A comparison of the effect of Polyvinylpyrrolidone (PVP) and SpermSlow™ on human spermatozoa

By Marlize Nel

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Medical Sciences (Reproductive Biology) in the Faculty of Medicine and Health Sciences, University of Stellenbosch.

Supervisor: Dr Marie-Lena Windt De Beer

March 2015

DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2015

ABSTRACT

Intracytoplasmic sperm injection (ICSI), as well as other micromanipulation assisted reproductive technology methods, such as physiologic ICSI (PICSI) and intracytoplasmic morphologically selected sperm injection (IMSI), are routinely used in many fertility laboratories around the world. An integral part of these methods is the manipulation of spermatozoa in preparation of the injection into the oocyte. It is common practice to place prepared spermatozoa in a viscous holding medium to facilitate the handling, manipulation and slowdown of spermatozoon movement during the immobilization and injection processes of ICSI. The possible effect of these holding mediums on basic semen parameters, as well as the sperm deoxyribonucleic acid (DNA) and structural integrity of spermatozoa, is of importance.

Hamilton Thorne IVOS® developed an automated software solution for live sperm morphology evaluation under high magnification, called IMSI Strict™. It combines Tygerberg Strict Criteria morphological classification of human spermatozoa with motile sperm organelle morphology examination (MSOME) and provides software-based categorization. The IMSI Strict™ software was developed to aid in the IMSI spermatozoon selection process that enables objective classification of spermatozoa to remove inter-technician variation. For good optics and spermatozoon evaluation in IMSI Strict™, spermatozoa need to be moving very slowly or be immotile, but still viable. This can be achieved by placing spermatozoa in a viscous holding medium, either polyvinylpyrrolidone (PVP) or SpermSlow™, sometimes for a substantial time period. Before marketing the clinical use of IMSI Strict™, the possible toxicity or deleterious effect of PVP and SpermSlow™ on spermatozoa needs to be excluded.

The primary objective of this study was to evaluate the effect of PVP and SpermSlow™ on human spermatozoa after different exposure times using a viability stain, CASA motility and kinetic parameters, chromatin packaging analysis (CMA_3 staining analysis) and DNA fragmentation

analysis (TUNEL analysis). The secondary objective was to evaluate the effect of PVP and SpermSlow™ on human spermatozoa's ultrastructure with Transmission Electron Microscopy.

This prospective analytical study was conducted at Drs Aevitas Fertility Clinic (Vincent Pallotti Hospital, Cape Town, South Africa) as well as the Fertility Unit at Tygerberg Hospital (Cape Town, South Africa) between July 2013 and October 2014. A total of 90 separate (no duplication) semen samples were analysed for the quantitative analysis (primary objective) and 1 sample for the descriptive analysis (secondary objective).

Results showed that although PVP and SpermSlow™ treated sperm outcomes often differed significantly after typical statistical analysis, clinically these two mediums were shown to be equivalent (using a specific statistical test for equivalence) for the tested outcomes. PVP and SpermSlow™ had no detrimental effect clinically on sperm viability, motility parameters, chromatin packaging and DNA fragmentation rate. The secondary investigation indicated that SpermSlow™ might exert a disintegrating effect on various sperm membranes, and as a secondary consequence of the eventual necrotic process, alteration of chromatin and cytoskeletal components. PVP medium on the other hand did not show these disintegrating effects. This finding needs to be further investigated since only one semen sample was evaluated.

Based on this study's results, either PVP or SpermSlow™ can be used for IMSI Strict™ purposes. However, the study did not include the technical aspects of the usage of PVP and SpermSlow™.

OPSOMMING

Intrasitoplasmiese sperm inspuiting (ICSI), sowel as ander mikro-manipulasie voortplantings tegnieke, soos fisiologiese ICSI (PICSI) en intrasitoplasmiese morfologies geselekteerde sperm inspuiting (IMSI), word in baie fertiliteitsklinieke regoor die wêreld gebruik. 'n Integrale deel van hierdie metodes is die manipulasie van spermatosoa ter voorbereiding van die inspuitproses. Dit is algemeen om voorbereide spermatosoa in 'n viskose medium te plaas om die hantering, manipulasie en vertraging van spermatosoön beweging tydens die immobilisasie en inspuitproses van ICSI te fasiliteer. Die effek van hierdie mediums op basiese semenparameters, sowel as die sperm deoksiribonukleïensuur (DNS) en strukturele integriteit van spermatosoa, is van belang.

Hamilton Thorne IVOS® het 'n sagteware oplossing, IMSI Strict™, vir lewende sperm morfologie evaluering onder hoë vergroting ontwikkel. Hierdie sagteware bied sagteware-gebaseerde morfologiese klassifikasie deur die Tygerberg streng kriteria morfologiese klassifikasie met beweeglike spermorganel morfologie ondersoek (MSOME) te kombineer. Die IMSI Strict™ sagteware is ontwikkel om die objektiewe klassifikasie van spermatosoa vir IMSI spermatosoön seleksie moontlik te maak. Spermatosoa moet baie stadig beweeg of immotiel, maar steeds lewensvatbaar wees om goeie optika en spermatosoön evaluering vir IMSI Strict™ te verseker. Dit sal bereik kan word deur spermatosoa in 'n viskose medium, hetsy PVP (“polyvinylpyrrolidone”) of SpermSlow™, vir 'n aansienlike tydperk te inkubeer. Voordat IMSI Strict™ vir kliniese gebruik bemark kan word moet die moontlike toksisiteit of nadelige effek van PVP en SpermSlow™ op spermatosoa uitgesluit word.

Die primêre doel van hierdie studie was om die effek van PVP en SpermSlow™ op menslike spermatosoa na verskillende inkubasie tye te evalueer deur ’n lewensvatbaarheid kleuring toets, twee sperm DNS toetse (CMA_3 en TUNEL) en rekenaar geëvalueerde sperm beweeglikheid toetse te gebruik. Die sekondêre doel was om die effek van PVP en SpermSlow™ op menslike spermatosoa se ultrastruktuur deur middel van Transmissie Elektronmikroskopie te evalueer.

Hierdie studie is by Drs Aevitas Fertiliteitskliniek (Vincent Pallotti Hospitaal, Kaapstad, Suid-Afrika) sowel as die Fertiliteitseenheid by Tygerberg Hospitaal (Kaapstad, Suid-Afrika) tussen Julie 2013 en Oktober 2014 uitgevoer. 'n Totaal van 90 semenmonsters vir die kwantitatiewe analise (primêre doel) en een vir die beskrywende analise (sekondêre doel) is ontleed.

Resultate het getoon dat alhoewel PVP en SpermSlow™ geïnkubeerde spermuitkomste dikwels na 'n tipiese statistiese analise betekenisvol verskil, hierdie twee mediums vir die geëvalueerde uitkomste klinies ekwivalent (bepaal deur middel van spesifieke statistiese toetse vir ekwivalensie) is. Die mediums het ook nie klinies 'n nadelige effek op sperm lewensvatbaarheid, beweeglikheid parameters, chromatien verpakking en DNS fragmentasie koers getoon nie. Die sekondêre ondersoek het getoon dat SpermSlow™ hoofsaaklik 'n effek van disintegrasie op verskeie spermmembrane getoon het. Hierdie nekrotiese proses kan lei tot verandering van chromatien en sitoskelet komponente. PVP medium het egter nie dieselfde disintegrerende effek getoon nie. Hierdie bevinding moet egter verder ondersoek word, aangesien slegs een semenmonster geëvalueer is.

Alhoewel hierdie studie nie die tegniese aspekte van die gebruik van PVP en SpermSlow™ geëvalueer het nie, kan aanbeveel word dat óf PVP óf SpermSlow™ op grond van geëvalueerde uitkomste tydens die IMSI Strict™ sperm seleksie proses gebruik word.

ACKNOWLEDGEMENTS

It is a great privilege to convey my most sincere gratitude and appreciation to the following people who have each made a tremendous contribution to the completion of this thesis:

Firstly, I would like to thank **Dr ML Windt-De Beer** for her endless support, active and expert guidance in her role as my promoter and her willingness and helpfulness at all times.

A very special word of thanks to **Prof TF Kruger** for the wonderful opportunity I have been afforded to undertake my postgraduate studies and to be part of the Department of Obstetrics and Gynaecology at the University of Stellenbosch.

Thank you to **Evelyn Erasmus** for her support and advice on writing this thesis.

I would like to thank **ORIGIO®**for sponsoring the SpermSlow™ medium used in this study. Thank you also to **Drs Aevitas Fertility Clinic**and the **Harry Crossley foundation** for the financial contribution for other experimental materials.

My sincere appreciation also goes to the personnel of the Vincent Pallotti Fertility Clinic,**GregTinney-Crook, Nicole Lans, Marlene Levin and Cherree Thwaits**, for their assistance during the collection of experimental material for this study.

Thank you to**Nolan Muller**for the TEM evaluations and **Prof M Sousa** who was willing to analyses the TEM micrographs.

I also wish to thank **Prof C Lombard** for analyzing the statistical data of this study.

To **Estee van Zyl and Emma Wilson Poe**, for their continuous support throughout the two years.

I would like to thank the following foundations and scholarships for the financial assistance:
National Research Foundation(NRF) Scarce Skills Scholarshipand **Merck Serono Scholarship**.

Words alone are not enough to express my gratitude and appreciation towards my mother and sister, **Marietjie and Joline Nel,** for their love and encouragement.

Lastly, I convey my sincere gratitude towards my **Heavenly Father**, through Whom everything is possible.

DEDICATION

This thesis is dedicated to my father and the greatest influence on my life, the late Theuns Nel.

His memory will forever be a comfort and a blessing.

TABLE OF CONTENTS

ABBREVIATIONS

ALH	Amplitude of lateral head displacement
AOT	Acridine orange test
ART	Assisted reproduction technology
ATP	Adenosine triphosphate
BCF	Beat cross frequency
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted sperm analysis
CMA_3	Chromomycin A_3
COC	Cumulus-oocyte complex
COMET	Single-cell gel electrophoresis assay
DBD-FISH	DNA breakage detection-fluorescent *in situ* hybridization assay
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
dUDP	Deoxyuridine triphosphate
FDA	Food and drug administration
HA	Hyaluronic acid
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
ICSI	Intracytoplasmic sperm injection
IMSI	Intracytoplasmic morphologically selected sperm injection
ISNT	*In situ* nick translation

IUI	Intra-uterine insemination
IVF	*In vitro* fertilization
LIN	Linearity
MACS	Magnetic activated cell sorting
MSOME	Motile-sperm organelle morphology examination
PGD	Preimplantation genetic diagnosis
PICSI	Physiologic ICSI
PLCζ	Phospholipase C zeta
PVP	Polyvinylpyrrolidone
PVS	Perivitelline space
PZD	Partial zona dissection
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCSA	Flow cytometric-based sperm chromatin structure assay
SNDF	Sperm nucleus decondensing factor
STR	Straightness
SUZI	Subzonal insemination
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TUNEL	Terminal deoxynucleotidyl transferase-mediated fluorescein-deoxyuridine triphosphate nick-end labelling
VAP	Average path velocity

VCL	Curvilinear velocity speed
VSL	Straight line velocity
WHO	World Health Organization
ZP	Zona pellucida

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Background Information and Literature Review

Intracytoplasmic sperm injection (ICSI), as well as other micromanipulation assisted reproductive technology (ART) methods, such as physiologic ICSI (PICSI) and intracytoplasmic morphologically selected sperm injection (IMSI), are routinely used in many fertility laboratories around the world. An integral part of these methods is the manipulation of spermatozoa in preparation of the injection into the oocyte. It is common practice to place prepared spermatozoa in a viscous holding medium to facilitate the handling, manipulation and slowdown of spermatozoon movement during the immobilization and injection processes of ICSI. The possible effect of these holding mediums on basic semen parameters, as well as the sperm deoxyribonucleic acid (DNA) and structural integrity of spermatozoa, is of importance.

1.1 History and development of ICSI

The development and successful introduction of ICSI into clinical practise was a breakthrough in ART and has revolutionized the treatment of male factor infertility (Tarlatzis and Bili, 1998). This micromanipulation technique entails the mechanical insertion of a single selected spermatozoon directly into the cytoplasm of a mature oocyte after ovarian superovulation and oocyte retrieval.

Initially this technique was used in veterinary medicine to investigate the different steps during fertilization. The first procedure where a spermatozoon was injected into an oocyte was done by Hiramoto (1962) in the sea urchin. In 1966, Lin reported the first mammalian oocyte injection procedure where mouse oocytes were microinjected. Ten years later, Uehara and Yanagimachi (1976) described the microinjection of human and hamster spermatozoa into hamster oocytes. Despite the use of fine micropipettes, only a limited number of the injected oocytes survived this

technical procedure (Thadani, 1981). However, in 1988, Hosoi *et al.* obtained live offspring after the transfer of microinjected rabbit oocytes.

It became apparent that the results of conventional IVF were much less efficient when the semen characteristics of the male partner were well below the reference values (Devroey and Van Steirteghem, 2004). By the end of the 1980's several procedures of ART were developed to overcome these problems. Since fertilization of normal animal gametes and live offspring after microinjection techniques were described, scientists experimentally applied microinjection techniques to human gametes. PZD (partial zona dissection) was one of the techniques developed in an attempt to improve fertilization and pregnancy rates with male factor infertility patients (Cohen *et al.*, 1988). This technique allowed the spermatozoon to have direct access to the oocyte's oolemma by means of a small opening that was made in the zona pellucida (see Figure 1.1). The results obtained from PZD were generally inconsistent and disappointing. It was thought that the PZD process reduced the protection of the oocyte which the intact zona pellucida offered against waste substances and oxygen radicals produced by the non-motile and defective spermatozoa.

Another micromanipulation technique called SUZI (subzonal insemination) was developed where a few motile spermatozoa were microinjected through the zona pellucida into the perivitelline space (Ng *et al.*, 1988). However, the overall experience with PZD and SUZI was that the percentage normal fertilization as well as pregnancy rates was too low to consider it for routine clinical application. Although ICSI was first applied to human gametes in 1988, Palermo *et al.* (1992) was the first to publish human pregnancies and deliveries generated after ICSI. Since then the use of the ICSI procedure has resulted in fertilization and pregnancy rates comparable to those obtained in patients with normal semen parameters undergoing conventional IVF (Palermo *et al.*, 1995a).

ICSI quickly became the favoured technique for cases of male-factor infertility, as it was discovered that basic semen parameters (low sperm count or motility) had little impact on its success (Nagy *et al.*, 1995). ICSI also enabled scientists to achieve high levels of fertilization in the presence of

multiple morphological and dysfunctional sperm defects. ICSI started to be used for cryopreserved-thawed sperm as well as sperm extracted from the testis (Devroey and Van Steirteghem, 2004). Today, ICSI indications have expanded beyond just male-factor infertility and include multiple failed IVF cycles, mixed factor infertility, poor fertilization for unknown reasons, patients that require preimplantation genetic diagnosis (PGD) and patients using vitrified oocytes for a IVF cycle (Sauer and Chang, 2002; Palermo *et al.*, 2009; De Mouzon *et al.*, 2010).

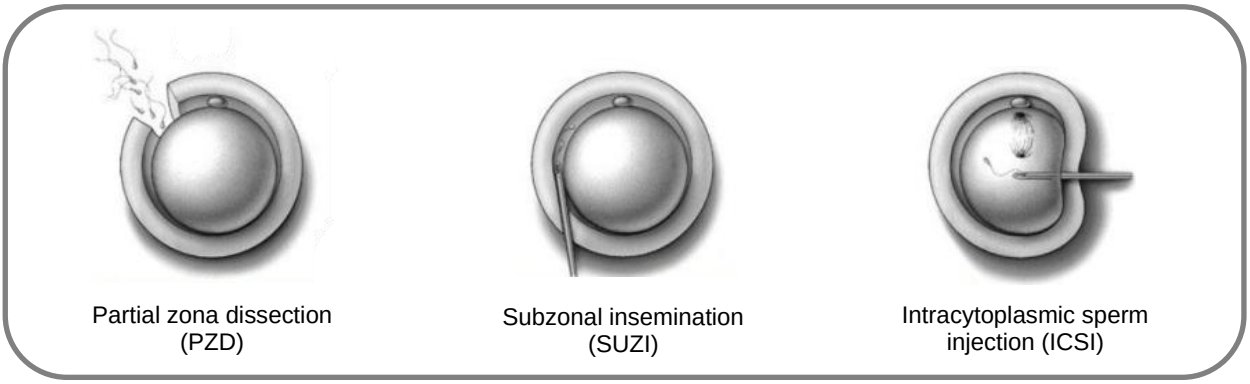


Figure 1.1 Illustration of the differences between PZD, SUZI and ICSI techniques. (Available at: http://www.sotiriskoukos.gr/content.php?content_id=54&lg=)

1.2 Differences involved during natural and ICSI fertilization processes

1.2.1 Natural fertilization process

The fertilization processes involved during natural conception and IVF are complex and still researched today. In order for a morphologically normal and mature spermatozoon to fertilize a mature oocyte, the spermatozoon needs to undergo capacitation. This process refers to the ability of a spermatozoon to initiate the fertilization process including the physiological acrosome reaction. In the human, spermatozoa capacitate *in vivo* upon exposure to the female reproductive tract secretions, but capacitation of spermatozoa can be induced *in vitro* in the presence of various

synthetic media during semen preparation techniques (Fenichel *et al.*, 1995). Although the spermatozoon does not portray any visual signs of capacitation, it involves the post ejaculatory mobilization and/or removal of certain spermatozoon plasma membrane surface compounds, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor and acrosin inhibitor. These changes in the distribution of intramembranous particles result in areas over the acrosome that become free of these particles during capacitation (Tesarik, 1984). Other intracellular changes during capacitation include increases in membrane fluidity, protein tyrosine phosphorylation, and cAMP (cyclic adenosine monophosphate) concentrations. It also includes decreases in cholesterol/phospholipid ratio of the plasma membrane and net surface charge, as well as changes in spermatozoon swimming patterns. These alterations to the membrane phospholipids initiate modest rises in intracellular Ca^{2+} and Na^{+} ions prior to the acrosome reaction.

When a spermatozoon reaches the cumulus-oocyte complex (COC), it penetrates the cumulus mass, binds to and penetrates the zona pellucida (ZP) and fuses with the oocyte oolemma in order to fertilize the oocyte. The acrosome that caps the spermatozoon's head contains enzymes required for this penetration and fusion processes. Some of the enzymes include hyaluronidase, acrosin, proacrosin, phosphatase arylsulfatase, collagenase, phospholipase C and β-galactosidase (Dale, 1996). Hyaluronidase is required to digest the hyaluronic acid (HA) matrix that forms the cumulus cloud around the oocyte. *In vitro*, the hyaluronidase enzyme from spermatozoa is also released from the acrosomes of dying or dead spermatozoa that will cause the digestion of the whole cumulus mass around the oocyte.

Before or during the cumulus penetration process, the spermatozoon becomes hyperactivated. The spermatozoon's tail movement frequency increases and its movement pattern changes from two-dimensional to three-dimensional (Cleine, 1996). These changes in movement assist the spermatozoon to penetrate the cumulus. The HA present in the cumulus matrix has a vital function in selecting only mature sperm which ultimately binds and penetrates the ZP and fertilize the oocyte during *in vivo* or *in vitro* fertilization (Parmegiani *et al.*, 2010b).

The human ZP consists of three glycoproteins; ZP1, ZP2 and ZP3. These glycoproteins, together with protein-bound progesterone from the cumulus mass, form a complex in the outer layer of the ZP. Some have postulated a three stage model for sperm-zona pellucida interaction; the first comprises primary binding of acrosome-intact spermatozoa to the ZP, followed by secondary binding of the acrosome-reacted spermatozoa to the zona pellucida and finally penetration of the acrosome-reacted sperm through the zona pellucida into the perivitelline space (Saling *et al.*, 1979; Swenson and Dunbar, 1982; Redgrove *et al.*, 2012). Spermatozoa contains a large number of ZP receptor candidates that accounts for the sequence of both low and high affinity interactions (Thaler and Cardullo, 2002). When the spermatozoon reaches and attaches to the ZP, this protein complex of the ZP has an inducing effect on the acrosome reaction. For the spermatozoon to be able to attach, the acrosome should still be intact and the spermatozoon should have a normal morphology. A morphologically abnormal spermatozoon will not bind to the ZP due to its shape or a resulting defect in its receptor area. The morphologically normal spermatozoon's head binds loosely to the ZP and the acrosome reaction is initiated (Kopf *et al.*, 1991).

During the acrosome reaction the contents of the acrosome are released by exocytosis. A Ca^{2+} influx causes the spermatozoon head's lipid membrane to change and more receptors to be exposed. This will cause the spermatozoon to bind more tightly to the ZP proteins (Cleine, 1996). The Ca^{2+} influx also induce the fusion between the phospholipids of the outer acrosomal membrane and the overlying plasma membrane. These multiple fusion areas form pores through which the hydrolytic enzymes from the acrosome are released. These enzymes (mostly acrosin) digest the ZP, allowing the spermatozoon to approach the oocyte for fertilization (Takano *et al*., 1993). Finally the acrosome cap falls away and only the equatorial segment remains. Only spermatozoa that are able to undergo the acrosome reaction when bound to the ZP can penetrate the zona by enzyme digestion from the acrosome and vigorous hyperactivated motility (Cleine, 1996).

After the acrosome-reacted sperm reaches the perivitelline space (PVS), it fuses with the oocyte oolemma. Spermatozoa that contain the appropriate oolemma fusion receptors will be anchored by firm binding between the inner acrosomal membrane and the microvilli. Here too it is important for the spermatozoon to have a normal shaped head, so that the receptors can make good contact with the oocyte. The binding between the oocyte oolemma and the equatorial segment of the spermatozoon's head begins, after which fusion starts (Cleine, 1996; Wassarman *et al.*, 2005).

Once fusion occurs, the spermatozoon is incorporated into the ooplasm and the acrosome membrane and tail are disrupted from the zygote. Oocyte-activating proteins, such as phospholipase C zeta (PLCζ), are released into the oocyte's cytosol by the spermatozoon which triggers calcium oscillations (Saunders *et al.*, 2002). The intracellular calcium rise will start within a few minutes of spermatozoa fusion and is largely due to the release of calcium from intracellular stores within the endoplasmic reticulum (Wakai and Fissore, 2013). This increase in oocyte cytoplasmic Ca^{2+} levels activates the oocyte. The oocyte extrudes cortical granules into the PVS (Sathananthan and Trounson, 1982) to prevent any secondary spermatozoa from transit further in the ZP and into the PVS. This process, known as the cortical reaction, prevents polyspermy as well as protects and supports the embryo.

After the spermatozoon and oocyte fuses, the envelope around the spermatozoon nucleus disintegrates. The nucleus decondenses to a haploid set of chromosomes and a nuclear membrane around the chromosomes developes. This forms the male pronucleus. The oocyte extrudes the second polar body, also undergo chromatin decondensation and form the female pronucleus (Sathananthan *et al.*, 1993). Both pronuclei migrate to the centre if the oocyte until syngamy takes place. The microtubules of the spermatozoon aster play a role in this migration process. This organelle grows out into a star shape from the centrosome from the mid-piece of the spermatozoon. The centrosome and the male pronucleus are driven by the growing sperm aster from the cell cortex towards the centre of the oocyte where syngamy will take place (Barroso and Oehninger, 2007).

1.2.2 Fertilization process during ICSI

With ICSI, a single spermatozoon is injected directly into a denuded oocyte's cytoplasm. This method bypasses the penetration through the cumulus mass, binding to the ZP, penetration through the ZP and fusion with the oocyte's oolemma. By bypassing these spermatozoon-oocyte interaction processes, spermatozoa are not naturally selected before spermatozoon-oocyte fusion (Sathananthan *et al.*, 1989). Motile spermatozoa with normal morphology have an increased chance of fertilization and embryo development (Sathananthan and Trounson, 1999) and one of the drawbacks in ICSI is the inability to select a morphologically normal, viable spermatozoon with absolute certainty.

Since ICSI bypasses so many penetration and binding processes through the cumulus mass, ZP and oolemma, the acrosome may be seen as unnecessary. However, Katayama *et al.* (2002) found that the delay of sperm chromatin decondensation is associated with that of acrosome disassembly. They also found that acrosomes appear to disintegrate in the oocyte ooplasm whether or not the acrosome reaction has taken place. As previously discussed, the acrosome reaction occurs spontaneously during incubation in a defined medium and is also time dependent (Schill *et al.*, 1988). Based on these observations, Mansour *et al.* (2008) examined the impact of spermatozoa preincubation time and spontaneous acrosome reaction in ICSI and found that the optimum incubation time of spermatozoa before ICSI was three hours, which resulted in a higher fertilization rate. However, the only statistically significant difference between the different incubation period groups was the rate of acrosomal reacted spermatozoa. This rate was the highest after five hours of incubation and lowest after one hour of incubation. They stated that it seems to be physiologically correct to use acrosome-reacted spermatozoa for ICSI to avoid irregular sperm chromatin decondensation that adversely affect fertilization.

The same activation processes of the oocyte (during IVF or natural conception) occur when spermatozoa are microinjected into oocytes during ICSI (Homa and Swann, 1994; Tesarik *et al.*,

1994). During IVF the initial calcium increase starts within a few minutes of spermatozoa fusion, whereas following ICSI this trigger is provoked immediately during injection by the artificial calcium influx from the surrounding injection medium (Tesarik *et al.*, 2000).

Since ICSI is an invasive fertilization technique that bypasses multiple steps of the natural sperm selection and fertilization process by introducing spermatozoa into the ooplasm, it is important to consider the immediate safety of ICSI as well as any possible long term implications. According to Wong and Ledger (2013), the potential concerns regarding ICSI offspring relate to four general areas; transmission of genetic anomalies, imprinting disorders, congenital malformations and developmental abnormalities. In 2013, Davies *et al.* presented data from the largest registry to date, comparing 6163 births from ART. Their finding showed that IVF and ICSI is associated with a significant increase in risk of birth defects, but that this risk persists only after the use of ICSI, not IVF, after adjustment for parental and other factors. This finding can be explained by one of two ways; either the ICSI procedure itself is dangerous by causing structural damage during the procedure or there may be some underlying factors associated with the couples chosen for ICSI.

Wong and Ledger (2013) however reasoned that there are several explanations for why the results of this study recorded such high risks of birth defects in comparison to similar studies in the past. The authors did not separate twin from singleton pregnancies, single from double embryo transfers and included cerebral palsy as a birth defect. A meta-analysis done in 2012 by Wen *et al.* is only one of the many studies that differ from the findings of Davies. In this analysis the outcome of 46 studies covering 125 000 IVF and IVF/ICSI babies demonstrated a small increased risk of congenital abnormalities with IVF and ICSI babies compared to natural conception, but no difference in the risk between IVF and ICSI conceptions.

1.3 Different factors that can influence ICSI outcome

The success rate of ICSI is thought to be influenced by a number of factors, some which include oocyte quality (Loutradis *et al.*, 1999), sperm quality (Nagy *et al.*, 1998), ICSI technique as well as the injection needles being used (Svalander *et al.*, 1995).

1.3.1 Sperm count

Nagy *et al.* (1998) found that only semen with an extremely low sperm count (no spermatozoa seen in initial semen sample; cryptozoospermia) showed a significant lower fertilization rate during ICSI than semen with a sperm count of >5 million per milliliter. Strassburger *et al.* (2000) also reported a significant decrease in fertilization and pregnancy rate in cryptozoospermic ICSI cycles when compared to the control group (sperm concentration of $>1x10^5$ and $<10x10^6$/ml).

1.3.2 Sperm motility and viability

During natural conception or IVF, spermatozoa need to be motile and be hyperactive to penetrate the oocyte's cumulus as well as the zona pellucida. Men without any motile spermatozoa are infertile without ICSI (Aitken *et al.*, 1983). Motility might be a critical factor when selecting spermatozoa for ICSI, considering clinical results by Nagy *et al.* (1995). Shen *et al.* (2003) also found that one of the important laboratory variables that affected the fertilization rate during ICSI was sperm motility. However, sometimes spermatozoa have to be selected for ICSI from a complete immotile sperm population (absolute asthenozoospermia). For this particular group of patients, non-harmful methods to identify live/viable spermatozoa suitable for ICSI were pursued, since sperm vitality is a prerequisite for successful ICSI (Nagy *et al.*, 1995). These include hypo-

osmotic swelling test, mechanical touch technique, exposure to Pentoxifylline, laser-assisted sperm selection and birefringence-polarization microscopy (Ortega *et al.*, 2011)**.**

The sperm centrosomal material contains centrioles and centrosomal components onto which oocyte centrosomal proteins assemble after sperm incorporation to form the sperm aster that is essential for uniting sperm and oocyte pronuclei (Schatten and Sun, 2009). This organelle contributes to the assembly of microtubule within the penetrated oocyte, which contributes to the formation of the first mitotic spindles during the initial fertilization phase and initiates embryonic cell division. Sathananthan *et al.* (1996a; 1997) reported that fertilization with spermatozoa with abnormal or damaged centrioles could lead to embryos with cleavage arrest or irregular cleavage patterns, since embryos' centriolar apparatus and mitotic potential are paternally derived. Unfortunately these defects are not visible in spermatozoa, but it is believed that immotile spermatozoa as well as poorly motile spermatozoa with no forward progression are more likely to have these defective centrosomes (Sathananthan, 1996b).

Since it is recognized that the microscopic method for assessing motility is subjective to large errors, many researchers use commercial computer systems that provides objective assessment of sperm motility and kinetic characteristics (time-varying geometric aspects of motion; Drobnis *et al*., 1988; Mortimer, 2000). With a computer assisted semen analysis (CASA) system, spermatozoa are recognized based on the size and brightness of the head. The computer will take consecutive video recordings and evaluate spermatozoa's difference in position in several consecutive frames.

Kinetics of sperm motility such as average sperm velocities, curvilinear velocity speed (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), percentage straightness (STR) and percentage linearity (LIN) are some of the characteristics CASA systems can evaluate (Addendum I). Some of these characteristics have been significantly correlated with fertilization rates *in vitro*(Mortimer, 1989), but there are no consistent findings and it is still not clear which characteristics of sperm movement are

useful for predicting fertility (Hirano *et al.*, 2001). However, various studies have used some of these motion parameters to analyse spermatozoon hyperactivation. Mortimer *et al.* (1990) defined hyperactivated spermatozoa by the following criteria: ALH ≥5.0µm, LIN ≤60% and VCL ≥100µm/s.

1.3.3 Sperm morphology

The importance of sperm morphology in IVF was first described by Kruger *et al.* (1986) and today it is seen as one of the most important semen parameters of male infertility assessment that correlates with natural *in vivo* fertilization, *in vitro* fertilization as well as pregnancy outcome (Kruger *et al.*, 1996; Coetzee *et al.*, 1998; Avendaño *et al.*, 2009). Menkveld and colleagues developed the Tygerberg Strict Criteria morphological classification of human spermatozoa (Kruger *et al.*, 1986; Menkveld *et al.*, 1990; Kruger *et al.*, 1996) which is still internationally used today. Three prognostic categories with the Strict Criteria was described by Kruger *et al.* (1996) and include the poor-prognosis or p-pattern group (1-4% morphologically normal spermatozoa), good-prognosis or g-pattern group (5-14% morphologically normal spermatozoa) and normal-prognosis or n-pattern group (>14% morphologically normal spermatozoa).

Previous studies reported positive predictive values for IVF success rates using the 5% and 15% sperm morphology thresholds as described by the Tygerberg Strict Criteria. They observed a significant decrease in pregnancy rates in the p-pattern morphology group (Coetzee *et al.*, 1998; Van der Merwe *et al.*, 2005). On the other hand, Nagy *et al.* (1995; 1998) showed that the outcome of ICSI is not related to strict morphology of the sperm used for microinjection. They reported that the fertilization rate obtained with semen samples in the p-pattern morphology group do not differ from samples with better overall sperm morphology. This observation can be explained by the fact that the embryologist selects a motile, morphologically normal-looking spermatozoon for injection within the limits of the microscope used for micromanipulation (x400 magnification). Mansour *et*

al.(1995) stated that as long as morphologically well-shaped viable spermatozoa are used for injection, fertilization and pregnancy rates after ICSI are not affected.

