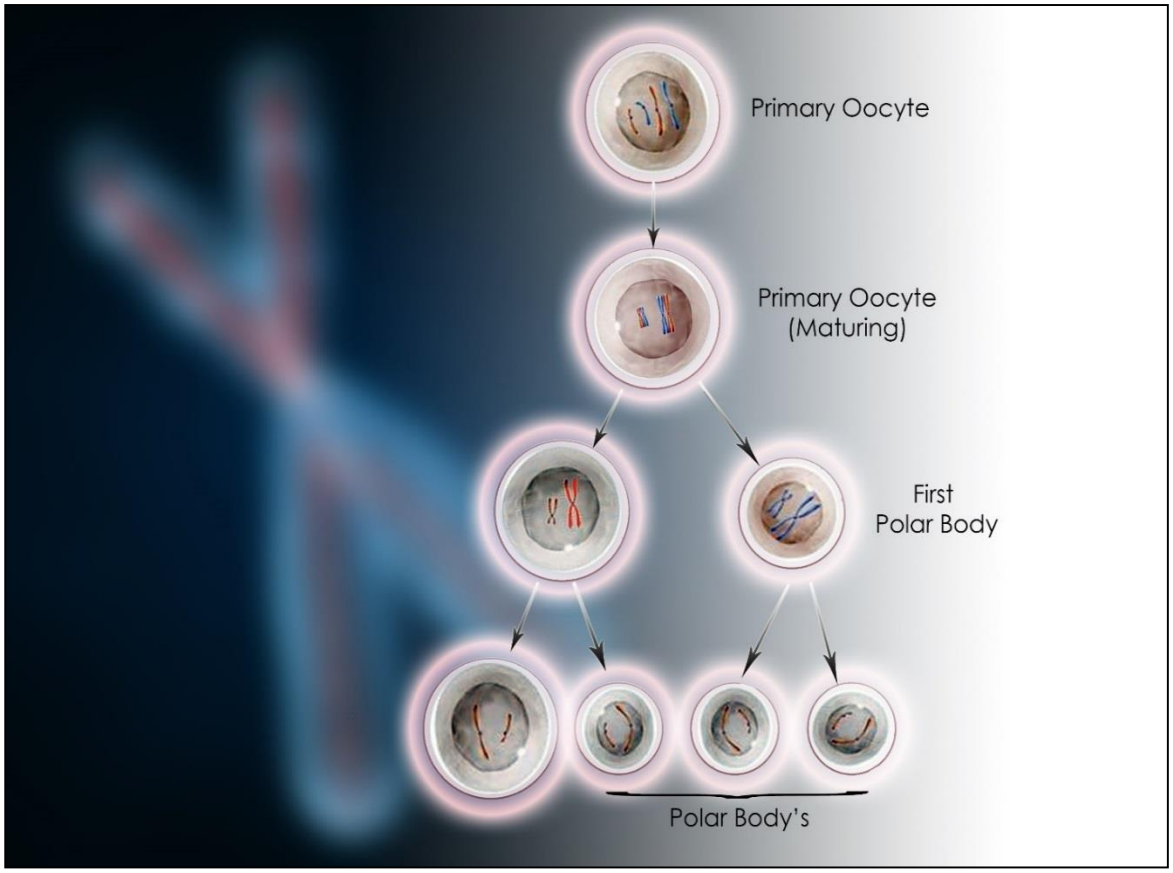

Figure 4: A diagram depicting the maturation on an oocyte and its polar bodies **Picture by J Wilde**

The maturation processes are not just determined by the nucleus, but also by its vestments (Hong *et al.* 2004; Sousa *et al.* 1997) and this is evident by the hormonal impact that oestrogen (changes the reactivity of Ca^{2+} release systems) and progesterone have on the maturity of the cumulus matrix as well as the ooplasm (Overstreet, 1996; Sousa *et al.* 1997). Cumulus cells have been shown to play an imperative role in oocyte maturity, specifically with regard to the nuclear and cytoplasmic maturity (Tesarik *et al.* 1997) and it has been shown that oestrogen (E_2) exertion results in Ca^{2+} oscillations during maturation.

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The cumulus oophorus complex (COC) is made of two components, the inner stratum, made of trypsin-sensitive granules embedded in a viscoelastic matrix comprised of hyaluronic acid (HA) (Cooper & Yeung, 2000; Fatchi *et al.* 2002) and the outer stratum, consisting of the glycoproteins called the zona pellucida (ZP). The importance of the cumulus matrix is mainly responsible for secreting cAMP which is key in maintaining meiosis after the second division and protecting the oocyte from environmental effects (Overstreet, 1996). The cAMP acts via gap junctions and these mechanisms are cut off when the cumulus cells retract from the ZP near the time of ovulation (Menkveld & Coetzee, 1995; Overstreet, 1996; Fatchi *et al.* 2002). The COC is also known to facilitate oocyte transport in the fallopian tubes, as well as improving the spermatozoon's fertilizing ability (Yanagimachi 1994**)** by among others activating capacitation and thus leading to the AR of the spermatozoon both through hormonal (oestrogen and progesterone) and mechanical mechanisms (Foresta *et al.* 1992).

This is theorized to aid in the selection of morphologically normal spermatozoa (Fatchi *et al.* 2002). Research has corroborated this (Carrell *et al.* 2000; Fetterolf *et al.* 1994; Mansour *et al.* 1995; Stock *et al.* 1989; White *et al.* 1990); spermatozoa that have been retrieved from the cumulus matrix were partially acrosome reacted (Cooper & Yeung, 1995; Hong *et al.* 2004; Rijsdijk *& Franken*, 2007) and the presence of cumulus cells has resulted in selection of better spermatozoa (Carrell *et al.* 2000; Corselli & Talbot, 1987; Stock *et al.* 1989; Suarez *et al.* 1983). This selection of morphologically superior spermatozoa can still not be fully explained as the matrix has many components, namely, hormonal/steroidal impact, mechanical and dimensional. The chemotactic/chemokinetic factors (hormonal/steroidal) can be demonstrated in follicular fluid (Ralt *et al.* 1994; Cohen-Dayag *et al.* 1994) but this does not explain the effect of the cumulus and more importantly the matrix as a whole on the selection of the spermatozoa.

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The extracellular matrix of the COC is formed primarily of hyaluronan (a dermantan sulphate proteoglycan), but also contains other proteins such as inter-alpha-trypsin inhibitor, adermatan sulphate proteoglycan, and a pentraxin-3. (Drahorad *et al.* 1991; Relucenti *et al.* 2005). The matrix is comprised of the hyaluronan and the proteins and thus aids in gamete interactions (Relucenti *et al*. 2005).

The effect of HA has been extensively researched and has been shown to:

1. improve developmental maturity of spermatozoa, increase the quantity of acrosome-reacted spermatozoa (by increasing intracellular Ca^{2+}),
2. improve spermatozoa chromatin integrity,
3. increase spermatozoa morphology,
4. increase functional competence of spermatozoa (by stimulating spermatozoa motility),
5. decrease chromosomal aneuploidy and decrease active caspase-3 of spermatozoa (Bains *et al.* 2001; Cayli *et al.* 2003; Jakab *et al.* 2005)

Carell (2000) showed that zygotes derived from ICSI oocytes showed better embryo development when co-cultured with cumulus cells. It has also been shown in mice that in the presence of a hyaluronidase inhibitor, cumulus cell dispersal is inhibited (Kim *et al.* 2005); this possibly influences oocyte maturity.

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1.3 Fertilization *In Vivo*

Fertilization *in vivo* incorporates several processes in a specific order of events including spermatozoa penetration through the cumulus matrix, spermatozoon binding to the ZP, acrosomal exocytosis, spermatozoa penetration through the ZP and fusion of the spermatozoa and oocyte (Yanagimachi *et al.* 1994). Capacitation of the spermatozoa is required, with the acrosome still intact, to facilitate the spermatozoa to transverse the cumulus matrix. The matrix therefore acts as a natural 'selector' of spermatozoa by allowing only a selected spermatozoon populace through to the ZP. The exact mechanisms and the extent to which the cumulus acts are still not clear. As seen in IVF, the natural selection process of spermatozoa leads to fertilization (Benchaib *et al.* 2003; Jakab *et al.* 2005) unlike that of ICSI.

The cumulus then stimulates the beginning of the AR (this takes place at the ZP) as well as increased motility. This increased motility is needed for the spermatozoa to travel through the cumulus as well as the ZP. The extracellular matrix of the cumulus mass is high in HA and it is believed that this acid facilitates the spermatozoa's passage through the matrix. Binding of the acrosome-intact spermatozoa to the ZP induces the fusion of the plasma and outer acrosomal membranes, also known as the acrosome reaction (AR). This process allows for interaction with the ZP and allows entry into the oocyte (Kim *et al.* 2005). The process of spermatozoa incorporation into the oolemma is phagocytotic in nature and only acrosome-reacted spermatozoa have the capability to fuse with the oolemma. A cascade of subsequent events leads to a rise in intracellular Ca^{2+} levels, which in turn results in the exocytosis of cortical granules from the ooplasm, which

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causes chemical changes in the ZP, which in turn make the ZP impenetrable to other spermatozoa. This fusion has therefore led to the 'activation' of the oocyte.

A few hours later, the pronuclei of both the gametes can be visualized and this is called the pronuclear stage (Veeck & Zaninovic 2003). The zygotic centrosome is then assembled, a vital process that leads to the final progression of the fertilization procedure whereby there is reorganization and union of the maternal and paternal chromosomes and formation of the zygote (Veeck *et al.* 2003). The human zygote remains single-celled for 24 hours and the fertilization process is completed when the first cleavage occurs.

In vitro fertilization leads to a 'natural' selection process of spermatozoa, leading to fertilization (Benchaib *et al.* 2003; Jakab *et al.* 2005). The fertilization process may require as little as one hour of spermatozoa exposure to the oocyte (Gianaroli *et al.* 1996) in comparison to ICSI.

1.4 Intracytoplasmic Sperm Injection (ICSI)

1.4.1 Spermatozoa Selection for ICSI

ICSI manages to bypass many processes during fertilization compared to *in vivo* fertilization.

The exceptional fertilization rate of ICSI is offset by the occasional lack of blastocyst development if damaged spermatozoa have been injected into the oocytes. Damaged spermatozoa have been shown to cause adverse events such as: fertilization failure, early

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embryo death, spontaneous abortion, and childhood cancers, as well as infertility in resulting offspring due to chromosomal aneuploidies and propagation disorder linked to Y-chromosome deletions (Jakab *et al.* 2005). This can be explained by the natural selective nature of the cumulus matrix during IVF (Benchaib *et al.* 2003; Jakab *et al.* 2005) as ICSI shows a fivefold increase in chromosomal aberrations, 8% increase in spontaneous abortions (IVF has abortion rate of 10% and ICSI 18%) and increases in malformation of embryos (Jakab *et al.* 2005).

It has been postulated that spermatozoon DNA damage (Muratori *et al.* 2000; Sakkas *et al.* 2003) as the result of aborted apoptosis, although this has been challenged. Sakkas *et al*. (2003) suggested that spermatozoa with DNA fragmentation, 'escaped' apoptosis. Along the same lines, Marcon and Boissoneault (2004) proposed that DNA damage was due to the incorrect reparation of transient DNA nicks that occur during spermatogenesis. Many other factors have been shown to cause DNA fragmentation including advanced paternal age, certain drugs (e.g. chemotherapy), smoking, genital tract inflammation and varicoceles (Sakkas and Alvarez, 2010). DNA fragmentation influences fertilization rates for the threshold value above 10% as well as implantation rates (Benchaib *et al.* 2003). The DNA fragmentation index (DFI) is a predictive factor for ART outcome (Bungnum *et al.* 2007). DFI below 30% showed no increased risk in early pregnancy loss but, when the DFI was more than 30%, ICSI was indicated as the preferred method of treatment (Bungnum *et al.* 2007; Parmegiani *et al.* 2010a and Parmegiani *et al.* 2010b; Zini *et al.* 2008). Benchaib *et al.* (2003) compared ICSI to IVF and stated that DNA fragmentation only plays a role in the embryo development of ICSI embryos when DNA fragmentation is severe, where Tomsu *et al.* (2002) was in disagreement.

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They stated that ICSI patients receive ICSI as preferred treatment because of their decreased parameters (such as motility, forward progression and morphology) and therefore displayed increased spermatozoa defragmentation rates (Benchaib *et al.* 2003; Gandini *et al.* 2000; Irvine *et al.* 2000; Sun *et al.* 1997;. Gandini *et al*. (2004) however suggested that the stringent spermatozoa selection process in ICSI as well as the embryo selection mitigated the negative effects of the DNA damage. This was evident in the research of Hirsch *et al.* (1999) who stated that ICSI patients with high rates of DNA fragmentation had longer time to pregnancy (TTP) than other ICSI patients; this was confirmed by the meta-analysis of Zini *et al.* (2009) that showed that both ICSI and IVF patients with high DFI had an increased rate of pregnancy loss.

There have been several attempts to try to improve ICSI results, especially when spermatozoa parameters are exceptionally low. These research studies have resulted in several novel techniques. One such technique, as in this study, is using the PICSI® dish for spermatozoa selection. The PICSI® dish has been shown to select spermatozoa with better DNA chromatin packing as described by Huszar *et al*. (Huszar *et al.* 1997*;* Huszar *et al.* 2000, Huszar *et al.* 2007). The mechanisms are explained on from page 15.

Another technique, intracytoplasmic morphologically selected sperm injection (IMSI) (Bartoov *et al.* 2002), with the emphasis on spermatozoa morphology (at ultra-high magnifications), has seen a significant improvement in pregnancy rates. Contrasting views and results have been published relating to the results of IMSI. Khattabi *et al.* (2013) reported improved pregnancy rates but this was only seen when poor spermatozoa parameters led to embryo degradation after day 3 of development, indicating the impact of the ‘late’ paternal effect (Bartoov *et al.* 2002; Khattabi *et al.*

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2013). In contrast Oliveira *et al.* (2011) could not show an improvement in fertilization, embryo quality, pregnancy and implantation although a statistical trend was noted toward improved ongoing pregnancies, live births and lower miscarriage rates. The equipment required for IMSI is however costly, and laboratories with limited budgets will not be able to implement this new technique very easily.

In comparison, another technique in use is the selection of ZP-bound spermatozoa for ICSI (Liu *et al.* 2011). This Chinese and Australian collaboration (Liu *et al.* 2011) reported higher numbers of good quality embryos and higher implantation rates. The spermatozoa were allowed to bind to immature oocytes and after manipulation the bound spermatozoa were used for ICSI. The results obtained were as theoretically expected, as spermatozoa that bind to the ZP have been shown to have normal morphology as well as normal DNA quality (Menkveld *et al.* 1991). This study has limitations as the study group was small (a total of 53 couples per group).

Gianaroli *et al.* (2008) used yet another technique: 'sperm head birefringence' (SHBF) to select spermatozoa for ICSI. Spermatozoa have anisotrophic properties and the mature spermatozoa's nucleus can be determined using double refraction of light to view the pattern of human SHBF and DNA damage (Gianaroli *et al.* 2008; Petersen *et al.* 2010). Gianaroli *et al.* (2008) studied several different groups including oligozoospermic patients with no progressive motility, as well as patients undergoing testis biopsy. This study (Gianaroli *et al*., 2008) showed a higher number of grade 1 embryos on day 3 (higher rate of good quality embryos) as well as a higher implantation rate than the control group in all the different spermatozoa quality samples. The pregnancy rate for the control group was initially higher; however the abortion rate was also increased, thus illustrating that abnormal genetic complement leads to spontaneous

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abortion. A later study (Gianaroli *et al.* 2010) showed that there was an increase in the implantation rate of the cycles where the reacted spermatozoa were being used. This is a method that has merit because it can be used across the male diagnosis spectrum. The equipment required for this method is however expensive and not suited to laboratories with limited budgets.