However, the overall appearance of a sperm sample does not always reflect the quality of the spermatozoon injected. De Vos *et al.* (2003) evaluated the influence of individual spermatozoon morphology on the outcome of ICSI in terms of fertilization, embryo development on day two and implantation rate after embryo transfer. They found that individual sperm morphology assessed at the moment of ICSI correlated well with fertilization outcome, but did not affect embryo development up until day two cleavage stage. However, previous studies found that male factor infertility affect blastocyst formation and quality and decrease the ability of ICSI-derived embryos to develop to blastocyst compared with IVF-derived embryos (Jones *et al.*, 1998; Shoukir *et al.*, 1998; Loutradi *et al.*, 2006). Furthermore, De Vos *et al.*(2003) also found that the implantation rate was significantly lower when only embryos resulting from injection of abnormal spermatozoa were available. They speculated that when the morphology of ICSI semen samples are evaluated, many spermatozoa are classified as abnormal on the basis of morphologic details that might not interfere with their fertilizing capacity, since they will be directly introduced into the oocyte and bypass the zona pellucida and oolemma barriers.

1.3.4 Sperm DNA

Sperm chromatin abnormalities as a cause of male infertility have been studied extensively in the past two decades. Evidence indicates that a negative correlation exists between disturbances in the organization of the genomic material in sperm nuclei and the fertility potential of spermatozoa, whether *in vivo* or *in vitro*. This emphasizes that stable DNA is one of the criteria needed for a spermatozoon to be considered as fertile (Amann *et al.,* 1989*;* Sun *et al.*, 1997; Spano *et al.*, 2000).

A poor quality sperm chromatin structure may be an indication of male subfertility, regardless of the number, motility or morphology of spermatozoa. Various studies reported that sperm DNA damage is useful as a predictor of treatment success, as suggested by its association at numerous points in the reproductive process, including impaired fertilization, disrupted pre-implantation embryo development, miscarriage and birth defects in the offspring (Simon *et al.*, 2011; Zini, 2011; Bungum *et al.*, 2012). Thus, sperm chromatin structure evaluation can provide good diagnostic and prognostic capabilities and may be considered as a reliable predictor of a couple's ability to conceive (Evenson *et al.*, 1999; Aitken, 1999).

1.3.4.1 Human sperm chromatin structure

The function of a spermatozoon is to successfully transport and deliver the paternal chromosomes to the oocyte during reproduction (Sousa *et al.*, 2009). The spermatozoon contains a haploid set of DNA that is situated in the nucleus in the post acrosomal part of the sperm head. The DNA molecules consist of many nucleotides that are involved in carrying the paternal genetic information, which includes the sex-determining X or Y chromosome (Hoogendijk *et al.*, 2007).

The status of a spermatozoon's nucleus depends on two events during spermiogenesis; the final nuclear shape acquisition and the replacement of histones with protamines (P1 and P2) for the formation of compact and stable chromatin. This organized, condensed and compacted chromatin packaging almost occupies the entire volume of the small sperm nucleus and ensures that the DNA transferred to the oocyte is delivered in a physical and chemical form that allows the developing embryo to access the genetic information (Poccia, 1986). The condensation of sperm DNA is also important for the repression of gene expression during spermiogenesis and to protect the paternal genetic message, during transport through the male and female reproductive tracts, making it inaccessible to nucleases or mutagens (Oliva, 2006).

The organization of the chromatin for packaging in the spermatozoon takes place at four different levels: 1) chromosomal anchoring where the DNA attaches to the nuclear annulus; 2) DNA loop domain formation as the DNA attaches to the newly added nuclear matrix; 3) histone replacement with protamines which condense the DNA structure and 4) chromosomal positioning (Ward and Coffey, 1991).

1.3.4.2 Origin of sperm DNA damage

Sperm DNA abnormalities can be induced during any of its developmental, storage or transport stages (Fernández *et al.*, 2008; Sakkas and Alvarez, 2010) and can be triggered by internal or external factors. Abnormal genomic material (internal factor) may cause abnormalities such as DNA compaction, nuclear maturity defects, DNA strand breaks, DNA integrity anomalies or sperm chromosomal aneuploidies (Shafik *et al.*, 2006). In 2009, Varghese *et al.* concluded that the amount of spermatozoa with damaged DNA is predominantly higher in the ejaculate of men with poor quality semen. Their study supported the results from Shafik *et al.* (2006) who stated that anomalies within the sperm DNA may be associated with abnormal semen parameters.

The aetiology of sperm DNA damage is probably multi-factorial, but the three main theories by which DNA damage arise in human spermatozoa have been proposed as defective sperm chromatin packaging, oxidative stress and abortive apoptosis (Schulte *et al.*, 2010).

a) Incomplete chromatin packaging

There are a few events associated with spermiogenesis that can cause genetic instability in mature spermatozoa (Leduc *et al.*, 2008). Spermatozoa with an altered protamine 1 and 2 (P1/P2) ratio are normally more susceptible to stressors and have been correlated with sperm DNA

fragmentation (Garcia-Peiró *et al.*, 2011). However, the cause of the deregulation of protamine expression in male infertility remains unclear.

Histone-protamine replacement, epididymal maturation and chromatin stability during ejaculation are events where sperm chromatin abnormalities can occur. Chromatin protein modification or absence can lead to abnormal chromatin packaging and influence the quality of spermatozoa and fertilization potential (Kazerooni *et al.*, 2009).

When spermatozoa with improper chromatin packaging are exposed to stressors such as extreme temperatures or frequent pH changes, the DNA can fragment and lead to single strand chromatin (Varghese *et al.*, 2009).

b) Oxidative stress

Oxidative stress seems to have a critical influence on male reproduction. In fact, Aitken and De Iuliis (2010) reported that the most common cause of DNA fragmentation in spermatozoa is oxidative stress. It is caused by excessive reactive oxygen species (ROS) which include highly oxidative radicals, such as hydroxyl radicals (OH^-), and nonradical species, such as hydrogen peroxide (H_2O_2; Aitken and Bennetts, 2007). Both ROS species are normal by-products of metabolism (Valko *et al.*, 2007) and are required in low concentrations for many cellular processes. In spermatozoa, ROS plays an important role in a number of essential functions, i.e. chromatin compaction in maturing spermatozoa during epididymal transit (Wright *et al.*, 2014), capacitation (Doshi *et al.*, 2012), hyperactivation (Nassar *et al.*, 1999), binding of the spermatozoon to the zona pellucida (Liu *et al.*, 2006) and acrosome reaction (Dona *et al.*, 2011). This explains why spermatozoa produce ROS themselves. These ROS are relatively harmless to spermatozoa under normal concentrations and conditions where antioxidant mechanisms help maintain the balance required for ROS-related functions (Sharma and Agarwal, 1996).

Cellular components such as proteins, lipids, RNA (ribonucleic acid) and DNA molecules are chemically modified by excess ROS and causes normal cell function impairment. Spermatozoa are particularly vulnerable to ROS for three reasons: (i) their plasma membrane is largely composed of polyunsaturated fatty acids that renders it highly susceptible to oxidative and other chemical modifications; (ii) they have inherent deficiencies in intracellular antioxidant enzyme protection; and (iii) they have a limited capacity for DNA damage detection and repair, unlike most cell types (Lewis *et al.*, 2013).

ROS is produced by morphologically abnormal, immature or dead spermatozoa as well as seminal leukocytes and can damage sperm DNA by inducing double- and single-stranded DNA breaks (Thomson *et al.*, 2011) that has been linked to male infertility (Laberge and Boissonneault, 2004). Clinical studies done by Moskovtsev *et al.* (2007) demonstrated a weak correlation between leukocyte concentration and sperm DNA damage, suggesting that leukocyte-derived ROS are not as important as those derived from spermatozoa. ROS can also cause DNA damage such as chromatin cross-linking, chromosome deletion, mutations and base oxidation (Agarwal *et al.*, 2003).

Spermatozoa are especially vulnerable for damage caused by oxidative stress, since they have no defence mechanism, apart from the characteristic tight packaging of the DNA and anti-oxidants present in the seminal plasma. DNA repair is also limited in spermatozoa and only occurs during specific stages of spermiogenesis. Spermatozoa are exposed to oxidative damage in the epididymis and during the transport in seminal fluid. However, its repair mechanism is no longer activated during nuclear condensation in the epididymis (Leduc *et al.*, 2008). The next opportunity for DNA damage repair is by the oocyte. Not all DNA damage may be repairable by the oocyte and can have a significant impact on fertilization, embryo quality and clinical pregnancy outcome after IVF and ICSI (Lewis and Simon, 2010; Gonzalez-Marin *et al.*, 2012).

c) Abortive apoptotic DNA degradation

Apoptosis is more commonly described as programmed cell death and has been associated with sperm chromatin condensation and DNA fragmentation (Jégou *et al.*, 2002). This event is induced by specialized Sertoli cells, within the seminiferous tubules of the testes, which will prevent the overproduction of germ cells and selectively destroy injured germ cells. These germ cells are assigned with apoptotic markers and are supposed to be phagocytised and be removed by the Sertoli cells (Sakkas and Alvarez, 2010). However, some damaged germ cells may escape the apoptotic pathway, known as abortive apoptosis, enter spermiogenesis and end up in the ejaculate (Sakkas *et al.*, 2002).

Apoptotic DNA degradation in spermatozoa may be caused during internal (*in vivo*) or external (*in vitro*) conditions. Some of these conditions include hormonal depletion, irradiation, toxic agents, chemicals, heat exposure, the presence of leukocytes, the production of free radicals during migration, inflammation, infection, ROS and antioxidant depletion (Barroso and Oehninger, 2007; Bronet *et al.*, 2012).

d) External factors

Apart from the above mentioned three main theories that can cause DNA damage in human spermatozoa, external factors may also affect sperm DNA integrity. These include lifestyle factors, such as diet (Leduc *et al.*, 2008), alcohol (Varshini *et al.*, 2012) and caffeine consumption (Schmid *et al.*, 2006), cigarette smoking (Valavanidis *et al.*, 2009), antibiotics (Wright *et al.*, 2014), air pollution (Rubes *et al.*, 2005) and hyperthermia that can lead to excessive ROS production (Mieusset *et al.*, 1987; Ollero *et al.*, 2001). Other conditions that are increasingly linked to sperm DNA abnormalities include obesity (Dupont *et al.*, 2013), cancer (Shafik *et al.*, 2006), genital tract

inflammation, semen infections, hormonal disorders (American Society of Reproductive Medicine, 2008; Sakkas and Alvarez, 2010) and ageing (Schmid *et al.*, 2006).

1.3.4.3 Evaluation of sperm nuclear DNA damage

There are different assays available to evaluate sperm DNA abnormalities, immaturity or damage. Some of these assays include:

a) simple staining techniques such as the acidic aniline blue (AB) and toluidine blue (TB) stains,

b) fluorescent staining techniques such as the sperm chromatin dispersion (SCD) test, chromomycin A_3 (CMA_3), DNA breakage detection-fluorescent *in situ* hybridization assay (DBD-FISH), *in situ* nick translation (ISNT), flow cytometric-based sperm chromatin structure assay (SCSA), acridine orange test (AOT) together with terminal deoxynucleotidyl transferase-mediated fluorescein-deoxyuridine triphosphate nick-end labelling (TUNEL) assay, and lastly high-performance liquid chromatography (HPLC),

c) an electrophoretic technique - the single-cell gel electrophoresis (COMET) assay (Henkel, 2007a, Muratori *et al.*, 2010).

Despite extensive research for the successful implementation of sperm DNA tests into routine practice, a universal agreement on the ultimate technique for the accurate evaluation of human sperm DNA integrity has not yet been reached (Cohen *et al.*, 2004; Shafik *et al.*, 2006; Muratori *et al.*, 2010).However, of all the methods currently available, the COMET assay is considered the most sensitive (Villani *et al.*, 2010). Not only does it detect single- and double-strand breaks as well as abasic sites (DNA sites with neither a purine nor a pyrimidine base), but it is the only test that quantifies DNA fragmentation in an individual spermatozoon.

Simon *et al.* (2014) reported that the development of novel diagnostic approaches that will allow non-invasive evaluation of individual sperm with minimal DNA damage could be a potentially valuable tool to improve ART success.

a) Chromomycin A_3 assay

CMA_3 is a guanine-cytosine-specific fluorochrome that competes with protamines for binding to the minor groove of the DNA helix. CMA_3 fluorescence has been interpreted as an indirect demonstration of decreased presence of protamine leading to poor protamination (Lolis *et al.*, 1996). It reveals chromatin that is poorly packaged in human spermatozoa by detecting protamine-deficiency in loosely packed chromatin. CMA_3 stains the post acrosomal part of the sperm head. Spermatozoa with immature chromatin packaging will be fluorescent yellow after staining. A low protamination state will therefore be indicated by high levels of CMA_3 fluorescence. Dull or no fluorescent stain indicates mature chromatin packaging. (Esterhuizen *et al.*, 2000; Agarwal and Said, 2004).

As a discriminator of IVF success (>50% oocytes fertilized), CMA_3 staining has a sensitivity of 73% and specificity of 75%. Therefore, it can distinguish between IVF success and failure (Esterhuizen *et al.*, 2000). In 1996, Sakkas *et al.* reported that spermatozoa from patients with high CMA_3 fluorescence and a higher level of endogenous DNA nicks are not limited in their ability to achieve fertilization using ICSI when compared to patients exhibiting low levels of these two parameters However, in 1998 Sakkas *et al.* concluded that patients with poor chromatin packaging (>30% CMA_3 fluorescence), had more than double the number of unfertilized oocytes during ICSI. They also concluded that DNA fragmentation may be linked to an increase in early embryo death. In 2009, Kazerooni *et al.* also showed that the number of CMA_3 positive spermatozoa is significantly higher in patients with spontaneous recurrent abortions.

The CMA_3 assay is reliable as it strongly correlates with other assays used to evaluate sperm chromatin (Manicardi *et al.*, 1995). It is however important to remember that this assay is limited by observer subjectivity.

b) Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay

The TUNEL assay was first developed by Ausubel *et al.* in 1992 and Gorczyca *et al.* (1993b) was the first to apply this method to spermatozoa. This assay determines the existing DNA damage of spermatozoa and is based on the ligation of labelled dUTP-nucleotides to the 3'-OH phosphate ends of single- as well as double-stranded DNA breaks (Nakamura *et al.*, 1995). This reaction is catalysed by the template-independent enzyme, terminal deoxynucleotidyl transferase (TdT). By using labelled dUTP-nucleotides, DNA fragmentation in spermatozoa can be identified by using bright field microscopy, fluorescent microscopy as well as flow cytometry (Gorczyca *et al.*, 1993a; Muratori *et al.*, 2010). Spermatozoa with normal DNA have only background staining, while those with fragmented DNA (multiple chromatin 3' –OH ends) stain brightly (Gorczyca *et al.*, 1993a).

The TUNEL assay provides useful information in many cases of male infertility. Researchers found a negative correlation between the DNA fragmented sperm percentage and the motility, morphology and concentration in the ejaculate (Lopes *et al.*, 1998; Henkel, 2007b; Aitken and De Iuliis, 2007). This assay appears to be potentially useful as a predictor for Intra-uterine insemination (IUI) pregnancy rate, IVF embryo cleavage rate and ICSI fertilization rate. It also provides an explanation for recurrent pregnancy loss (Sun *et al.*, 1997; Duran *et al.*, 2002; Carrell *et al.*, 2003; Muratori *et al.*, 2003; Benchaib *et al.*, 2003). Avendaño *et al.* (2009) was the first to simultaneously examine individual spermatozoa for normal morphology and DNA fragmentation and demonstrated that infertile men can present DNA fragmentation in the morphologically normal sperm population assessed by strict criteria. Furthermore, the evaluation of DNA integrity in morphologically normal spermatozoa after sperm selection was proposed to be a better approach

to evaluate the impact of sperm DNA fragmentation on ICSI outcome than the assessment of the total sperm population (Avendaño and Oehninger, 2011).

Although the reliable thresholds for TUNEL require further conformation, a working threshold of 15-20% is considered high and correlates with reduced fertility potential (Sharma *et al.*, 2010). However, the threshold value commonly used is that from a study by Evenson *et al.* (1999) where they concluded that >30% DNA fragmentation was a threshold not considered compatible with fertility. Although the flow cytometric method is thought to be generally more accurate and reliable, it is more expensive and complex. This method may also detect DNA of cells other than spermatozoa. The epi-fluorescent TUNEL assay has demonstrated good quality control parameters with an intra-observer variability of less than 8% and an inter-observer variability of less than 7% (Barroso *et al.*, 2000). When using this method, DNA fragmentation of other cells will not be included in your analysis, as one will only count spermatozoa.

1.3.4.4 The role of sperm DNA in fertilization and ART outcomes

a) Diagnosis of male infertility

There is a consensus based on numerous publications that male partners in infertile couples have a higher level of sperm DNA fragmentation compared with those in the general population and proven fertile males (Lewis *et al.*, 2013). Studies suggest a negative impact on both natural (Evenson *et al.*, 1999; Spano *et al.*, 2000) and ART conception (Larson *et al.*, 2000). A significant DNA fragmentation level can be present in normozoospermic subfertile men, but it also coexists with reduced sperm count, increased abnormal morphology (Virro *et al.*, 2004; Chi *et al.*, 2011), and reduced motility (Lin *et al.*, 2008). However, like most scientific research, results are controversial. A recent publication by the Practice Committee of the American Society for

Reproductive Medicine (2013) reported that recent methods for assessing sperm DNA integrity do not reliably predict treatment outcomes and cannot be recommended routinely for clinical use.

Excessive ROS does not only damage sperm nuclear DNA, but can also damage mitochondrial DNA. Since male mitochondrial DNA is degraded by the oocyte, leaving only maternal inheritance of mitochondrial DNA, these sperm mitochondrial DNA mutations do not impact the health of the offspring (Sutovsky *et al.*, 2004). However, it can limit ATP (adenosine triphosphate) production and energy provision for motility in spermatozoa, reducing fertility (Shamsi *et al.*, 2008a).

Even if a spermatozoon appears morphologically normal at high-power magnification, fertilization of a mature oocyte is still not guaranteed. This can be explained in that the fertilization potential of human spermatozoa also depends on its chromatin condensation ando DNA integrity. Sperm chromatin condensation does not play a direct role in the shaping of the sperm head. However, during the binding of protamine to DNA the DNA molecules are enabled to be condensed resulting in a more hydrodynamic sperm head and thus contribute indirectly to the shape of the head. It has been reported that sperm containing poor chromatin packaging frequently have enlarged or abnormal head shapes (Balhorn, 2007). Furthermore, associations between sperm chromosomal abnormalities and sperm morphology and head defects (amorphous, elongated or round heads and the presence of large nuclear vacuoles) have been reported (Chemes *et al.*, 2007; Cassuto *et al.*, 2009; Varghese *et al.*, 2009). Cohen-Bacrie *et al.* (2009) also reported a positive correlation between sperm DNA fragmentation and sperm morphological abnormalities that included broken necks, abnormal necks and curled tails. Studies that were conducted to test the relationship between sperm morphology and IVF rates showed a positive correlation for normal morphology and IVF rates. A highly negative correlation was shown between percentage normal sperm morphology and poor chromatin packaging (Esterhuizen *et al.*, 2000). However, some studies have reported that spermatozoa with normal morphology could have chromosomal aberrations and that sperm dimensions or shape are not reliable attributes in selection of haploid sperm for ICSI (Burrello *et al.*, 2004; Celik-Ozenci *et al.*, 2004).

b) Assisted reproductive technology (ART)

The selection of spermatozoa with abnormal DNA during ART procedures may influence the genetic quality of the embryo (Sakkas and Alvarez, 2010). This can lead to impaired implantation and poor embryogenesis (Shafik *et al.*, 2006; Lazaros *et al.*, 2011) that may have an impact on a couple's fertility potential and ART outcome (Sakkas *et al.*, 1995; Shafik *et al.*, 2006). Abnormal DNA has also been associated with high abortion incidences and disease in offspring (Lewis and Agbaje, 2008; Thomson *et al.*, 2011). However, despite a significant amount of research associating sperm DNA damage with ART outcomes, the results remain controversial. These discrepancies suggest that the type of assay used to measure sperm DNA damage may influence the detection of associations with ART outcomes (Simon *et al.*, 2014).

According to the sperm chromatin structure assay (SCSA) threshold for fertility, a DNA fragmentation index (DFI) higher than 30% is statistically significant and is associated with poor fertilization rates in IUI, IVF and ICSI (Lopes *et al*., 1998; Fernández *et al.*, 2003; Apedaile *et al.*, 2004; Fernández *et al.*, 2005; Oleszczuk *et al.*, 2011). The DFI describes the number of spermatozoa with fragmented DNA expressed as a percentage of the total sample. As early as 1998 Sakkas *et al.* found that patients with poor chromatin packaging (>30% CMA_3 fluorescence), had more than double the number of unfertilized oocytes after ICSI. They also concluded that DNA fragmentation may be linked to an increase in early embryo death. However, a meta-analysis done by Li *et al.* (2006) found no relationship between sperm DNA damage (using SCSA or TUNEL) and fertilization rates during IVF or ICSI. According to them this finding was not completely unexpected since the paternal genome is not expressed until the four- to eight-cell stage, thus fertilization may not be dependent on sperm DNA integrity. This late paternal effect has been mainly attributed to abnormalities in the organization of the sperm chromatin, such as reduced chromatin condensation, chromosome anomalies and increased DNA strand breaks or fragmentation (Tesarik *et al*., 2004; Wiener-Megnazi *et al*., 2012).

Studies have also shown that sperm DNA abnormalities may influence the embryo at various levels, including nuclear, cytoskeletal and at the organelle level, thus influencing the embryo's morphology and development (Thomson *et al.*, 2011). The DNA integrity of human spermatozoa contributes significantly to embryonic growth and fetal health (Kumar *et al.*, 2012). Some scientists believe that embryonic failure may occur as a result of DNA repair failure in the oocyte. It has been stated that when spermatozoaonly show slight DNA damage, the oocyte might have the capability to repair this nuclear damage (Meseguer *et al.*, 2011). The extend of the repair will however depend on the quality of the oocyte as well as the severity of the sperm DNA damage. If the DNA damage is too severe, the human embryo's natural protection mechanisms and pathways will prevent further embryonic development (Sakkas and Alvarez, 2010). However, some lesions may be repaired incorrectly or remain impaired. This can lead to fertilization failure, poor embryo quality, non-viable embryos or pregnancy loss (Shafik *et al.*, 2006; Avendaño *et al.*, 2010; Balasuriya *et al.*, 2011).

There is controversy as to the influence of sperm DNA damage on pregnancy rates with IVF and ICSI. Various studies reported a significant reduction of pregnancy rates following IVF or ICSI for patients with increased levels of sperm DNA damage (Henkel *et al.*, 2003; Henkel *et al.*, 2004; Virro *et al.*, 2004; Frydman *et al.*, 2008), while other contradicting studies reported no significant differences in pregnancy rates after IVF or ICSI between patients with high or low levels of sperm DNA damage (Huang *et al.*, 2005; Zini *et al.*, 2005; Benchaib *et al.*, 2007). A meta-analysis by Zini and Sigma (2009) who analysed nine IVF and eleven ICSI studies showed a modest increased pregnancy chance after IVF in cases when the proportion of DNA damaged spermatozoa was below the threshold values for SCSA or TUNEL. Lewis *et al.* (2013) reported that sperm DNA damage has not been found to be predictive for ICSI treatment (Zini, 2011), except for one study (Bungum *et al.*, 2007) who reported no significant difference in IVF and ICSI pregnancy outcomes between low and high DFI groups. When there were high levels of sperm DNA damage (DFI >30%), they reported pregnancy rates with ICSI were significantly higher than IVF. However, this

was not a randomised control study. Therefore, the impact of factors contributing to the choice of treatment cannot be excluded.

Lewis *et al.* (2013) reported four hypotheses that may explain the finding that poor sperm DNA does not appear to impact ICSI outcomes adversely. Firstly, in ICSI treatment cycles, contrary to women in IVF cycles where the fertility problem often resides with the female partner, up to 30% of women with subfertile partners have no detectable fertility problems. Their oocytes may have more capacity to repair DNA damage even if the injected spermatozoon is of poor quality. Secondly, during ICSI, spermatozoa are injected into the optimal environment of the oocyte's ooplasm within a few hours after ejaculation. This may protect the spermatozoon from laboratory-induced damage, since it has been found that even the birthweight of IVF babies can be markedly influenced by minor differences in culture conditions (Dumoulin *et al.*, 2010). Thirdly, since it has been reported that spermatozoa from up to 40% of infertile men have high levels of ROS (Aitken *et al.*, 2012), oocytes can be exposed to ROS released from the 0.5 million spermatozoa overnight during IVF. This may impair the oocyte's functional ability, including its capacity to repair sperm DNA fragmentation post fertilization. Finally, it has been reported that embryos with high sperm DNA damage levels are associated with early pregnancy loss (Zini *et al.*, 2008). Therefore, ICSI success rates are sometimes adversely affected by sperm DNA damage, but at a later stage. In fact, Robinson *et al.* (2012) reported that high levels of sperm DNA damage are associated with increased risk of pregnancy loss regardless of the *in vitro* technique applied.

Despite the large number of controversial studies, more studies should be done to limit the degree of uncertainty on these issues and to establish robust clinical test systems.

1.3.4.5 Treatment modalities for high levels of sperm DNA damage

Pons and colleagues (2013) reported that sperm DNA fragmentation can be decreased by reducing the days of sexual abstinence to only one abstinence day. They suggested that reducing

the time of abstinence can be a simple, low-cost and non-invasive technique to reduce DFI in comparison with strategies like antioxidants, antibiotics, testicular biopsy, magnetic activated cell sorting (MACS), hyaluronic acid binding (physiologic ICSI) or intracytoplasmic morphologically selected sperm injection (IMSI). However, it is known that when the abstinence time is reduced, the volume, concentration and motility will diminish (Pons *et al.*, 2013).

a) Antioxidants and antibiotics

Some studies have reported improved sperm DNA integrity with the use of oral antioxidants (Geva *et al.*, 1998; Comhaire *et al.*, 2000; Greco *et al.*, 2005a), but few of these studies reported on the effect of pregnancy rates. Before one can reach any definite conclusion on the clinical usefulness of antioxidants in treatment of male infertility, large, prospective, randomized control studies will be necessary (Schulte *et al.*, 2010). More information is also required about which antioxidants are more successful and which patients will benefit from its use. Furthermore, antibiotics are effective in reducing the DFI when it is raised due to an infection (Gallegos *et al.*, 2008). Reducing the abstinence period may be a co-treatment, since the contact time between spermatozoa and leucocytes will be reduced (Pons *et al.*, 2013).

b) Testicular biopsy

Greco and colleagues (2005b) reported a statistical significant increase in pregnancy (44.4% vs. 5.6%) and implantation rate (20.7% vs 1.8%) with the use of testicular spermatozoa compared to ejaculated spermatozoa in males with increased DFI. However, this technique is invasive and it also provides immature spermatozoa for ICSI which have been suggested have an inferior ability to produce normal zygotes (Palermo *et al.*, 1995b).

c) Magnetic activated cell sorting (MACS)

Magnetic activated cell sorting (MACS) allows the reduction of both apoptotic sperm and DFI (Said *et al.*, 2008; Rawe *et al.*, 2010). This magnetic separation technique is not yet routinely used, since it requires expensive equipment and its secondary effect on spermatozoa is still unknown. Another limitation is that MACS separate apoptotic sperm cells, but fragmented spermatozoa that have not yet started apoptosis may be present. These cells would not be retained by the column and would still be available for the use in ART.

d) Physiologic ICSI

During *in vivo* fertilization hyaluronic acid (HA), secreted by the cumulus cells, plays a vital role in the selection of mature spermatozoa that will penetrate the zona pellucida and fertilize the oocyte. Parmegiani *et al.* (2010a) reported that the use of HA to select mature spermatozoa appears to be another way of treating patients with a high DFI. However, this technique can only be applied in ICSI cases. Two ready-to-use sperm-HA binding selection systems that have received the CE mark, indicating their conformity with health and safety requirement in the European Economic Area, as well as FDA (Food and Drug Administration) clearance, are currently available. One being the PICSI® Sperm Selection Device (MidAtlantic Devices, Origio, Harrilabs, South Africa) and the other being SpermSlow™ (MediCult, Origio, Harrilabs, South Africa). The PICSI® Sperm Selection Device is a plastic culture dish with microdots of HA hydrogel attached to the bottom of the dish, while SpermSlow™ is a viscous medium containing HA.

Mature spermatozoa that bind to HA *in vitro*, are viable spermatozoa having completed the spermiogenetic process of plasma membrane remodelling, cytoplasmic extrusion and nuclear maturation (Huszar *et al.*, 2003). Furthermore, these spermatozoa show low chromosomal aneuploidies and DNA fragmentation, and good nuclear morphology (Jakab *et al.*, 2005). It has been reported that HA sperm selection for ICSI contribute to improved embryo quality, fertilization

rate, implantation rate, pregnancy rate and reduce miscarriage rate (Nasr-Esfahani *et al.*, 2008; Parmegiani *et al.*, 2010a; Worrilow *et al.*, 2010).

e) Intracytoplasmic morphologically selected sperm injection (IMSI)

In 2001, Bartoov *et al.* introduced the motile-sperm organelle morphology examination (MSOME) by which the fine nuclear morphology of unstained motile spermatozoa is examined in real time using an inverted light microscope, equipped with high power optics enhanced by digital imaging, to achieve a total magnification of over 6000x. MSOME assess six sperm organelles, the acrosome, post-acrosomal lumina, neck, tail, mitochondria and nucleus, of which the sperm nucleus seems to be the most important. According to Bartoov *et al.* (2002) the morphological normalcy of the nucleus is analysed in terms of shape (smooth, symmetric and oval) and chromatin content (vacuole occurrence). Although the origin of sperm nuclear vacuoles is still unknown, studies reported that the presence of large vacuoles in motile spermatozoa are related to male subfertility (Mundy *et al.*, 1994), and higher incidence of chromosomal, chromatin packaging and DNA abnormalities (Boitrelle *et al.*, 2011; Perdrix *et al.*, 2011; Wilding *et al.*, 2011; Cassuto *et al.*, 2012). In contrast, several authors suggested that sperm vacuoles should be regarded as normal features of the sperm head (Pedersen, 1969; Watanabe *et al.*, 2011; Tanaka *et al.*, 2012).

The incorporation of MSOME together with a micromanipulation system has introduced a modified ICSI method called intracytoplasmic morphologically selected sperm injection (IMSI). This technique requires a high level of technical expertise and inter-observer reproducibility (Said and Land, 2011). IMSI allows embryologists to identify subtle sperm morphology features, such as abnormal head size, midpiece defects and the presence of vacuoles, and select motile spermatozoa with an ideal morphology for oocyte injection.

Various studies compared ICSI versus IMSI outcomes and found that IMSI significantly improves the percentage of top-quality embryos, implantation and pregnancy rates, and significantly reduced

miscarriage rate when compared with ICSI (Bartoov *et al.*, 2003; Hazout *et al.*, 2006; Antinori *et al.*, 2008; Vanderzwalmen *et al.*, 2008; Wilding *et al.*, 2011). They believed that these findings can be explained by the fact that during conventional ICSI, sperm morphology assessment takes place at only 400x magnification, while IMSI enables sperm selection higher than 6000x magnification. A mock ICSI trial by Wilding and colleagues (2011) determined that 64.8% of spermatozoa selected for conventional ICSI, had morphological abnormalities and would not be selected for IMSI.

The paternal effect phenomenon refers to sperm-derived factors that can influence preimplantation embryo development (Shoukir *et al.*, 1998; Tesarik *et al.*, 2004). This effect may display early at the zygote stage, which is known as the early paternal effect. Studies however also demonstrated that the paternal effect can negatively influence preimplantation embryo development and clinical outcomes in the absence of any detectable impairment in zygote development. This is referred to as the late paternal effect (Tesarik *et al.*, 2004). Sperm vacuoles have been found to be negatively correlated with IMSI outcome (pregnancy, implantation and live-birth rates). This suggests that sperm vacuoles are related to the late paternal effect (Greco *et al.*, 2013).

Recently Cassuto *et al.* (2014) conducted a prospective population-based study to investigate the major malformation rate of children born after ICSI and IMSI. They found a significant lower incidence of major birth defects in children born after IMSI (1.33%) compared with those born from ICSI (3.8%).

However, like most scientific research, results are controversial. Several authors observed no statistical significant difference in any of the outcomes when IMSI and ICSI were compared (Mauri *et al.*, 2010; Balaban *et al.,* 2011; Knez *et al.*, 2011; Oliveira *et al.*, 2011; Setti *et al.*, 2011). It has been proposed that these conflicting results might be due to differences in inclusion criteria, simulation protocols, seminal and oocyte qualities and many other variables within the IVF cycle (Setti *et al.*, 2013). A Cochrane Review done in 2013 included only randomised control trials comparing ICSI and IMSI. It was concluded that the results do not support the clinical use of IMSI

and that further trials are necessary to improve the evidence quality before IMSI can be recommended in clinical practice (Teixeira *et al.*, 2013). Furthermore, De Vos and colleagues (2013) stated that IMSI is a time-consuming procedure at the expense of oocyte ageing and the lack of proof and understanding of its benefit does not justify its routine clinical application at present. Since IMSI's priority over ICSI is still under debate, a literature review concluded that the only confirmed indication for IMSI is recurrent implantation failure following ICSI. All of the other potential indications of IMSI require further investigation (Boitrelle *et al.*, 2014).

1.3.5 ICSI technique

Although the technique of ICSI appears to be simple, it involves the expertise of a trained embryologist, sophisticated instruments, fine glass needles and pipettes, as well as an inverted microscope with a heated stage. Since ICSI is an invasive assisted reproduction technique, the use of good quality micro-tools is important for minimal trauma to the oocyte and optimal results (Clarke *et al.*, 1988). Today ICSI micro needles are supplied by numerous companies and are globally and commercially available.

1.3.5.1 Sperm immobilization

Many scientists have described the importance and necessity of the spermatozoon plasma membrane damage process prior to ICSI (Dozortsev *et al.*, 1995b; Gerris *et al.*, 1995; Van den Bergh *et al.*, 1995; Palermo *et al.*, 1996). The technique causes destabilization and increased permeabilization of the spermatozoon's membrane that induce changes in the acrosome and sperm head plasma membrane (Fishel *et al.*, 1995; Palermo *et al.*, 1996 Takeuchi *et al.*, 2004). It is also believed that this immobilization process induces the release of spermatozoon-associated oocyte activating factors, such as oscillin, into the ooplasm that will lead to the initiation of calcium oscillations and subsequent oocyte activation (Dozortsev *et al.*, 1995b; Parrington *et al.*, 1996;

Palermo *et al.*, 1997). Tesarik *et al.* (1994) alsoreported that the depolymerisation of the spermatozoon's membrane is necessary for sperm nucleus decondensing factors (SNDF), released from the ooplasm, to reach the spermatozoon's nucleus in order to initiate chromatin decondensation and pronuclei formation. The immobilization of the spermatozoon may also prevent the interference of the spermatozoon with the cytoskeleton and metaphase spindle of the oocyte. However, Vanderzwalmen *et al.* (1996) speculated that the process of aspiration of the cytoplasm may be more deleterious for the organelles inside the cytoplasm, than the possible damage that a motile spermatozoon can cause. The immobilized spermatozoon may also be injected into the ooplasm with less medium than required than more motile spermatozoa.

Several immobilization techniques can be used to induce sperm membrane permeabilization. The conventional method consists of striking the tail of the spermatozoon against the bottom of the dish with the micro injection needle until a clear bend in the tail is seen (Palermo *et al.*, 1996). Other more aggressive techniques include: permanently crimping the tail in the mid-piece region (Palermo *et al.*, 1996), cutting the tail below the mid-piece region (Fishel *et al.*, 1995), between the head and the tip of the tail (Gerris *et al.*, 1995), or at the tip of the tail (Chen *et al.*, 1996) and also the application of lasers (Debrock *et al.*, 2003) or piezo-pulses (Yanagida *et al.*, 1999) to sperm tails. Higher post ICSI fertilization rates after more aggressive mechanical (Fishel *et al.*, 1995; van den Bergh *et al.*, 1995) or piezo-pulse induced (Yanagida *et al.*, 1999) sperm immobilization compared to the conventional method have been reported. However, a more recent randomised control trial by Velaers *et al.* (2012) compared the triple touch with the single touch sperm immobilization technique and found that the triple touch technique had no advantages compared to the single touch technique. They speculated that the amount of damage needed to immobilize a motile spermatozoon is not dependant on the number of strokes, but rather on the region where the spermatozoon is immobilized. Sathananthan (1999) reported that the spermatozoon's neck and proximal midpiece region should be kept intact, since the centrosome is located beneath the basal plate of the spermatozoon's head. The sperm centrosomal material contains centrioles and

centrosomal components onto which oocyte centrosomal proteins assemble after sperm incorporation to form the sperm aster that is essential for uniting sperm and oocyte pronuclei (Schatten and Sun, 2009). This organelle contributes to the assembly of microtubule within the penetrated oocyte, which contributes to the formation of the first mitotic spindles during the initial fertilization phase and initiates embryonic cell division.

1.3.5.2 Oocyte position during microinjection

One of the important technical parameters of ICSI is the awareness of the Metaphase-II spindle location during the injection procedure. It is believed that the meiotic spindle with metaphase II chromosomes is located in the periphery of the ooplasm subjacent to the first polar body (Cohen *et al.*, 1998). Therefore, the polar body marks the approximate spindle location. It became traditional to position the oocyte with the first polar body at the 6 or 12 o'clock position and inject the spermatozoon into the oocyte at the 3 or 9 o'clock position (Lanzendorf *et al.*, 1988; Van Steirteghem *et al.*, 1993) with the opening of the needle away from the polar body position. These orientations stabilized the presumed spindle position farthest from the path of the injection needle, aiming to minimize the potential for spindle damage. In contradiction, studies have found that the first polar body does not accurately predict the location of the metaphase II meiotic spindle (Silva *et al.*, 1999). However, one of the suggested reasons is that the polar body can be displaced from its original extrusion site during the manipulation required for cumulus and corona removal (Rienzi *et al.*, 2003).

1.3.5.3 Oocyte ooplasm aspiration

A technique which dramatically increased success rates, involves the aspiration of the ooplasm just before sperm injection (Van Steirteghem *et al.*, 1993; Van Steirteghem *et al.*, 1994). This causes

the oolemma to rupture and Ca^{2+} to be released, which facilitates and assists oocyte activation (Tesarik and Sousa, 1995; Ebner *et al.*, 2004; Vanden Meerschaut *et al.*, 2014).This technique also ensures that the oocyte is penetrated and that the spermatozoon will be deposited into the ooplasm and not into the perivitelline space.

Cytoplasmic aspiration may be influenced by the oocyte quality and maturity, as well as the type of microtool used (Vanderzwalmen *et al.*, 1996).

1.3.5.4 Head first or tail first sperm injection

Traditionally an immobilized spermatozoon is aspirated into the injection pipette tail first, so that it can be microinjected head first into to ooplasm. The rationale for this may however be questioned since ICSI bypasses the steps involved with normal fertilization for which sperm direction is necessary. A study done by Woodward *et al.* (2008) showed that the direction of sperm microinjection at ICSI appears to have no effect on fertilization or development, provided that the sperm is successfully deposited into the ooplasm without damage to the oocyte. Although studies concluded that sperm head or tail first injection into the ooplasm does not affect human oocyte survival, fertilization or embryo development (Suh *et al.*, 1997; Hiraoka *et al.*, 2014), Woodward*et al.* (2008) discussed that the embryologist may prefer to microinject spermatozoa head first purely for technical reasons, rather than biological considerations. Since the sperm head is larger than the tail and more visible under standard microscopy, the head first injection method may allow for more precise placement of the sperm head at the tip of the injection pipette just before ICSI. This will allow greater precision than the tail first microinjection and may assist the scientist in injecting as little as possible medium into the oocyte during ICSI. Some also believe that by injecting the spermatozoon head first, the head will serve as an anchor and prevent the spermatozoon to be drawn out from the ooplasm with the injection pipette after microinjection (personal communication; Mr GM Tinney-Crook). However, there is a lack of published information with regards to this theory.

1.4 The role of PVP during ICSI

Polyvinylpyrrolidone, also known as PVP, is widely and commonly used in a variety of applications in the fields of medicine, pharmacy, cosmetics and industry (Sanner and Straub, 1985). This synthetic, viscous medium is commercially available from most companies supplying ART media. PVP (7-10%) is routinely used during ICSI to facilitate the handling, manipulation and slowdown of spermatozoon movement during the immobilization process (Gerris and Khan, 1987; Akerlöf *et al.*, 1991). It is also part of density gradients such as Sil-Select®.

Since the introduction of ICSI for human ART, more than five thousand ICSI babies have been born annually in the United Kingdom (NHS Lothian, 2012). This data clearly suggest that the embryo can develop to fetal stages and into a healthy child following the injection of spermatozoa and a small volume of PVP into the oocyte during ICSI. However, various studies have reported adverse effects as a result of its *in vivo* and *in vitro* use. It is thus vital to confirm the safety of PVP application for human ART.

Research has shown that PVP stabilizes the sperm plasma membrane (Dozortsev *et al.*, 1995a), causing a delay in calcium oscillations in the sperm-penetrated oocyte and resulting in a delayed fertilization process. Other studies indicated a prevention of nuclear decondensation (Clarke *et al.*, 1988; Dozortsev *et al.,* 1995b) and DNA lesions (Ray *et al.*, 1995). However, researchers found that PVP reduce bull sperm membrane integrity (De Leeuw *et al.,* 1993) and is toxic when injected into mouse zygotes (Bras *et al.*, 1994). Palermo *et al.* (1995b) also suggested that when injecting oocytes, as little as possible medium should be injected together with the spermatozoon into the ooplasm, since this can lead to swelling of the oocyte and be deleterious as well as reduce the chance of sperm decondensation. Although Motoishi *et al.* (1996) and Saha *et al.* (1996) concluded that PVP is neither detrimental to bovine embryonic development nor to embryo quality, it is believed that since PVP is a large polymer, it probably cannot diffuse out of the oocyte or be

digested by lysosomal enzymes and will remain in the oocyte for a prolonged period. (Jean *et al.*, 2001)

With the use of transmission electron microscopy, Strehler *et al.* (1998)found that the exposure of sperm to PVP cause sub-microscopic changes in the sperm structure. Damage in the sperm nucleus was observed, both in terms of shape and texture of chromatin, which was frequently decondensed. They speculated that the nuclear deterioration was caused by a general breakdown of sperm membranes, and subsequently necrotic processes. Their findings contradicted the theory proposed by Clarke *et al.* (1988) and Dozortsev *et al.* (1995a) that PVP stabilizes the plasma membrane of spermatozoa. It is also believed that the detrimental effects of PVP on spermatozoa are dependent on the length of exposure time (Kato and Nagao, 2009) during the sperm selection and immobilization process of ICSI.

1.5 Alternative PVP free ICSI methods

It is clear that there are some concerns about the safety of the usage of PVP in ART. To eliminate the potential damaging effect of PVP, various techniques have been developed which do not necessitate slowing sperm motility (Harari *et al.*, 1995; Hlinka *et al.*, 1998; Tsai *et al.*, 2000). However, the absence of PVP made the ICSI procedure more laborious and difficult for trained embryologists since fast swimming spermatozoa were difficult to immobilise. Therefore, an alternative medium to PVP has been sought for reducing spermatozoon motility.

Barak *et al.* (1999; 2001) advocated that hyaluronic acid (HA) could be used as a physiological replacement for PVP. HA is a natural readily degradable glycosaminoglycan component of the extracellular matrix of the cumulus oophorus surrounding the oocyte. As previously described, HA seems to play a pivotal role in the physiological sperm selection process, since it only enables mature spermatozoa with a high affinity of HA receptor to bind and eventually penetrate the zona pellucida and fertilize the oocyte. They reported similar fertilization and pregnancy rates after ICSI

with sperm exposed to either HA or PVP. Several authors also have observed that HA-containing products have no negative effect on postinjection zygote development and can be metabolised by the oocyte (Barak *et al.*, 2001; Balaban *et al.*, 2003); unlike what is believed for PVP.

1.5.1 SpermSlow™

Recently ORIGIO® released a natural biodegradable alternative to PVP; SpermSlow™ (Medicult, Origio, Harrilabs, South Africa). This viscous medium is one of the two ready-to-use systems (the other being the PICSI® Sperm Selection Device) specially designed for sperm-HA binding selection that have received the CE mark, indicating their conformity with health and safety requirement in the European Economic Area, as well as FDA clearance. A prospective randomized control trial compared these two systems and found that both PICSI®and SpermSlow™ allow comparable clinical efficiency in selecting HA-bound spermatozoa (Parmegiani *et al.*, 2012). The only significant difference observed, was the ICSI procedure duration which was almost three times longer in the PICSI® group. Since the design of the two systems differs, mature HA-bound spermatozoa have a different appearance. PICSI® HA-bound spermatozoa are bound by the head to the bottom of the dish, spin around their heads with vigorous tail motility and can be selected by their degree of motility. SpermSlow™ HA-bound spermatozoa on the other hand, appear to be slowed with stretched tail appearance and narrow beat amplitudes. HA-unbound spermatozoa are less slowed down by the viscous medium and swim around with wider tail-beat amplitude. Specific training is needed to recognize this motility pattern (Parmegiani *et al.*, 2012). SpermSlow™ is more versatile than PICSI® in that it can be used on a glass-bottom culture dish for high-magnification microscopy. This procedure would then be called physiologic IMSI (Parmegiani *et al.*, 2010a).

Since SpermSlow™mainly consists of HA, it is said to be a safer alternative to PVP (Parmegiani *et al.*, 2010a). In SpermSlow™, mature sperm cells' heads bind to the HA via HA receptors and is effectively slowed down and could be visualized for IMSI. Parmegiani *et al.* (2010a) conducted a

study by using SpermSlow™for ICSI. SpermSlow-bound spermatozoa showed a significant twofold reduction in sperm DNA fragmentation rate compared with spermatozoa recovered from PVP. By favouring selection of spermatozoa with normal nucleus and intact DNA, a statistical significant improvement in embryo quality and development were observed when oocytes were injected with SpermSlow™-selected spermatozoa. They also demonstrated that HA may help to select a subpopulation of spermatozoa with normal nuclei, thus speeding up the time-consuming IMSI procedure. A trend towards better fertilization, pregnancy and implantations in the SpermSlow™-ICSI group was observed, however, these findings were not significant. With a large retrospective comparison of SpermSlow™-ICSI versus conventional PVP-ICSI, Parmegiani *et al.* (2010b) confirmed their initial observation. They reported a trend towards better fertilization, pregnancy and abortion rates in the SpermSlow™-ICSI group. Furthermore, injection of SpermSlow™-bound spermatozoa significantly improved embryo quality and implantation rates.

In contrast, some authors found no statistically significant differences in fertilization rates, embryo quality, pregnancy rates or implantation rates when comparing SpermSlow™-ICSI with conventional PVP-ICSI treatments (Van Den Berg *et al.*, 2009; Ménézo *et al.*, 2010). At the very least, in all of the studies HA-sperm injection never caused a detrimental effect on ICSI outcome parameters.

1.6 IMSI Strict™

Hamilton Thorne IVOS® developed an automated software solution for live sperm morphology evaluation under high magnification, called IMSI Strict™. It combines Tygerberg Strict Criteria morphological classification of human spermatozoa (Kruger *et al.*, 1986) with motile sperm organelle morphology examination (MSOME) and provides software-based categorization (IMSI Strict™ brochure, 2013). As discussed previously, IMSI is based on MSOME where the

examination of motile spermatozoa is performed in real time using an inverted light microscope equipped with high power optics enhanced by digital imaging. The IMSI Strict™ software was developed to aid in the IMSI spermatozoon selection process by measuring the size and shape of the spermatozoon head, detecting mid-piece abnormalities and identifying vacuoles on the live sperm sample. This software enables objective classification of spermatozoa that removes inter-technician variation. The analysis does not require manual measurements of spermatozoa and produce immediate results. Currently this software is available for research use only and not used for diagnostic purposes (IMSI Strict™ brochure, 2013).

For good optics and spermatozoon evaluation in IMSI Strict™, spermatozoa need to be moving very slowly or be immotile, but still viable. This will be achieved by either incubating spermatozoa in PVP or SpermSlow™ medium for a substantial time period. Before marketing the clinical use of IMSI Strict™, the possible toxicity or deleterious effect of PVP and SpermSlow™ on spermatozoa needs to be excluded. There are conflicting data regarding the effect of these two mediums on spermatozoon parameters. Salian *et al.* (2012) reported a significant increase in sperm DNA fragmentation when spermatozoa were incubated with PVP, while spermatozoa incubated with SpermSlow™ did not induce DNA damage. In contrast, Rougier and colleagues (2013) reported that spermatozoa incubated with PVP and SpermSlow™ showed similar increases in sperm DNA fragmentation over time. This analytical study evaluated the possible effect of PVP and SpermSlow™ on human spermatozoa.

1.7 Objectives of this study

The primary objective of this study was to evaluate the possible effect of 7% Ready-to-use PVP Solution (SAGE, Cooper Surgical, Harrilabs, Paulshof, South Africa) and SpermSlow™ (Origio, Cooper Surgical, Harrilabs, Paulshof, South Africa) on human spermatozoa after different incubation periods using a viability stain, CASA motility and kinetic parameters, chromatin packaging analysis (CMA_3 staining analysis) and DNA fragmentation analysis (TUNEL analysis).

The secondary objective was to evaluate the possible effect of 7% Ready-to-use PVP Solution (SAGE, Cooper Surgical, Harrilabs, Paulshof, South Africa) and SpermSlow™ (Origio, Cooper Surgical, Harrilabs, Paulshof, South Africa) on human spermatozoa's ultrastructure with Transmission Electron Microscopy (TEM) after one hour incubation period.

1.8 Hypothesis

It was hypothesized that PVP and SpermSlow™ will perform the same (thus be equivalent) in all tested outcomes and will not be detrimental to sperm viability, motility, ultrastructure, chromatin packaging or DNA fragmentation.

CHAPTER 2

Materials and Methods

2.1 Study population and semen sample collection

This prospective analytical study was conducted at Drs Aevitas Fertility Clinic(Vincent Pallotti Hospital, Cape Town, South Africa) as well as the Fertility Unit at Tygerberg Hospital (Cape Town, South Africa) between July 2013 and October 2014. The Health Research Ethics Committee (HREC) of the University of Stellenbosch approved the study protocol (Ethics Reference number: S13/05/114). A total of 90 semen samples without duplication were analysed for the quantitative analysis (Experiment A) and 1 sample for the descriptive analysis (Experiment B). Excess semen from routine semen analysis samples, that would normally be discarded, was used in this study. It must be noted that semen samples used in this study was not used for any ART procedures (ICSI/IVF/IUI). For this reason, the HREC approved a waiver of consent for the semen samples that were used for this study.

After semen samples were collected by masturbation, it was allowed to liquefy at 37°C. Routine semen analysis (see Addendum II),using the World Health Organization (WHO, 2010) as guideline, was performed to decide whether to include the semen sample in this study. Samples with one or more of the following properties were excluded from the study:

1) <1ml semen
2) increased viscosity (≥10cm)
3) <40% motility
4) <2+ forward progression
5) $<50x10^6$/ml spermatozoa (concentration after swim up of $\geq 20x10^6$/ml ideally needed)
6) increased amount of debris and cells in semen sample
7) HIV positive samples

2.2 Semen sample preparation and handling

The excess semen from samples used for routine semen analysis was used and prepared with a routine swim up method (see Addendum III) as for ART. Modified Tygerberg Hospital andrology manual based on WHO (2010) was used as guideline for the preparation of semen. Spermatozoa swam up in 1ml Quinn's Sperm Washing medium (SAGE, Cooper Surgical, Harrilabs, South Africa). The exclusion criteria were chosen in order to obtain an ideal swim up concentration of ≥20x10^6/ml.

Nine 1,5ml Eppendorf tubes were labelled as P1, P2, P3, S1, S2, S3, C1, C2 and C3. 80µl of 7% Polyvinylpyrrolidone (PVP) medium (SAGE, Cooper Surgical, Harrilabs, South Africa) was aliquoted into tubes P1-P3. 80µl of SpermSlow™ medium (Origio, Cooper Surgical, Harrilabs, South Africa) was aliquoted into tubes S1-S3. Tubes C1-C3 contained 80µl aliquots of Sperm Washing medium and acted as the control group. All mediums were kept at room temperature. 80µl of the swum up sperm sample were mixed and incubated at 37°C with the above mentioned mediums in the nine separate Eppendorf tubes. Tubes P1, S1 and C1 were incubated for 5 minutes, tubes P2, S2 and C2 for 30 minutes and tubes P3, S3 and C3 were incubated for 60 minutes (see Addendum IV).

After the respective incubation periods, each sample was washed to remove the PVP and SpermSlow™ medium. 500µl Sperm Washing medium was added to every sample (including the control samples) after which it was centrifuged (450g, 5minutes). The resulting pellet was resuspended in ≥50µl Sperm Washing medium. The volume of the Sperm Washing medium used for resuspension was calculated for each sperm sample to achieve a final sperm concentration of ±20x10^6/ml.

After resuspension, the quantitative (Experiment A) and descriptive (Experiment B) analyses were done on the nine different sperm solutions.

2.3 Experiment A

2.3.1 Computer assisted semen analysis (CASA) - Motility analysis

A standardized method for spermatozoa motility determination was followed using the Hamilton Thorn CASA system (IVOS® Hamilton Thorne CASA system equipped with HT-CASA II software version 1.1). The nine different sperm solutions per semen sample were loaded onto a labelled Leja® slide with a fixed, consistent chamber depth (Tomlinson *et al*., 2001) and inserted into the CASA system for the motility analyses (see Addendum V). The following additional spermatozoa motion parameters were also analysed and notated for all the sperm samples: Curvilinear velocity (VCL); Average path velocity (VAP); Straight-line velocity (VSL); Amplitude of lateral head displacement (ALH); Beat cross frequency (BCF); Linearity (LIN) and Straightness (STR). A total of 90 semen samples were analysed.

2.3.2 Viability stain analysis

The viability stain analysis is a test to determine the percentage of viable spermatozoa by assessing the membrane integrity of the cells. This staining technique uses eosin (Merck, Modderfontein, South Africa) as a vital stain. This method is based on the principle that damaged plasma membranes, such as non-vital spermatozoa, allow entry of this membrane-impermeant stain. Nigrosin (Merck, Modderfontein, South Africa) is used to increase the contrast between the background and the sperm heads. This makes it easier to discern faintly stained spermatozoa. It also permits slides to be stored for re-evaluation and quality control purposes (Björndahl *et al.*, 2003).

With bright field optics, viablespermatozoa have white or light pink heads and dead spermatozoa have heads that are stained red or dark pink. If the stain is limited to only a part of the neck region, and the rest of the head area is unstained, this is considered a ‘leaky neck membrane’. This is not

a sign of cell death and total membrane disintegration and these cells should be assessed as viable.

Frosted slides were labelled according to the sample number and the nine sperm solutions (P1-P3, S1-S3, C1-C3) per semen sample. The spermatozoon viability stain was done on each sperm solution (see Addendum VI). Modified Tygerberg Hospital andrology manual based on WHO (2010) was used as guideline for the spermatozoon viability stain. A total of 100 spermatozoa were randomly assessed for each sperm solution slide using a light microscope with the 100x oil immersion lens. A percentage of viable spermatozoa (not stained, white spermatozoa) were notated. A total of 90 semen samples were analysed.

2.3.3 Chromomycin A_3 (CMA_3) staining test

As previously discussed in the introduction, CMA_3 is a guanine-cytosine-specific fluorochrome that exposes chromatin that is poorly packaged via direct visualization of protamine deficient loosely packaged chromatin (Bianchi *et al.*, 1996). The CMA_3 and protamines compete for the same binding sites (minor groove) in the DNA. A previous study my Manicardi *et al*. (1995) found a strong correlation between sensitivity to CMA_3 staining and sensitivity to endogenous *in situ* nick translation.

Smears of each of the nine test sperm solutions per semen sample were made for the CMA_3 staining test (see Addendum VII). The slides were allowed to air dry and were fixed for 20 minutes in methanol/acetic acid. Following the fixation, slides were stained with 15µl CMA_3 staining medium (Sigma-Aldrich, Johannesburg, South Africa) and kept in a dark chamber for 20 minutes. Slides were then rinsed in McIlvaine's buffer and mounted with Dabco anti-fade solution (Sigma-Aldrich, Johannesburg, South Africa). An Olympus BX40 fluorescence microscope (Wirsam Scientific,

Cape Town, South Africa), equipped with the appropriate filter and excitation of 465-495nm was used for the slide evaluation (Esterhuizen *et al.*, 2000).

A total of 100 spermatozoa were randomly assessed for each sperm solution slide. Bright yellow stained spermatozoa (CMA_3 positive) were regarded as immature spermatozoa with decondensed chromatin. Spermatozoa with dull yellow staining (CMA_3 negative) contained good chromatin packaging and were therefore regarded as mature spermatozoa. Chromatin packaging of samples with CMA_3 values of ≤40% were considered as normal. Values of 41-60% were considered as dubious abnormal chromatin packaging and values ≥61% were considered as abnormal sperm chromatin packaging (Esterhuizen *et al.*, 2000). A total of 90 semen samples were analysed.

2.3.4 Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay - Epi-fluorescence

As previously discussed in the introduction, TUNEL determines the existing DNA damage of spermatozoa and is based on the ligation of labelled dUTP-nucleotides to the 3'-OH phosphate ends of single- as well as double-stranded DNA breaks (Nakamura *et al.*, 1995). This reaction is catalysed by the template-independent enzyme, terminal deoxynucleotidyl transferase (TdT). By using labelled dUTP-nucleotides, DNA fragmentation in spermatozoa can be identified by using bright field microscopy, fluorescent microscopy as well as flow cytometry (Gorczyca *et al.*, 1993a; Muratori *et al.*, 2010). Spermatozoa with normal DNA have only background staining, while those with fragmented DNA (multiple chromatin 3' –OH ends) stain brightly (Gorczyca *et al.*, 1993a).

An aliquot (≥30µl) of each of the nine test sperm solutions per semen sample was frozen in liquid nitrogen using a custom made storage device consisting of microtips sealed with high security CBS® straws (see Addendum VIII). The tips were labelled and directly dunked into the nitrogen after each time interval. The tips were stored in a nitrogen tank reserved for experimental samples only.

Sperm DNA fragmentation was assessed using a modified method previously described by Avendaño *et al.* (2010). Dr Venesa Y. Rawe (Centro de Estudios de Gynecología y Reproducción, Buenos Aires, Argentina)was also contacted in connection with the TUNEL methodology and evaluation. The In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) was used for the analysis. The kit contains an enzyme solution (TdT) that catalyse the addition of the label solution (fluoresceine-dUTP) at free 3'-OH groups in single- and double-stranded DNA that allows the detection of single- and double-stranded DNA breaks that occur at the early stages of apoptosis.

Since we had limited funding for reagents as well as limited time to do the analyses, it was decided to only assess 29 of the 90 sperm samples at two time intervals (30 and 60 minutes). These sperm samples were randomly selected. Test sperm solutions were thawed for 10 minutes at 37°C and pipetted onto two duplicate 21 well Teflon printed diagnostic slides (Protea Laboratory solutions, Johannesburg, South Africa). One of the slides was kept as a back-up. The slides were allowed to air dry.

4% Formaldehyde/PBS solution was used as a fixative by adding 5µl to each well and incubating the slide for 45 minutes at room temperature in a humidified chamber. After the incubation period, the fluid form each well was slowly siphoned by using cut tissue paper pieces. Each well was washed with 10µl of 1% HSA/PBS solution after which the fluid was siphoned again.

Spermatozoa were permeabilized with 5µl of 0.1% Triton X-100/PBS for 10 minutes at room temperature in a humidified chamber. Again the fluid was siphoned from each well and the wells were washed twice, siphoning between each washing step.

After siphoning the fluid from each well after the second washing step, 5µl of the Roche kit label solution was pipetted onto one of the wells. This well served as a negative control. 10µl Roche kit enzyme solution was then mixed with 90µl Roche kit label solution and 5µl of this reaction mixture was carefully pipetted onto the remaining 20 wells. The slide was incubated for 90 minutes at 37°C

in a dark humidified chamber. After the incubation period the wells were siphoned, washed twice and siphoned again. Dabco anti-fade solution was added to the slide and it was covered with a coverslip.

Slides were analysed immediately. A total of 100 spermatozoa were randomly assessed in each well under a fluorescence microscope (Olympus BX40). Spermatozoa were classified according to intact or fragmented DNA. Spermatozoa showing no fluorescence had intact DNA (TUNEL negative spermatozoa), while spermatozoa with DNA damage fluoresced green (TUNEL positive spermatozoa). A sperm DNA status was seen as normal with TUNEL positive spermatozoa values ≤30%. TUNEL values >30% indicated fragmented sperm DNA (Evenson *et al.*, 1999; Henkel *et al.,* 2004).

2.4 Experiment B

2.4.1 Transmission Electron Microscopy (TEM)

Three hundred micro liters (300µl)of the swum up sperm samplewas mixed with 300µl PVP, SpermSlow™ and Sperm Washing medium respectively to ensure enough sperm cells for the analyses. Only three test sperm solutions per semen sample (not nine as described in Experiment A) were prepared for TEM evaluation. These samples were incubated for one hour only. After the incubation period with PVP, SpermSlow™ and sperm washing medium, the samples were washed with 500µl sperm washing medium, centrifuged and the pellets were resuspended in 100µl sperm washing medium. These resuspended spermatozoa samples were carefully suspended into 2.5% Glutaraldehyde/PBS solution (Sigma-Aldrich, Johannesburg, South Africa) with a ratio of 1:5 (test sample:glutaraldehyde). Samples were stored at 4°C for 24 hours during which time a pellet formed at the bottom of each Eppendorf. These pellets were used for the Transmission Electron Microscopy (TEM) evaluation. TEM was done by Mrs. Nolan Muller (Anatomical Pathology –

Electron microscopy division) using a standard TEM technique. Evaluation of micrographs was done by expert, Prof M Sousa (University of Porto, Portugal). Prof Sousa previously collaborated with the Tygerberg Fertility Clinic in publishing an atlas (Shafie *et al.*, 2000) on TEM images of human spermatozoa and unfertilized oocytes. Only one semen sample's micrographs were evaluated.

2.5 Data management and statistical analysis

A data sheet (see Addendum IX) was used to record all the relevant information. This information was transferred to a Microsoft Excel sheet in order for the statistical analysis to take place. The statistician, Prof Carl Lombard [The Biostatistics Unit of the South African Medical Research Council (MRC)] was consulted.