Said *et al.* (2005a, 2005b, 2006 and 2008) highlighted the use of another non-invasive selection technique called magnetic activated cell sorting (MACS). This cell sorting uses annexin V-conjugated microbeads to remove spermatozoa that are apoptotic based on the externalization phosphatidylserine residues. During one specific apoptotic event, phosphatidylserine, which is normally present in the inner leaflet of the sperm-plasma membrane, is externalized, and this binds to the annexin V beads therefore 'marking' the spermatozoon as apoptotic (Paasch *et al*. 2003). A case study by Rawe *et al.* (2010) showed a pregnancy result for a patient with high levels of spermatozoon DNA fragmentation when the MACS spermatozoa selection method was used. More recently Troya *et al*. (2015) showed that using spermatozoa with intact membranes and a normal nucleas (after MACS selection) for ICSI delivered a higher pregnancy rate (58.1%) compared to PICSI (40.4%) as well as to normal ICSI (27.3%) (Troya *et al*. 2015)

1.4.2 Fertilization *In Vitro*

During ICSI, spermatozoa are selected by the embryologist and injected into the ooplasm. Previously it was thought that the oocyte supplied all the primary materials, in the form of proteins and mRNA, and that spermatozoa played a secondary role. The activated maternal genome (by the spermatozoon) is in force during the fertilization of the oocyte and the first cleavage of the embryo, the paternal genes are 'switched on' at the 4-cell stage until the

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blastocyst stage, when the embryonic genome is activated (Ahmadi & Ng, 1999; Seli *et al.* 2004).

Lopes *et al.* (1998) showed that with DNA damage of more than 25%, the fertilization rate is less than 20% (normal fertilization is seen to be between 60 and 98%). Oocytes do however have a limited capacity to repair DNA damage (originating from the spermatozoa) but this is related to the extent of the spermatozoa damage (Ahmadi & Ng, 1999), as well as the maternal age. Elevated levels of spermatozoa DNA damage or advanced maternal age have been shown to result in increased embryonic fragmentation (Sakkas *et al.* 1996) or arrest. The presence of vacuoles in the spermatozoon head have also been shown to negatively affect fertilization rates as reported by Sakkas *et al.* (1998) and Lopes *et al.* (1998), who were investigating the selection of spermatozoa using IMSI. The vacuoles have been thought to be correlated to spermatozoa DNA packaging as well as quality. Conflicting reports have been published in recent years, showing increases in fertilization rates with no effect on pregnancy, and others showing improvements in pregnancy rates, but not in embryo development when PICSI® was used for spermatozoa selection (Nasr-Esfahani *et al.* 2008; Parmegiani *et al.* 2010; Tarozzi *et al.* 2009; Ye *et al.* 2006). This discrepancy may be due to different study designs, data analysis and patient populations (Said & Land, 2011). Oocytes have been shown to have the capacity to repair spermatozoa DNA damage (Ahmadi &Ng, 1999; Genesca *et al.* 1992) but this depends on the extent of the damage as well as the age and competence of the oocyte (Sakkas *et al.* 1996).

1.4.3 Cleavage

Cleavage of cells is a universal feature which occurs after nuclear replication and segregation (Veeck *et al.* 2003). The human prezygote undergoes mitotic divisions

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every 12-18 hours (Veeck *et al.* 2003) and this process is controlled by the spermatozoa centrosome. The cytoplasm characteristically elongates and the exterior membrane contracts around the smallest circumference with the narrowing continuing until the zygote is divided into two separate blastomeres (Veeck *et al.* 2003).

Bartoov *et al.* (2002) showed that the spermatozoa nucleus is the most important factor affecting ART outcome. It is very rare for cleavage not to take place once pronuclear formation has been noted, but this event occurs in less than 5% of normally fertilized oocytes. This has been attributed to centrosomal problems of the spermatozoon. This is evident when oocytes inseminated using ICSI have failed to progress past the pronuclear stage, but when the same oocytes are inseminated using IVF, they cleave normally. This is due to insufficient spermatozoa aster formation (Chemes & Sedo, 2011). Because the spermatozoa head-neck junction cannot be visualized by the operator performing the ICSI, the spermatozoa selection is less than optimal.

1.4.4 Blastocyst Formation

Blastocyst formation starts between 114 and 120 hours after injection of the spermatozoon into the oocyte. The hatching of a human blastocyst takes place at approximately 144 hours (Veeck *et al*. 2003) and can be seen in Figure 5. Blastocyst development was originally thought to be controlled only by the embryonic genome, but it was recently made evident that blastocycst formation genetically also falls into the realm of the ‘late paternal’ effect of the spermatozoon (Seli *et al.* 2004). The rate of blastocyst development varies significantly from 20.9% to 52.3% (Rubio *et al*. 2014). Shoukir *et al.* (1998) reported that blastocyst rates decrease in ICSI patients versus patients undergoing IVF and this was confirmed in subsequent studies by Sakkas *et al.* (1998), Miller & Smith (2001). Embryos of good quality (the grading on day 2 and 3)

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that subsequently degenerate reflect the effects of abnormalities in the paternal genome (Jones *et al.* 1998). Vanderzwalmen *et al.* (2008) observed that blastocyst rates increased with the use of IMSI. This was explained by the decrease in spermatozoa DNA fragmentation (and thus the late paternal effect) resulting in better blastocyst formation rates (Jones *et al.* 1998).

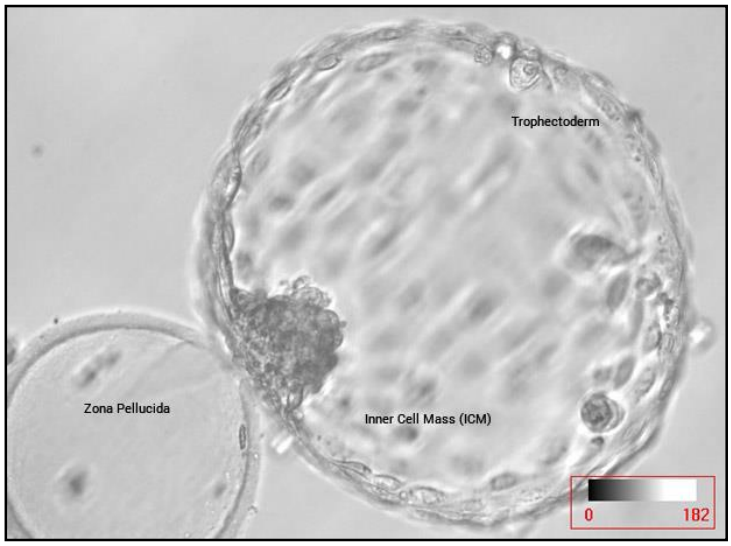

Figure 5: The hatching blastocyst on Day 5 of development

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Chapter 2: Materials and Methods

2.1 Patients and Study Groups

2.1.2 Inclusion and Exclusion Criteria

All couples (n = 223) who were included in the study underwent ICSI as the method of treatment (teratozoospermic patients). The patients were randomly divided into three groups (73 in the swim-up group; 73 in the cumulus group and 77 in the PICSI group).

The group allocation was as follows:

1. ICSI using the conventional double wash swim-up method (Group 1)
2. ICSI using the cumulus oophorus capillary model (Group 2)

   (As described by Hong *et al.* 2004 and later modified by Rijsdijk and Franken, 2007)
3. ICSI using the hyaluronan droplets for selection (PICSI® dish) (Group 3)

Once the patient had been allocated a spermatozoa selection technique using the randomization table (as seen on page 53), slides were made of the raw (Before) and prepared samples (After). The slides were then treated appropriately for each of the tests as laid out in Section 2.3. All patients in the study signed an informed consent form after Institutional Review Board approval. Patients were only granted access to their results once the study was completed.

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2.1.3 Ovulation Induction

Female patients had a wide variety of diagnosis including anovulation and endometriosis. Female patients were not subject to any exclusion criteria as we wanted to report research results based on a general IVF arena. Ovarian stimulation in an agonist cycle (long protocols) was carried out by the administration on day 18 (of previous cycle) of gonadotrophin-releasing hormone (GnRHa) (Lucrin Depot®; Abbott Laboratories South Africa (Pty) Ltd), followed by 14-15 days of pure follicle-stimulating hormone (FSH, 75 IU) and luteinizing hormone (LH, 75 IU) (Menopur®, Ferring Pharmaceuticals, South Africa, (Pty) Ltd) from day 2 of the cycle. Patients were followed up by doing a series of ultrasonographical measurements of the Graafian follicles. Ovulation was then induced by the administration of human chorionic gonadotrophin (hCG) (10 000 IU) (Ovidrel®; Serono, South Africa (Pty) Ltd) when the leading Graafian follicles reached 18-20mm. The oocytes were then aspirated 36 hours after administration of the hCG.

Stimulation in the antagonist cycles (short protocols) was carried out by the administration of pure FSH (75 IU) and LH (75 IU) (Menopur®, Ferring Pharmaceuticals, South Africa, Pty. Ltd.) from day 2 of the cycle. LH suppression was achieved using Cetrotide 3mg (Merck (Pty) Ltd) on day 8 of the cycle and Cetrotide 0.25mg (Merck (Pty) Ltd) on day 11. The antagonist cycle then proceeded to aspiration with administration of hCG (Ovidrel®; Serono, South Africa (Pty) Ltd).

Progesterone support was started for all patients on the day of ovum pick-up (OPU), Cyclogest® (Alpha Pharm, South Africa, (Pty) Ltd) was administered eight hourly.

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2.1.4 Oocyte Retrieval, Preparation and Fertilization

Female patients underwent transvaginal follicle aspiration using a Cook® Oocyte Needle (Cook Medical, Australia) to obtain the oocytes. The resultant embryos were then incubated (37^{o}C, 6% CO_2 in 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 stripped of their cumulus matrix using hyaluronidase at the concentration of 70 IU (Quinns Advantage^{TM} Sequential Embryo Media Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA). Metaphase II oocytes were then injected with a spermatozoon from one of the three pre-prepared groups using the most morphologically normal spermatozoon (Figure 6). After 18 hours, fertilization of the oocytes was assessed by the presence of two pronuclei or two polar bodies and the resulting embryos were then placed in sequential media until transfer.

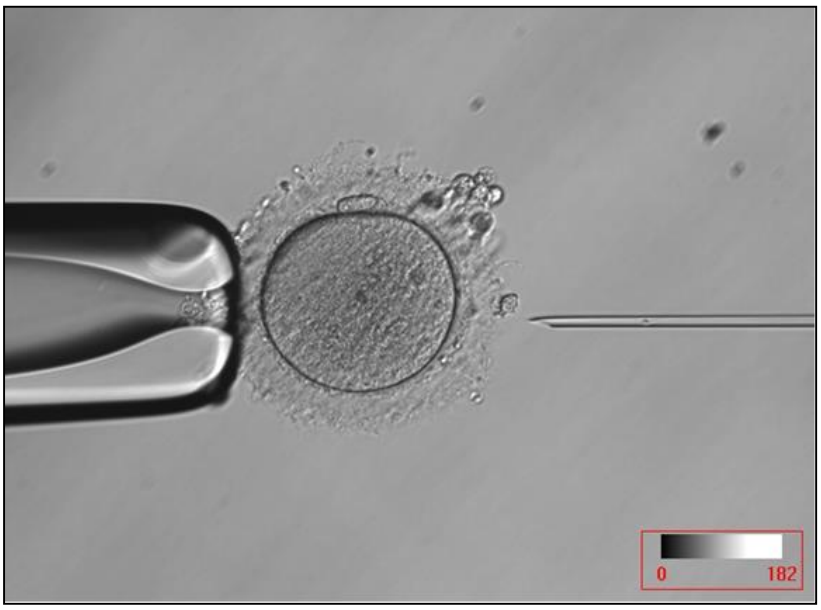

Figure 6: Photo of the ICSI process whereby one spermatozoon is injected into every metaphase II oocyte

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2.1.5 Embryo Culture

On day 2, the embryos were evaluated for cleavage and quality (morphology). On day 3, embryos were evaluated for those patients having a day 3 transfer. Embryos destined for culture to the blastocyst stage, were transferred to blastocyst medium and evaluated for embryo quality. Embryos that cultured to the blastocyst stage were evaluated on day 5 and suitable embryos were transferred. Embryos not transferred (whether day 3 or day 5) were cultured to day 6 and re-evaluated for possible vitrification. Figure 7 shows the stages of embryo culture.

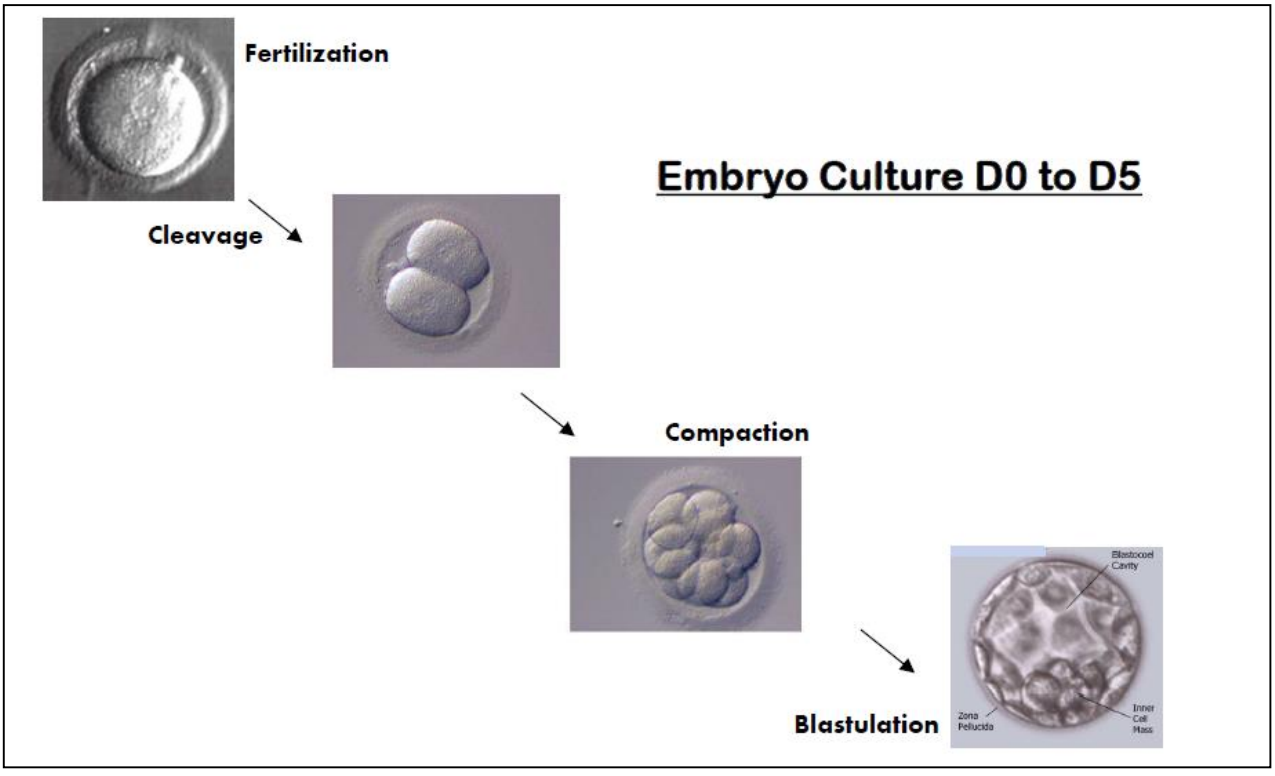

Figure 7: Embryo Culture from Day 0 to Day 5

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2.1.6 Transfer

One, two or three, day 3 or 5 embryos were transferred into the uterus with a Cook® K-JETS-SIVF. Transfers were performed using ultrasound guidance of the uterus, thus ensuring that the embryos were deposited approximately 1cm from the fundus. The laboratory personnel then checked the loading catheter as well as the feeding catheters to ensure the embryos had been safely deposited.