Statistical analysis was carried out using the Stata version 13.0 software for Windows. A Binomial Regression Model with Chi-square tests was used for the viability, CMA_3 and TUNEL parameters to estimate the difference in proportions between the two active mediums (PVP and SpermSlow^{TM}) as well as the difference with the control (sperm washing medium). The denominator used for each sample was n=100. The statistical analysis was adjusted for correlated experimental design in a Population Averaged Model. The CASA parameters (motility and progressive spermatozoa percentage) were analysed as if they were a continuous variable using a Linear Regression Model. These analyses took the experimental design into account; the nine test sample replications within the same sperm donor. The within donor design also controls for the between donor variability. Good precision is thus achieved as is evident from the narrow confidence intervals of the contrast estimates.

Statistical analyses with P-values of <0.05 were considered statistically significant. However, whether or not PVP andSpermSlow^{TM},sdata sets were equivalent had to be established. The

conventional significant test has little relevance in an equivalence trial. A failure to detect a difference does not imply equivalence (Altman *et al.*, 1995). On the other hand, a detected difference may not have any clinical relevance and may correspond to practical equivalence. The confidence interval defines a range for the possible true difference between two treatments. If every confidence interval point within this range corresponds to a difference of no clinical importance, then only may the treatment be considered to be equivalent. Therefore, a range of equivalence had to be predefined and then it could be established whether the confidence interval (centred on the observed difference) lies within these set margins (Jones *et al.*, 1996). Senior scientists in the reproductive biology field were approached to establish an equivalence margin for all the variables tested. Using the equivalence margins, it was possible to establish whether or not PVP and SpermSlow™ were clinically equivalent. The equivalence margins for sperm viability, motility and percentage progressive spermatozoa were set at ±20%. The equivalence margins for both CMA_3 and TUNEL positive parameters were set at ±10%.

The transmission electron microscopy (TEM) results were statistically analysed by Prof M Sousa through the IBM SPSS Statistics 20 program for Windows. Means were compared by the t-test for independent samples. Categorical variables were analysed using descriptive statistics and the Chi-square test, with continuity correction. In some variables, in the presence of cells with expected <5 value in contingency tables, the Fisher exact test was used. All statistical tests were two-tailed, with significance level of 0.05 (P-value <0.05).

CHAPTER 3

Results

Average sperm parameters (semen volume, semen viscosity, sperm motility, sperm forward progression and sperm concentration) of the semen samples (n=90) used in the study is presented in Addendum X.

3.1 Primary objective: The effect of PVP and SpermSlow™ on basic human semen parameters and sperm DNA integrity

Descriptive statistics for the viability, CMA_3 and TUNEL variables are presented in Table 3.1. Descriptive statistics for the motility and progressive sperm CASA (computer assisted semen analysis) variables are presented in Table 3.2. A Box-and-Whisker plot diagram was used for all the tested variables indicating the median, minimum and maximum parameters. The correlation between the incubation time and the tested variables was presented in a Lowess Smoother graph. The default bandwidth of 0.8 was used for all the Lowess Smoother graphs.

Table 3.1 Descriptive statistics of sperm viability, CMA_3 and TUNEL parameters after the incubation in PVP, SpermSlow™ and Sperm Washing medium for three incubation periods (5, 30 and 60 minutes).

Method variable		Viability (%)		CMA_3 (%)		TUNEL (%)	
Incubation time (minutes)	Medium	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
5	PVP	68.4	13.2	17.5	8.9	-	-
	SS	62.7	14.1	16.8	9.0	-	-
	SW	64.1	12.5	16.9	8.2	-	-

	PVP	67.4	13.3	18.2	8.1	11.6	7.8
30	**SS**	61.2	12.8	17.2	8.9	12.1	5.5
	SW	62.4	12.6	18.0	8.3	11.9	7.4
	PVP	63.0	14.6	19.1	9.4	10.9	6.2
60	**SS**	59.4	13.0	17.1	8.1	13.4	7.5
	SW	58.9	12.6	17.1	8.4	11.8	6.7

PVP = PVP 7% Ready-to-Use solution; SS = SpermSlow™; SW = Quinn's Sperm Washing Medium

Table 3.2 Descriptive statistics of CASA variables after the incubation in PVP, SpermSlow™ and Sperm Washing medium for three incubation time periods (5, 30 and 60 minutes).

Method variable		**CASA**			
		Motility (%)		**Progressive sperm (%)**	
Incubation time (minutes)	**Medium**	**Mean**	**Standard Deviation**	**Mean**	**Standard Deviation**
	PVP	73.6	14.0	51.4	18.5
5	**SS**	73.6	14.2	53.9	18.1
	SW	74.4	13.7	52.1	17.8
	PVP	73.6	13.6	58.7	13.9
30	**SS**	74.0	13.6	59.2	14.5
	SW	77.1	13.6	61.0	13.5
	PVP	68.7	15.2	54.5	13.0
60	**SS**	70.8	14.1	56.2	14.1
	SW	75.8	12.9	59.4	14.5

PVP = PVP 7% Ready-to-Use solution; SS = SpermSlow™; SW = Quinn's Sperm Washing Medium

3.1.1 Sperm viability (n=90)

Results for sperm viability (eosin viability analysis) after incubation are presented in Figure 3.1 and 3.2.

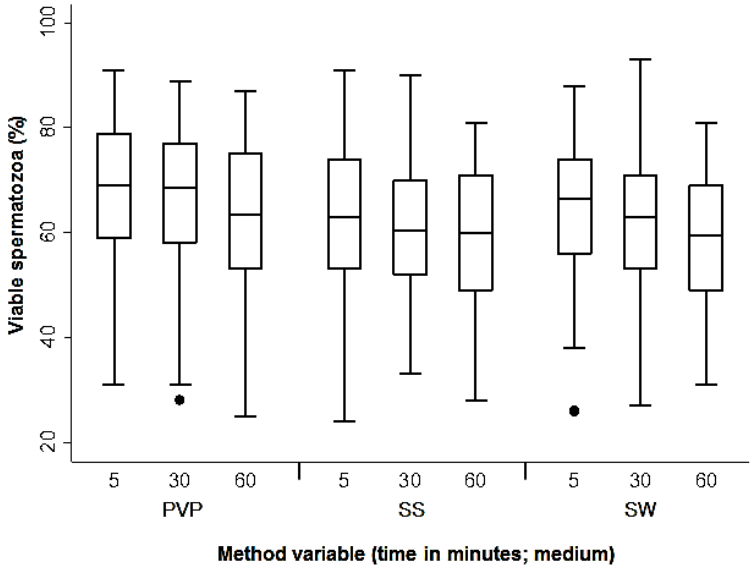


Figure 3.1 Box-and-Whisker plot diagram indicating sperm viability profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

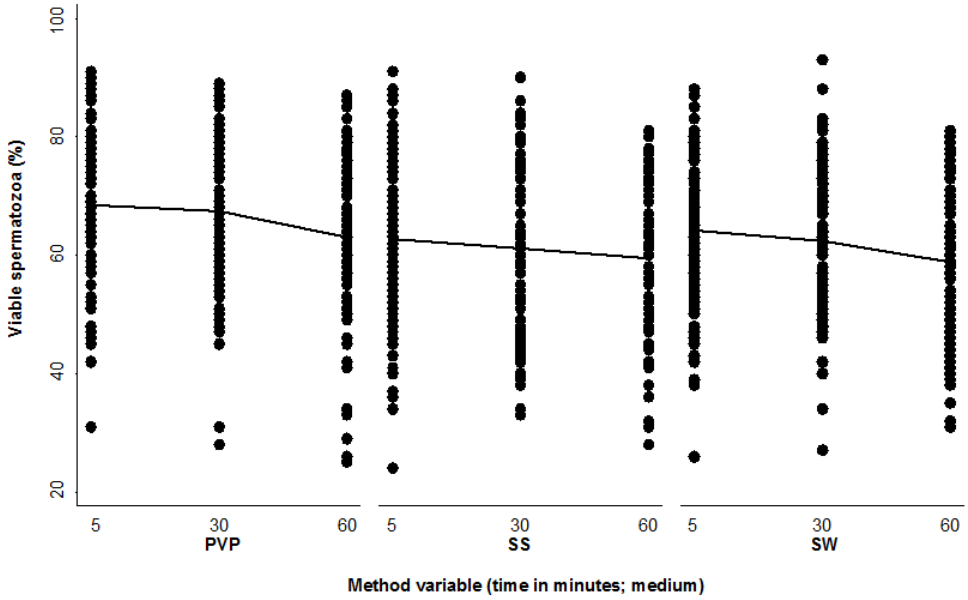


Figure 3.2 Lowess Smoother graph representing the correlation between the three incubation time periods (5, 30 and 60 minutes) and sperm viability after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW).

Statistical analysis on the **interaction between incubation time periods and incubation mediums** showed that the viability profile of the three mediums were parallel over time. There was no significant interaction between the time periods and incubation mediums (P = 0.532).

Statistical analysis on the **differencebetween the control (Sperm Washing medium) and two active mediums (PVP and SpermSlow™)** showed that there was a significant time effect on sperm viability. At the 30 minutes incubation period, the viability was 1.4% lower than at the 5 minute incubation period (P = 0.035). At the 60 minutes incubation period, the viability was 4.7% lower than at the 5 minute incubation period (P < 0.001). PVP incubation showed a significant higher viability (4.5%) compared to the control (P < 0.001). SpermSlow™ viability, was however not significantly different from the control (P = 0.35).

Statistical analysis on the **equivalence between PVP and SpermSlow™** showed that PVP incubation resulted in a significantly higher (5.2%) viability outcome than SpermSlow™ (P < 0.05). Although these mediums' viability outcomes were statistically different from each other, these two mediums are however still clinically equivalent in terms of viability profile, since the limit of the 95% confidence interval falls within the chosen ±20 % equivalence margin.

The original statistical analyses for sperm viability are available in Addendum XI.

3.1.2 Sperm chromatin packaging (n=90)

Results for sperm chromatin packaging (CMA_3 analysis) after incubation are presented in Figure 3.3 and 3.4.

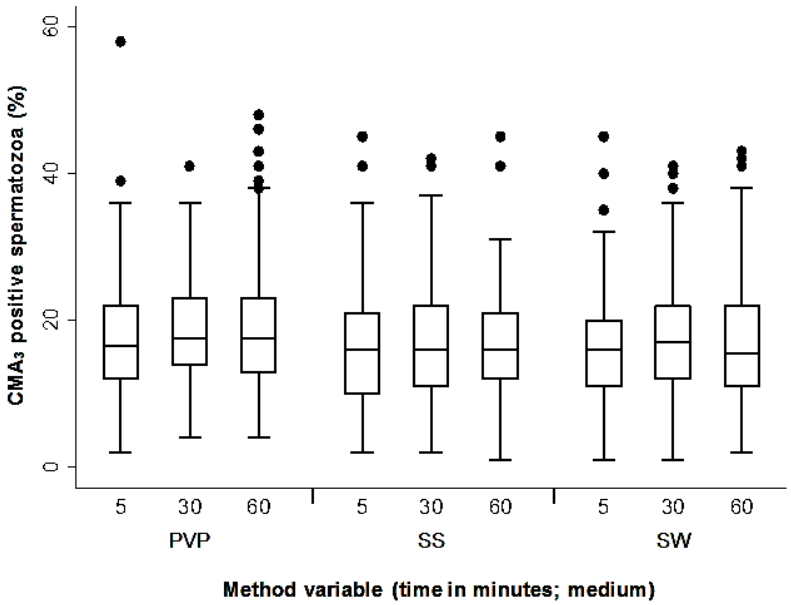


Figure 3.3 Box-and-Whisker plot diagram indicating the CMA_3 positive sperm profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

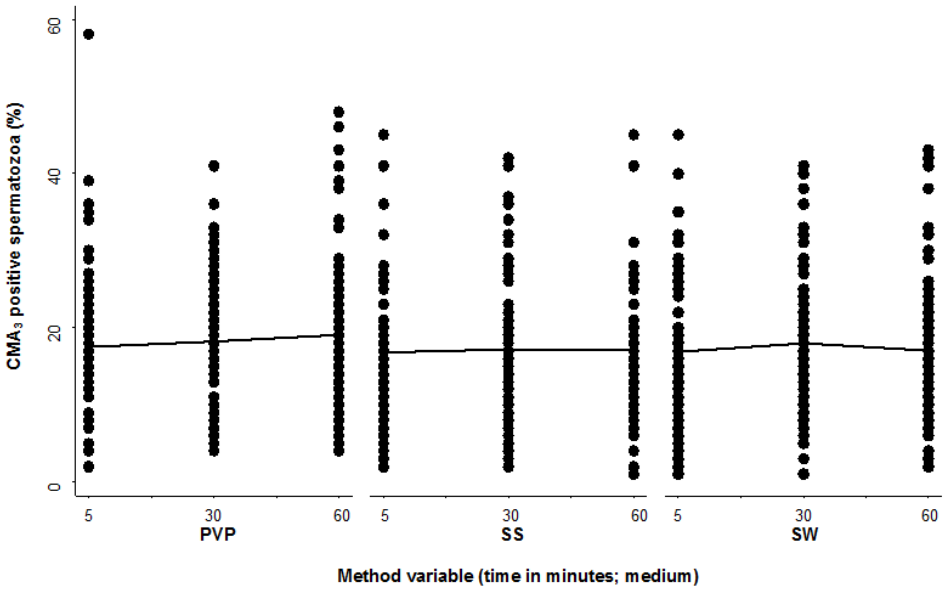


Figure 3.4 Lowess Smoother graph representing the correlation between the three incubation time periods (5, 30 and 60 minutes) and sperm CMA_3 profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW).

Statistical analysis on the **interaction between incubation time periods and incubation mediums** showed that the CMA_3 profile of the three mediums were parallel over time. There was no significant interaction between the time periods and incubation mediums (P = 0.1787).

Statistical analysis on the **differencebetween the control (Sperm Washing medium) and two active mediums (PVP and SpermSlow™)** showed that there was no significant time effect on the CMA_3 profile (P = 0.1515). PVP and SpermSlow™ incubation did not differ significantly from the control (P = 0.073 and 0.629 respectively).

Statistical analysis on the **equivalence between PVP and SpermSlow™** showed that PVP incubation resulted in a significantly higher (1.2%) CMA_3 outcome (less mature sperm DNA) than SpermSlow™ (P = 0.022). Although these mediums' CMA_3 outcome were statistically different from each other, these two mediums are however still clinically equivalent in terms of CMA_3 profile, since the limit of the 95% confidence interval falls within the chosen ±10 % equivalence margin.

The original statistical analyses for sperm chromatin packaging are available in Addendum XI.

3.1.3 Sperm DNA fragmentation (n=29)

Results for sperm DNA fragmentation (TUNEL analysis) after incubation are presented in Figure 3.5 and 3.6.

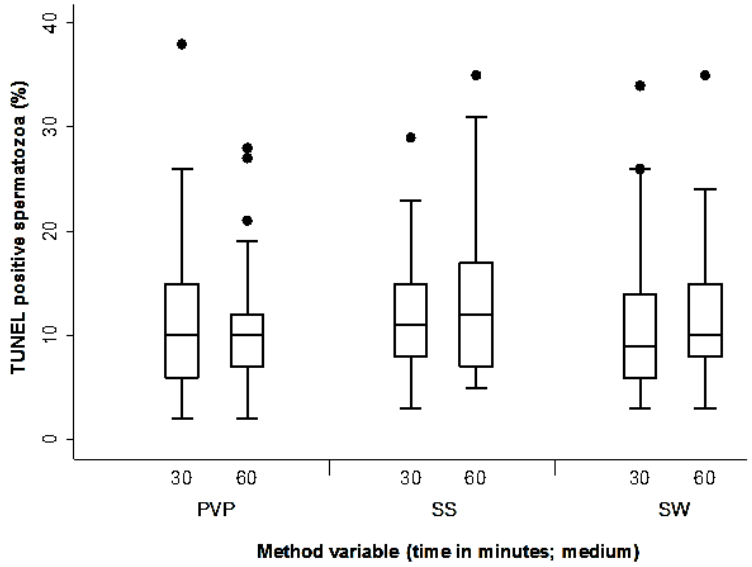


Figure 3.5 Box-and-Whisker plot diagram indicating the TUNEL positive sperm profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the two time periods (30 and 60 minutes).

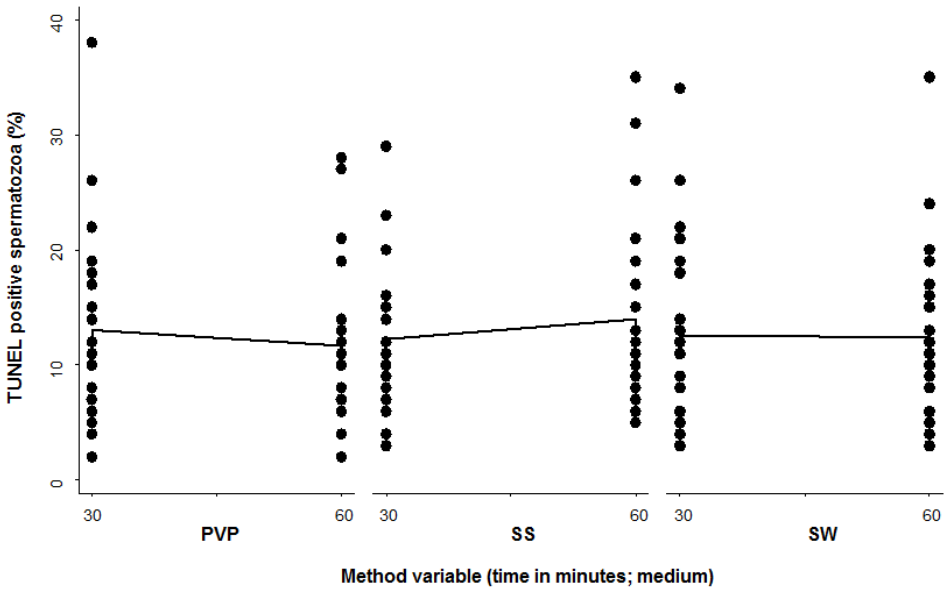


Figure 3.6 Lowess Smoother graph representing the correlation between the two incubation time periods (30 and 60 minutes) and sperm TUNEL profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW).

Statistical analysis on the **interaction between incubation time periods and incubation mediums** showed that the TUNEL profile of the three mediums were parallel over time. There was no significant interaction between the time periods and incubation mediums (P = 0.3959).

Statistical analysis on the **differencebetween the control (Sperm Washing medium) and two active mediums (PVP and SpermSlow™)** showed that there was no significant time effect on the TUNEL profile (P = 0.794). PVP and SpermSlow™ incubation did not differ significantly from the control (P = 0.299 and 0.145 respectively).

Statistical analysis on the **equivalence between PVP and SpermSlow™** showed that PVP incubation resulted in a significantly lower (1.5%) TUNEL outcome (less fragmented sperm DNA) than SpermSlow™ (P = 0.021). Although these mediums' TUNEL outcome were statistically different from each other, these two mediums are however still clinically equivalent in terms of TUNEL profile, since the limit of the 95% confidence interval falls within the chosen ±10 % equivalence margin.

The original statistical analyses for sperm DNA fragmentation are available in Addendum XI.

3.1.4 Sperm motility (n=90)

Results for sperm motility (CASA) after incubation are presented in Figure 3.7 and 3.8.

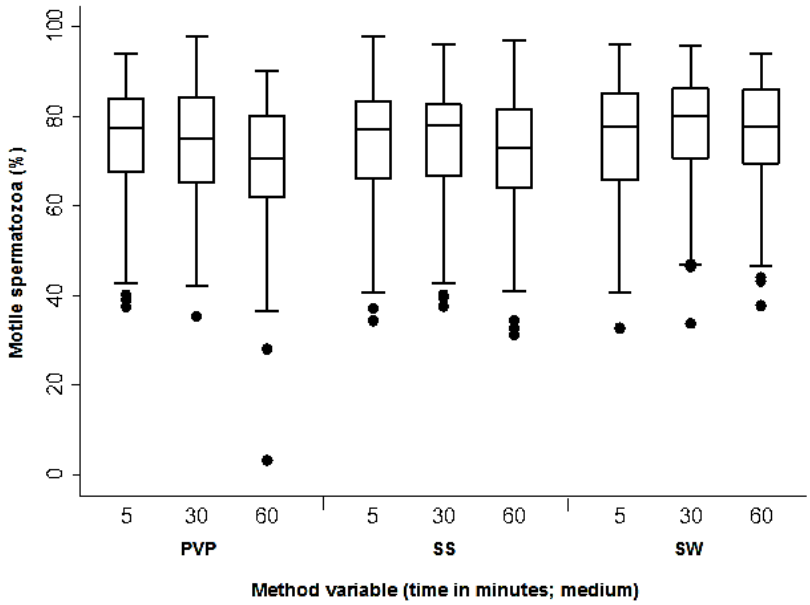


Figure 3.7 Box-and-Whisker plot diagram indicating sperm motility profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

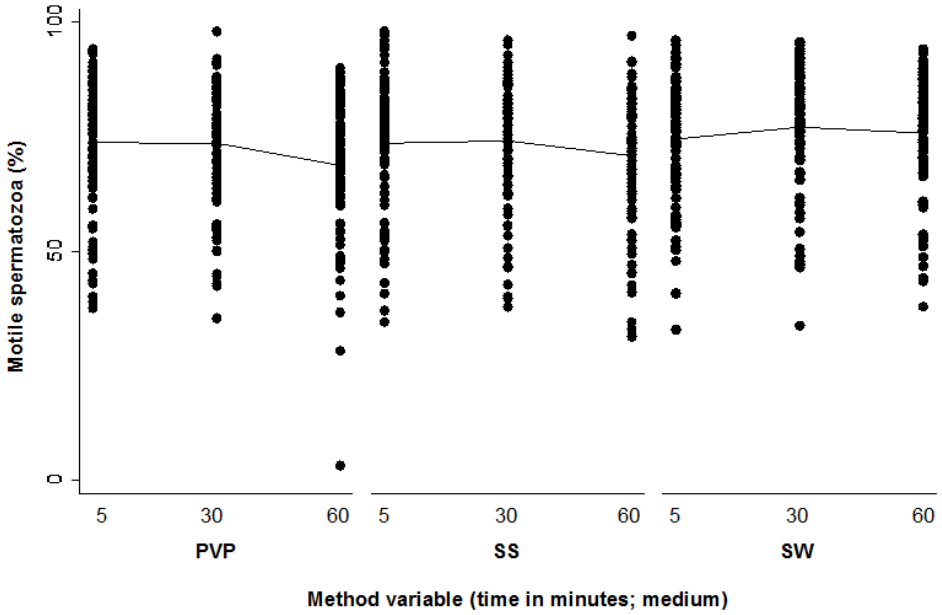


Figure 3.8 Lowess Smoother graph representing the correlation between the three incubation time periods (5, 30 and 60 minutes) and sperm motility after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW).

Statistical analysis on the **interaction between incubation time periods and incubation mediums** showed that the motility profile of the three mediums were not parallel over time. There was a statistically significant interaction between the incubation time periods and mediums (P = 0.0003).

Since there was interaction between the incubation time periods and mediums, time specific **comparisons betweenthe control (Sperm Washing medium) and two active mediums (PVP and SpermSlow™)** were made. Statistical analysis showed that there was no significant difference in motility between PVP and control, or SpermSlow™ and control at the 5 minute incubation time periods (P = 0.226 and P = 0.257 respectively). However, at the 30 minute incubation period, there was a significant difference between PVP and control, as well as SpermSlow™ and control (both P < 0.001). PVP showed a significantly lower motility percentage of 3.5% than the control, while SpermSlow™ showed 3.0% lower motility when compared to the control. At the 60 minute incubation time, once again there was a significant difference between PVP and control, as well as SpermSlow™ and control (both P < 0.001). PVP showed a 7.1% lower motility than the control, while SpermSlow™ showed a 5.0% lower motility when compared with the control.

Statistical analysis on the **equivalence between PVP and SpermSlow™** showed there was no statistically significant difference in motility between PVP and SpermSlow™ at any of the time points, since all of the P-values were > 0.05. The overall difference, taking all three time points into account, between PVP and SpermSlow™ was 0.9% with 95% confidence interval (- 0.3% to 2.0% confidence interval). There was also no significant difference between the overall profiles of PVP and SpermSlow™ (P = 0.147). These two mediums are clinically equivalent in terms of motility profile, since the 95% confidence interval of the difference between PVP and SpermSlow™ falls within the chosen ±20 % equivalence margin.

The original statistical analyses for sperm motility are available in Addendum XI.

3.1.5 Progressive spermatozoa (n=90)

Results for progressive spermatozoa (CASA) after incubation are presented in Figure 3.9 and 3.10.

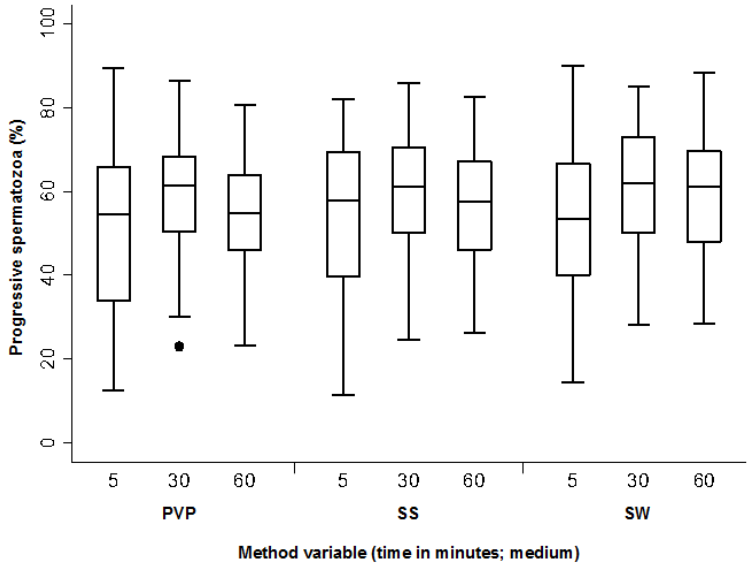


Figure 3.9 Box-and-Whisker plot diagram indicating progressive sperm profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

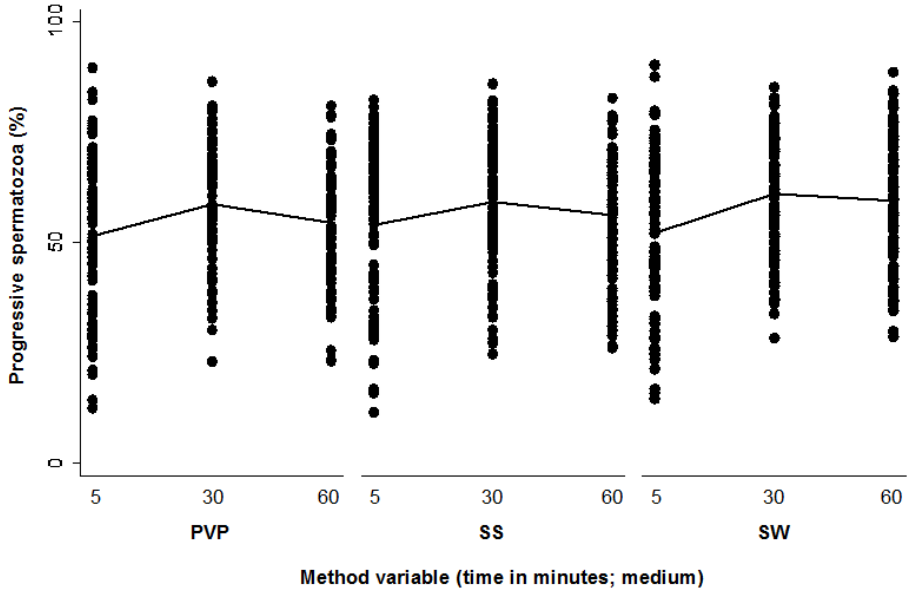


Figure 3.10 Lowess Smoother graph representing the correlation between the three incubation time periods (5, 30 and 60 minutes) and progressive sperm percentage after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW).

Statistical analysis on the **interaction between incubation time periods and incubation mediums** showed that the progressive sperm profile of the three mediums were not parallel over time. There was a statistically significant interaction between the incubation time periods and mediums (P = 0.0003).

Since there was significant interaction between the incubation time periods and mediums, time specific **comparisons betweenthe control (Sperm Washing medium) and two active mediums (PVP and SpermSlow™)** were made. Statistical analysis showed that there was no significant difference in the progressive sperm profile between PVP and control, or SpermSlow™ and control at the 5 minute incubation time periods (P = 0.47 and P = 0.118 respectively). However, at the 30 minute incubation period, there was a significant difference between PVP and control, as well as SpermSlow™ and control (both P < 0.001). PVP showed a significantly lower progressive sperm percentage of 2.3% than the control, while SpermSlow™ showed 1.8% lower progressive sperm when compared to the control. At the 60 minute incubation time, once again there was a significant difference between PVP and control, as well as SpermSlow™ and control (P < 0.001 and P = 0.002 respectively). PVP showed 5.0% lower progressive sperm than the control, while SpermSlow™ showed 3.3% lower progressive sperm when compared with the control.

Statistical analysis on the **equivalence between PVP and SpermSlow™** showed there was no statistically significant difference in percentage progressive sperm between PVP and SpermSlow™ at any of the specific time points, since all of the P-values were > 0.05. However, the overall difference between the two mediums was 1.6% and shows that PVP has a significant higher percentage progressive sperm compared to SpermSlow™ (P = 0.013). Although the two mediums do differ statistically, they are however still clinically equivalent in terms of progressive sperm profile, since the 95% confidence interval of the difference between PVP and SpermSlow™ falls within the chosen ±20 % equivalence margin.

The original statistical analyses for progressive sperm outcome are available in Addendum XI.

3.1.6 Sperm motility kinetics (n=90)

Various sperm motility kinetic parameters (VAP, VSL, BCF, VCL, STR, LIN) after incubation were also analysed during Experiment A using the CASA system. Since the clinical equivalence margins could not be set for these variables, it was decided that these CASA variables will not be statistically analysed. Instead, the mean values for each variable was calculated for each of the different incubation periods as well as the three mediums (see Figure 3.11 – 3.17).

There was a small increase at the 30 minute incubation period which either decreased or plateaued at the 60 minute incubation period in all of the mediums (PVP, SpermSlow™ and Sperm Washing medium) for all these motility kinetic parameters (VAP, VSL, BCF, VCL, STR, LIN).

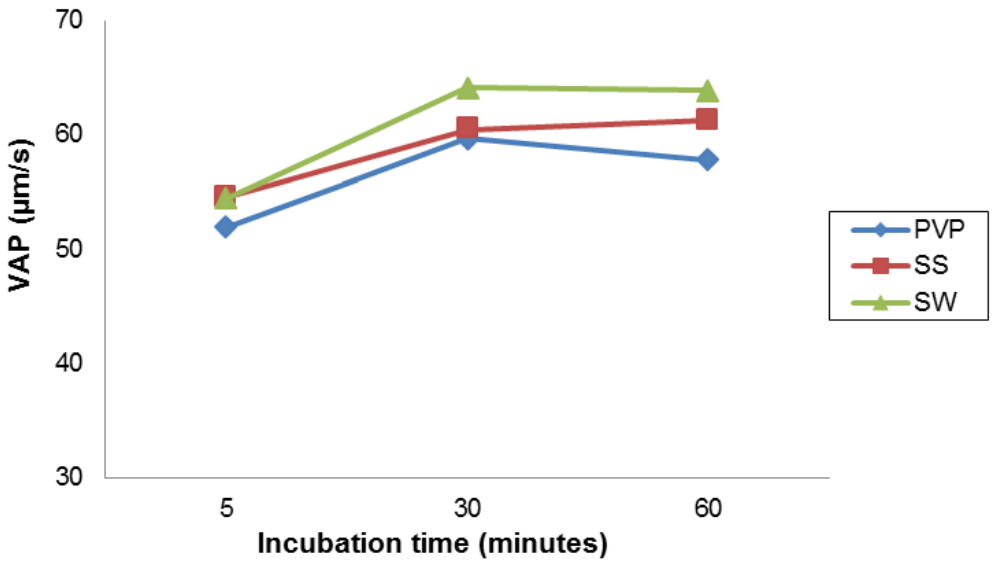


Figure 3.11 Line graph indicating the mean sperm VAP profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

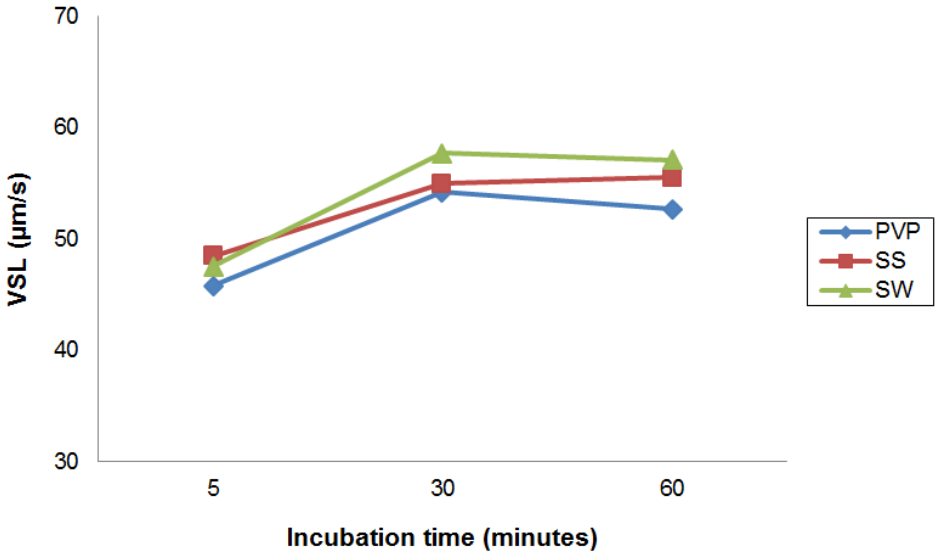


Figure 3.12 Line graph indicating the mean sperm VSL profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

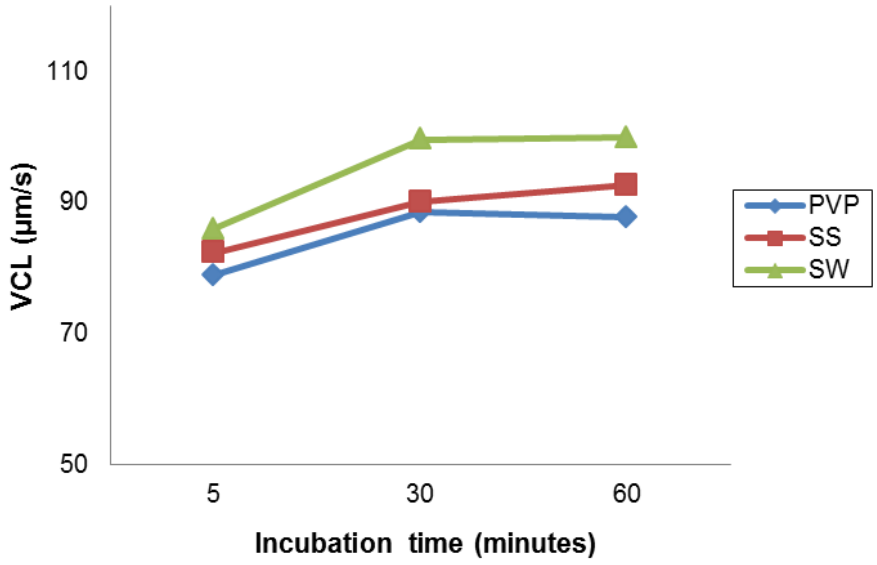


Figure 3.13 Line graph indicating the mean sperm VCL profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

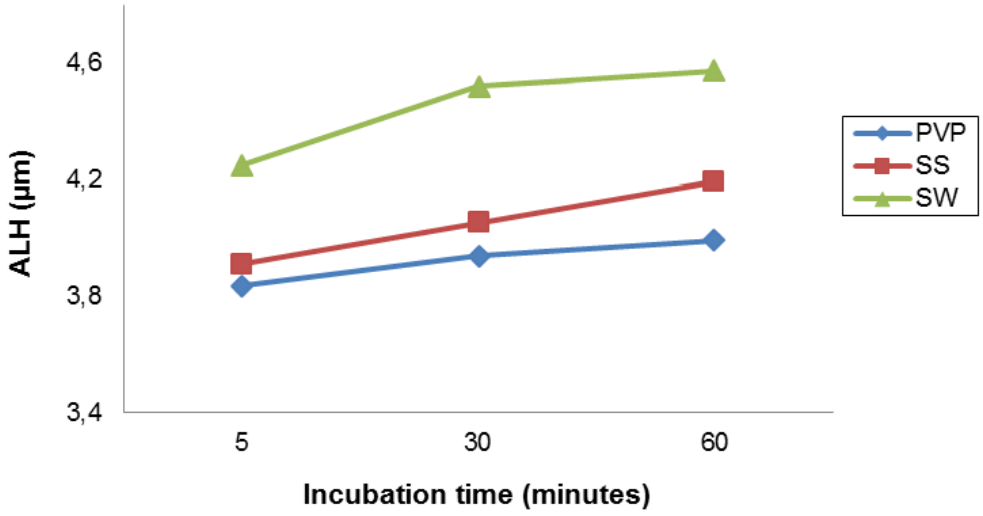


Figure 3.14 Line graph indicating the mean sperm ALH profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

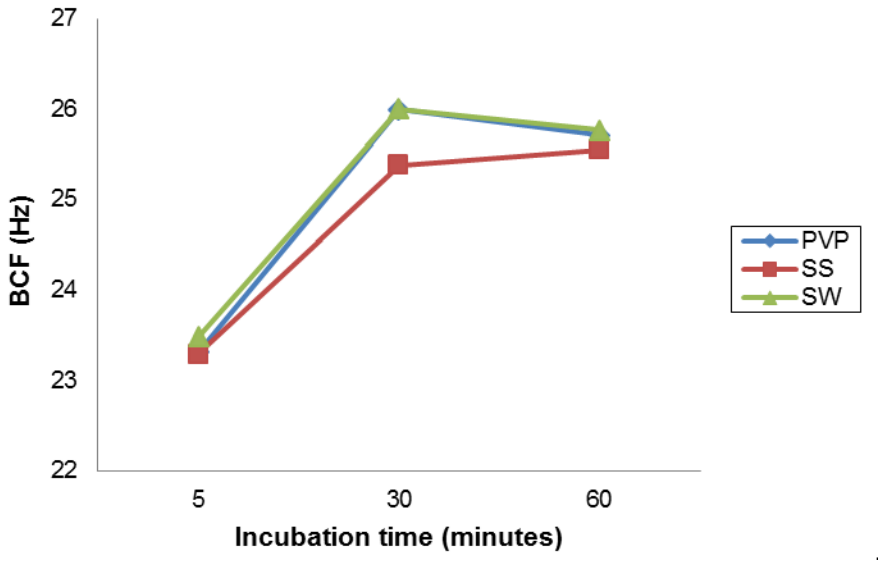


Figure 3.15 Line graph indicating the mean sperm BCF profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

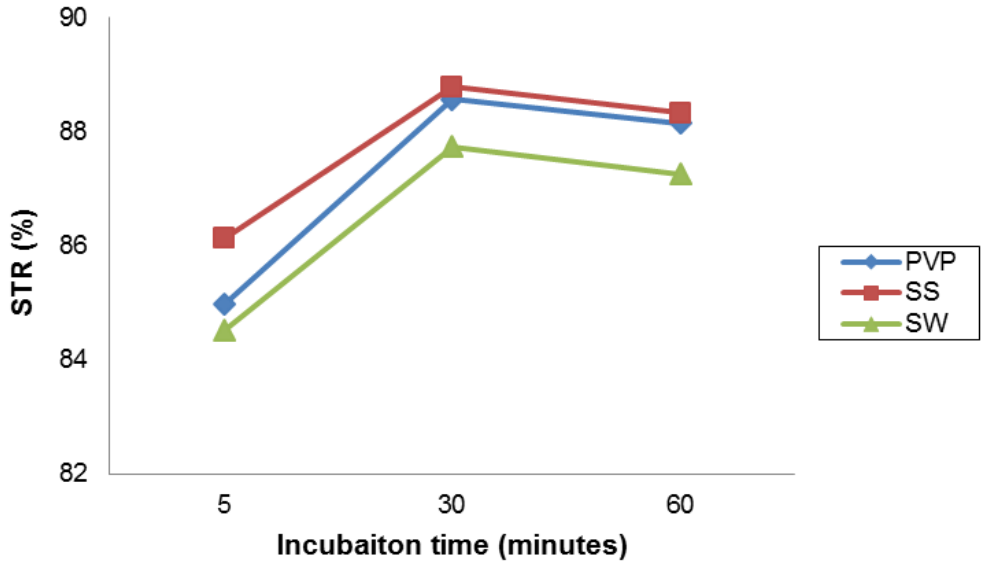


Figure 3.16 Line graph indicating the mean sperm STR profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

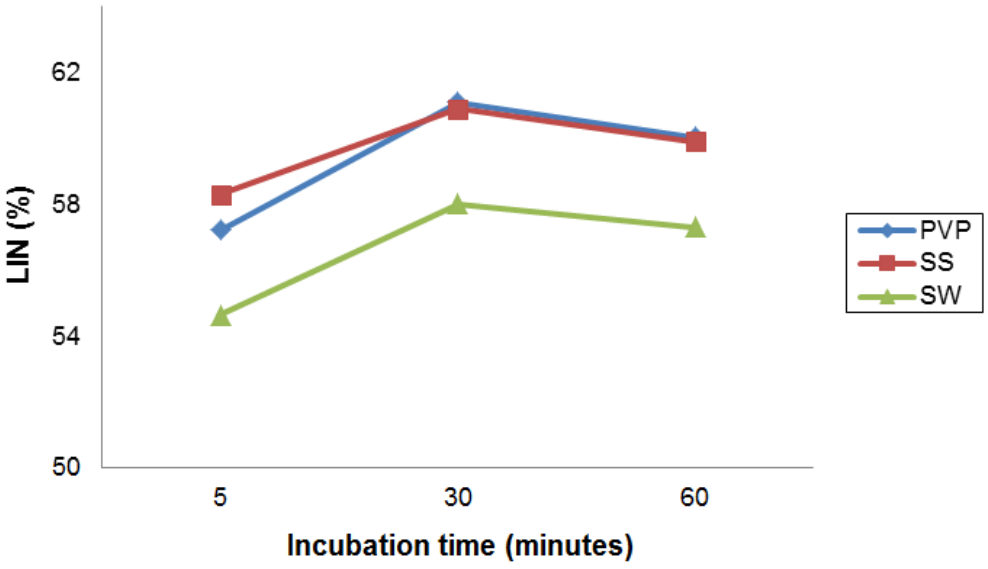


Figure 3.17 Line graph indicating the mean sperm LIN profile after the incubation in PVP, SpermSlow™ (SS) and Sperm Washing medium (SW) for the three time periods (5, 30 and 60 minutes).

3.2 Secondary objective: The effect of PVP and SpermSlow™ on human sperm ultrastructure (Transmission electron microscopy) (n=1)

Unfortunately due to the high cost of the transmission electron microscopy analysis, only one sample's micrographs were evaluated. The results of this analysis are presented in Table 3.3. Micrographs with classification annotations are presented in Figure 3.18 – 3.20.

Table 3.3 The effect of the three mediums (PVP, SpermSlow™ and Sperm Washing medium) on sperm ultrastructure after one hour incubation time.

Sperm characteristics			PVP	SS	SW	PVP vs SS	PVP vs SW	SS vs SW
						P-value	P-value	P-value
Cytoplasmic membrane	**Neck-piece**	**Conserved**	62/73 (84.9)	73/111 (65.8)	32/36 (88.9)	0.004	0.573	0.008
		Ruptured	*11/73 (15.1)*	*38/111 (34.2)*	*4/36 (11.1)*	*0.004*	*0.573*	*0.008*
	Mid-piece	**Conserved**	0	0	4/14 (28.6)	-	-	-
		Not well defined	*10/10 (100)*	*39/39 (100)*	*10/14 (71.4)*	-	*0.064*	*0.001*
Acrosomal vesicle	**Acrosomal vesicle**	**Well attached to the nucleus**	20/138 (14.5)	5/221 (2.3)	12/105 (11.4)	<0.001	0.484	0.001
		Abnormal	*118/138 (85.5)*	*216/221 (97.7)*	*93/105 (88.6)*	*<0.001*	*0.484*	*0.001*
	Presence	***Short-absent / Amorphous-reacted / not totally defined***	*87/138 (63.0)*	*200/221 (90.5)*	*68/105 (64.8)*	*<0.001*	*0.782*	*<0.001*
	Position	***Partially detached / Totally detached***	*31/138 (22.5)*	*16/221 (7.2)*	*25/105 (23.8)*	*<0.001*	*0.805*	*<0.001*

Nucleus	**Shape**	**Normal**	9/138 (6.5)	10/221 (4.5)	12/105 (11.4)	0.411	0.177	0.02
		Abnormal	*129/138 (93.5)*	*211/221 (95.5)*	*93/105 (88.6)*	*0.411*	*0.177*	*0.02*
	Content	**Dense**	100/138 (72.5)	182/221 (82.4)	104/105 (99.0)	0.026	<0.001	<0.001
		Not dense	*38/138 (27.5)*	*39/221 (17.6)*	*1/105 (1.0)*	*0.026*	*<0.001*	<0.001
	Pale areas	**Small nuclear space (also called nuclear vacuoles)**	90/138 (65.2)	132/221 (59.7)	84/105 (80)	0.298	0.011	<0.001
		Large pale areas	*48/138 (34.8)*	*89/221 (40.3)*	*21/105 (20)*	*0.298*	*0.011*	<0.001
Neck-piece / mid-piece	**Basal plate**		Normal	Normal	Normal	-	-	-
	Striated columns		Normal	Normal	Normal	-	-	-
	Outer dense fibre		Normal	Normal	Normal	-	-	-
Mitochondrial helix	**Regular helix, moderate dense with small pale areas**		29/62 (46.8)	45/87 (51.7)	14/25 (56)	0.551	0.436	0.706
	Enlarged and pale (swollen) / Displaced not forming a helix		*33/62 (53.2)*	*42/87 (48.3)*	*11/25 (44)*	*0.551*	*0.436*	*0.706*
Axoneme-principal piece	**9d + 2s**	**Normal pattern**	11/61 (18.0)	0	17/27 (63.0)	-	<0.001	-
		Abnormal pattern / Absence, reduced or displaced number of doubled, nexin links, dynein arms, radial spokes, central pair	*50/61 (82.0)*	*23/23 (100)*	*10/27 (37)*	*0.029*	*<0.001*	*<0.001*
	Annulus		Normal	Normal	Normal	-	-	-
	Outer dense fibre		Normal	Normal	Normal	-	-	-
	Fibrous sheath		Normal	Normal	Normal	-	-	-

Values in brackets are the value expressed in percentage

Micrographs were taken with at least 10 000x magnification and sperm characteristics were identified and annotated. 73 neck-piece cytoplasmic membranes were identified and evaluated in the PVP exposed spermatozoa micrographs. 84.9% of these were conserved and 15.1% were ruptured. 111 neck-piece cytoplasmic membranes were identified and evaluated in the SpermSlow^{TM} exposed spermatozoa micrographs. 65.8% of these were conserved and 34.2% were ruptured. Only 36 neck-piece cytoplasmic membranes were identified and evaluated in the control group's micrographs. 88.9% of these were conserved and 11.1% were ruptured.

The sperm **cytoplasmic membrane** was evaluated by examining both the neck-piece and mid-piece sperm characteristics. The neck-piece characteristics showed no significant difference ($P > 0.05$) between PVP exposed spermatozoa and the control (Sperm Washing medium). SpermSlow^{TM} incubated spermatozoa showed significantly lower conserved and significantly higher ruptured neck-piece cytoplasmic membranes when compared to both PVP incubated spermatozoa ($P < 0.05$) and the control ($P < 0.05$). The mid-piece cytoplasmic membranes showed no difference between PVP and SpermSlow^{TM} incubated spermatozoa. There were significantly higher less defined mid-piece cytoplasmic membrane characteristics in the SpermSlow^{TM} group compared to the control ($P < 0.01$).

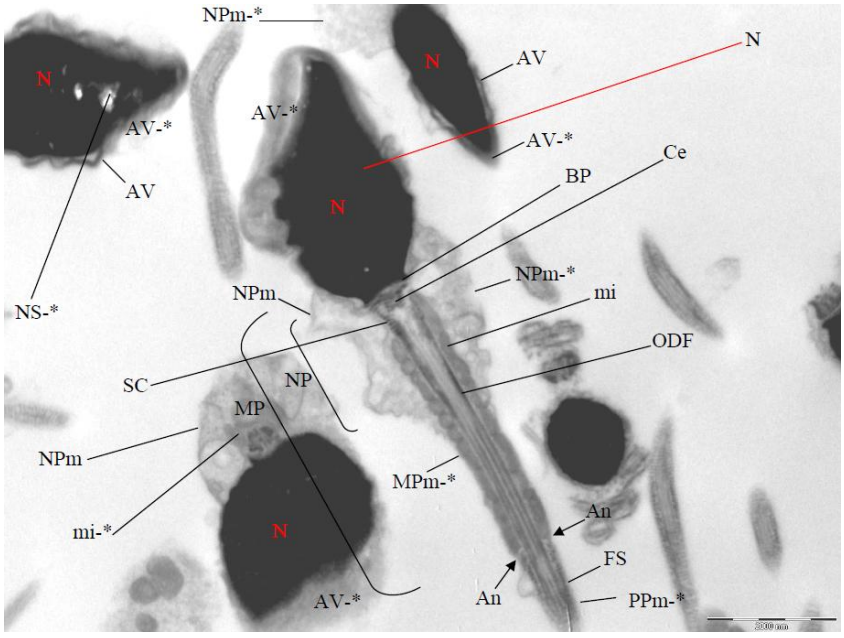
The **acrosomal vesicle** sperm characteristics were evaluated by examining the vesicle's attachment to the nucleus, the presence as well as the position of the vesicle. The attachment as well as the presence and position characteristics showed no significant difference between PVP incubated spermatozoa and the control ($P > 0.05$). SpermSlow^{TM} incubated spermatozoa showed significant lower well attached acrosomal vesicles, lower partially/totally detached acrosome vesicles and higher abnormal acrosomal vesicle sperm characteristics compared to both PVP and the control ($P < 0.001$).

The sperm **nucleus** characteristics were evaluated by examining its shape, content and pale areas. PVP exposed spermatozoa showed no significant different nucleus shape characteristics

compared to both SpermSlow™ and the control ($P > 0.05$). There were significantly less normal shaped nuclei in the SpermSlow™ group compared to the control ($P < 0.05$). The nucleus content density differed for each medium ($P < 0.05$). Sperm Washing medium (control) showed the best density, followed by SpermSlow™ exposed spermatozoa and then PVP exposed spermatozoa. PVP compared to SpermSlow™ incubated spermatozoa showed no significant difference in the nucleus pale area sperm characteristics (large pale areas and nuclear vacuoles). However, both SpermSlow™ and PVP groups were significantly different ($P < 0.05$) when compared to the control for nuclear vacuoles and large pale areas.

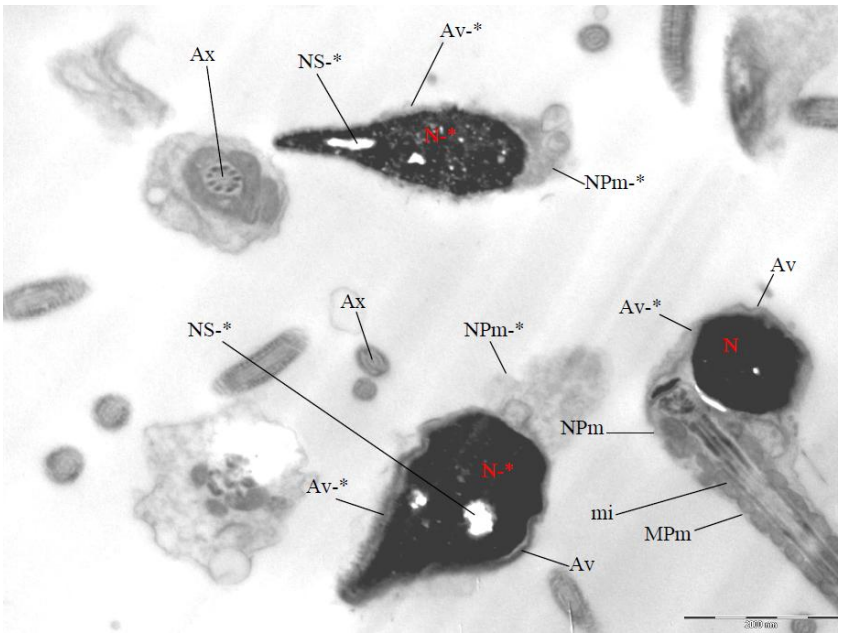
No significant differences were observed in the **mitochondrial helix** sperm characteristics between groups.

The **axoneme-principal piece** sperm characteristics were evaluated by examining the 9d + 2s pattern, annulus, outer dense fibre and fibrous sheath. Both PVP and SpermSlow™ exposed spermatozoa showed significant lower 9d + 2s normal pattern sperm characteristics when compared to the control ($P < 0.001$).



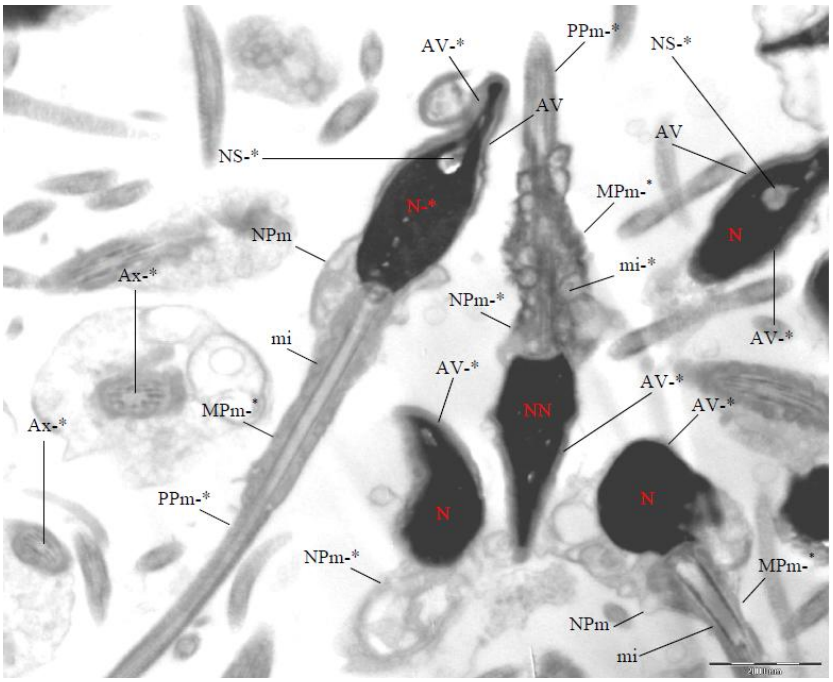
NP	: neck-piece
NPm	: intact neck-piece
NPm-*	: ruptured neck-piece membrane
MP	: mid-piece
MPm-*	: ruptured mid-piece
PP	: principal-piece
PPm-*	: ruptured principal-piece
N	: dense nuclear density : abnormal nucleus shape
NS-*	: large pale nuclear spaces
AV	: intact acrosomal vesicle
AV-*	: short-absent-amorphous-not totally defined acrosomal vesicle
mi	: mitochondrial helix
mi-*	: enlarged, swollen or displaced mitochondrial helix
BP	: normal basal plate
Ce	: normal centriole
SC	: normal striated columns
ODF	: normal outer dense fibres
An	: normal annulus
FS	: normal fibrous sheath

Figure 3.18 Transmission electron microscopy (TEM) micrograph with classification annotations of Sperm Washing medium (control) incubated spermatozoa.



NPm	: intact neck-piece
NPm-*	: ruptured neck-piece membrane
MPm	: intact mid-piece
N	: dense nuclear density
N-*	: decondensed nuclear density : abnormal nucleus shape
NS-*	: large pale nuclear spaces
AV	: intact acrosomal vesicle
AV-*	: short-absent-amorphous-not totally defined acrosomal vesicle
mi	: mitochondrial helix
Ax	: normal 9d+2s axoneme pattern

Figure 3.19 TEM micrograph with classification annotations of PVP incubated spermatozoa.



NPm	: intact neck-piece
NPm-*	: ruptured neck-piece membrane
MPm-*	: ruptured mid-piece
PPm-*	: ruptured principal-piece
N	: dense nuclear density
N-*	: decondensed nuclear density
NN	: normal nucleus shape
NS-*	: large pale nuclear spaces
AV	: intact acrosomal vesicle
AV-*	: short-absent-amorphous-not totally defined acrosomal vesicle
mi	: mitochondrial helix
mi-*	: enlarged, swollen or displaced mitochondrial helix
Ax-*	abnormal axoneme pattern

Figure 3.20 TEM micrograph with classification annotations of SpermSlow^{TM} incubated spermatozoa.

CHAPTER 4

Discussion and Conclusion

IMSI is seen as one of the techniques available to treat patients where the male partner presents with an elevated sperm DNA fragmentation rate. This modified ICSI technique allows embryologists to identify subtle sperm morphology features and requires a high level of technical expertise and inter-observer reproducibility. Hamilton Thorne IVOS® developed a software solution (IMSI Strict™) for sperm morphology categorization and IMSI sperm selection. This software aims to enable objective classification of spermatozoa, eliminating inter-technician variation.

For good optics and spermatozoon evaluation and selection in IMSI Strict™, spermatozoa need to be moving very slowly or be immotile, but still viable. This can be achieved by incubating prepared spermatozoa in a viscous holding medium, either PVP or SpermSlow™ medium, for the duration of the selection period. Before marketing the clinical use of IMSI Strict™, the possible toxicity or deleterious effect of these holding mediums on basic semen parameters, as well as sperm DNA and structural integrity of spermatozoa, need to be excluded.

In this study the effect of PVP and SpermSlow™ on human spermatozoa's viability (n=90), motility parameters (n=90), chromatin packaging (n=90), DNA fragmentation (n=29) and ultrastructure (n=1) was evaluated. All semen samples were prepared with a routine swim up method, since this represents the type of sample used during IMSI. Prepared, motile sperm samples were incubated for three time periods in PVP, SpermSlow™ and Sperm Washing medium (control).

Statistical analyses with P-values of <0.05 were considered statistically significant. However, whether or not PVP and SpermSlow™'s data sets were equivalent had to be established. The conventional significant test has little relevance in an equivalence trial. A failure to detect a difference does not imply equivalence (Altman *et al.*, 1995). On the other hand, a detected

difference may not have any clinical relevance and may correspond to practical equivalence. The confidence interval defines a range for the possible true difference between two treatments. If every confidence interval point within this range corresponds to a difference of no clinical importance, then only may the treatment be considered to be equivalent. Therefore, a range of equivalence had to be predefined and then it could be established whether the confidence interval (centred on the observed difference) lies within these set margins (Jones *et al.*, 1996). Senior scientists in the reproductive biology field were approached to establish an equivalence margin for all the variables tested. Using the equivalence margins, it was possible to establish whether or not PVP and SpermSlow™ were clinically equivalent. The equivalence margins for sperm viability, motility and percentage progressive spermatozoa were set at ±20%. The equivalence margins for both CMA_3 and TUNEL positive parameters were set at ±10%.

To our knowledge, this is the first study that compared the effect of PVP and SpermSlow™ on human spermatozoa's viability, motility parameters and chromatin packaging profiles. Therefore, we could not compare our results to any other published studies.

The interaction between the incubation time and the tested mediums showed that the **sperm viability** profile of the three mediums were parallel over time. There was no significant interaction between the incubation time and the tested mediums for the sperm viability outcome and, hence, the effect of PVP or SpermSlow™ on sperm viability was independent of the length of exposure time. The outcome of the specific comparison between the two active mediums (PVP and SpermSlow™) and the control (Sperm Washing medium) indicated that there was a significant time effect on sperm viability. Sperm viability significantly decreased over time, with the 60 minute incubation time period resulting in the lowest viability, as could be expected. PVP incubated spermatozoa showed a significant higher viability when compared to the control. SpermSlow™ on the other hand, did not differ significantly from the control with regards to viability. Furthermore, PVP incubated spermatozoa showed a statistically significant higher viability when compared to SpermSlow™ incubated spermatozoa. However, this viability difference was only 5.2% and not of

any clinical significance. The reason for the better performance of PVP regarding sperm viability is difficult to explain.

The interaction between the incubation time and tested mediums showed that the **CASAmotility and percentage progressive sperm** profiles of the three mediums differed over time. There was a significant interaction between the incubation time and the tested mediums for the CASA motility and progressive sperm outcomes and, hence, the effect of PVP or SpermSlow™ on sperm motility outcomes was dependent of the length of exposure time. The outcome of the specific comparison between the active mediums (PVP and SpermSlow™) and the control (Sperm Washing medium) indicated that PVP as well as SpermSlow™ incubation showed a significant lower motile and percentage progressive spermatozoa at the 30 and 60 minute incubation time periods compared to the control. There was a negative correlation between incubation time and the two motility parameters. The longer the incubation time, the more the motility and progressive sperm percentages decreased, as could be expected. Although all test sperm solutions were washed and resuspended in Sperm Washing medium before CASA motility analyses, we speculate that this decrease in motility and percentage progressive sperm parameters are due to the fact that the PVP and SpermSlow™ medium are viscous. The sperm energy mechanism may be depleted during the attempt of the spermatozoa to move in this medium, which may lead to the decrease in motility and percentage progressive sperm after a prolonged incubation period. The outcome of the specific comparison between PVP and SpermSlow™ was analysed in order to evaluate the equivalence of the two mediums. No statistically significant difference between the overall motility profiles of PVP and SpermSlow™ incubated spermatozoa was observed. PVP did however show a statistically significant higher overall progressive sperm profile compared to SpermSlow™. However, thisprogressive sperm difference was only 1.6% and not of any clinical significance. The reason for the better performance of PVP regarding progressive sperm outcome is difficult to explain.

Since the clinical equivalence margins could not be set for the different **CASA sperm motility kinetics** (VAP, VSL, BCF, VCL, STR, LIN), these variables were not statistically analysed. The

mean value for each variable was calculated for each of the different incubation periods as well as the three mediums. These results showed a small increase at the 30 minute incubation period which either decreased or plateaued at the 60 minute incubation period in all three mediums for all of these motility kinetics (VAP, VSL, BCF, VCL, STR, LIN). Therefore, PVP and SpermSlow™ medium were clinically equivalent with regards to its effect on sperm motility kinetics.

The interaction between the incubation time and mediums showed that the **sperm chromatin packaging** profile (CMA_3 analysis) of the three mediums were parallel over time. There was no significant interaction between the incubation time and the tested mediums for the CMA_3 outcome and, hence, the effect of PVP or SpermSlow™ on sperm chromatin packaging was independent of the length of exposure time. The outcome of the specific comparison between the two active mediums (PVP and SpermSlow™) and the control (Sperm Washing medium) indicated that neither PVP nor SpermSlow™ incubation showed a significant difference in chromatin packaging when compared to the control. Furthermore, PVP showed a statistically significant higher CMA_3 outcome than SpermSlow™ when the outcome of the specific comparison between PVP and SpermSlow™ was analysed. This indicates that PVP incubation resulted in less mature sperm chromatin packaging comparing to SpermSlow™. However, this CMA_3difference was only 1.2% and not of any clinical significance. The reason for the better performance of SpermSlow™ regarding sperm chromatin packaging outcome is difficult to explain.