2.2 Semen Samples

Teratozoospermic semen samples with normal count and good progressive motility parameters (>30%) according to the World Health Organization (WHO, 2010) criteria were used. After liquefaction a routine semen analysis including evaluation of spermatozoa motility, concentration, vitality and morphology was performed.

2.2.1 Semen Analysis

Spermatozoa concentration was determined with the use of a Bright field Neubauer hemocytometer and adjusted to a concentration of 20×10^6 motile spermatozoa/ml balanced salt solution (Earle's buffered saline solution, EBSS or Hams' F10).

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Motility assessment began after complete liquefaction of the semen to avoid temperature drop or dehydration of the preparation (Menkveld and Kruger 1996; Menkveld and Coetzee, 1995). The freshly made wet preparation was left to stabilize for approximately one minute. Since spermatozoa motility and velocity are highly dependent on temperature, the assessment was performed at 37ºC, using a heated stage. Examinations were carried out at 20-24ºC, with the temperature in the laboratory standardized, as it could affect the classification of the grades of motility.

1. Using a micro pipette 10µl undiluted semen (37°C) was placed on a clean microscope slide (37°C) and covered by a coverslip (22 x 22 mm).
2. Examination of the "wet preparation" began as soon as the "flow" had stopped (40 x, phase contrast).
3. At least 200 spermatozoa were classified in at least 5 fields.

Forward progression was assessed and quantified as seen below

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)

Morphological assessment was done according to the WHO (2010) criteria and is explained in full on page 42. A normal spermatozoon is defined as normal oval spermatozoon head

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with a smooth contour. The head should be 4-5µm in length and 2.5-3µm in width leading to a length to width ratio of 1.5:1.75 (Franken and Oehninger, 2012). There should also be a well-defined acrosomal region (40-70% of the head), the mid-piece should be lean and the tail should be straight, uniform and leaner than the mid-piece (explained in section 2.3.1 on page 42).

2.2.2 Semen Preparation for ICSI

Patients undergoing ICSI as method of treatment in the ART arena were randomized and allocated into 3 groups. Each couple was assigned a semen preparation technique according to their randomization and the preparation was as follows:

2.2.2.1 Swim-up or Gradient Preparation

Semen samples that were assigned to this group had either the swim-up or gradient method used for sample preparation. The gradient method was selected if the sample displayed excessive leukocytes in the raw sample. Semen samples were prepared by adding 2ml Sperm Preparation Media (Quinns Advantage^{TM} Sequential Embryo Media Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA) to the sample which was centrifuged for 10 minutes at 400G using a Heraeus Centrifuge. Once centrifuged, the supernatant was removed and another 2ml of Sperm Preparation Media added repeating the centrifugation process. After the second spin, the supernatant was again removed and 0.7ml of Sperm Preparation Media was loaded above the pellet (without disturbing it). The spermatozoon was allowed to swim from the pellet into the media above for 1 hour. This supernatant was removed and used for ICSI.

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The gradient method of semen preparation was performed using 100% Density Gradient Medium (Quinns Advantage™ Sequential Embryo Media Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA) and diluting it to 90%, 70% and 45% respectively using Sperm Preparation Media (Quinns Advantage™ Sequential Embryo Media Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA). The different densities were then layered on top of each other, starting with the 90% first (at the bottom), then the 70% and lastly 45% (as seen in Figure 8). The semen sample was subsequently loaded on top of the three layers and centrifuged for 20 minutes at 400G using a Heraeus Centrifuge. After centrifugation, the top two layers of density gradient (45% and 70%), as well as the seminal fluid, were removed and discarded. The spermatozoon pellet that remained was mixed with 2ml of spermatozoa preparation solution and centrifuged for 10 minutes at 400G. The supernatant was removed and another 2ml of spermatozoa preparation media was added to the pellet and again centrifuged. The supernatant was again removed and 0.3ml of spermatozoa preparation media was added to the pellet and mixed thoroughly. These spermatozoa were then used for ICSI.

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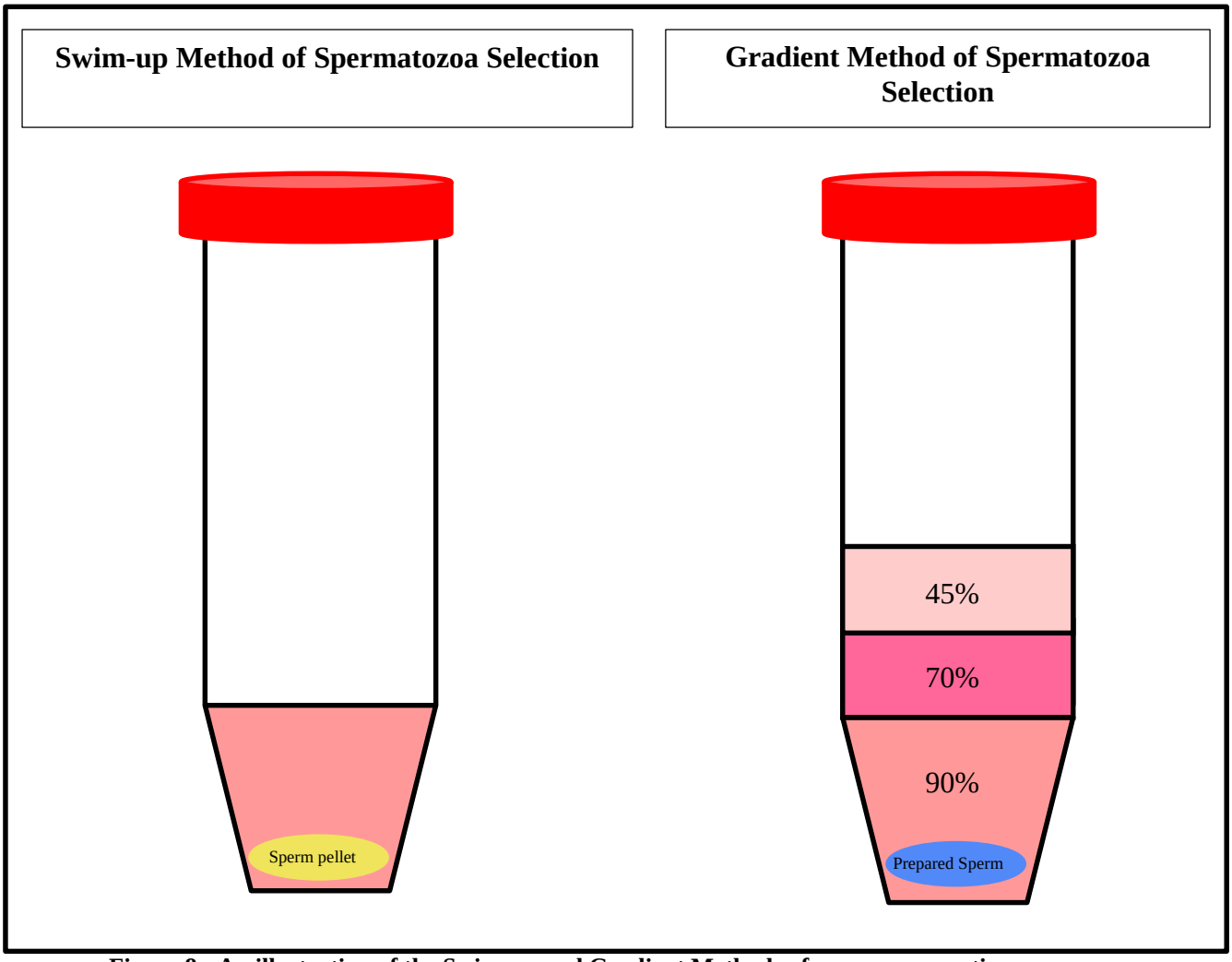

Figure 8: An illustration of the Swim-up and Gradient Methods of semen preparation

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2.2.2.2 Cumulus Oophorus Model

COCs were obtained from females undergoing treatment ICSI. After oocyte retrieval, the cumulus oophorus was dissected mechanically using a sterile syringe needle and a glass pipette. The cumulus oophorus from each patient was pooled (individually) in culture medium supplemented with bovine serum albumin (BSA) before experimentation.

A sterile glass capillary with an inner diameter of ±0.7mm was attached onto a 1ml disposal syringe and pre-warmed in an IVF incubator at 37°C with humidified air containing 6% CO_2 in air. Culture medium supplemented with BSA was aspirated to a length of 3 cm from the end of the capillary. This was followed by aspiration of cumulus oophorus, thus forming a cumulus oophorus column of a length determined by the experimental design (Figure 9). This end of the capillary was then placed into a 100µl droplet of spermatozoa suspension containing 10×10^6 motile spermatozoa/ml overlaid with mineral oil. The experimental apparatus was kept in the IVF chamber for 1 hour. After incubation, the capillary was cut with a diamond tip pen at the interface between the cumulus oophorus column and the medium column. The medium column thus contained the spermatozoa that had passed through the cumulus oophorus (penetrated spermatozoa). These spermatozoa were expelled into a 0.5ml Eppendorf tube. This sample was then used during the ICSI procedure.

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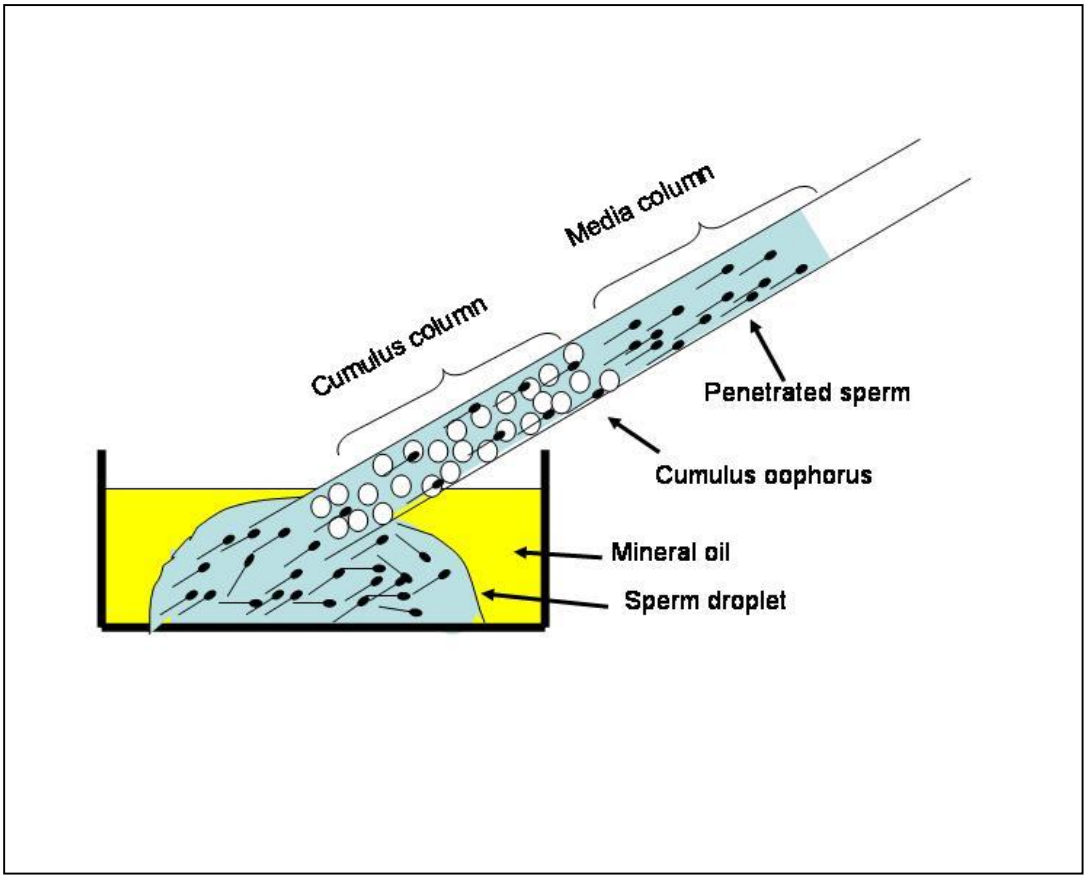

Figure 9: Experimental cumulus oophorus model (Rijsdijk *et al.* 2007)

2.2.2.3 PICSI® Dish

The PICSI® Dish Spermatozoa Selection Devices (Biocoat Inc. Horsham, PA, USA with distributors MidAtlantic Diagnostic) are commercially available and the spermatozoa isolation was done as follows: The hyaluronan microdots were rehydrated using Sperm Preparation Media (Quinns Advantage™ Sequential Embryo Media Sage *in vitro* Fertilization, CooperSurgical Company, Trumbull CT, USA) and then covered in tissue culture oil (room temperature). One to 2µl of spermatozoa was added to the media surrounding the first microdot and left for 5 minutes so as to allow the

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spermatozoa to bind to the hyaluronan droplets. Good levels of adherence can be seen in Figure 10, and adhered spermatozoa was selected from the middle of the droplet area as seen by the graphical representation in Figure 11. Once the spermatozoa injection was performed, other spermatozoa were removed from the HA droplet with the same injection needle, and placed on a slide to air-dry for further testing.