To our knowledge, only two previous, similar studies compared the effect of PVP and SpermSlow™ on human spermatozoa's DNA fragmentation (Salian *et al.*, 2012, Rougier *et al.*, 2013). Therefore, we were able to compare our results with their findings.

The interaction between the incubation time and mediums showed that the **sperm DNA fragmentation** profile (TUNEL analysis) of the three mediums were parallel over time. There was no significant interaction between the incubation time and the tested mediums for the TUNEL outcome and, hence, the effect of PVP or SpermSlow™ on sperm DNA fragmentation was

independent of the length of exposure time. The outcome of the specific comparison between the two active mediums (PVP and SpermSlow™) and the control (Sperm Washing medium) indicated that neither PVP nor SpermSlow™ incubated spermatozoa showed a significant difference in DNA fragmentation when compared to the control. Furthermore, PVP incubation did result in a statistically significant lower TUNEL outcome specifically compared to the SpermSlow™ outcome. This indicates that PVP incubation results in less sperm DNA fragments comparing to SpermSlow™. However, thisTUNELdifference was only 1.5% and not of any clinical significance.

Salian *et al.* (2012) compared the effect of PVP and SpermSlow™ incubation on the DNA integrity of fresh and frozen-thawed spermatozoa during conventional ICSI conditions from normozoospermic and oligozoospermic ejaculates. Prepared spermatozoa (after a routine swim up technique) were incubated for 30 minutes and assessed for sperm DNA fragmentation using a single-cell gel electrophoresis assay. They observed a detrimental effect of PVP medium on sperm DNA of oligozoospermic semen samples and a further increase in sperm DNA fragmentation when frozen-thawed spermatozoa from both normozoo- and oligozoospermic semen samples were exposed to PVP. However, SpermSlow™ did not notably induce DNA fragmentation in any of the tested groups. Their results differed from our study. We observed no significant difference in DNA fragmentation (TUNEL analysis) when PVP exposed spermatozoa were compared to the control (Sperm Washing medium). However, similar to Salian *et al.*'s study, SpermSlow™ exposed spermatozoa also showed no significant increased DNA fragmentation when compared to the control. When comparing PVP specifically with SpermSlow™ exposed spermatozoa, the only significant difference Salian *et al.* reported was sperm DNA fragmentation observed in the oligozoospermic frozen-thawed samples. They observed a significant higher extent of DNA fragmentation in the PVP group compared to the SpermSlow™ group. Again the results from our study differ from this result, since we found a significant lower (1.5%) extent of DNA fragmentation in the PVP group compared to the SpermSlow™ group. Although this 1.5% difference was statistically significant, we concluded after equivalence statistical analysis that clinically the

difference was not significant and that the induction of sperm DNA fragmentation by either PVP or SpermSlow™ is equivalent. The reason for the difference in results could possibly be explained by the fact that different sperm DNA fragmentation tests were used.

Rougier *et al.* (2013) compared the changes in sperm DNA fragmentation after incubating prepared spermatozoa (after a routine double-layer gradient) in PVP and SpermSlow™ for different time periods. They used TUNEL staining for the sperm DNA fragmentation evaluation and divided the sample population into two groups; original semen samples with <20% or ≥20% positive TUNEL evaluation. They found a more rapid DNA damage increase over time in samples with ≥20% DNA fragmentation than samples with <20% DNA fragmentation. However, PVP and SpermSlow™ incubation had similar effects and did not differ significantly. Once again, although our study showed a statistically significant, but small (1.5%) decreased DNA fragmentation outcome in the PVP group compared to the SpermSlow™ group, clinically this difference was not significant. We agree with Rougier *et al.*'s findings and conclude that clinically PVP and SpermSlow™ show equivalent DNA fragmentation in spermatozoa when incubated for different time periods.

Another study that also investigated the effect of PVP was that of Ray *et al.* (1995). They tested the effect of PVP on cultured human somatic cells that acted as a model to investigate the mutagenic potential of PVP by sister chromatid exchange analysis prior to use in intracytoplasmic sperm injection procedures. They found that PVP do not cause DNA lesions resulting insisterchromatid exchanges.

As a secondary objective the effect of PVP and SpermSlow™ on human **spermatozoa's ultrastructure** was evaluated using Transmission Electron Microscopy (TEM).

The outcome of the specific comparison between the three mediums (PVP, SpermSlow™ and Sperm Washing medium) was analysed. The sperm characteristics negatively affected by PVP incubation were the nucleus density, presence of large nuclear pale areas, as well as the 9d + 2s pattern of the axoneme principal piece. The sperm characteristics negatively affected by

SpermSlow™ incubation included the cytoplasmic membrane of the neck-piece and mid-piece, most acrosomal features, most nucleus features as well as the 9d + 2s pattern of the axoneme principal piece. The only sperm characteristics not influenced by SpermSlow™ were the mitochondrial helix features. However, these features were also not affected by PVP. Interestingly the SpermSlow™ exposed sperm group showed the lowest amount of partially detached or totally detached acrosomal vesicles when compared to PVP and Sperm Washing medium exposed spermatozoa. SpermSlow™ incubated spermatozoa also showed no significant difference in the amount of nuclear vacuoles when compared to the PVP group. Furthermore, the Sperm Washing medium group showed the highest amount of nuclear vacuoles between the three groups.

Only one previous study by Strehler *et al.* (1998) evaluated the effect of PVP medium on the ultrastructure of human spermatozoa. They found that the sperm characteristics negatively affected by PVP included the acrosomal features (position, shape, dimension, content and presence of reacted acrosomes), some nuclear features (shape, condensation and presence of disrupted chromatin), mitochondria features (shape and helix assembly), some general features of the axoneme (9d + 2s pattern, accessory fibres and fibrous sheath) as well as plasma membrane integrity. It was concluded that PVP incubation led to the disintegration of various sperm membranes that caused the alteration of chromatin and cytoskeletal components.

It is clear that PVP treatment did not nearly affect the ultrastructure of spermatozoa to the same extend in our study as reported by Strehler *et al*. (1998). The reason for the difference in results could be explained by the fact that only one semen sample was analysed in our study, while Strehler andcolleagues analysed a total of 12 semen samples. Another possible reason can be the fact that the sperm population used for these studies differed. Strehler *et al.* only washed the semen samples before PVP incubation, while we did a routine swim up on the semen sample before PVP and SpermSlow™ incubation. We also do not know whether our results represent the total ‘health’ of the sperm population, since the number of spermatozoa analysed for each of the sperm characteristics, varied extensively.

Although not similar to our study, De Leeuw *et al.* (1993) found that the addition of PVP to cryoprotective agents significantly reduced bull sperm membrane integrity after cooling and freezing. Dozortsev *et al.* (1995b) also found that PVP possibly impede sperm nucleus decondensation. In contrast, our study found that PVP exposed spermatozoa expressed significantly less dense nucleus content than the control (Sperm Washing medium).

According to our knowledge, this was the first study that compared the effect of PVP and SpermSlow™ on the ultrastructure of human spermatozoa. We found that PVP incubation (compared to the control) only showed significant differences regarding disrupted sperm for a few sperm components, while SpermSlow™ exposure led to most sperm components being deteriorated (compared to the control). The acrosome, plasma membrane and axoneme characteristics, together with the nucleus, whose condensation was affected, were shown to be the most affected sperm components. It is thought that since SpermSlow™ mainly consists of Hyaluronic acid (HA), spermatozoa bind to this molecule with HA-receptors located on the sperm surface. We speculate that this physiologic reaction may cause sperm membrane destabilization that may lead to sperm membrane breakdown. Membrane breakdowns together with necrotic processes may lead to nuclear deterioration. The deterioration of the axonemal tubules can also be explained as a consequence of the membrane disintegration.

Based on this study's results, either PVP or SpermSlow™ can be used for IMSI Strict™ purposes. However, the study did not include the technical aspects of the usage of PVP and SpermSlow™. In the SpermSlow™ instruction manual it is stated that "SpermSlow™ is not a general immobilising agent (such as PVP); therefore it has quite different characteristics". Comparing the different specifications, shelf life, incubation requirements, cost and ease of use of the two holding solutions (Addendum XII), PVP seems to have better properties than SpermSlow™. PVP's self-life is four times longer than SpermSlow™ and SpermSlow™ is almost three times more expensive than PVP. However, it is important to take into account that our study did not test SpermSlow™'s ability to improve non-fragmented sperm DNA selection. Since SpermSlow™ allows the active selection of

mature spermatozoa when performing ICSI (Parmegiani *et al.*, 2010a) and PVP does not, it can be argued that SpermSlow™ will theoretically be the more obvious choice between the two mediums for IMSI strict™. Using SpermSlow™ will allow the easy application of physiologic IMSI, where spermatozoa can first be selected based on their maturity (ability to bind onto SpermSlow™'s HA) and then be selected based on their morphological appearance.

Although this study did not include any ART procedures, Parmegiani *et al.* (2010a) conducted a study comparing SpermSlow™-ICSI and PVP-ICSI outcomes. A statistical significant improvement in embryo quality and development were observed when oocytes were injected with SpermSlow™-selected spermatozoa compared to PVP-ICSI. A trend towards better fertilization, pregnancy and implantations in the SpermSlow™-ICSI group was observed, however, these findings were not significant. With a large retrospective comparison of SpermSlow™-ICSI versus conventional PVP-ICSI, Parmegiani *et al.* (2010b) confirmed their initial observation. They reported a trend towards better fertilization, pregnancy and abortion rates in the SpermSlow™-ICSI group. Furthermore, injection of SpermSlow™-bound spermatozoa significantly improved embryo quality and implantation rates. In contrast, some authors found no statistically significant differences in fertilization rates, embryo quality, pregnancy rates or implantation rates when comparing SpermSlow™-ICSI with conventional PVP-ICSI treatments (Van Den Berg *et al.*, 2009; Ménézo *et al.*, 2010).

Various limitations and recommendations for future research can be identified. We did not establish the inter- and intra-technician variation for the different sperm parameters tested. Only 29 samples were used for the TUNEL analysis compared to 90 test samples for the sperm viability, motility and CMA_3 analysis. The TUNEL analysis also did not include the 5 minute incubation samples for the 29 test samples. As previously mentioned, only one sample was used for the transmission electron microscopy (TEM) evaluation. This was due to a lack of expertise (evaluation was outsourced to an expert) and funding. Future studies can be improved by including inter- and intra-technician variation for the different sperm parameters tested, analysing more samples with the TUNEL

evaluation, as well as including more TEM analyses. We also recommend that the technical application of PVP and SpermSlow™ in the IMSI Strict™ technique should be investigated before a final recommendation can be made.

From these findings we conclude that although PVP and SpermSlow™ incubated sperm outcomes often differed significantly after statistical analysis, clinically these two mediums were shown to be equivalent for the tested outcomes. These mediums also did not have a clinical detrimental effect on sperm viability, motility parameters, chromatin packaging and DNA fragmentation rate, as seen when compared to the control (Sperm Washing medium). The hypothesis that PVP and SpermSlow™ medium will perform the same in all the tested outcomes can be accepted.

The secondary investigation indicated that SpermSlow™ primarily exerted a disintegrating effect on various sperm membranes, and as a secondary consequence of the eventual necrotic process, alteration of chromatin and cytoskeletal components. PVP medium on the other hand did not show these disintegrating effects. The hypothesis that SpermSlow™ will not be detrimental to sperm ultrastructure can be rejected, although with caution until more samples have been analysed.

The difference in methodology (including semen preparation method), analyses (DNA fragmentation analysis method), sample size and sample type (gamete or somatic cells and human or animal spermatozoa) are some of the possible explanations why our study differed from findings of other authors.

Since there remain many uncertainties and controversies regarding the effect of PVP and SpermSlow™ on human spermatozoa in the literature, we suggest that more investigations on the effect of PVP medium on human spermatozoa are needed. Moreover, we also suggest that the effect of SpermSlow™ on human spermatozoa needs further investigation.

REFERENCES

Agarwal A, Said TM. Sperm chromatin assessment. In: Textbook of ART, 2nd Edition. Gardner DK, Weissman A, Howles CM, Shoham Z (eds). Taylor & Francis Group, PLC, London, UK. 2004. p. 93-106.

Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction.*Fertil Steril* 2003;79:829-843.

Aitken R. The Amoroso lecture.The human spermatozoa – a cell in crisis.*J Reprod Fertil* 1999;115:1-7.

Aitken RJ, Bennetts LE. Reactive oxygen species and their impact on fertility. In: Male Infertility, Diagnosis and Treatment. Oehninger S, Kruger T (eds). Informa UK Ltd. 2007. p. 255-268.

Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa.*Mol Hum Reprod* 2010;16:3-13.

Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online* 2007;14:727–33.

Aitken RJ, Jones KT, Robertson SA. Reactive oxygen species and sperm function -- in sickness and in health. *J Androl* 2012;33:1096-1106.

Aitken RJ, Ross A, Lees MM. Analysis of sperm function in Kartagener's syndrome.*Fertil Steril* 1983;40:696-698.

Akerlöf E, Fredricsson B, Gustafson O, Lunell NO, Nylund L, Rosenburg L, Slotte H, Pousette A. Sperm count and motility influence the result of human fertilization in vitro. *Int J Androl* 1991;14:79-86.

Altman DG, Bland JM. Absence of evidence is not evidence of absence. *Brit Med J* 1995;311:485.

Amann R. Can fertility potential of a seminal sample be predicted accurately? *J Androl* 1989;16:89-98.

American Society of Reproductive Medicine.The clinical utility of sperm DNA integrity testing.*Fertil Steril* 2008;90:178-180.

Antinori M, Licata E, Dani G, Cerusico F, Versaci C, D'Angelo D, Antinori S. Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. *Reprod Biomed Online* 2008;16:835-841.

Apedaile AE, Garett C, Liu DY, Clarke GN, Johnston SA, Baker HWG. Flow cytometry and microscopic acridine orange test: relationship with standard semen analysis. *Reprod Biomed Online* 2004;4:398-407.

Ausubel MF, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Strahl K (eds). Short protocols in Molecular Biology, 2nd Edition.Greene Publishing Association and John Wiley and Sons, New York, USA. 1992.

Avendaño C, Franchi A, Duran H, Oehninger S. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 2010;94:549-557.

Avendaño C, Franchi A, Taylor S, Morshedi M, Bocca S, Oehninger S. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 2009;91:1077–1084.

Avendaño C, Oehninger S. DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era? *J Androl* 2011;32:356-363.

Balaban B, Lundin K, Morrell JM, Tjellström H, Urman B, Holmes PV. An alternative to PVP for slowing sperm prior to ICSI.*Hum Reprod* 2003;18:1887-1889.

Balaban B, Yakin K, Alatas C, Oktem O, Isiklar A, Urman B. Clinical outcome of intracytoplasmic injection of spermatozoa morphologically selected under high magnification: a prospective randomized study. *Reprod Biomed Online* 2011;22:472-476.

Balhorn R. The protamine family of sperm nuclear proteins.*Genome Biology* 2007;8:227.

Barak Y, Ménézo Y, Veia A, Elder K. A physiological replacement for polyvinylpyrrolidone (PVP) in assisted reproductive technology. *Hum Fertil* 2001;4:99-103.

Barak Y, Ménézo Y, Veia A, Kogosowski A, Nevo Z. Hyaluronic acid – a physiological replacement for polyvinylpyrrolidone (PVP) in intracytoplasmic sperm injection (ICSI) procedure. Alpha Symposium.1999.

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

Barroso G, Oehninger S. Contribution of the male gamete to fertilization and embryogenesis. In: Male Infertility, Diagnosis and Treatment. Oehninger S, Kruger T (eds). Informa UK Ltd. 2007. p. 49-72.

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

Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl* 2002;23:1-8.

Bartoov B, Berkovitz A, Eltes F. Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. *New Engl J Med* 2001;345:1067-1068.

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

Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B, Guerin JF. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertil Steril* 2007;87:93-101.

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

Björndahl L, Söderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment.*Hum Reprod* 2003;18:813-816.

Blasuriya A, Speyer B, Serhal P, Doshi A, Harper JC.Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa.*Reprod Biomed Online* 2011;22:428-436.

Boitrelle F, Ferfouri F, Petit JM, Segretain D, Tourain C, Bergere M, Bailly M, Vialard F, Albert M, Selva J. Large human sperm vacuoles observed in motile spermatozoa under high magnification: nuclear thumbprints linked to failure of chromatin condensation. *Hum Reprod* 2011;26:1650-1658.

Boitrelle F, Guthauser B, Alter L, Bailly M, Bergere M, Wainer R, Vialard F, Albert M, Selva J. High-magnification selection of spermatozoa prior to oocyte injection: confirmed and potential indications. *Reprod Biomed Online* 2014;28:6-13.

Bras M, Dumoulin JCM, Pieters MHEC, Michaels AHJC, Geraedts JPM, Evers JLH.The use of a mouse zygote quality control system for training purposes and toxicity determination in an ICSI programme.*Hum Reprod* 1994;9:23-24.

Bronet F, Martinez E, Gaytán M, Liñán A, Cernuda D, Ariza M, Nogales M, Pacheco A, San Celestino M, Garcia-Velasco JA. Sperm DNA fragmentation index does not correlate with the sperm or embryo aneuploidy rate in recurrent miscarriage or implantation failure patients. *Hum Reprod* 2012;27:1922-1929.

Bungum M, Bungum L, Lynch KF, Wedlund L, Humaidan P, Giwercman A. Spermatozoa DNA damage measured by sperm chromatin structure assay (SCSA) and birth characteristics in children conceived by IVF and ICSI. *Int J Androl* 2012;35:485-490.

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

Burrello N, Arcidiacono G, Vicari E, Asero P, Di Benedetto D, De Palma A, Romeo R, D'Agata R, Calogero AE. Morphologically normal spermatozoa of patients with secretory oligo-astheno-teratozoospermia have an increased aneuploidy rate. *Hum Reprod* 2004;19:2298-2302.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 2003;49:49-55.

Cassuto NG, Hazout A, Bouret D, Balet R, Larue L, Benifla JL, Viot G. Low birth defects by deselecting abnormal spermatozoa before ICSI. *Reprod Biomed Online* 2014;28:47-53.

Cassuto NG, Hazout A, Hammoud I, Balet R, Bouret D, Barak Y, Jellad S, Plouchart JM, Selva J, Yazbeck C. Correlation between DNA defects and sperm-head morphology. *Reprod Biomed Online* 2012;24:211-218.

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

Chemes HE, Rawe VY. Sperm pathology: pathogenic mechanisms and fertility potential in assisted reproduction. In: Male Infertility, Diagnosis and Treatment. Oehninger S, Kruger T (eds). Informa UK Ltd. 2007. p. 85-103.

Chen SU, Ho HN, Chen HF, Huang SC, Lee TT, Yang YS. Intracytoplasmic sperm injection (ICSI) for severe semen abnormalities: dissecting the tail of spermatozoa at the tip. *Hum Reprod* 1996;11:2640-2644.

Chi HJ, Chung DY, Choi SY, Kim JH, Kim GY, Lee JS, Lee HS, Kim MH, Roh SI. Integrity of human sperm DNA assessed by the natural comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program. *Clin Exp Reprod Med* 2011;38:10-17.

Clarke RN, Johnson LA. Factors related to successful sperm microinjection of hamster eggs: the effect of sperm species, technical experience, needle dimensions, and incubation medium on egg viability and sperm decondensation following microinjection. *Theriogenology* 1988;30:447-460.

Cleine JH. Fertilization / theory.In IVF, laboratory aspects of in-vitro fertilization. Bras M, Lens JW, Piederiet MH, Rijnders PM, Verveld M, Zeilmaker GH (eds). Organon. 1996. p. 127-145.

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

Cohen J, Malter H, Fehilly C, Wright G, Elsner C, Kort H, Massey J. Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. *Lancet* 1988;2:162.

Cohen J, Scott R, Alikani M, Schimmel T, Munné S, Levron J, Wu L, Brenner C, Warner C, Willadsen S. Ooplasmic transfer in mature human oocytes. *MolHum Reprod* 1998;4:269-280.

Cohen KR, Griffin JT, Carrell DT.Evaluation of chromatin integrity in human sperm using acridine orange staining with different fixatives and after cryopreservation.*Andrologia* 2004;36:321-326.

Cohen-Bacrie P, Belloc S, Ménézo YJR, Clement P, Hamidi J, Benkhalifa M. Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients. *Fertil Steril* 2009;91:1801-1805.

Comhaire FH, Chrisophe AB, Zalata AA, Dhooge WS, Mahmoud AM, Depuydt CE. The effect of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men.*Prostag Leukot Ess* 2000;63:159-165.

Cooper TG and Yeung CH. Computer-aided evaluation of assessment of “grade a” spermatozoa by experienced technicians.*Fertil Steril* 2006;85:220-224.

Dale B. Fertilization. In: Comprehensive Human Physiology, Vol 2. Greger R, Windhorst U (eds). Springer-Vertag, Berlin, Germany. 1996. p. 2265.

Davies MJ, Moore VM, Willson KJ, Van Essen P, Priest K, Scott H, Haan EA, Chan A. Reproductive technologies and the risk of birth defects. *N Engl J Med* 2012;366:1803-1813.

De Leeuw FE, De Leeuw AM, Den Daas JH, Colenbrander B, Verkleij AJ. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing.*Cryobiology* 1993;30:32-44.

De Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG, Nyboe Anderson A. Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. *Hum Reprod* 2010;25:1851-1862.

De Vos A, Polyzos NP, Verheyen G, Tournaye H. Intracytoplasmic morphologically selected sperm injection (IMSI): a critical and evidence-based review. *Basic Clin Androl* 2013;23:10.

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

Debrock S, Spiessens C, Afschrift H, Van der Auwere I, D'Hooghe TM. Application of the Fertilase-laser system versus the conventional mechanical method to immobilize spermatozoa for intracytoplasmic sperm injection: a randomised controlled trial. *Gynecol Obstet Invest* 2003;56:102-105.

Devroey P, Van Steirteghem A. A review of ten years experience of ICSI.*Hum Reprod Update* 2004;10:19-28.

Dona G, Flore C, Tibaldi E, Frezzato F, Andrisani A, Ambrosini G, Florentin D, Armanini D, Bordin L, Clari G. Endogenous reactive oxygen species content and modulation of tyrosine phosphorylation during sperm capacitation. *Int J Androl* 2011;34:411-419.

Doshi SV, Khullar K, Sharma RK, Agarwal A. Role of reactive nitrogen species in male infertility. *Reprod Biol Endocrin* 2012;10:109

Dozortsev D, De Sutter P, Rybouchkin A, Dhont M. Oocyte activation and ICSI. *Assist Reprod Rev*1995a;5:32-39.

Dozortsev D, Rybouchkin A, De Sutter P, Dhont M. Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. *Hum Reprod* 1995b;10:2960-2964.

Dozortsev D, Rybouchkin A, De Sutter P, Qian C, Dhont M. Fertilization and early embryology: Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. *Hum Reprod* 1995c;10:403-407.

Drobnis EZ, Yudin AI, Cherr GN, Katz DF. Kinematics of hamster sperm during penetration of the cumulus cell matrix.*Gamete Res* 1988;21:367-383.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP, Evers JL. Effect of in vitro culture of human embryos on birthweight of newborns.*Hum Reprod* 2010;25:605-612.

Dupont C, Faure C, Sermondade N, Boubaya M, Eustache F, Clement P, Briot P, Berthaut I, Levy V, Cedrin-Durnerin I, Benzacken B, Chavatte-Palmer P, Levy R. Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl*2013;15:622–625.

Duran E, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002;17:3122-3128.

Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. *Hum Reprod*2004;19:1837–1841.

Esterhuizen AD, Franken DR, Lourens JGH, Prinsloo E, van Rooyen LH. Sperm chromatin packaging as an indicator of in vitro fertilization rates. *Hum Reprod* 2000;15:657-661.

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

Fenichel P, Donzeau M, Cervoni F, Menezo Y, His BL. Expression of complement regulatory proteins on human eggs and preimplantation embryos.*Am J Reprod Immunol* 1995;33:155-164.

Fernández JL, Dela Calle JFV, Tamayo M, Cajigal D, Agarwal A, Gosálves J. Sperm DNA integrity and male infertility: current perspectives. *Arch Med Sci* 2008;4:1-8.

Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, LaFromboise M, De Jong C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 2005;84:833-842.

Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *J Androl* 2003;24:59-66.

Fishel S, Lisi F, Rinaldi L, Green S, Hunter A, Dowell K, Thornton S. Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. *Hum Reprod* 1995;10:497-500.

Frydman N, Prisant N, Hesters L, Frydman R, Tachdjian G, Cohen-Bacrie P, Fanchin R. Adequate ovarian folicular status does not prevent the decrease in pregnancy rates associated with high sperm DNA fragmentation. *Fertil Steril* 2008;89:92-97.

Gallegos G, Ramos B, Santiso R, Goyanes V, Gosalvez J. Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. *Fertil Steril* 2008;90:328-334.

Garcia-Peiró A, Martinez-Heredia J, Oliver-Bonet M, Abad C, Amengual MJ, Navarro J, Jones C, Coward K, Gosálves J, Benet J. Protanime 1 to protamine 2 ration correlates with dynamic aspects of DNA fragmentation in human sperm. *Fertil Steril* 2011;95:105-109.

Gerris J, Mangelschots K, van Royen E, Joostens M, Estermans W, Ryckaert G. ICSI and severe male factor infertility: breaking the sperm tail prior to injection. *Hum Reprod* 1995;10:484-504.

Gerris V, Khan I. Correlation between in vitro fertilization and human sperm density and motility. *J Androl* 1987;8:48-54.

Geva E, Lessing JB, Lerner-Geva L, Amit A. Free radicals, antioxidants and human spermatozoa: clinical implications. *Hum Reprod* 1998;13:1422-1424.

Gonzalez-Martin C, Gosalvez J, Roy R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *Int J Mol Sci* 2012;13:14026-14052.

Gorczyca W, Gong J, Darzynkiewics Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993a;53:1945-1951.

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

Greco E, Romano S, Iacobelli M, Ferrero S, Beroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. *Hum Reprod* 2005a;20:2590-2594.

Greco E, Scarselli F, Fabozzi G, Colasante A, Zavaglia D, Alviggi E, Litwicka K, Varricchio MT, Minasi MG, Tesarik J. Sperm vacuoles negatively affect outcomes in intracytoplasmic morphologically selected sperm injection in terms of pregnancy, implantation, and live-birth rates. *Fertil Steril* 2013;100:379-385.

Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C, Tesarik J. Efficient treatment in infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod* 2005b;20:226-230.

Harari O, Bourne H, Baker G, Gronow M, Johnston I. High fertilization rate with intracytoplasmic sperm injection in mosaic Klinefelter's syndrome. *Fertil Steril* 1995;63:182-184.

Hazout A, Bumont-Hassan M, Junca AM, Cohen Bacrie P, Tesarik J. High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. *Reprod Biomed Online* 2006;12:19-25.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004;81:965-972.

Henkel R, Kierspel E, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Schill W, Kruger TF. DNA fragmentation of spermatozoa and assisted reproduction technology.*Reprod Biomed Online* 2003;7:477-484.

Henkel R. Detection of DNA damage in sperm. In: Male infertility, Diagnosis and Treatment. Oehninger SC, Kruger TF (eds). Informa UK Ltd. 2007a. p. 225–238.

Henkel R. DNA fragmentation and its influence on fertilization and pregnancy outcome. In: Male infertility, Diagnosis and Treatment. Oehninger SC, Kruger TF (eds). Informa UK Ltd. 2007b. p. 277–290.

Hiramoto Y. Microinjection of the live spermatozoa into sea urchin eggs.*Exp Cell Res* 27, 416-426.

Hirano Y, Shinahara H, Obara H, Suzuki T, Takamizawa S, Yamaguchi C, Tsunoda H, Sato I. Andrology: Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. *J Assist Reprod Genet* 2001;18:215-220.

Hiraoka K, Kitamura S, Kuwayama M. Effect of sperm head first or sperm tail first injection into ooplasm on oocyte survival, fertilization and embryo development with piezo-ICSI in human oocytes. *Fertil Steril* 2014;102:e319.

Hlinka D, Herman M, Vesela J, Hredzak R, Horvath S, Pacin J. A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone.*Hum Reprod* 1998;13:1922-1927.

Homa S, Swann K. A cytosolic sperm factor triggers calcium oscillation and membrane hyperpolarizations in human oocytes. *Hum Reprod* 1994;9:2356-2361.

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

Hosoi T, Miyake M, Utsumi K, Iritani A. Development of rabbit oocytes after microinjection of spermatozoon. In: Proceeding of the 11th Congress on Animal Reproduction and Artificial Insemination. University College Dublin, Ireland. 1988. p. 331-333.

HT-CASA II Software Manual, Version 1.1. Hamilton Thorne Inc. 2013. p. 4-31.

Huang CC, Lin DPC, Tsao HM, Cheng TC, Liu CH, Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. *Fertil Steril* 2005;84:130-140.

Huszar G, Celik-Ozenci C, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril* 2003;79:1616-1624.

IMSI Strict™ brochure [Online] [s.a.] [access 2015, February 2015]; Available: http://www.hamiltonthorne.com/index.php/imsi-strict-brochures.

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

Jean M, Mirallié S, Boudineau M, Tatin C, Barriére P. Intracytoplasmic sperm injection with polyvinylpyrrolidone: a potential risk. *Fertil Steril* 2001;76:419-420.

Jégou B, Pineau C, Toppari J. Spermatogenesis in vitro in mammals. In: Assisted Reproductive Technology – Accomplishments and Horizons. De Jonge CJ, Barratt CLR (eds). Cambridge University Press, UK. 2002. p. 3-5.

Jones B, Jarvis P, Lewis JA, Ebbutt AF. Trials to assess equivalence: the importance of rigorous methods. *Brit Med J* 1996;313:36-39.

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

Katayama M, Koshida M, Miyake M. Fate of the acrosome in ooplasm in pigs after IVF and ICSI. *Hum Reprod* 2002;17:2657-2664.

Kato Y, Nagao Y. Effect of polyvinylpyrrolidone on sperm function and early embryonic development following intracytoplasmic sperm injection in human assisted reproduction.*Reprod Med Biol* 2012;11:165-176.

Kato Y, Nagao Y. Effect of PVP on sperm capacitation status and embryonic development in cattle.*Theriogenology* 2009;72:624-635.

Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Kazerooni Y, Zolghadr J. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Gen* 2009;26:591-596.

Knez K, Zorn B, Tomazevic T, Vrtacnik-Bokal E, Virant-Klun I. The IMSI procedure improves poor embryo development in the same infertile couples with poor semen quality: a comparative prospective randomized study. *Reprod Biol Endocrin* 2011;9:123.

Kopf GS, Gerton GL.The mammalian sperm acrosome and the acrosome reaction. In: Elements of Mammalian Fertilization. Wasserman PM (ed). CRC Press, Boston, USA. 1991. p 153-203.

Kruger TF, Coetzee K. The role of sperm morphology in assisted reproduction. *Hum Reprod* 1996;5:172-178.

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

Kumar K, Deka D, Singh A, Mitra DK, Vanitha BR, Dada R. Predictive value of DNA integrity analysis in idiopathic recurrent pregnancy loss following spontaneous conception. *J Assist Reprod Gen* 2012;29:861-867.

Laberge R-M, Boissonneault G. Nature and origin of transient DNA strand breaks during spermiogenesis. *International Congress Series* 2004;1271:189-192.

Lanzendorf S, Maloney M, Ackerman S. Fertilizing potential of acrosome-defective sperm following microsurgical infection into eggs. *Gamete Res* 1988;19:329-337.

Larson KL, De Jonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod* 2000;15:1717-1722.

Lazaros LA, Vartholomatos GA, Hatzi EG, Kaponis AI, Makrydimas GV, Kalantaridou SN, Sofikitis NV, Stefos TI, Zikopoulos KA, Georgiou IA. Assessment of sperm chromatin condensation and ploidy status using flow cytometry correlates to fertilization, embryo quality and pregnancy following in vitro fertilization. *J Assist Reprod* 2011;28:885-891.