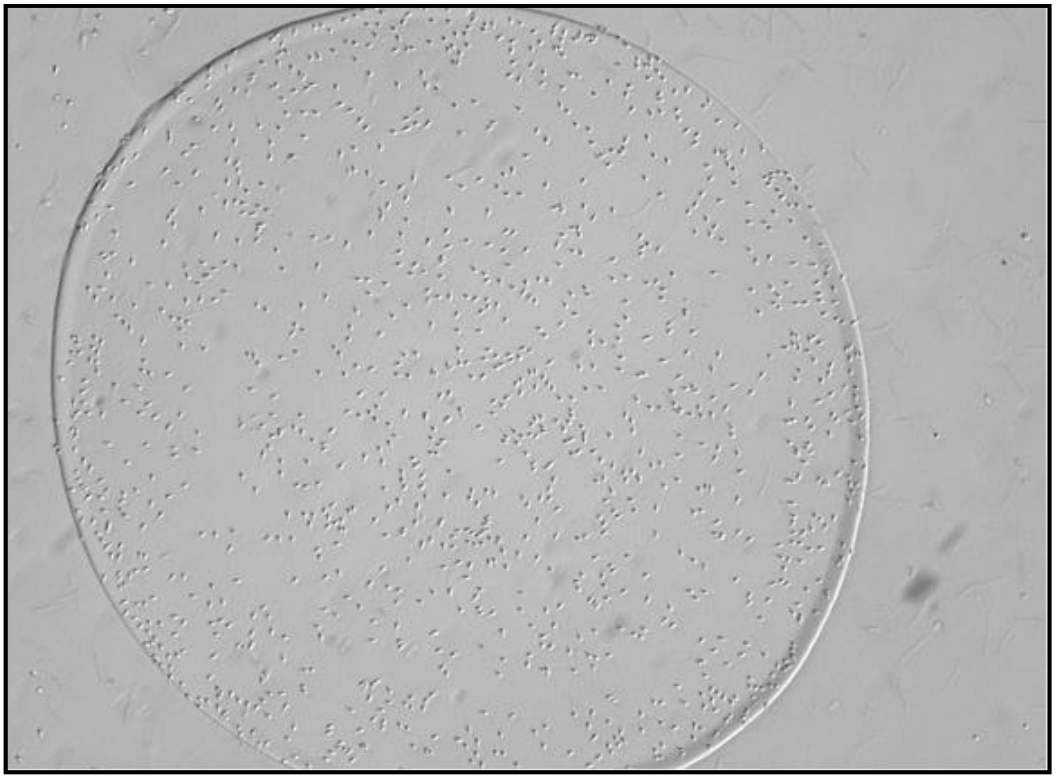

Figure 10: The microdot of the PICSI® dish to which the spermatozoa is adhering. This specific patient showed good adherence (magnification of x 440)

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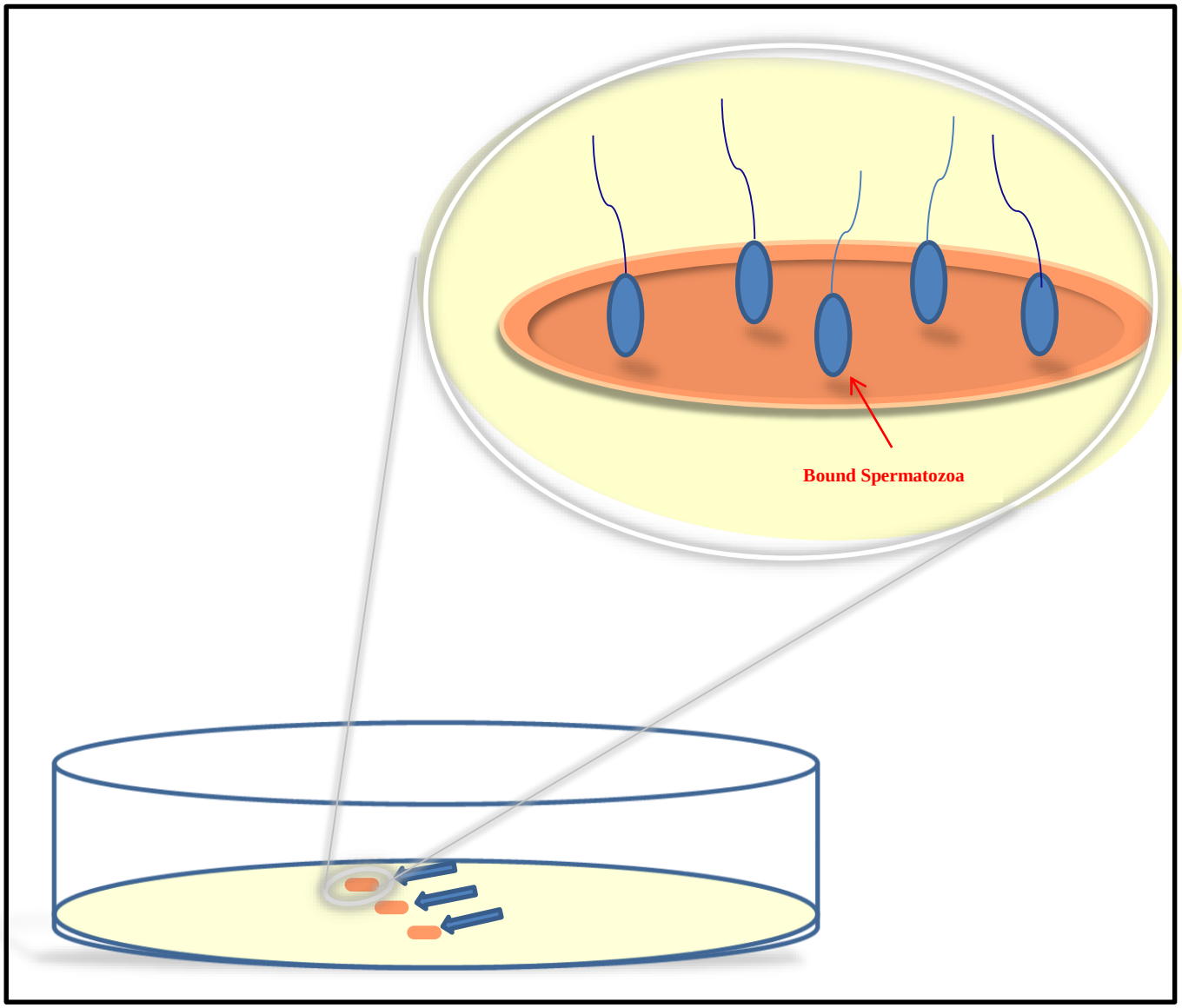

Figure 11: An Illustration of the use of the PICSI® dish

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2.3. Tests Performed on the Raw and Prepared Samples

The following tests were performed on an aliquot of both the raw and prepared samples of each male patient in each of the three groups.

2.3.1. Morphological Evaluation – Diff-Quick Method

Spermatozoa morphology was evaluated using the following guidelines: For a spermatozoon to be considered normal (Menveld *et al.* 1990), the spermatozoa head, neck, mid-piece and tail must be normal (Figure 12). The head should be oval in shape. Allowing for the slight shrinkage that fixation and staining induce, the length of the head should be 4.0-5.0µm and the width 2.5-3.5µm. The length-to-width ratio should be 1.50:1.75. These ranges are the 95% confidence intervals limits for Papanicolaou-stained spermatozoa heads (Katz *et al.* 1986). Estimation of the length and width of the spermatozoa were made with an ocular micrometer. There should be a well-defined acrosomal region comprising 40-70% of the head area. The mid-piece should be slender, less than 1µm in width, about one and a half times the length of the head, and attached axially to the head. The tail should be straight, uniform, thinner than the mid-piece, uncoiled and approximately 45µm long (WHO, 1999). This classification scheme requires that all "borderline" forms be considered abnormal (Kruger *et al.* 1986, Menkveld *et al.* 1990). Diff-Quick® staining was employed in the study since the author uses the stain as the standard staining method.

The spermatozoa of all patients in each respective group were collected and slides were made and left to air dry. The slides were all stained using the Diff- Quick® staining

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solutions, which consists of a fixative, staining solution 1 (eosinophillic xanthene) and staining solution 2 (basophilic thiazine). Two hundred spermatozoa were then evaluated for normal forms using the Tygerberg Strict Criteria (Kruger *et al.* 1986; Menkveld *et al.* 1990; Coetzee *et al.* 1998).

The reference limits for the criteria are as follows:

Normal ≥ 5%

Abnormal < 5%

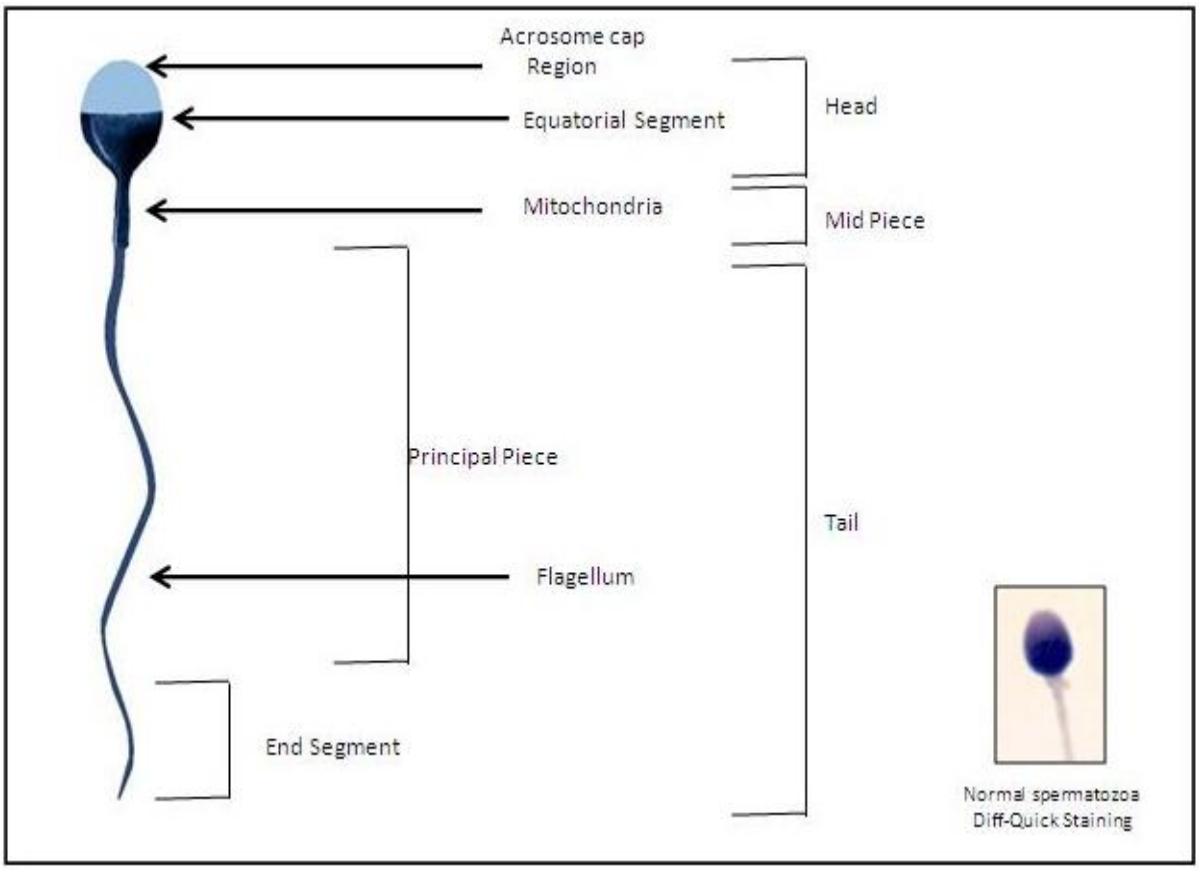

Figure 12: A schematic drawing of a morphologically normal spermatozoon

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2.3.2. Chromomycin A_3 (CMA_3)

Chromatin of mature spermatozoa has undergone stabilization when their histones are replaced by protamines and there is formation of molecular disulphide bonds. The nucleoprotein components can be modified during spermatogenesis and this leads to abnormalities in chromatin packaging. The CMA_3 staining technique utilizes the detection of protamine-deficient (or loosely packed) chromatin as well as nicked DNA, to indicate the likelihood of ART success due to possible DNA damage and the failure of the spermatozoon decondensation after ICSI (Bianchi *et al.* 1996; Lopes *et al.* 1998; Sakkas *et al.* 1995)

Spermatozoa was collected from all patients and slides were left to air dry. The slides were then fixed using 3:1 ratio of methanol to glacial acetic acid. A 250µl volume of CMA_3 – ethanol (Sigma Chemicals, St Louis, MO USA Cat 2659) was dropped onto the slide and then placed in a dark cupboard for 20 minutes. The slide was then washed in a magnesium chloride McIlvaines buffer solution (0.25mg/mL in McIlvane's buffer, pH 7.0 containing 10mM $MgCl_2$) and mounted while still wet with Dabco (Aldridge Chemicals Co, Milwaukee, US Cat No. 29,073-4) and a coverslip. The slides were then left overnight at 4°C and evaluated the following day using a fluorescein isothiocyanate (FITC) filter and an Eplan 100X objective. A total of 200 spermatozoa were randomly evaluated on each slide (Franken *et al.* 1999).

The spermatozoa are then evaluated as follows

1. No staining (no fluorescence)
2. Fluorescent band at the equatorial region of the spermatozoon head
3. Faint yellow fluorescence of the entire spermatozoon head
4. Bright yellow fluorescence of the entire spermatozoon head.

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The results are reported as classes 1 and 2 which together represent mature DNA and classes 3 and 4 are indicative of immature DNA as seen in Figure 13.

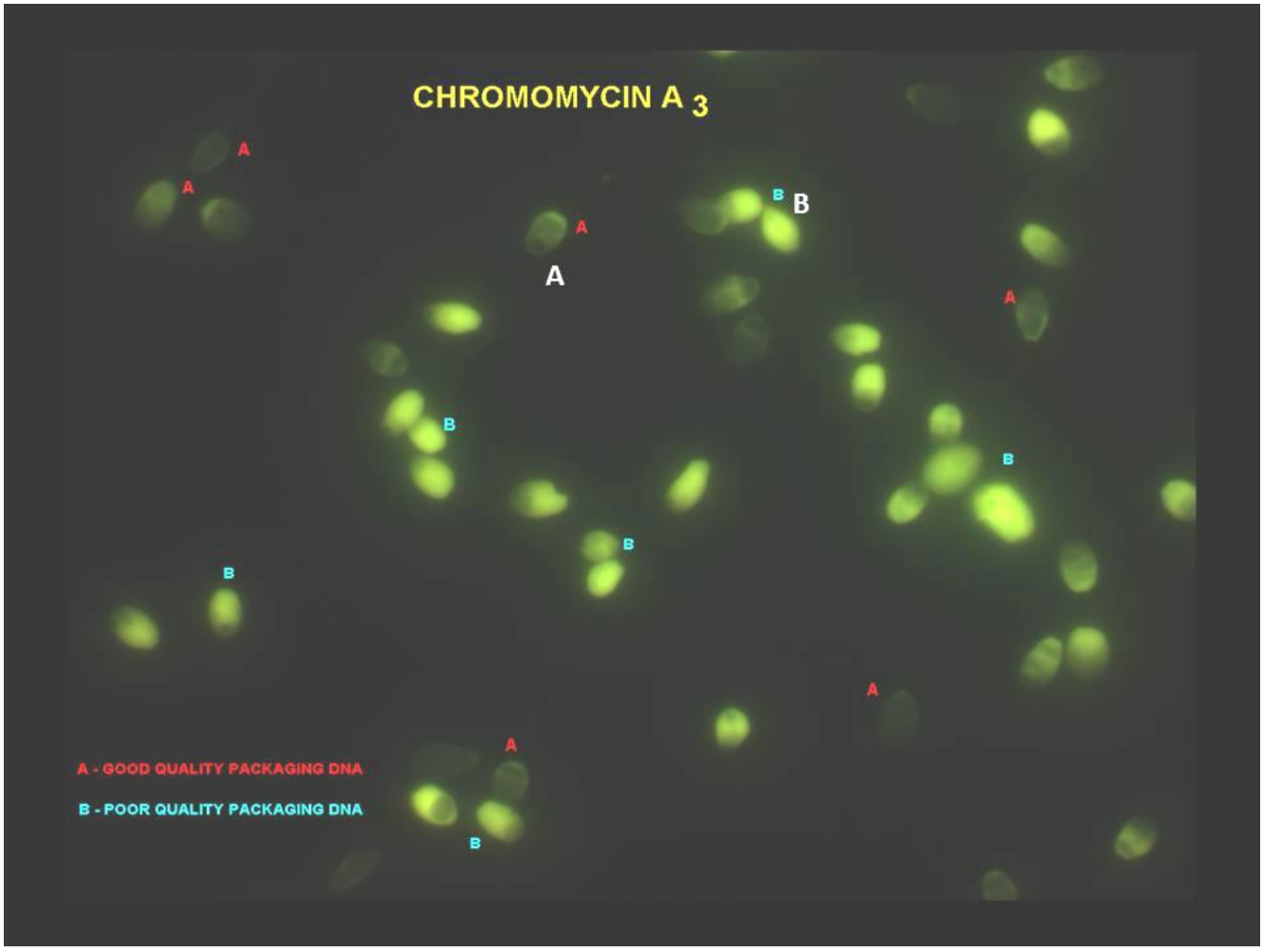

Figure 13: A photograph of Chromomycin A_3 staining illustrating the differences between the good (dull stain) and poor (bright) quality spermatozoa.

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2.3.2. Chlortetracycline (CTC) test

The acrosomal status of spermatozoa was determined by CTC staining as previously described (Yao *et al.* 2000). Spermatozoa was collected from all patients and slides were made. These slides were fixed using a 1:1 ratio of Tris buffer to 12.5% paraformaldehyde solution. The slides were stained with CTC solution and stored overnight at 4°C (750 µM of CTC in 20 mM of Tris buffer supplemented with 130mM of NaCl and 5mM of cysteine) until counting.

The acrosomal status of 200 spermatozoa was evaluated under a fluorescence microscope with a filter set consisting of an excitation filter BP 450-490, a chromatic beam splitter FT510, and a barrier filter LP520. CTC staining patterns of the spermatozoa head were identified according to the method of Perry *et al.* (1995).

They are: (1) CTC1, a fluorescent band in the postacrosomal region;

(2) CTC2, a bright fluorescent head with a non-fluorescent postacrosomal region;

(3) CTC3, a bright fluorescent head with a non-fluorescent thin band in the postacrosomal region;

(4) CTC4, uniform head fluorescence; and

(5) CTC5, a decrease in or loss of uniform fluorescence over the head.

CTC1, CTC2, and CTC3 were the uncapacitated patterns. CTC4 is the capacitated pattern and CTC5 is the acrosome-reacted pattern. Only CTC5 was counted in this study (Figure 14).

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Figure 14: The staining pattern CTC5 showing the acrosome-reacted spermatozoa

2.3.3. Acridine Orange (AO)

The DNA quality of the spermatozoa was determined using the acridine orange (AO) staining method as previously described (Claasens *et al.* 1992; Klun *et al.* 2002 and Eggert-Kruse *et al.* 1998). When AO binds to double-stranded DNA it emits a green fluorescence with single-stranded DNA (or RNA) emitting a red, orange or yellow fluorescence (Figure 15). Slides were fixed using Carnoy's solution overnight (methanol/acetic acid, v/v 3:1) and then left to air dry. Ten millilitres of 1% AO (diluted in distilled water) was mixed with 40ml of 0.1M citric acid and 2.5ml of 0.3M Na_27HPO_4 and the slides were stained for 5 minutes with this solution. The slides were then washed in distilled water and sealed using DPX mountant (Sigma) and counted immediately. Two hundred spermatozoa were

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evaluated for each patient and the result expressed as a percentage. The percentage of red, orange or yellow spermatozoa (single-stranded DNA) were recorded as well as the green (double-stranded) fluorescence spermatozoa.

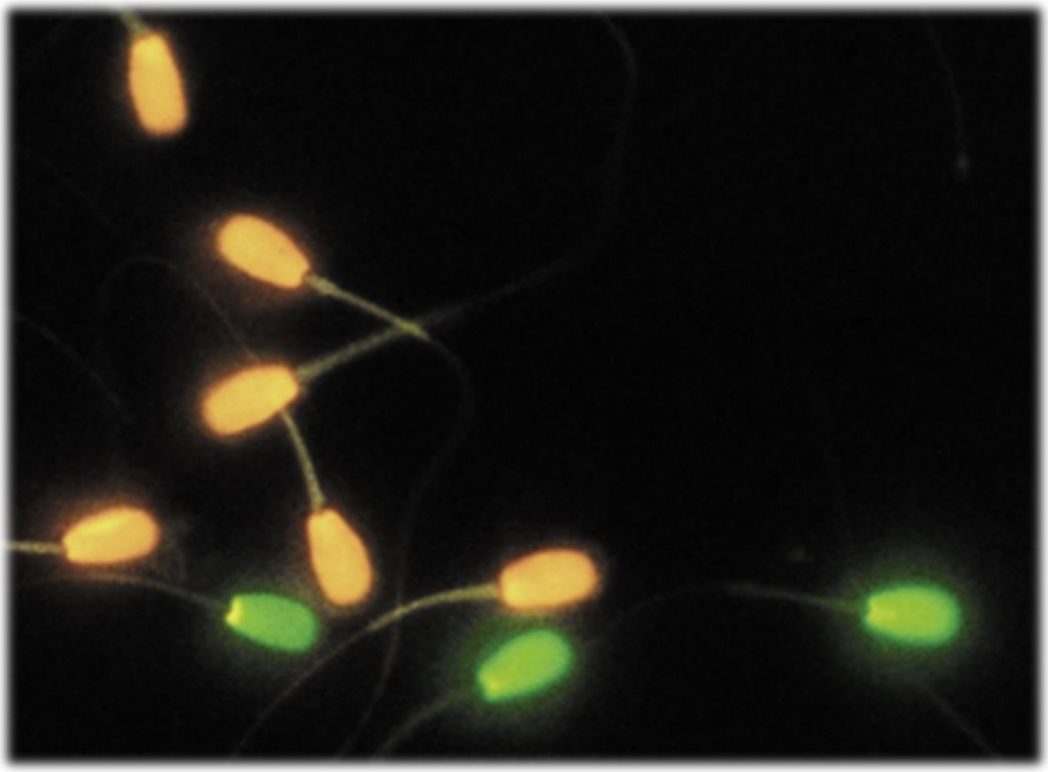

Figure 15 : A photo of the staining characteristics of the acridine orange stain (AO)
Green head = diploidy
Orange head = aneuploidy

2.4. The Rationale

In this study we aim to compare the efficacy of the wash and swim-up method, the cumulus oophorus spermatozoa selection model (Hong *et al*, 2004; Rijsdijk and Franken, 2007) and the PICSI™ dish in selecting spermatozoa for the ICSI procedure. The primary aim is to determine which spermatozoa selection method delivers the best results in terms of the chromatin packaging quality, DNA integrity, morphology, capacitation and AR. The secondary aim is to determine which method delivers the best results in the ICSI arena in terms in fertilization rate, cleavage rate,

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embryo quality, pregnancy rate, implantation rate and live birth rate. Figure 16 illustrates the study design.

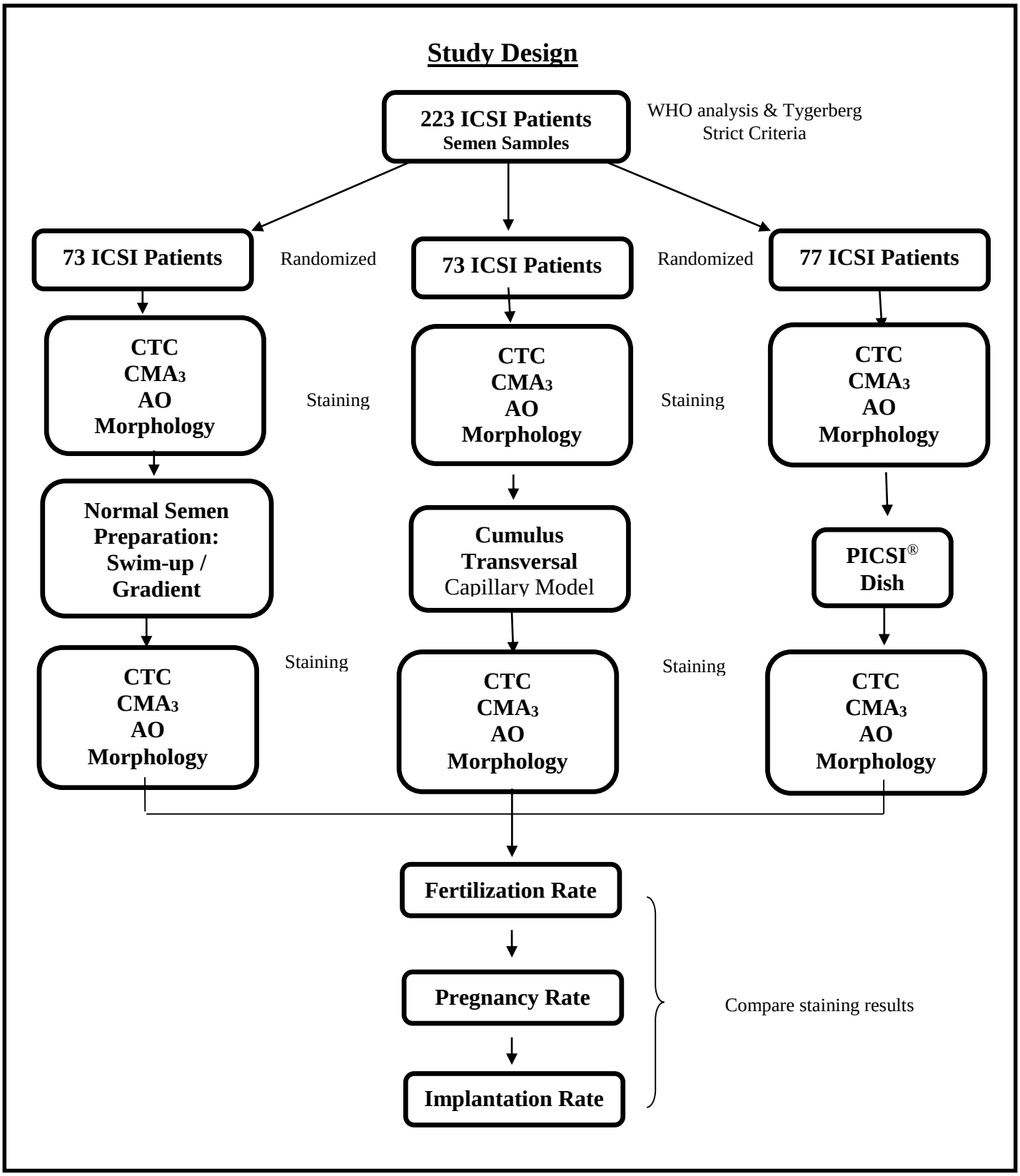

Figure 16: An illustration of the study design

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2.5. Statistics

Power calculations for this randomized trial were considered and based upon the results obtained from the pilot study; the sample size was determined to be a minimum of 60 samples within each arm (see Addendum 1). A randomization chart of patients entering the study was supplied by the same statistician and this was used to randomize the patients entering the study (Figure 17). All statistical analyses for the study results were performed using analysis of variance or ANOVA (Stata version 11. Stata Corp LP, 4905 Lakeway Drive, College Station, Texas 77845, USA). A repeated measure of variance has been done on CTC, CMA_3 and AO data. Results were analysed to calculate the mean difference, confidence interval and p-values of all 3 groups of the ICSI patients (patients undergoing normal "swim-up", the cumulus subjected semen samples and the PICSI® group) in relation to CTC, CMA_3,AO and morphology, with fertilization rate, cleavage rate, pregnancy rate and live birth rate being primary outcomes.

Quantile regression was used to compare the median changes between the three groups. This is a non-parametric test with good power, given the sample size of the study. Some of the observations had outliers and this method is designed to be robust and handle such values. Significance was set as $p<0.05$

Randomization Numbe	Method to be used
100001 1	Swim-up
100002 2	Cumulus Model
100003 3	PICSI Dish®
100004 1	Swim-up
100005 2	Cumulus Model
100006 3	PICSI Dish®
100007 1	Swim-up
100008 3	PICSI Dish®
100009 2	Cumulus Model
100010 2	Cumulus Model

Figure 17: An example of the randomization chart used to assign patients to the study

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Chapter 3: Results

The different methods of spermatozoa preparation were assigned a number for that specific technique. The swim-up/gradient method was Group 1, the cumulus oophorus model Group 2 and the PICSI dish method was Group 3..

3.1. Basic Comparison of Groups - Descriptive

The results of all the overall descriptive baseline values, for all three groups, reflects the success in randomization of the study patients as seen in Table 1.

Table 1: The results of the tests performed for each of the study groups

Group	Test	Mean	SD	Min	Percentile 25th	Median	Percentile 75th	Max
Group 1 Swim-up / gradient	**Morphology Before %**	3.12	1.67	1	2	3	4	8
	Morphology After %	5.52	2.25	1	4	5	7	13
	CTC Before %	8.11	4.58	0	4	8	10.5	21
	CTC After %	34.58	16.09	10	22	33	42.5	89
	CMA_3 Before %	54.51	18.25	16	40	51.5	68	99
	CMA_3 After %	71.18	16.48	40	58	70	86	99
	AO Before %	48.37	19.65	10	38	47.5	63	89
	AO After %	69.71	22.14	12	54.5	72.5	89	99
Group 2 Cumulus oophorus model	**Morphology Before %**	2.77	1.52	1	1	3	4	6
	Morphology After %	5.81	2.75	2	4	5	7	15
	CTC Before %	8.69	6.93	1	4	7	11.5	45
	CTC After %	43.26	19.48	8	28	44.5	56	89
	CMA_3 Before %	58.01	18.21	12	44.5	55.5	75	89
	CMA_3 After %	74.11	16.80	25	60	79	88	99
	AO Before %	47.76	20.43	2	35.5	48	61.5	88
	AO After %	71.50	21.69	12	56	81	89	98
Group 3 PICSI® dish	**Morphology Before %**	2.90	1.63	1	2	2.5	4	8
	Morphology After %	5.65	2.20	2	4	5	7.5	10
	CTC Before %	8.71	10.07	1	5	7	10	85
	CTC After %	26.40	15.23	4	15	22	39	60
	CMA_3 Before %	54.28	19.95	12	38	50.5	70.5	89
	CMA_3 After %	79.27	17.90	41	60	86	98	99
	AO Before %	45.26	21.65	2	29	48	60	89
	AO After %	65.56	26.57	0	48	71	89	100

CTC = Chlotetracycline stain CMA_3 = Chromomycin A_3 stain AO = Acridine Orange stain

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3.1.1. Morphology

The morphology results seen in Table 1 showed the pre-and post-preparation values in each of the three groups. The mean morphology for Group 1 (swim-up) is 3.125% ± 1.6778% (mean ± standard deviation (SD)) before and 5.52 ± 2.2515% after preparation (a mean difference of 2.40 ± 1.56 (Table 1), with a minimum value of 1% and a maximum value of 8% before, and a minimum value of 1% and maximum value of 13% after preparation.

In Group 2 (cumulus oophorus model) the morphology before preparation is 2.77 ± 1.521% and 5.81 ± 2.754% after (a mean difference of 3.04, Table 2). This group showed a minimum value of 1% and max value of 6% before preparation, and a minimum value of 2% and max after preparation of 15% ± 2.01 as seen in Table 2.