Leduc F, Nkoma GB, Boissonneault G. Spermiogenesis and DNA repair: a possible etiology of human infertility and genetic disorders. *Syst Biol Reprod Med* 2008;54:3-10.

Lewis SEM, Agbaje I. Sperm DNA test as useful adjuncts to semen analysis. *Syst Biol Reprod Med* 2008;54:111-125.

Lewis SEM, Aitken RJ, Conner SJ, Iuliis GD, Evenson DP, Henkel R, Giwercman A, Gharagozloo P. The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment. *Reprod Biomed Online* 2013;27:325-337.

Lewis SEM, Simon L. Clinical implications of sperm DNA damage. *Hum Fertil* 2010;13:201-207.

Li Z, Wang L, Cai J, Huang H. Correlation of sperm DNA damage with IVF and ICSI outcome: a systematic review and meta-analysis. *J Assist Reprod Genet* 2006;23:367-376.

Lin MH, Kuo-Kuang Lee R, Li SH, Lu CH, Sun FJ, Hwu YM. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril* 2008;90:352-359.

Lin TP. Microinjection of mouse eggs.*Science* 1966;151:333-337.

Liu DY, Clarke GN, Baker HW. Tyrosine phosphyrylation on capacitated human sperm tail detected by immune-fluorescence correlates strongly with sperm-zona pellucida (ZP) binding but not with the ZP-induced acrosome reaction.*Hum Reprod* 2006;21:1002-1008.

Lolis D, Georgiou I, Syrrou M, Zikopoulos K, Konstantelli M, Messinis I. Chromomycin A3- staining as an indicator of protamine deficiency and fertilization. *Int J Androl* 1996;19:23-27.

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

Loutradi KE, Tarlatzis BC, Goulis DG, Zepiridis L, Pagou T, Chatziioannou E, Grimbizis GF, Papadimas I, Bontis I. The effect of sperm quality on embryo development after intracytoplasmic sperm injection.*J Assist Reprod Genet* 2006;23:69-74.

Loutradis D, Drakakis P, Kallianidis K, Milingos S, Dendrinos S, Michalas S. Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection. *Fertil Steril* 1999;72:240-244.

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

Mansour RT, Aboulghar MA, Serour GI, Amin YM, Ramzi AM. The effect of sperm parameters on the outcome of intracytoplasmic sperm injection.*Fertil Steril* 1995;64:982-986.

Mansour RT, Serour MG, Abbas AM, Kamal A, Tawab NA, Aboulghar MA, Serour GI. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: a controlles randomized study. *Fertil Steril* 2008;90:584-591.

Maratori M, Tamburrino L, Tocci V, Constantino A, Marchiani S, Giachini C, Laface I, Krausz C, Mariggiola MC, Forti G, Baldi E. Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl* 2010;31:336-345.

Mauri AL, Petersen CG, Oliveira JB, Massaro FC, Baruffi RL, Franco JG Jr. Comparison of the day 2 embryo quality after conventional ICSI versus intracytoplasmic morphologically selected sperm injection (IMSI) using sibling oocytes. *Eur J Obstet Gynecol Reprod Biol* 2010;150:42-46.

Ménézo Y, Junca AM, Dumont-Hassan M, De Mouzon J, Cohen-Bacrie P, Ben Khalifa M. “Physiologic” (hyaluronic acid-carried) ICSI results in the same embryo quality and pregnancy rates as with the use of potentially toxic polyvinylpyrrolodone (PVP). *Fertil Steril* 2010;94:S232.

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

Meseguer M, Santiso R, Garrido N, Garcia-Herrero S, Remohi J, Fernandez JL. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertil Steril* 2011;95:124-128.

Mieusset R, Bujan L, Mondinat C, Mansat A, Pontonnier F, Grandjean H. Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertil Steril* 1987;48:1006–1010.

Mortimer D. Objective analysis of sperm motility and kinematic. In: The Handbook of the Laboratory Diagnosis and Treatment of Infertility. Keel BA, Webster BW (eds). CRC Press, Boca Raton, Florida, USA. 1989. p. 97.

Mortimer S. CASA – Practical aspects. *J Androl* 2000;21:515-524.

Mortimer ST, Mortimer D. Kinematics of human spermatozoa incubated under capacitating conditions. *J Androl* 1990;11:195-203.

Moskovtsev SI, Willis J, White J,Mullen JB. Leukocytospermia: relationship to sperm deoxyribonucleic acid integrity in patients evaluated for male factor infertility. *Fertil Steril* 2007;88:737-740.

Motoishi M, Goto K, Tomita K, Ookutsu S, Nakanishi Y. Examination of the safety of intracytoplasmic injection procedures by using bovine zygotes. *Hum Reprod* 1996;11:618-620.

Mundy AJ, Ryder TA, Edmonds DK.A quantitative study of sperm head ultrastructure in subfertile males with excess sperm precursors.*Fertil Steril* 1994;61:751-754.

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim up selected human spermatozoa during long term incubation. *J Androl* 2003;24:253-262.

Muratori M, Tamburrino L. Tocci V, Costantino A, Marchiani S, Giachini C, Laface I, Krausz C, Meriggiola MC, Forti G, Baldi E. Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl* 2010;31:336-345.

Nagy ZP, Liu H, Joris H, Verheyen G, Trournaye H, Camus M, Derde MC, Devroey P, Van Steirteghem AC. Andrology: The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum Reprod* 1995;10:1123-1129.

Nagy ZP, Verheyen G, Tournaye H, Van Steirteghem AC. Special applications of intracytoplasmic sperm injection: the influence of sperm count, motility, morphology, source and sperm antibody on the outcome of ICSI. *Hum Reprod* 1998;13:143-154.

Nakamura T, Sakai T, Hotchi M. Histochemical demonstration of DNA double strand breaks by in situ 3'-tailing reaction in apoptotic endometrium. *Biotech Histochem* 1995;70:33-39.

Nasr-Esfahani MH, Razavi S, Vahdati AA, Fathi F, Tavalaee M. Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. *J Assist Reprod Genet* 2008;25:197-203.

Nassar A, Mahony M, Morshedi M, Lin MH, Srisombut C, Oegninger S. Modulation of sperm tail protein tyrosine phosphorylation by pentoxifylline and its correlation with hyperactivated motility. *Fertil Steril* 1999;71:919-923.

Ng SC, Bongso A, Ratnam SS, Sathananthan H, Chan CL, Wong PC, Hagglund L, Anandakumar C, Wong YC, Goh VH. Pregnancy after transfer of sperm under zona.*Lancet* 1988;2:790.

NHS Lothian. Intracytoplasmic sperm injection. [Online] [s.a.] [access 2015, February 11]; Available: http://www.nhslothian.scot.nhs.uk/Services/A-Z/EdinburghAssistedConception Programme/Pages/ICSI.aspx.

Oleszczuk K, Giwercman A, Bungum M. Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples. *Hum Reprod* 2011;26:3244-3248.

Oliveira JB, Cavagna M,Petersen CG, Mauri AL, Massaro FC, Silva LF, Baruffi RL, Franco JG Jr. Pregnancy outcome in women with repeated implantation failure after intracytoplasmic morphologically selected sperm injection (IMSI). *Reprod Biol Endocrin* 2011;9:99.

Olivia R. Protamines and male infertility.*Hum Reprod Update* 2006;12:417-435.

Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, Evenson D, Thomas Jr AJ, Alvarez JG. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod*2001;16:1912–1921.

Ortega C, Verheyen G, Raick D, Camus M, Devroey P, Tournaye H. Absolute asthenozoospermia and ICSI: what are the options? *Hum Reprod Update* 2011;17:684-692.

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

Palermo GD, Avrech OM, Colombero LT, Wu H, Wolny YM, Fissore RA, Rosenwaks Z. Human sperm sytosolic factor triggers Ca^{2+} oscillations and overcome activation failure of mammalian oocytes. *MolHum Reprod* 1997;3:367-374.

Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Development and implementation of intracytoplasmic sperm injection (ICSI). *Reprod Fertil Dev* 1995a;7:211-218.

Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. *Fertil Steril* 1995b;63:1231-1240.

Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: Where we have been and where we are going. *Semin Reprod Med* 2009;27:191-202.

Palermo GD, Schlegel PN, Colombero LT, Zaninovic N, Moy F. Rosenwaks Z. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum Reprod* 1996;11:1023-1029.

Parmegiani L, Cognigni GE, Bernardi S, Troili E, Ciampaglia W, Filicori M. “Physiological ICSI”: Hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril* 2010a;93:598-604.

Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Taraborrelli S, Arnone A, Maccarini AM, Filicori M. Comparison of two ready-to-use systems designed for sperm-hyaluronic acid binding selection before intracytoplasmic sperm injection: PICSI vs. Sperm Slow: a prospective, randomized trial. *Fertil Steril* 2012;98:632-637.

Parmegiani L, Cognigni GE, Ciampaglia W, Pocognoli P, Marchi F, Filicori M. Efficiency of hyaluronic acid (HA) sperm selection. *J Assist Reprod Genet* 2010b;27:13-16.

Partial zona dissection (PZD), Subzonal insemination (SUZI) and Intracytoplasmic sperm injection (ICSI) techniques illustration [Online] [s.a.] [access 2014, February 20]; Available: http://www.sotiriskoukos.gr/content.php?content_id=54&lg= .

Pedersen H. Ultrastructure of the ejaculated human sperm.*Z Zellforsch Mik Ana* 1969;94:542-554.

Perdrix A, Travers A, Chelli MH, Escalier D, Do Rego JL, Milazzo JP, Mousset-Simeon N, Mace B, Rives N. Assessment of acrosome and nuclear abnormalities in human spermatozoa with large vacuoles. *Hum Reprod* 2011;26:47-58.

Perrington J, Swann K, Shevchenko VI, Sesay AK, Lai A. Calcium oscillations in mammalian egg triggered by a soluble sperm protein. *Nature* 1996;379:364-368.

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

Pons I, Cercas R, Villas C, Braña C, Fernández-Shaw S. One abstinence day decreases sperm DNA fragmentation in 90% of selected patients. *J Assist Reprod Gen* 2013;30:1211-1218.

Practice Committee of the American Society for ReproductiveMedicine. The clinical utility of sperm DNA integrity testing: a guideline. *Fertil Steril* 2013;99:673-677.

Rawe VY, Boudria HU, Alvarez Sedóa C, Cassoa M, Papiera S, Nodara F. Healthy baby born after reduction of sperm DNA fragmentation using cell sorting before ICSI. *Reprod Biomed Online* 2010;20:320-323.

Ray BD, Hawell RT, McDermott A, Hull MGR. Testing the mutagenic potential of polyvinylpyrrolidone and methyl cellulose by sister chromatid exchange analysis prior to use in intracytoplasmic sperm injection procedures. *Hum Reprod* 1995;10:436-438.

Redgrove KA, Aitken RJ, Nixon B. More than a simple lock and key mechanism: unraveling the intricacies of sperm-zona pellucida binding. In: Binding protein. Abhdelmosen K (ed). Intech, Open acess book. 2012. p. 73-122.

Riensi L, Ubaldi F, Martinez F, Iacobellin M, Minasi MG, Ferrero S, Tesarik J, Greco E. Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI. *Hum Reprod* 2003;18:1289-1293.

Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis SEM, Kirkman-Brown J, Coomarasamy A. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 2012;27:2908-2917.

Rougier N, Uriondo H, Papier S, Checa MA, Sueldo C, Alvarez Sedó C. Changes in DNA fragmentation during sperm preparation for intracytoplasmic sperm injection over time. *Fertil Steril* 2013;100:69-74.

Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, Perreault SD. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Hum Reprod* 2005;20:2776–2783.

Saha S, Otoi T, Takagi M, Boediono A, Suzuki T. Normal calves obtained after direct transfer of vitrified bovine embryos using ethylene glycol, trehalose, and polyvinylpyrrolidone. *Cryobiology*1996;33:291-299.

Said TM, Agarwal A, Zborowski M, Grunewald S, Glander HJ, Paasch U. Utility of magnetic cell separation as a molecular sperm preparation technique. *J Androl* 2008;29:134-142.

Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. *Hum Reprod Update* 2011;17:719-733.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanism of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010;93:1027-1036.

Sakkas D, Manicardi G, Bianchi PG, Bizzaro D, Bianchi U. Relationship between presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol Reprod* 1995;52:1149-1155.

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

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

Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y, Campana A. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum Reprod* 1998;13:11-19.

Salian SR, Kalthur G, Uppangala S, Kumar P, Adiga SK. Frozen-thawed spermatozoa from oligozoospermic ejaculates are susceptible to in situ DNA fragmentation in polyvinylpyrrolidone-based sperm-immobilization medium. *Fertil Steril* 2012;98:321-325.

Saling PM, Sowinski J, Storey BT. An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae in vitro: sequential relationship to the acrosome reaction. *J Exp Zool* 1979;209:229-38.

Sanner AHF, Straub F. Polymers of N-vinylpyrrolidone: synthesis, characterization and uses. *Polym J* 1985;17:143-152.

Sathananthan AH, Ng SC, Bongso A, Trounson AO, Ratnam SS (eds). Visual Atlas of Early Human Development for Assisted Reproductive Technology.National University of Singapore, Singapore. 1993.

Sathananthan AH, Ng SC, Jackson P, Dharmawardena V, Trounson AO. Sperm penetration, incorporation, fertilization and microfertilization in assisted reproduction. In: Visual Atlas of Human Sperm Structure and Function for Assisted Reproductive Technology. Sathananthan AH (ed). Serono, Singapore, 1996a. p. 193.

Sathananthan AH, Ng SC, Trounson AO, Laws-King A, Bongso A, Ratnam SS. Human microfertilization by injection of single or multiple sperm: ultrastructure. *Hum Reprod* 1989;4:574.

Sathananthan AH, Trounson A. Ultrastructure of ICSI. In: Handbook of In Vitro Fertilization, Second Edition. Trounson A, Gardner D (eds). CRC Press LLC, Florida, USA. 1999. p. 467.

Sathananthan AH, Trounson AO. Cortical granule release and zona interaction in monospermic and polyspermic human ova fertilized in vitro. *Gamete Res* 1982;6:225.

Sathananthan AH. Mitosis in the human embryo: the vital role of the sperm centrosome (centriole) – Review. *Histol Histopath.* 1997;12:827.

Sathananthan AH. The paternal centrosome: its role in human embryonic development and infertility (a recent discovery). In:Current Issues in Obstetrics and Gynaecology. Arulkumaran A, Ng SC (eds). Oxford University Press, Singapore. 1996b. p. 101.

Sathananthan AH.Ultrastructure of human gametes, fertilization and embryo development. In: Handbook of In Vitro Fertilization, Second Edition. Trounson A, Gardner D (eds). CRC Press LLC, Florida, USA. 1999. p. 431.

Sauer MV, Chang P. Establishing a clinical program for human immunodeficiency virus 1-seropositive med to father seronegative children by mean of in vitro fertilization with intracytoplasmic sperm injection.*Am J Obstet Gynecol* 2002;186:627-633.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* 2002;129: 3533-3544.

Schatten H, Sun Q-Y. The role of centrosomes in mammalian fertilization and its significance for ICSI.*Mol Hum Reprod* 2009;15:531-538.

Schill WB, Topfer-Petersen E, Heissler E. The sperm acrosome: functional and clinical aspects. *Hum Reprod* 1988;3:139-145.

Schmid T, Eskenazi B, Baumgartner A, Marchetti F, Young S, Weldon R, Anderson D, Wyrobek A. The effects of male age on sperm DNA damage in healthy non-smokers. *Hum Reprod* 2006;22:180–187.

Schulte RT, Ohl AQ, Sigman M, Smith GD. Sperm DNA damage in male infertility: etiologies, assays, and outcomes. *J Assist Reprod Gen* 2010;27:3-12.

Setti AS, Figueira RD, Braga DP, Iaconelli A Jr, Borges E Jr. Intracytoplasmic morphologically selected sperm injection benefits for patients with oligoasthenozoospermia according to the 2010 World Health Organization reference values. *Fertil Steril* 2011;95:2711-2714.

Setti AS, Paes de Almeida Ferreira Braga D, Iaconelli A Jr, Aoki T, Borges E Jr. Twelve years of MSOME and IMSI: a review. *Reprod Biomed Online* 2013;27:338-352.

Shafie Mel, Sousa M, Windt ML, Kruger TF (eds). An Atlas of the ultrastructure of human oocytes: A guide for assisted reproduction. CRC Press, New York, USA. 2000.

Shafik A, Shafik AA, Shafik I, El Sibai O. Sperm DNA Fragmentation. *Arch Andrology* 2006;52:197-208.

Shamsi MB, Kumar R, Bhatt A, Bamezai RN, Kumar R, Gupta NP, Das TK, Dada R. Mitochondrial DNA mutations in etiopathogenesis of male infertility. *Indian J Urol* 2008;24:150-154.

Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology* 1996;48:835-850.

Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A. TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology* 2010;76:1380-1386.

Shen S, Khabani A, Klein N, Battaglia D. Statistical analysis of factors affecting fertilization rates and clinical outcome associated with intracytoplasmic sperm injection. *Fertil Steril* 2003;79:355-360.

Shoukir Y, Chardonnes D, Campana A, Sakkas D. Blastocyst development from supernumerary embryos afterintracytoplasmic sperm injection: a paternal influence? *Hum Reprod* 1998;13:1632-1637.

Silva CP, Kommineni K, Oldenbourg R, Keefe DL. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertil Steril* 1999;71:719-721.

Simon L, Liu L, Murphy K, Ge S, Hotaling J, Aston KI, Emery B, Carrell DT. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment.*Hum Reprod* 2014;29:904-917.

Simon L, Lutton D, McManus J, Lewis SE. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril* 2011;95:652-657.

Sousa APM, Tavares RS, De la C, Alle JVF, Figueiredo H, Almeida V, Almeida-Santos T, Ramalho-Santos J. Duel use of Diff-Quik-like stains for the simultaneous evaluation of human sperm morphology and chromatin status. *Hum Reprod* 2009;24:28-36.

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

Strassburger D, Friedler S, Raziel A, Schachter M, Kasterstein E, Ron-El R. Very low sperm count affects the result of intracytoplasmic sperm injection. *J Assist Reprod Gen 2000;17:431-436.*

Strehler E, Baccetti B, Sterzik K, Captani S, Collodel G, De Santo M, Gambera L, Piomboni P. Detrimental effects of polyvinylpyrrolidone on the ultrastructure of spermatozoa (Notulae seminologicae 13). *Hum Reprod* 1998;13:120-123.

Suh TK, Moon JS, Kim KC, Lee TH, Chun SS. Effect of head or tail first sperm injection into

oocytes in intracytoplasmic sperm injection. *Fertil Steril* 1997;1001:152.

Sun J, Jurisicova A, Casper R. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol Reprod* 1997;56:602-607.

Sutovsky P, Van Leyen K, McCauley T, Day BN, Sutovsky M. Degradation of paternal mitochondria after fertilization: implications for heteroplasmy, assisted reproduction technologies and mtDNA inheritance. *Reprod Biomed Online* 2004;8:24-33.

Svalander P, Forsberg AS, Jakobsson AH, Wikland M. Factors of importance for the establishment of a successful program of intracytoplasmic sperm injection treatment for male infertility.*Fertil Steril* 1995;63:828-837.

Swenson CE and Dunbar BS.Specificity of sperm-zona interaction.*J Exp Zool* 1982;219:97-104.

Takano H, Yanagimachi R, Urch UA. Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with the oolemma: inhibitor studies using the golden hamster. *Zygote* 1993;1:79-91.

Takeuchi T, Colombero LT, Neri QV, Rosenwaks Z, Palermo GD. Does ICSI require acrosomal disruption? An ultrastructural study.*Hum Reprod* 2004;19:114-117.

Tanaka A, Nagayoshi M, Tanaka I, Kusunoki H. Human sperm head vacuoles are physiological structures formed during the sperm development and maturation process. *Fertil Steril* 2012;98:315-320.

Tarlatzis BC,Bili H. Survey on intracytoplasmic sperm injection: report from the ESHRE ICSI Task Force. European Society of Human Reproduction and Embryology.*HumReprod*1998;13:165-77.

Teflon Printed diagnostic slide photograph[Online] [s.a.] [access 2014, November 2]; Available: http://www.2spi.com/catalog/new/ptfesld.shtml

Teixeira DM, Barbosa MAP, Ferriani RA, Navarro PA, Raine-Fenning N, Nastri CO, Martins WP. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction.*Cochrane Db Syst Rev* 2013;Issue 7:Art N:CD010167.

Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004;19:611-615.

Tesarik J, Mandoza C, Greco E. The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertil Steril* 2000;74:1245-1247.

Tesarik J, Sousa M, Testart J. Human oocyte activation after intracytoplasmic sperm injection.*Hum Reprod* 1994;9:511-518.

Tesarik J, Sousa M. Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. *Fertil Steril* 1995;64:770-776.

Tesarik J. Topographical relations of intramembrane particle distribution patterns in human sperm membranes.*J Ultrastruct Res* 1984;89:42.

Thaler CD and Cardullo RA.Distinct membrane fractions from mouse sperm bind different zona pellucida glycoproteins. *Biol Reprod* 2002;66:65-9.

Thomson LK, Zieschang JA, Clark AM. Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. *Fertil Steril* 2011;4:843-847.

Tomlinson M, Turner J, Powell G, Sakkas D. One-step disposable chambers for sperm concentration and motility assessment: how do they compare with the World Health Organization's recommended methods? *Hum Reprod* 2001;16:121-124.

Tsai MY, Huang FJ, Kung FT, Lin YC, Chang SY, Wu JF, Chang HW. Influence of polyvinylpyrrolidone on the outcome of intracytoplasmic sperm injection. *J Reprod Med* 2000;45:115-120.

Uehara T, Yanagimachi R. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol Reprod.* 1976;15:467-470.

Valavanidis A, Vlachogianni T, Fiotakis K. Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. *Int J Environ Res Public Health*2009;6:445–462.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44-84.

Van den Bergh A, Bertrand E, Biramane J, Englert Y. Importance of breaking a spermatozoon's tail before intracytoplasmic injection: a prospective randomized trial. *Hum Reprod* 1995;10:2819-2820.

Van den Bergh M, Fahy-Deshy M, Hohl MK. Pronuclear zygote score following intracytoplasmic injection of hyaluronan-bound spermatozoa: a prospective randomized study. *Reprod Biomed Online* 2009;19:796-801.

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

Van Steirteghem AC, Naagy ZP, Liu J, Joris H, Smitz J, Camus M, Devroey P, Bonduelle M. Intracytoplasmic sperm injection – ICSI. *Reprod Med Rev* 1994;3:199-207.

Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smitz J, Wisanto A, Devroey P. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod* 1993;8:1061-1066.

Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI failure. *Reprod Biomed Online* 2014;28:560-571.

Vanderzwalmen P, Bertin G, Lejeune B, Nijs M, Vandamme B, Schoysman R. Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa after rupture of the oolemma. *Hum Reprod* 1996;11:540-547.

Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A, Uher P, Zintz M, Lejeune B, Vanderzwalmen S, Cassuto G, Zech NH. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online* 2008;17:617-627.

Varghese AC, Bragais FM, Mukhopadhyay D, Kundu S, Pal M, Bhattacharyya AK, Agarwal A. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Andrologia* 2009;41:207-215.

Varshini J, Srinag BS, Kalthur G, Krishnamurthy H, Kumar P, Rao SB, Adiga SK. Poor sperm quality and advancing age are associated with increased sperm DNA damage in infertile men. *Andrologia* 2012;44:642-549.

Velaers A, Paternot G, Debrock S, D'Hooghe T, Spiessens C. Triple touch sperm immobilization vs. single touch sperm immobilization in ICSI - a randomised trial. *Reprod Biol Endocrin* 2012;10:65-70.

Villani P, Eleuteri P, Grollino MG, Rescia M, Altavista P, Spano M, Pacchierotti F, Cordelli E. Sperm DNA fragmentation induced by DNAse I and hydrogen peroxide: an in vitro comparative study among different mammalian species. *Reproduction* 2010;140:445-452.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 2004;81:1289-1295.

Wakai T, Fissore RA. Ca^{2+} homeostasis and regulation of ER Ca^{2+} in mammalian oocytes/eggs.*Cell Calcium* 2013;53:63-67.

Ward W, Coffey D. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod* 1991;44:569-574.

Wassarman PM. Fertilization in mammals.*Scientific American* 1988;259:52-58.

Watanabe S, Tanaka A, Fujii S, Mizunuma H, Fukui A, Fukuhara R, Nakamura R, Yamada K, Tanaka I, Awata S, Nagayoshi M. An investigation of the potential effect of vacuoles in human sperm on DNA damage using a chromosome assay and the TUNEL assay. *Hum Reprod* 2011;26:978-986.

Wen J, Jiang J, Ding C, Dai J, Liu Y, Xia Y, Hu Z. Birth defects in children conceived by in vitro fertilization and intracytoplasmic sperm injection: a meta-analysis. *Fertil Steril* 2012;97:1331-1337.

Wiener-Megnazi Z, Auslender R, Dirnfeld M. Advanced paternal age and reproductive outcome. *Asian J Androl* 2012;14:69-76.

Wilding M, Coppola G, di Matteo L, Palagiano A, Fusco E, Dale B. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. *J Assist Reprod Genet* 2011;28:253-262.

Wong MYW, Ledger WL. Is ICSI risky? *Obstet Gynecol Int* 2013;2013:473289

Woodward BJ, Campbell KHS, Ramsewak SS.A comparison of headfirst and tailfirst microinjection of sperm at intracytoplasmic sperm injection.*Fertil Steril* 2008;89:711-714.

World Health Organisation: WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th Edition. Cambridge University Press, Cambridge, UK. 2010. p. 7-113.

Worrilow KC, Huynh T, Bower JB, Anderson AR, Schillings W, Crain JL. PICSI VS ICSI: statistically significant improvement in clinical outcomes in 240 in vitro fertilization (IVF) patients. *Fertil Steril* 2007;88:S37.

Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. *Reprod Biomed Online* 2014;28:684-703.

Yanagida K, Katayose H, Yazawa H, Kimura Y, Konnai K, Sato A. The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans.*Hum Reprod* 1999;14:448-453.

Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 2008;23:2663-2668.

Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. *Hum Reprod* 2005;20:3476-3480.

Zini A, Sigma M. Are tests of sperm DNA damage clinically useful? Pros and cons. *J Androl* 2009;30:219-229.

Zini A. Are sperm chromatin and DNA defects relevant in the clinic? *Syst Biol Reprod Med* 2011;57:78-85.

CHAPTER 5

Addenda

ADDENDUM I Computer assisted semen analysis (CASA) - Motion parameters (fromIVOS HT-CASA Software Manual, Version 1.1)

Table 5.1 Motion parameters and their definitions measured by CASA.

Data fields	Unit	Short description	Function
VAP	µm/s	Average path velocity	The average path is determined by smoothing the sperm head position in a running average. The resultant path length is determined and divided by the elapsed time.
VSL	µm/s	Straight line velocity	The distance between the first and last points on the sperm track, divided by the elapsed time.
VCL	µm/s	Curvilinear velocity	VCL is measured by summing the distance between the sperm head positions in each frame, divided by the elapsed time.
ALH	µm	Amplitude of lateral head displacement	The maximum value of the approximately sinusoidal oscillation of the sperm head about the track. It is measured as the maximum distance between the actual sperm position and the corresponding average sperm position for all points over the track.
BCF	Hz	Beat-cross frequency	The frequency with which the sperm head crosses the average path line during acquisition.
LIN	%	Linearity	The ratio VSL/VCL in percent and is a measure of track direction.

STR	%	Straightness	The ratio VSL/VAP in percent and is a measure of track compactness.

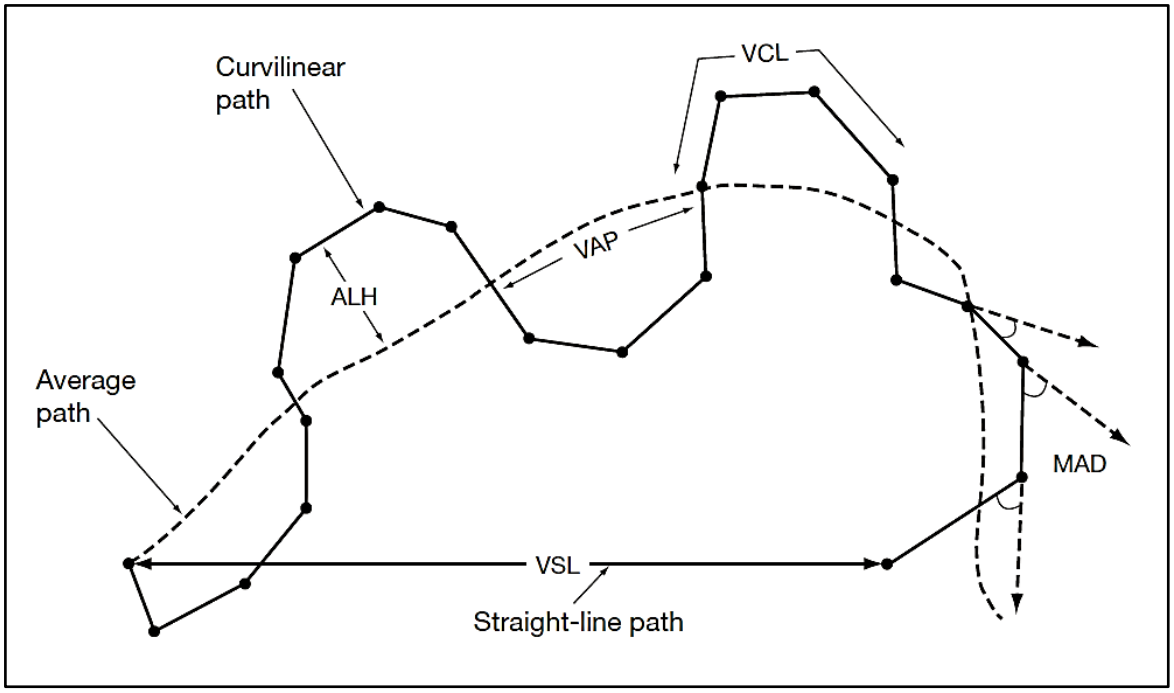


Figure 5.1 Illustration of different motion parameters measured by the CASA system. (Available in: WHO; 2010)

ADDENDUM II Routine semen analysis (WHO, 2010 used as guideline)

Semen viscosity

After liquefaction, the viscosity of the sample was estimated. A wide-bore (approximately 1.5mm diameter) plastic disposable pipette was used. Semen was gently aspirated into the pipette and allowed to drop by gravity. The length of the thread was observed. A normal sample left the pipette in small discrete drops. If viscosity was abnormal, the drop formed a thread more than 2cm long.

Semen volume

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymis. Precise measurement of volume is essential in any evaluation of semen. It allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated. The volume was measured directly from an equilibrated test tube (in millimetres).

Semen pH

Semen pH reflects the balance between the pH values of the different accessory gland secretions. These include mainly the alkaline seminal vesicle secretion and the acidic prostatic secretion. The pH was measured after liquefaction within one hour of ejaculation. A drop of semen was spread onto a pH paper. The colour was compared with the calibration strip to read the pH.

Wet preparation

One drop of semen (10µl) was placed onto a clean glass slide and covered with a coverslip (22mm × 22mm). The weight of the coverslip spread the sample evenly. Using a regular light microscope at a 400x magnification, the freshly made wet preparation was assessed as soon as the contents

settled under the coverslip. Motility, forward progression and other cell parameters were evaluated using the wet preparation.

Motility

In determining quantitative motility, the percentage of motile spermatozoa from the percentage of immotile spermatozoa was distinguished. The estimation of the percentage motile spermatozoa was made to the nearest 10%.