Table 2: The difference for each group for all the tests done between pre and post preparation

Group	Variable	Mean Difference	Standard deviation (SD)	95% Conf. Interval
1 **Swim-up/gradient**	Morphology Difference %	2.40 [a]	±1.56	1.63 – 2.36
	CTC Difference %	26.47 [a]	±16.64	20.26 – 31.74
	CMA_3 Difference %	16.66 [a]	±19.94	11.41 – 20.59
	AO Difference %	21.33 [a]	±22.31	13.96 – 28.03
2 **Cumulus model**	Morphology Difference %	3.04 [a] [ƛ]	±2.01	0.49 – 1.51
	CTC Difference %	34.56 [a]	±21.17	-0.11 – 16.11
	CMA_3 Difference %	16.09	±13.07	-9.49 – 3.49
	AO Difference %	24.21 [a]	±19.11	-10.98 – 8.98
3 **PICSI** ® dish	Morphology Difference %	2.75	±1.98	-0.51 – 0.51
	CTC Difference %	17.69 [a] [ƛ]	±16.98	-20.01 - -3.99
	CMA_3 Difference %	24.98 [a] [ƛ]	±20.23	2.59 – 15.41
	AO Difference %	19.32 [a]	±20.75	-12.98 – 6.99

[a] Significant $p < 0.005$ [ƛ] The significant change comparing the 3 groups ($p < 0.001$)

CTC = Chlotetracycline stain CMA_3 = Chromomycin A_3 stain AO = Acridine Orange stain

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Improvements in morphology post sample preparation were not as successful in the PICSI® dish (Group 3) as Group 2 ; the mean morphology before preparation was 2.90% ± 1.634% and after preparation 5.65% ±2.206% with a mean difference of 2.75% ±1.98 (Table 1; Table 2; Figure 18).

Quantile regression was used to compare the median change between the three groups. This is a non-parametric test but with good power given the sample size of the study. Overall there was a significant difference in the morphology changes achieved by the three groups ($p = 0.01240$ which can be seen in Table 2 and Figure 18. A greater difference of 1% (improvement) is seen in Group 2 (cumulus) with $p < 0.001$ (a difference of 3.04 compared to the 2.04 in Group 1 and 2.75 in Group 3 (95% CI (0.488 – 1.510) (Table 2)).

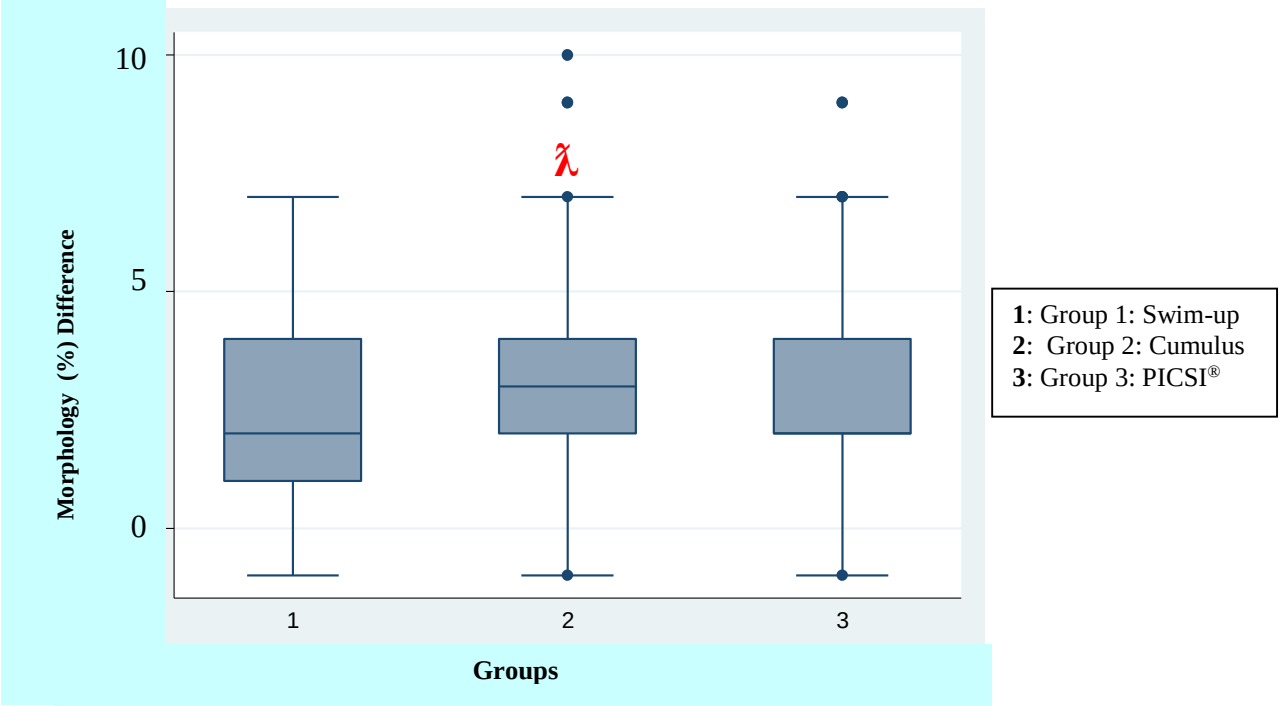

Figure 18: The significant improvement from pre- to post-preparation for morphology in all groups ($p < 0.05$) λ Significant ($p < 0.001$)

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3.1.2.CTC

Overall there is a significant difference ($p < 0.001$) in the CTC changes in all groups. The swim-up group showed a significant increase in the CTC value ($p < 0.05$: 95% CI 20.25 – 31.74). The cumulus group (Group 2) showed an improvement in value, irrespective of the baseline, as viewed in Figure 19 ($p = 0.053$; 95% CI (-0.11 – 16.11)) (Table1). PICSI® (a mean before value 8.71% compared to a mean after value of 26.40) also showed a significant improvement ($p <0.05$; 95% CI (-20.01 - -3.98), but only when the baseline values were higher. If the baseline values for the PICSI® dish group were low, then no improvement was noted (mean difference of 17.69% compared to the 26.47% in Group 1 and 34.56% in Group 2 – Table 2).

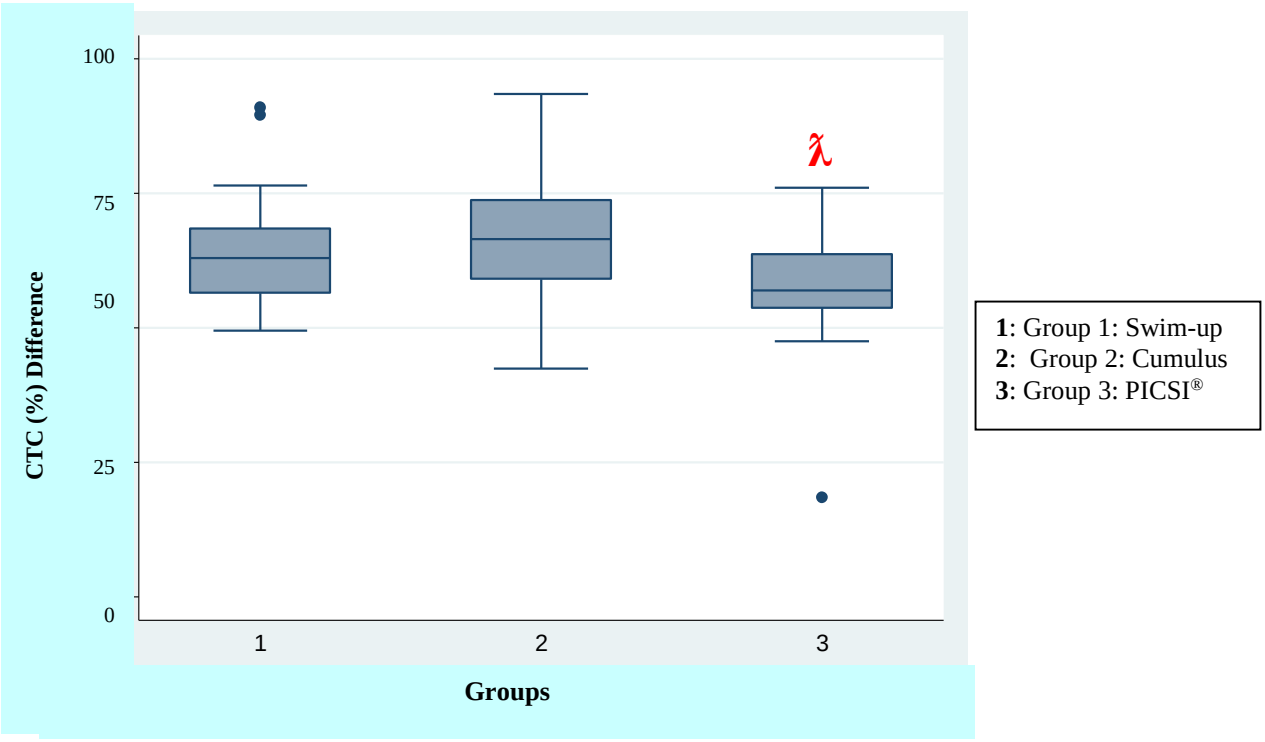

Figure 19: The measure of change from pre- to post-preparation for CTC in all groups

λ Significant (p<0.0001)

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3.1.3. CMA_3

The mean values for the CMA_3 for all groups are shown in Table 1. All three groups showed a significant improvement in CMA_3 results ($p = 0.0008$) (Figure 20) with PICSI® showing the highest rate of improvement compared to the other two groups (Table 2; Figure 20) (mean differences: Group 1: 16.66 ± 19.94%; Group 2: 16.09 ± 13.07% and Group 3: 24.98 ± 20.23%).

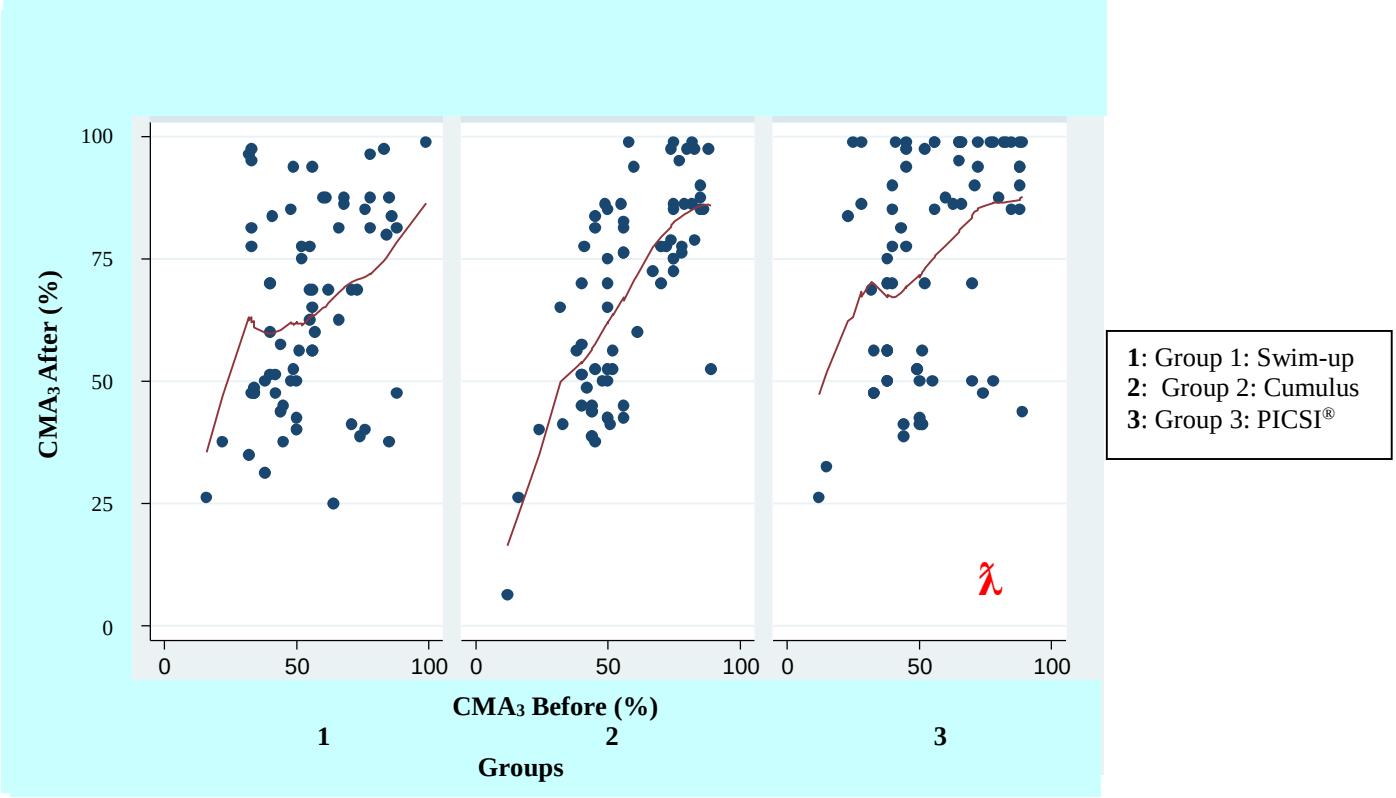

Figure 20: The measure of change from pre- to post-preparation for CMA_3 in all groups

λ **Significant ($p < 0.005$)**

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Overall there is a significant difference in CMA_3 changes between all three groups (p = 0.0008). There is no difference between Groups 1 and 2 with a p-value of 0.364. The absolute mean change for these two groups was: Group 1: 16.66 ± 19.94% and Group 2: 16.09 ± 13.07%. Group 3 (PICSI®) had the most significant increase in change of p-value (0.006 or 9 units) of the three groups between before and after preparation which is 24.98 ± 20.23% (Table 2) (Figure 20).

3.1.4. AO

All groups showed an improvement in spermatozoa selection for double-stranded DNA positive spermatozoa (Figure 21). The mean changes were Group 1: 48.37% (before preparation) to 69.70% (after preparation), Group 2: 47.76% (before preparation) to 71.50% (after preparation) and Group 3: 45.26% (before preparation) to 65.56% (after preparation) (Table 1).

The cumulus group (Group 2) had a higher rate of improvement with a mean change of (24.21 ± 19.11%) vs. Group 1 (21.33 ± 22.31%) and Group 3 (19.32 ± 20.75%) (Table 2); however, this was not significant. When the baseline values for the AO stain were lower, Group 1 also showed a better improvement. There is no significant difference between the three groups for AO results (p = 0.8341) (Figure 21).

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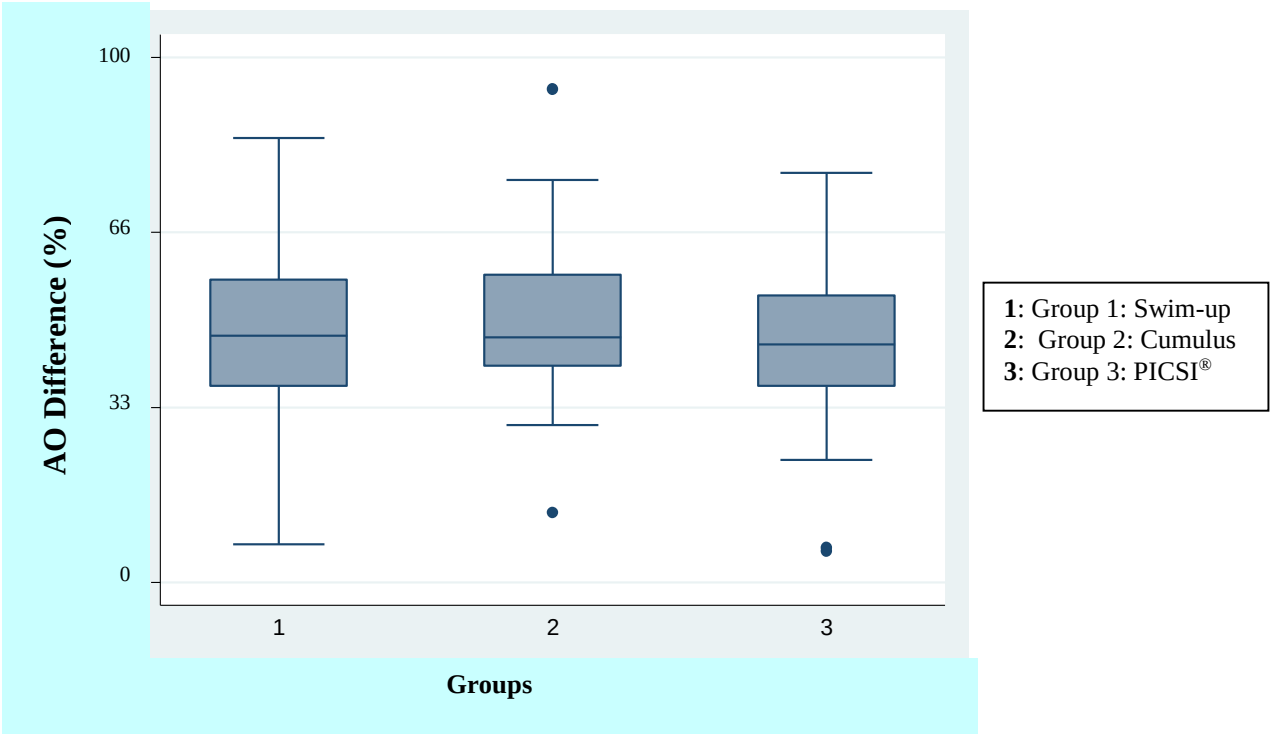

. Figure 21: The measure of change from pre to post preparation for AO in all groups

3.2. The Method Effect vs. Fertilization, Cleavage, Pregnancy and Implantation Rates

The fertilization of mature (metaphase II) oocytes showed a significantly higher likelihood of fertilization (75% as seen in Table 3) in the swim-up group (p = 0.0183; Figure 22) v. both the cumulus and the PICSI® groups (odds ratio = 0.72 (95% CI 0.55 – 0.94) and 0.70 (95% CI 0.53 – 0.92) for Group 2 and Group 3 respectively). The difference between the cumulus and PICSI® groups was not significant (p = 0.8123) (Figure 22). The difference between the swim-up and cumulus groups (in terms of fertilized ova) is p = 0.0015. The difference between Group 2 and 3 in terms of fertilized ova is not significant (p = 0.4414).

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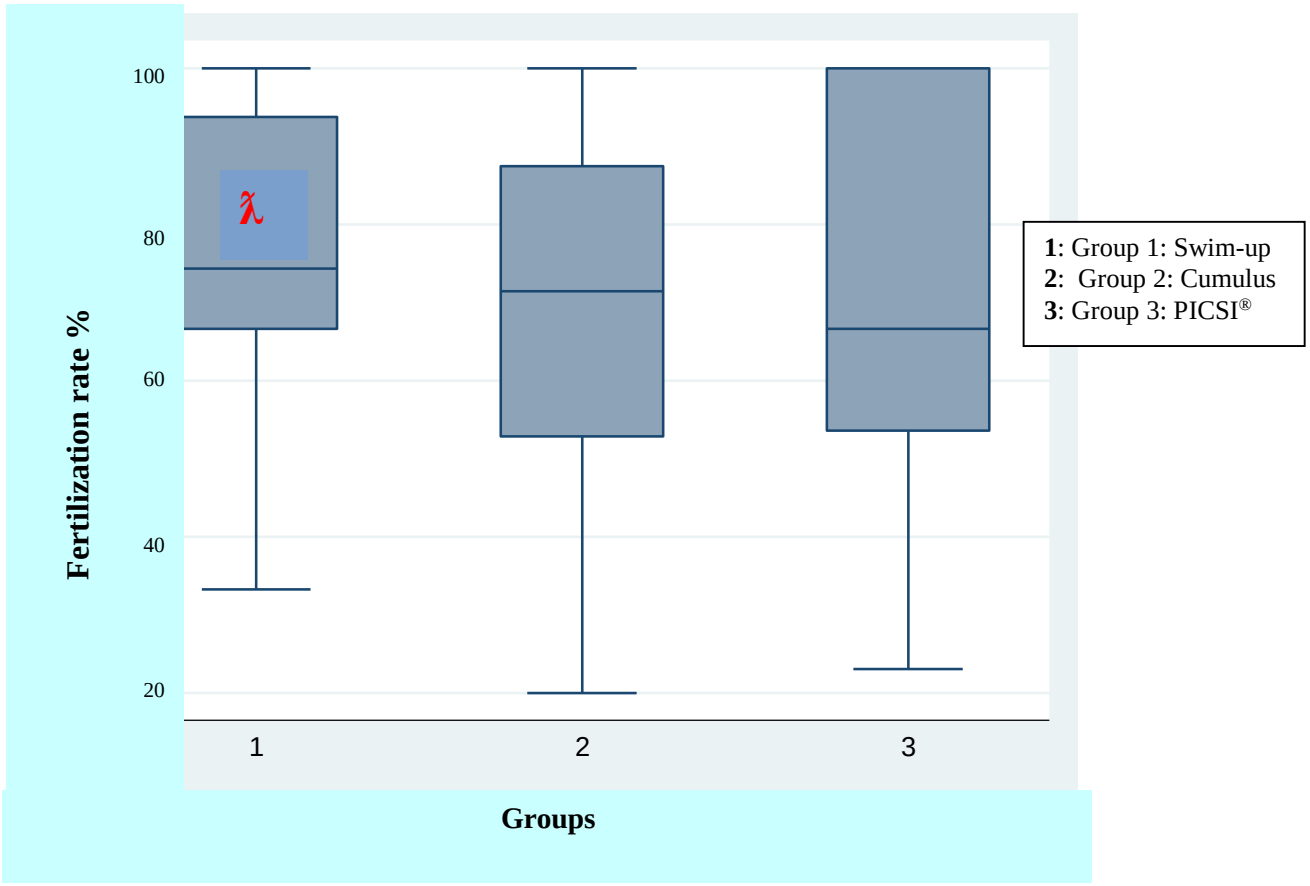

Figure 22: The fertilization rate of the 3 groups with the median value being the solid line λ Significant (p <0.005)

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Table 3: The comparison of ICSI outcomes in all 3 groups of semen preparation

Results	Group 1: Swim-up/Gradient	Group 2: Cumulus Model	Group 3 PICSI® Dish
Number of Patients (N)	72	72	76
Percentage of Total Patients (%)	32.73	32.73	34.55
Mean Female Age	32.1 ± 4.71	32.4 ± 4.68	34.1 ± 3.84
No. Ova (mean ± SD)	8.06 ± 5.48	9.63 ± 5.37	7.07 ± 4.76
Ova Injected (mean ± SD)	6.65 ±3.95	8.01 ± 4.72	5.98 ± 4.05
Ova Fertilized (mean ± SD)	4.91 ± 3.01	5.37 ± 3.07	3.97 ± 2.61
Fertilization Rate % (per ova retrieved)	75[a]	70.29	71.9
No. Embryos Cleaved (mean ± SD)	4.68 ± 2.71	5.33 ± 3.09	3.82 ± 2.55
Cleavage Rate %	95.2	99.2	96.36
No. Embryos Transferred (mean ± SD)	1.94 ± 0.28	1.97 ± 0.41	1.98 ± 0.53
Top Grade Embryos at ET (%)	47.22	35.21	40.83
Pregnancy Rate per Top Grade Embryo (%)	48.61	50.00	46.05
Pregnancy %	56.94	55.56	57.89
Implantation rate per Embryo Transferred% (n)	30.7 (43)	27.5 (38)	27.3 (38)
Pregnancy Loss Rate (PLR) (%)	8	6	6

[a] Significant $p < 0.05$; (N) = number; (n) = number of fetal hearts, cardiac activity confirmed ET = embryo transfer

The difference in cleavage rate based on the number of fertilized oocytes (95.2% for the swim-up group, 99.2% for the cumulus group and 96.36% for the PICSI® group) was not statistically significant.

No difference in pregnancy rate ($p = 0.5721$) was noted across all the groups per embryo transferred (swim-up 56.94%, cumulus 55.56% and PICSI® 57.89%) (Figure 23). Implantation rates are also similar across all three groups per embryo transferred (swim-up 30.7%, cumulus 27.5% and PICSI® 27.3%). The incidence of singleton pregnancies was 33.18% over the whole patient population and 10% for twin pregnancies. No single group

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showed a significant improvement in the fetal number (implantation rate) in terms of the group effect (p = 0.5721).

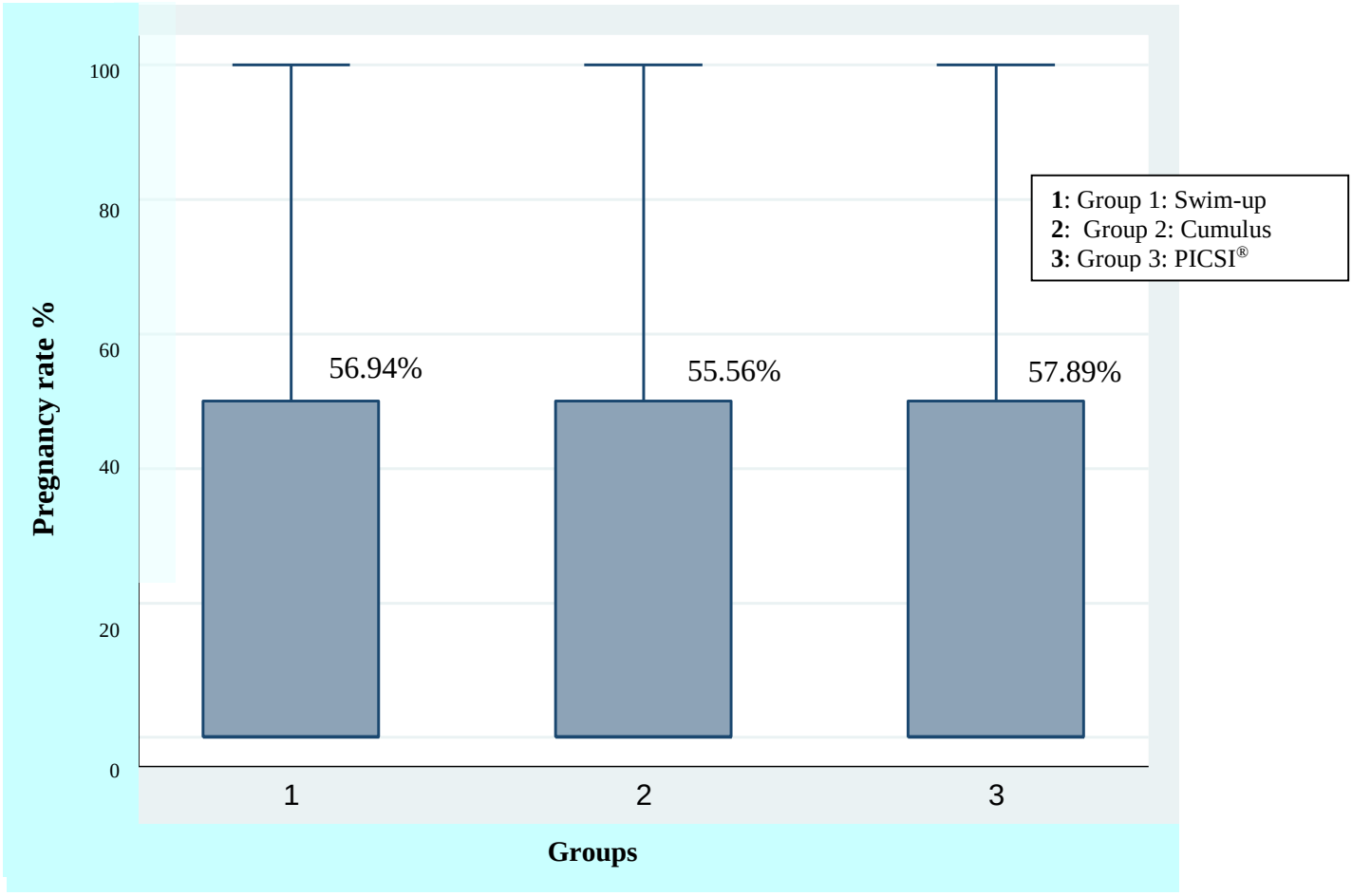

Figure 23: The pregnancy rate as distributed across the three groups per transfer

The pregnancy loss rate for the swim-up, cumulus model and PICSI® groups was 8%, 6% and 6% respectively (fetal loss per fetal heart). None of these values was statistically different from one another with all the p-values being 0.50.

No group effect was noted when using the intention-to-treat analysis with a p-value of 0.57. No group effect was noted even when adjusting for morphology, age and maximum and

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minimum grade of embryos (p = 0.9566). No significant difference was seen in terms of embryo quality in the three groups (p = 0.1819).

Age has a significant effect on outcome (p = 0.0036) with older women having a lower probability of pregnancy (p= 0.91) that declines further for every year in advancing age (Figure 24).

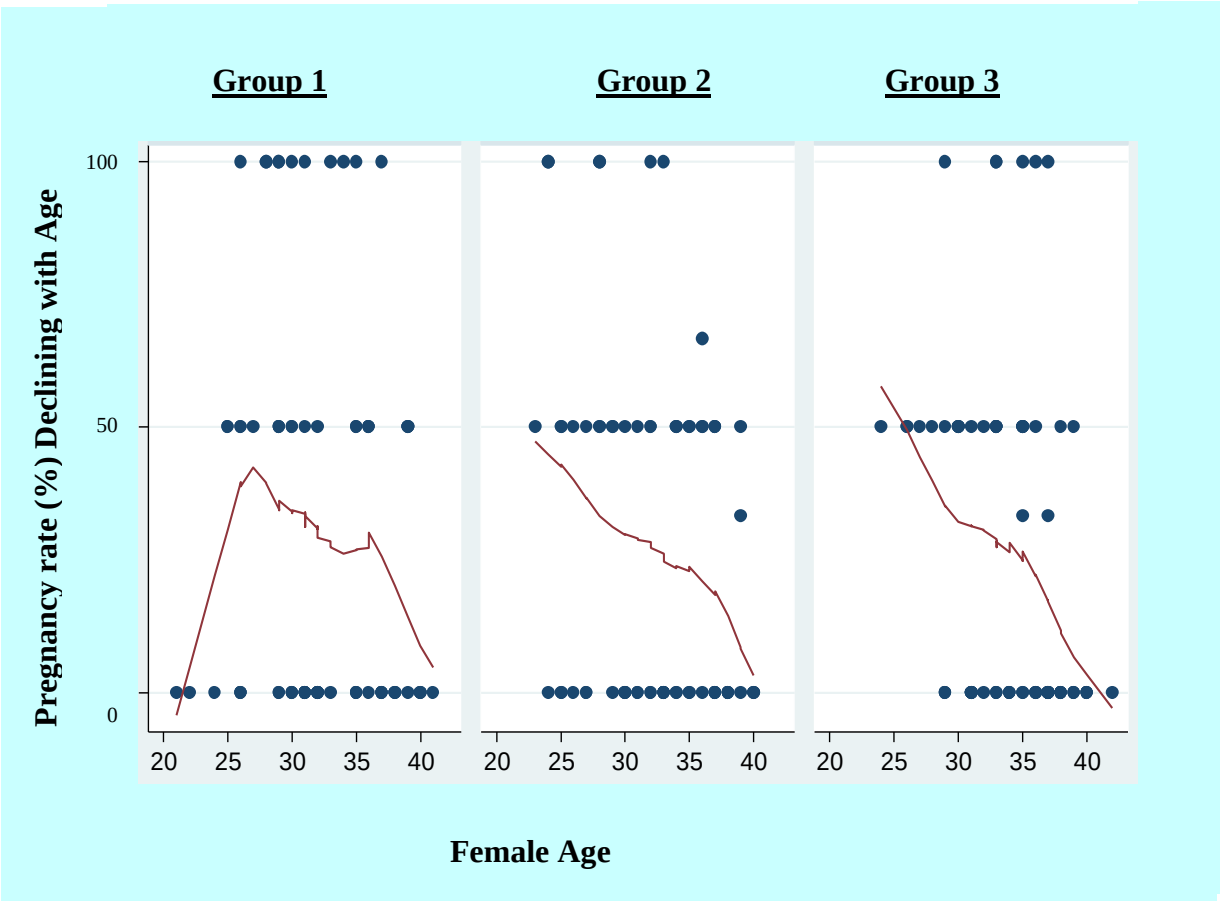

Figure 24: The decline in pregnancy rate as seen with increasing age in all three groups

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Chapter 4: Discussion and Conclusion

4.1. Discussion

ICSI has been the method of choice for male infertility since its inception into the ART arena. The shortcomings of this novel technique are that spermatozoa of unknown genetic quality, are now being injected into oocytes, leading to fertilization and cleavage (Palermo *et al*. 1992). The possible aneuploidy of the injected spermatozoon is not known, and subsequently, many ICSI cycles result in fertilization failure, cleavage aberrations and/or embryonic arrest. Strong predictors of ICSI outcome are morphology, chromatin packaging and DNA quality (double-stranded DNA) (Kruger *et al.* 1986; Sakkas *et al.* 2002) and the improvement of the above qualities, through selection of better spermatozoa for ICSI, can lead to an improvement in ICSI success.

During late spermiogenesis, the cytoplasmic extrusion and plasma membrane remodelling lead to the formation of ZP binding sites in mature, normal spermatozoa (Huszar *et al*,. 1994; Huszar *et al*., 1997). Morphology of spermatozoa has long been shown to influence the fertilization and pregnancy rates of IVF and/or ICSI cycles (Kruger *et al.*, 1986), and spermatozoa with impeded maturation have been shown to have aberrations in their shape, size of head, size of the mid-piece and tail length (Celik-Ozenci *et al*., 2003, Celik-Ozenci *et al*,. 2004); thus morphology has also been shown to indicate the degree of spermatozoa maturity (Celik-Ozenci *et al*,. 2003). This in turn, has been shown to have a relationship with cytoplasmic extrusion, apoptotic processes, DNA chain fragmentation and chromosomal aneuploidies (Huszar *et al*. 1994; Jakab *et al*. 2005; Cayli *et al*. 2003; Sati *et al*. 2008)). The exact mechanisms of the spermatozoa selection process *in vivo* have always been thought to be an oocyte-dominated selection process, but recently Huszar *et*

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al. (1994) and Prinosilova *et al.* (2009) have shown that membrane remodelling in the spermatozoa leads to the selection of mature spermatozoa that are also morphologically normal.

Morphology in the current study in all three groups showed significant improvement ($p=0.01240$) and interestingly the cumulus group (Group 2) showed significantly improved morphology by 1% ($p< 0.001$) as confirmed by the original work published by Hong *et al.* (2004), as well as Rijsdijk and Franken (2007). The results from Group 3 are in agreement with the research by Nijs *et al*. (2009) that showed a correlation between sperm morphology and HA binding.

The selection of morphologically normal spermatozoon at 400 x magnification (shown by French *et al.* 2010) showed no effect on blastocyst development and embryo morphology, but did show improved fertilization rates for teratozoospermic patients. The introduction of IMSI (6600 x magnification) by Bartoov *et al.* (2001) allowed for the identification of normal spermatozoon forms, as well as normal nuclear content. This technique was subsequently proven to result in better pregnancy rates, higher blastocyst development rates and higher delivery rates (Balaban *et al.* 2011; Bartoov *et al.* 2003; Berkovitz *et al.* 2005, 2006; Hazout *et al.* 2006; Vanderzwalmen *et al.* 2008). Petersen et al. (2010) in contrast, showed that there was no difference in the spermatozoa morphology of HA-bound or HA-unbound spermatozoa when using high magnification motile sperm organellar morphology examination (MSOME).

The capacitation of a spermatozoon is a complex reaction. This state was measured by using the CTC-test, ultimately looking at the AR as the measurable end point. The AR (although not needed for ICSI) is a key step for *in vivo* fertilization, and it has been

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reported in many trials (Fukui 1990; Yeo *et al.* 2000) that cumulus cells specifically induced capacitation. Although the AR should commence at the ZP (*in vivo* fertilization), the spermatozoa cannot be fully reacted as the zona does not allow passage of a fully reacted spermatozoon. The number of AR spermatozoa significantly increased in all three groups ($p < 0.001$) (Table 1). This indicates that all methods of semen preparation were activational as well as selective in nature in terms of morphology, as the process of capacitation is initiated by all three preparation techniques. This is in agreement with this author's previous study (Rijsdijk and Franken, 2007) which showed a significant increase in AR spermatozoa in the fraction that transversed the cumulus mass. Youssef *et al.* (1996) also found that cumulus cells increase the percentage of AR spermatozoa. This increase is also explained for Group 3 (PICSI®), as HA is a known component of the cumulus matrix, and although it showed a significantly lower increase compared to Groups 1 and 2 ($p = 0.003$), it still displays a stimulatory nature.

The influence of HA has been seen to induce spermatozoa motility and vigour, as shown by Hong *et al.* (2009), but these findings did note that it depended on the concentration of HA used. These findings could be of clinical significance since AR spermatozoa are more likely to be injected (using ICSI) into the oocyte and this is a natural progression in the fertilization cascade. Another factor to be considered is that Kato and Nagan (2012) showed that polyvinylpyrrolidone (PVP) also induces the AR, leading to enhanced pronuclear formation; PVP was used in all three groups. The manufacturers of the PICSI® dish do not advocate the use of PVP during spermatozoa manipulation of the spermatozoa; however this step was included in this study so as to exclude PVP as a variable from a statistical point of view. The PVP could however have an effect on the outcome although diminished blastocyst rate was not noted.

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Chromatin packaging quality has been related to fertilization outcomes (Sakkas *et al.* 2002) with a decreased fertilization rate or fertilization failure during IVF/ICSI as well as an increased risk of pregnancy loss (Zini *et al.* 2008). Kennedy *et al.* (2011) also recorded a significant positive correlation between chromatin structure and spontaneous abortion rates as well as multiple pregnancy rates. A significant increase in the CMA_3 values pre and post preparation in all three techniques was highlighted with the best results recorded in the PICSI® group (Table 2), especially when baseline values were low. One needs to remember that the CMA_3 stain only tests for the protamination of the spermatozoon chromatin and not the density thereof (as would be seen when using sperm chromatin structure assay (SCSA)). HA is normally present within the cumulus matrix and it plays a role in the selection of physiologically normal spermatozoa (Parmegiani *et al.* 2010) although this effect was not noteworthy in the cumulus group when compared to the other groups. The process of normal spermatozoa maturation includes plasma membrane remodelling and the formation of HA binding sites. There are several studies including Nijs *et al.* (2009) (and Ghosh *et al.* 2007; Prinosilova *et al.* 2009) that showed that with improved morphology, there was improved HA binding therefore leading to better sperm-egg binding. Nijs *et al*. (2009) could not confirm the findings of Huszar *et al*. (2003) that a mature spermatozoon has completed histone-protamine replacement at the completion of spermatogenesis.

Immature spermatozoa are devoid of these binding sites and therefore do not bind to the PICSI® dish (Huszar *et al.* 2004, Parmegiani *et al.* 2010). Immature spermatozoa have been known to have higher retention of CK which in turn increases lipid peroxidation and subsequently results in DNA fragmentation (Huszar *et al.* 2007; Parmegiani *et al.* 2010). It is well known that mature spermatozoa can be initiated in the active processes of apoptosis and subsequently fail to result in viable embryos or pregnancies (Vinatier *et al.* 1996). The most reported characteristics of apoptosis are DNA double-stranded breaks which are

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formed by the activation of endogenous nucleases (Gorczyca *et al.* 1993) and have been shown to be caused by ROS from both spermatozoa in the ejaculate and leukocytes within the seminal fluid.

The rate of DNA damage (as measured by the AO test) was seen to significantly decrease in all three groups. Studies by Sakkas *et al.* (1996) showed that spermatozoa with chromatin and/or DNA damage could influence spermatozoa decondensation after ICSI and thus affect embryo quality. This was confirmed but a multitude of later studies including those done by Zini *et al*. (2001) and Evenson *et al*. (2002). Virant-Klun *et al.* (2002) also showed reduced embryo quality following ICSI, where spermatozoa with single-stranded DNA was used. It would also be very difficult to determine the extent to which the oocyte exerts its propensity to repair DNA strand breaks before the commencing the first cleavage (Troya et al. 2015).

In this study there was no significant difference in embryo quality, pregnancy, implantation and miscarriage rates in any of the three groups. There was, however, a significant difference in the fertilization rates ($p = 0.0183$) but this parameter did not translate in improved pregnancy rates. The embryo degeneration rate, however, was not measured and this could possibly have shown some difference although this can only be confirmed in subsequent studies.

Previous researchers (Nasr-Esfahani *et al.* 2008; Menezo *et al.* 2010; Parmegiani *et al.* 2010; Van Den Bergh *et al.* 2009) have showed similar improved fertilization as well as cleavage rates when HA-bound spermatozoa was used in ICSI. This was contradicted by work by Castilo-Baso *et al.* (2011), who reported improved embryo quality but only when the baseline morphology was less than4%. Choe *et al.* (2012) also reported no difference

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in fertilization and cleavage rates when treating sibling oocytes comparing conventional ICSI to HA-bound ICSI. This could indicate that PICSI® is only favourable when morphology is very low (less than 3%) although this study failed to confirm this, since there was no difference in the pregnancy rates. The research did however show that when CMA_3 values were low (less than 40%, which is abnormal), the biggest improvement was seen in the selection of spermatozoa with normal chromatin packaging (Table 2) although this was not reflected in the pregnancy outcome. Troya *et al*. (2015) did however, showed an improved pregnancy rate using PICSI when compared to ICSI. This same group showed an even higher pregnancy rate using MACS for spermatozoa selection and also a reduced miscarriage rate. (Troya *et al*. 2015). Nijs *et al*. (2009), in agreement with this study, could not confirm the results in terms of pregnancy rates.

The selection of spermatozoa using the PICSI® dish can be advocated as it is relatively inexpensive to implement compared to IMSI which requires expensive instrumentation and high levels of skill (Said *et al.* 2011). The PICSI dish also requires less equipment and reagents than compared to the MACS procedure.

4.2. Conclusion

Failure in the ART arena is multi-factorial in nature, but the selection of spermatozoa with proper chromatin condensation and a lower aneuploidy rate ensures a better clinical outcome (Huszar *et al*. 1994; Jakab *et al.* 2005; Worrilow *et al.* 2009).

The advantages of any of the three techniques used in this study, are limited, as clinical outcome could not be confirmed when compared. A multi-centred prospective study could shed more light in this field, especially in terms of the cumulus model and the PICSI® dish for spermatozoa selection.

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Terminology

Deviations from normal semen variables lead to this international nomenclature. As different values have been allocated to them in different laboratories it is very important that, when used, 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%)

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Equipment Required

Microscope:	phase contrast and bright field (10 x, 40X phase objectives and 100x bright field), with a heated stage, immersion oil, ICSI micro-manipulation arms
Microscope	fluorescence microscope with a filter set consisting of an excitation filter BP 450-490, a chromatic beam splitter FT510, and a barrier filter LP520
Microscope slides:	standard size
PICSI Dish	commercially manufactured dish
Coverslips:	(22 mm x 22 mm, No 1½ [thickness]; 24 x 24 mm; No 1½)
Counting Chamber	Neubauer counting chamber
Plastic Pasteur Pipettes:	air displacement for making wet preparation and vitality smears
Pipette tips:	plastic, for air displacement pipettes, volume 5-50 μL
Glass Pasteur Pipettes	air displacement for oocyte and embryo manipulation
Centrifuge:	1000-3000 g
Plastic gloves:	for laboratory use
Test tube rack	for laboratory use
Incubators	Nu Air incubator, with 5% CO_2 and 96% humidity,

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Frequently used Abbreviations

AR:	Acrosome reaction/ acrosome reacted
Ca^{2+}:	Calcium
cAMP:	Cyclic adenosine monophosphate
CMA_3:	Chromomycin A_3
CTC:	Chlorotetracycline test
AO	Acridine orange
COC:	Cumulus oophorus complex
ZP:	Zona pellucida.
HSA:	Human serum albumin
ART	assisted reproductive technology / -ies
ICSI:	Intracytoplasmic sperm injection
IVF:	*In vitro* fertilization
IMSI	Intracytoplasmic morphologically selected sperm injection
PICSI®	A dish that uses hyaluronan droplets to bind normal sperm
HA	Hyaluronic acid
MACS	Magnetic cell sorting
HspA2	Heat shock protein
CK	Creatine kinase
ROS	Reactive oxygen species
HABP-1	Hyaluronan-binding protein 1
E_2	Oestrogen
Ca^{2+}-ATPase pumps	A membrane transporter protein

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Addendum 1: Sample size Calculations

Chromatin Packaging

Assumptions

Group1	Group2	Group3
Mean=45	58	60
SD=6.5		

When the sample size in each of the 3 groups is 6, a one-way analysis of variance will have 90% power to detect at the 0.050 level a difference in means characterized by a Variance of means of 44.222, assuming that the common standard deviation is 6.500.

Total sample size required =18

A sample size of 37 in each group will have 90% power to detect a difference in means of -5.000 (the difference between a Group 1 meanof 55.000 and a Group 2 meanof 60.000) assuming that the common standard deviation is 6.500 using a two group t-test with a 0.050 two-sided significance level.

Morphology

Comparison using anova

Group1	Group2	Group3
7	8	9
SD=2		

When the sample size in each of the 3 groups is 27, a one-way analysis of variance will have 90% power to detect at the 0.050 level a difference in means characterized by a Variance of means of 0.667, assuming that the common standard deviation is 2.000.

Total sample size required=81

A sample size of 86 in each group will have 90% power to detect a difference in means of -1.000 (the difference between a Group 1 mean of 8.000 and a Group 2 mean of 9.000) assuming that the common standard deviation is 2.000 using a two group t-test with a 0.050 two-sided significance level.

Again for comparison between groups 2 and 3 morphology drives the sample size

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Acrosome reaction

Comparison using anova

Group1	Group2	Group3
14	28	34
SD=6		

When the sample size in each of the 3 groups is 5, a one-way analysis of variance will have 90% power to detect at the 0.050 level a difference in means characterized by a Variance of means of 70.222, assuming that the common standard deviation is 7.000.

Total sample size required=15

The sample size requirement for the semen parameters is driven by morphology. The small sample size requirements for two of the parameters is due to the difference expected between group 1 and groups 2 and 3.

For comparisons between group 2 and 3 the sample size requirement will be larger for chromatin packing and acrosome reaction

A sample size of 23 in each group will have 90% power to detect a difference in means of -6.000 (the difference between a Group 1 mean of 28.000 and a Group 2 mean of 34.000) assuming that the common standard deviation is 6.000 using a two group t-test with a 0.050 two-sided significance level.

Summary

60 women per group will give you a reasonable chance to detect most differences between the three groups.