Forward progression

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

0	No movement
1	Movement (twitching) - none forward
1+	Movement - every now and then
2	Movement - undirected and slow
2+	Movement - slowly but directly forward
3-	Movement - fast but not direct
3	Movement - fast and direct
3+	Movement - very fast and direct
4	Movement - extremely fast and direct

Cells

Somatic cells (leukocytes, histiocytes and epithelium cells) were observed and expressed as follow:

±	A few cells on the slide
+	1 – 5 cells per high power field
++	6 – 10 cells per high power field

+++ >10 cells per high power field

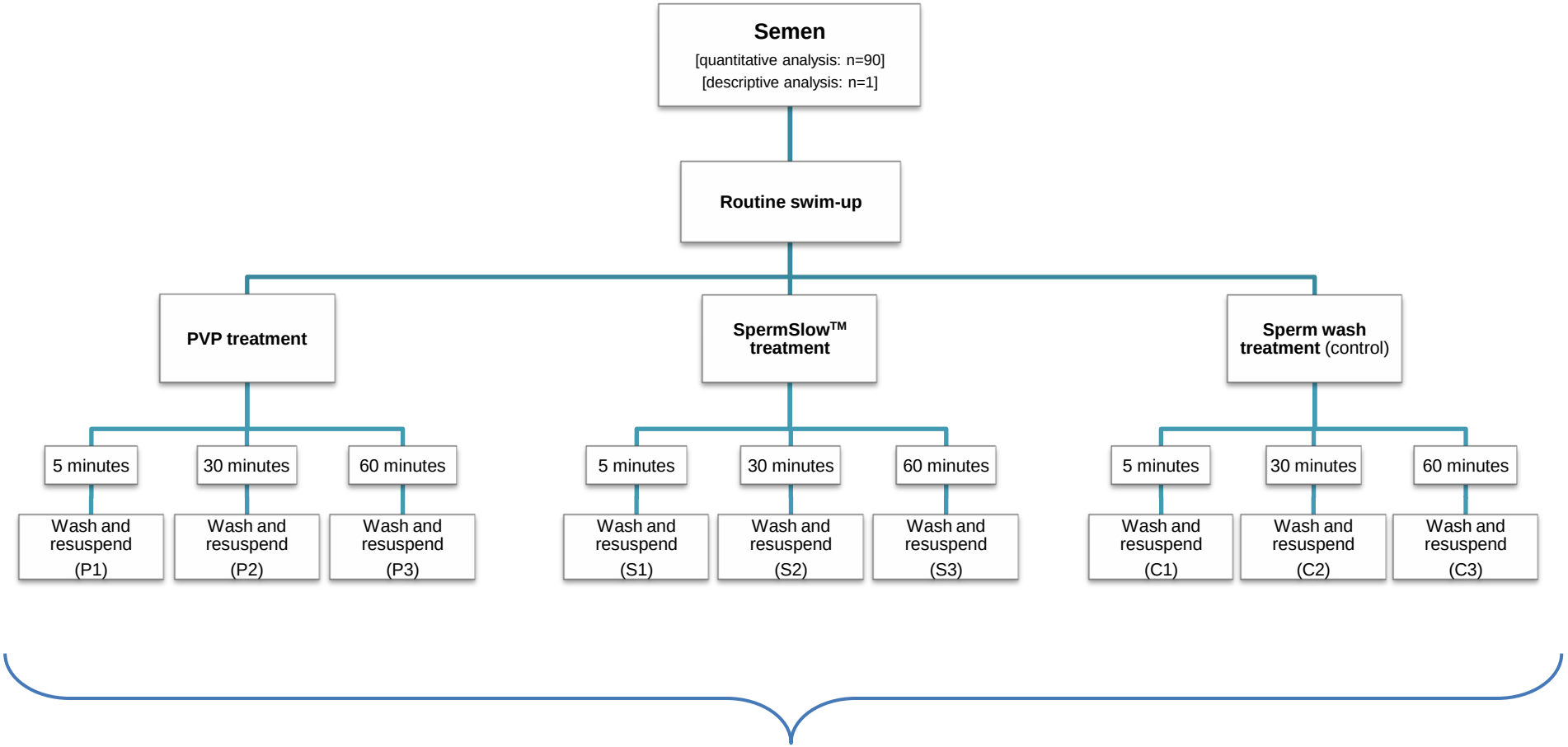
Sperm concentration

The wet preparation was used to determine the appropriate dilution necessary for the sperm concentration assessment. Either 10 or 100 time dilutions were made with water. The diluted sample was loaded on a coverslip covered Neubauer haemocytometer, kept in a moist chamber and the sperm concentration assessed 10 minutes after loading. The total number spermatozoa in 32 blocks were counted and the sperm concentration was calculated using a formula based on the dilution used.

ADDENDUM III Routine swim up sperm preparation method (modified from Tygerberg Hospital Andrology manual)

1) Allow the semen sample to liquefy.
2) Make a wet preparation and determine the sperm parameters (estimated concentration, motility and forward progression).
3) Dilute 1.5ml semen with 2ml sperm washing medium in a test tube and centrifuge at 450g for 10 minutes.
4) Remove the supernatant, resuspend the remaining pellet in 2ml sperm washing medium and centrifuge at 450g for 10 minutes.
5) Remove the supernatant and carefully overlay the pellet with 1ml sperm washing medium.
6) Incubate the tube at an angled position at 37°C for one hour to ensure a maximum area for the sperm to swim into.
7) After the one hour incubation period, aspirate 1ml of the top medium that contains the motile sperm with a pipette and place into a clean and well labelled test tube.
8) Prepare a wet preparation slide and determine the sperm parameters (estimated concentration, motility and forward progression).

ADDENDUMIV Diagrammatic presentation of methodology



Quantitative analysis [Experiment A]: CASA Motility analysis
Viability Stain analysis
CMA_3 staining analysis
TUNEL analysis

Descriptive analysis [Experiment B]: Transmission Electron Microscopy (TEM) analysis

ADDENDUM V Computer assisted semen analysis (CASA) - Motility and Kinematics analysis method (from IVOS HT-CASA Software Manual, Version 1.1)

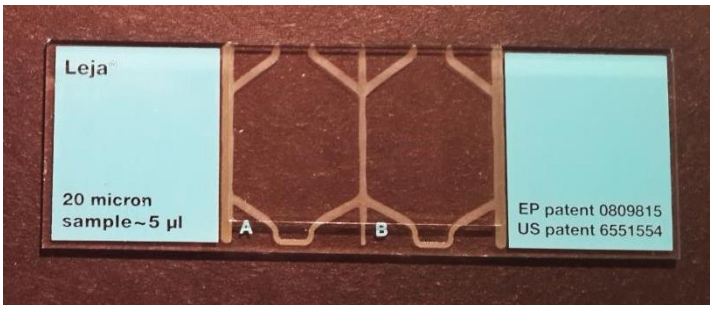


Figure 5.2 Photograph of a Leja® loading chamber used for CASA motility analysis. (Photo by M Nel)

Method:

1) Load ±5µl of the well mixed sperm solutions (after resuspension) into both 20µm deepchambers of a Leja® slide (see Figure 5.2).
2) Allow the solution to spread by capillary action.
3) Set the CASA machine (IVOS® Hamilton Thorne CASA system equipped with HT-CASA II software version 1.1) up for sperm motility evaluation according to the software manual.
4) Place the slide into the loading chamber. Use the LOAD, JOG IN and JOD OUT buttons to ensure that the slide is in the correct position for the analysis.
5) Use the focus knob to adjust the focus and analyse the sperm motility and motion parameters using the STANDARD HUMAN MOTILITY setup. (Use the software manual where needed)
6) Assess 6 fields per chamber (12 fields in total). The tracks of at least 200 motile spermatozoa per specimen should be counted.

ADDENDUM VI Eosin-Nigrosin vital staining method (modified from WHO, 2010)

1) Mix the test sperm solution well.
2) Use the porcelain spot plate and mix 10µl of the well mixed sperm solution with 10µl eosin for 15 seconds.
3) Add 10µl nigrosin and mix for another 15 seconds.
4) Make a smear by applying a drop of the mixed solution to the end of a labelled frosted glass slide. Use a second glass slide to pull the drop along the surface of the slide as shown in Figure 5.3. Allow the smear to air dry.

Figure 5.3 Schematic representation of the semen smearing method
(Available in: WHO, 2010)

5) Examine each slide with bright field optics at ×1000 magnification and oil immersion.
6) Evaluate one hundred spermatozoa on each slide (using a laboratory counter) and annotate the percentage of stained (dead) and unstained (vital) spermatozoa.

ADDENDUM VII Chromomycin A_3 (CMA_3) staining method (Esterhuizen *et al.*, 2000)

Materials:

- **McIlvaine's Buffer Stock Solutions**

  Solution 1: Dissolve 3.8424g citric acid (anhydrous) in 200ml distilled water

  Solution 2: Dissolve 25.5564g sodium phosphate dibasic (anhydrous) in 900ml distilled water

- **McIlvaine's Buffer Working Solution (1L)**

  Dilute 176.5ml of solution 1 with 823.5ml of solution 2

- **Chromomycin A_3**

  Dissolve 10mg CMA_3 in 1ml ethanol

  Store at -20°C in 50µl aliquots

  Add 250µl of McIlvaine's buffer (working solution) to each aliquot before using

- **Fixative**

  Methanol/acetic acid (3:1)

- **Dabco Anti-Fade Solution**

Method:

CMA3 Staining:

1) Prepare smears of all nine test samples (see Figure 5.3), and allowed to air dry.
2) Fix smears for 20 minutes at room temperature in methanol/acetic acid and allow to air dry.

The following steps must take place in the dark:

3) Stain slides for 20 minutes with 15µl CMA_3 in a dark chamber.
4) Rinse slides in McIlvaine's buffer.

Mounting:

5) Mount slides immediately with Dabco anti-fade solution.
6) Cover slides with coverslips; the occurrence of air bubbles must be avoided.

7) Store slides at room temperature in a dark chamber overnight and evaluate the next morning.

Evaluation:

8) Evaluate slides using a fluorescence microscope with 100x oil immersion phase contrast objective.

9) Evaluate one hundred spermatozoa on each slide. The percentage bright yellow stained spermatozoa must be counted as CMA_3 positive cells. These spermatozoa are regarded as cells with poor chromatin packaging quality. Poor chromatin packaging quality in the sperm head are indicated by:

   - faintly yellow fluorescent staining
   - bright yellow fluorescent staining

   Dull yellow stained spermatozoa must be counted as CMA_3 negative cells. These spermatozoa are regarded as cells that contain good chromatin packaging. Good chromatin packaging quality in the sperm head are indicated by:

   - no fluorescent staining
   - fluorescence band at equatorial segment
   - fluorescent stain around periphery of head (stain did not permeate membrane)

   (Esterhuizen *et al.,* 2000)

10) Annotate the percentage of CMA_3 positive cells and classify sperm samples as mature (good) versus immature (poor) DNA packaging spermatozoa.

ADDENDUM VIII Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assaymethod – Epi-fluorescence (modified from Avendaño *et al.*, 2010)

Materials:

- **PBS solution**

  Dissolve one phosphate buffered saline tablet in 1l distilled water

- **4% formaldehyde/PBS solution**

  Mix 1ml formaldehyde with 9ml PBS (phosphate buffered saline)

- **1% HSA/PBS**

  Mix 0.1ml HSA (human serum albumin) in 9.9ml PBS

- **0.1% Triton X-100/PBS**

  Mix 0.01ml Triton X-100 in 9.9ml PBS

- **TUNEL reaction mixture**

  Thaw 2 vials (enzyme and label solution) from the Roche In Situ Cell Death Detection Kit, Fluorescein. Aliquot 10µl enzyme solution and 95µl labelling solution into a total of ten Eppendorf tubes (respectively). Label the tubes and refreeze all the tubes. Remember that these solutions must always be kept in the dark. During the staining method, mix enzyme/label (1:9) solution that will serve as the TUNEL reaction mixture.

- **Dabco Anti-Fade Solution**

Method:

Sample collecting:

1) Freeze an aliquote (≥30µl) of each of the nine test sperm solutions after each time interval in pipette tips (see Figure 5.4) by dunking it directly into liquid nitrogen.
2) Store the samples in a nitrogen tank specifically reserved for experimental samples only.

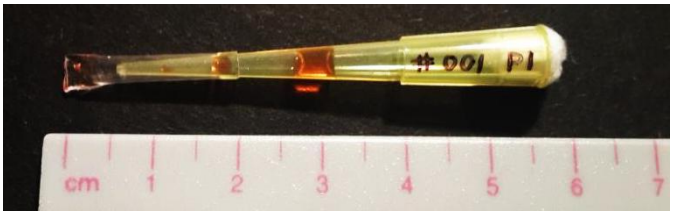


Figure 5.4 Photograph of pipette tips used to freeze sperm test sample for TUNEL analysis. (Photo by M Nel)

Sample preparation:

3) Thaw 20 test sample solutions for 10 minutes at 37°C.
4) Aliquot the 20 samples onto two duplicate 21 well Teflon printed diagnostic slides (see Figure 5.5). The 21st well will serve as a negative control. The extra duplicate slide will be used if the first slide evaluations were not effective.
5) Allow the slides to air dry.

Figure 5.5 Teflon Printed diagnostic slide with negative control well indicated in red. (Available at: http://www.2spi.com/catalog/new/ptfesld.shtml)

TUNEL staining:

6) Add 5µl of 4% formaldehyde/PBS to each well of one slide and incubate for 45 minutes at room temperature in a humidified chamber.
7) Siphon the fluid from all the wells.
8) Add 10µl of 1% HSA/PBS to each well and siphon the fluid after about 2 minutes. This will serve as a washing step.

9) Add 5µl of 0.1% Triton X-100/PBS to each well and incubate for 10 minutes at room temperature in a humidified chamber.
10) Siphon the fluid from all the wells.
11) Wash the wells twice (see step 8).

The following steps must take place in the dark:

12) During the last 2 minute washing step, thaw 2 Eppendorf tubes (enzyme and label solution) previously aliquoted.
13) Add 5µl label solution to the negative control well.
14) Mix the remaining label solution (90µl) to the 5µl thawed enzyme solution.
15) Add 5µl of the TUNEL reaction mixture to the remaining 20 wells.
16) Incubate the slide for an hour and a half in a dark humidified chamber at 37°C.
17) Siphon the fluid from all the wells.
18) Wash the wells twice (see step 8).
19) Add Dabco anti-fade solution to the wells, cover the slide with a coverslip and evaluate immediately.

Evaluation:

20) Evaluate slides using a fluorescence microscope with 100x oil immersion phase contrast objective. Use an excitation wavelength in the range of 450 – 500nm and detection in the range of 515 – 565nm (green).
21) Evaluate one hundred spermatozoa on each well. The percentage green fluorescent stained spermatozoa must be counted as TUNEL positive cells. These spermatozoa are regarded as cells with DNA fragmentation/damage. Spermatozoa with no fluorescent staining must be counted as TUNEL negative cells. These spermatozoa are regarded as cells that contain intact DNA.
22) Annotate the percentage of TUNEL positive cells.

ADDENDUM IX Data sheet

Date	
Patient name	
Patient sample number	

P	S	C

	Before S/U	After S/U
Volume (ml)		
Viscosity (cm)		
Count (x 10^6 / ml)		
Motility (%)		
FP		
Cells		
Agglutination		

Time passed	
Time analysed	
Time S/U fin	

Volume to resuspend (µl)	

MOTILITY and KINEMATIC MEASUREMENTS

	p1	p2	p3	s1	s2	s3	c1	c2	c3
Motility (%)									
Progressive (%)									
VAP (µm/s)									
VSL (µm/s)									
VCL (µm/s)									
ALH (µm)									
BCF (Hz)									
STR (%)									
LIN (%)									
Comments									

VIABILITY

	p1	p2	p3	s1	s2	s3	c1	c2	c3
Viable sperm cells (%)									
Comments									

CMA_3

	p1	p2	p3	s1	s2	s3	c1	c2	c3
CMA_3 positive sperm (%)									
Comments									

TUNEL

	p1	p2	p3	s1	s2	s3	c1	c2	c3
TUNEL positive sperm (%)									
Comments									

ADDENDUM X Summary of sperm parameters of donors

Table 5.2 Summary of sperm parameters of donors used with reference to the WHO (2010).

Semen parameter	**WHO (2010) lower reference limit**	**Exclusion criteria of study**	**Average sperm parameters of donors used**
Volume (ml)	1.5	< 1	2.3
Viscosity (cm)	> 2	≥ 10	0.6
Motility (%)	40	< 40	55.9
Forward progression	*	< 2+ forward progression	3-
Concentration (10^6/ml)	15	< 50	91.8

* The previous edition of this manual recommended that progressively motile spermatozoa should be categorized as rapid or slow, with a speed of >25 µm/sec at 37 °C defining “grade a” spermatozoa. However, it is difficult for technicians to define the forward progression so accurately without bias (Cooper and Yeung, 2006).

Although the exclusion criteria is not in accordance with the WHO (2010) reference values, the criteria were chosen in order to obtain an ideal swim up concentration of ≥20x10^6/ml.

ADDENDUM XI Statistical analysis results

Sperm viability

```
. by time, sort : tabstat vs , statistics( mean sd min p25 p50 p75 max    ) by(method) varwidth(8)
colum
>ns(statistics) nototal
------------------------------------------------------------------------------------------

medium variable |      mean        sd       min       p25       p50       p75       max
----------------+-------------------------------------------------------------------------
->time = 5      |
PVO          vs |  68.42222  13.17981        31        59        69        79        91
SPS          vs |  62.74444  14.10599        24        53        63        74        91
SPW          vs |  64.13333  12.54314        26        56      66.5        74        88
->time = 30     |
PVO          vs |  67.42222  13.33491        28        58      68.5        77        89
SPS          vs |  61.18889   12.8342        33        52      60.5        70        90
SPW          vs |  62.38889  12.59454        27        53        63        71        93
->time = 60     |
PVO          vs |  63.02222  14.59643        25        53      63.5        75        87
SPS          vs |  59.44444  12.95117        28        49        60        71        81
SPW          vs |  58.88889  12.55858        31        49      59.5        69        81
------------------------------------------------------------------------------------------
```

Models

1. Interaction between time and medium

```
. test _ItimXmet_2_2 _ItimXmet_2_3 _ItimXmet_3_2 _ItimXmet_3_3
( 1)  _ItimXmet_2_2 = 0
( 2)  _ItimXmet_2_3 = 0
( 3)  _ItimXmet_3_2 = 0
( 4)  _ItimXmet_3_3 = 0
chi2(  4) =    3.16
         Prob > chi2 =    0.5320
```

2. Contrast between control (Sperm Washing) and active mediums (PVP& SpermSlow)

```
xi: binreg vs b3.method i.time , rd n(n) vce(cluster id)
i.time            _Itime_1-3          (naturally coded; _Itime_1 omitted)
Generalized linear models                         No. of obs      =       810
Optimization     : MQL Fisher scoring             Residual df     =       805
                   (IRLS EIM)                     Scale parameter =         1
Deviance         =  6212.112098                   (1/df) Deviance =  7.716909
Pearson          =  6042.957493                   (1/df) Pearson  =  7.506779
Variance function: V(u) = u*(1-u/n)               [Binomial]
Link function    : g(u) = u/n                     [Identity]
                                                  BIC             =  820.9995
                                    (Std. Err. adjusted for 90 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
vs | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
method |
PVP  |   .0449073   .0085113     5.28   0.000     .0282256    .0615891
SPS  |  -.0069281   .0074057    -0.94   0.350    -.0214429    .0075868
             |
    _Itime_2 |  -.0142496   .0067652    -2.11   0.035    -.0275092     -.00099
    _Itime_3 |  -.0466244   .0082645    -5.64   0.000    -.0628225   -.0304263
       _cons |   .6383719   .0127914    49.91   0.000     .6133013    .6634425
------------------------------------------------------------------------------
. test _Itime_2 _Itime_3
( 1)  _Itime_2 = 0
( 2)  _Itime_3 = 0
chi2(  2) =   32.02
         Prob > chi2 =    0.0000
```

3. Equivalence between two active mediums

```
xi: binreg vs i.method i.time , rd n(n) vce(cluster id)
i.method          _Imethod_1-3        (naturally coded; _Imethod_1 omitted)
i.time            _Itime_1-3          (naturally coded; _Itime_1 omitted)
Generalized linear models                         No. of obs      =       810
Optimization     : MQL Fisher scoring             Residual df     =       805
                   (IRLS EIM)                     Scale parameter =         1
Deviance         =  6212.112098                   (1/df) Deviance =  7.716909
Pearson          =  6042.957493                   (1/df) Pearson  =  7.506779
Variance function: V(u) = u*(1-u/n)               [Binomial]
Link function    : g(u) = u/n                     [Identity]
                                                  BIC             =  820.9995
                                     (Std. Err. adjusted for 90 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
vs | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imethod_2 |  -.0518354    .007858    -6.60   0.000    -.0672367    -.036434
  _Imethod_3 |  -.0449073   .0085113    -5.28   0.000    -.0615891   -.0282256
    _Itime_2 |  -.0142496   .0067652    -2.11   0.035    -.0275092    -.00099
    _Itime_3 |  -.0466244   .0082645    -5.64   0.000    -.0628225   -.0304263
       _cons |   .6832793   .0136328    50.12   0.000     .6565595    .709999
------------------------------------------------------------------------------
```

CMA_3 analysis

```
. by time, sort : tabstat cma , statistics( mean sd min p25 p50 p75 max    ) by(method) varwidth(8)
colu
>mns(statistics) nototal
------------------------------------------------------------------------------------------

medium variable |      mean        sd       min       p25       p50       p75       max
----------------+-------------------------------------------------------------------------
->time = 5
----------------+-------------------------------------------------------------------------
PVP         cma |  17.52222  8.890378         2        12      16.5        22        58
SPS         cma |  16.83333  8.968422         2        10        16        21        45
SPW         cma |  16.87778  8.188547         1        11        16        20        45
------------------------------------------------------------------------------------------
->time = 30
----------------+-------------------------------------------------------------------------
PVP         cma |  18.18889  8.073948         4        14      17.5        23        41
SPS         cma |  17.23333  8.920435         2        11        16        22        42
SPW         cma |  17.95556  8.343619         1        12        17        22        41
------------------------------------------------------------------------------------------
->time = 60
----------------+-------------------------------------------------------------------------
PVP         cma |      19.1  9.354504         4        13      17.5        23        48
SPS         cma |  17.11111  8.130183         1        12        16        21        45
SPW         cma |  17.05556  8.447273         2        11      15.5        22        43
------------------------------------------------------------------------------------------
```

Models

1. Interaction between time and medium

```
. test _ItimXmet_2_2 _ItimXmet_2_3 _ItimXmet_3_2 _ItimXmet_3_3
( 1)  _ItimXmet_2_2 = 0
( 2)  _ItimXmet_2_3 = 0
( 3)  _ItimXmet_3_2 = 0
( 4)  _ItimXmet_3_3 = 0
chi2(  4) =    6.29
          Prob > chi2 =    0.1787
```

2. Contrast between control (Sperm Washing) and active mediums (PVP& SpermSlow)

```
. xi: binreg cma b3.method i.time , rd n(n) vce(cluster id)
i.time            _Itime_1-3          (naturally coded; _Itime_1 omitted)
    Generalized linear models                         No. of obs      =       810
Optimization     : MQL Fisher scoring                 Residual df     =       805
                   (IRLS EIM)                         Scale parameter =         1
Deviance         =  4106.459126                       (1/df) Deviance =  5.101191
Pearson          =  4106.163228                       (1/df) Pearson  =  5.100824
Variance function: V(u) = u*(1-u/n)                   [Binomial]
Link function    : g(u) = u/n                         [Identity]
                                                      BIC             = -1284.653
                                        (Std. Err. adjusted for 90 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
cma | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
method |
PVP  |   .0097143   .0054169     1.79   0.073    -.0009025    .0203312
SPS  |  -.0023465   .0048598    -0.48   0.629    -.0118716    .0071786
             |
    _Itime_2 |   .0071409   .0040785     1.75   0.080    -.0008528    .0151346
    _Itime_3 |   .0066357   .0040568     1.64   0.102    -.0013154    .0145868
       _cons |   .1683719   .0079709    21.12   0.000     .1527493    .1839945
------------------------------------------------------------------------------
. test _Itime_2 _Itime_3
( 1)  _Itime_2 = 0
( 2)  _Itime_3 = 0
chi2(  2) =    3.77
          Prob > chi2 =    0.1515
```

3. Equivalence between two active mediums

```
. xi: binreg cma i.method i.time , rd n(n) vce(cluster id)
i.method          _Imethod_1-3        (naturally coded; _Imethod_1 omitted)
i.time            _Itime_1-3          (naturally coded; _Itime_1 omitted)
Generalized linear models                         No. of obs      =       810
Optimization     : MQL Fisher scoring             Residual df     =       805
                   (IRLS EIM)                     Scale parameter =         1
Deviance         =  4106.459126                   (1/df) Deviance =  5.101191
Pearson          =  4106.163228                   (1/df) Pearson  =  5.100824
Variance function: V(u) = u*(1-u/n)               [Binomial]
Link function    : g(u) = u/n                     [Identity]
                                                  BIC             = -1284.653
                                    (Std. Err. adjusted for 90 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
cma | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imethod_2 |  -.0120609   .0052848    -2.28   0.022    -.0224188   -.0017029
  _Imethod_3 |  -.0097143   .0054169    -1.79   0.073    -.0203312    .0009025
    _Itime_2 |   .0071409   .0040785     1.75   0.080    -.0008528    .0151346
    _Itime_3 |   .0066357   .0040568     1.64   0.102    -.0013154    .0145868
       _cons |   .1780863   .0087013    20.47   0.000      .161032    .1951405
------------------------------------------------------------------------------
```

TUNEL analysis

```
. by time, sort : tabstat tun , statistics( mean sd min p25 p50 p75 max    ) by(method) varwidth(8)
colu
>mns(statistics) nototal
------------------------------------------------------------------------------------------
->time = 30

method variable |      mean        sd       min       p25       p50       p75       max
----------------+-------------------------------------------------------------------------
PVP         tun |  11.62069  7.775482         2         6        10        15        38
SPS         tun |  12.06897  5.541603         3         8        11        15        29
SPW         tun |  11.86207  7.361697         3         6         9        14        34
------------------------------------------------------------------------------------------
->time = 60

method variable |      mean        sd       min       p25       p50       p75       max
----------------+-------------------------------------------------------------------------
PVP         tun |  10.89655  6.189535         2         7        10        12        28
SPS         tun |  13.44828  7.467005         5         7        12        17        35
SPW         tun |   11.7931  6.678212         3         8        10        15        35
------------------------------------------------------------------------------------------
```

Models

1. Interaction between time and medium

```
. test _ItimXmet_2_2 _ItimXmet_2_3 _ItimXmet_3_2 _ItimXmet_3_3
( 1)  _ItimXmet_2_2 = 0
( 2)  _ItimXmet_2_3 = 0
( 3)  o._ItimXmet_3_2 = 0
( 4)  o._ItimXmet_3_3 = 0
       Constraint 3 dropped
       Constraint 4 dropped
chi2(  2) =    1.85
         Prob > chi2 =    0.3959
```

2. Contrast between control (Sperm Washing) and active mediums (PVP& SpermSlow)

```
xi: binreg tun b3.method b2.time , rd n(n) vce(cluster id)
Iteration 5:   deviance =  695.2197
Generalized linear models                         No. of obs      =       174
Optimization     : MQL Fisher scoring             Residual df     =       170
                   (IRLS EIM)                     Scale parameter =         1
Deviance         =   695.219654                   (1/df) Deviance =  4.089527
Pearson          =  762.9021553                   (1/df) Pearson  =   4.48766
Variance function: V(u) = u*(1-u/n)               [Binomial]
Link function    : g(u) = u/n                     [Identity]
                                                  BIC             = -181.8197
                                    (Std. Err. adjusted for 29 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
tun | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
method |
PVP  |  -.0056693   .0054591    -1.04   0.299    -.0163689    .0050302
SPS  |   .0092718   .0063587     1.46   0.145    -.0031912    .0217347
             |
time |
60  |   .0015699   .0060185     0.26   0.794    -.0102262     .013366
       _cons |   .1174974   .0132298     8.88   0.000     .0915675    .1434274
------------------------------------------------------------------------------
```

3. Equivalence between two active mediums

```
. xi: binreg tun i.method i.time , rd n(n) vce(cluster id)
i.method          _Imethod_1-3        (naturally coded; _Imethod_1 omitted)
i.time            _Itime_1-3          (naturally coded; _Itime_1 omitted)
note: _Itime_3 omitted because of collinearity
Generalized linear models                         No. of obs      =       174
Optimization     : MQL Fisher scoring             Residual df     =       170
                   (IRLS EIM)                     Scale parameter =         1
Deviance         =   695.219654                   (1/df) Deviance =  4.089527
Pearson          =  762.9021553                   (1/df) Pearson  =   4.48766
Variance function: V(u) = u*(1-u/n)               [Binomial]
Link function    : g(u) = u/n                     [Identity]
                                                  BIC             = -181.8197
                                    (Std. Err. adjusted for 29 clusters in id)
------------------------------------------------------------------------------
             |             Semirobust
tun | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imethod_2 |   .0149411   .0064618     2.31   0.021     .0022761    .0276061
  _Imethod_3 |   .0056693   .0054591     1.04   0.299    -.0050302    .0163689
    _Itime_2 |  -.0015699   .0060185    -0.26   0.794     -.013366    .0102262
       _cons |   .1133979   .0125166     9.06   0.000     .0888659      .13793
------------------------------------------------------------------------------
```

CASA Analysis – Sperm motility

```
. by time, sort : tabstat mot , statistics( mean sd min p25 p50 p75 max    ) by(method) varwidth(8)
columns(statistics) nototal

->time = 5

method variable | mean     sd       min       p25       p50       p75       max
----------------+--------------------------------------------------------------
PVP    mot |  73.59667  13.99066      37.4      67.5     77.35        84        94
SPS    mot |  73.58556  14.21284      34.4      66.1     77.25      83.4        98
SPW    mot |  74.43889  13.71803      32.8      65.9      77.6      85.2        96
-------------------------------------------------------------------------------
->time = 30

method variable | mean     sd       min       p25       p50       p75       max
----------------+--------------------------------------------------------------
PVP   mot |  73.57667  13.56704      35.3      65.4     75.05      84.3        98
SPS   mot |  74.03333  13.64179      37.7      66.5     78.15      82.7        96
SPW   mot |  77.05667  13.56274      33.7      70.7      80.1      86.5      95.7
-------------------------------------------------------------------------------
->time = 60
method variable | mean    sd       min       p25       p50       p75       max
----------------+--------------------------------------------------------------
PVP    mot |  68.70444  15.17682       3.2      61.9      70.7        80        90
SPS    mot |  70.82556  14.11112      31.3        64     73.05      81.6        97
SPW    mot |  75.76778  12.86855      37.8      69.5     77.85      86.1        94
-------------------------------------------------------------------------------
```

Models

1. Interaction between time and medium

```
test _ItimXmet_2_2 _ItimXmet_2_3 _ItimXmet_3_2 _ItimXmet_3_3

( 1)  [mot]_ItimXmet_2_2 = 0
( 2)  [mot]_ItimXmet_2_3 = 0
( 3)  [mot]_ItimXmet_3_2 = 0
( 4)  [mot]_ItimXmet_3_3 = 0
chi2(  4) =   21.12
         Prob > chi2 =    0.0003
```

2. Contrast between control (Sperm Washing) and PVP

a) 5 minute incubation

```
. lincom _Imethod1_2
( 1)  [mot]_Imethod1_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.8422222   .6953636    -1.21   0.226     -2.20511    .5206654
------------------------------------------------------------------------------
```

b) 30 minute incubation

```
. lincom _Imethod1_2+ _ItimXmet_2_2
( 1)  [mot]_Imethod1_2 + [mot]_ItimXmet_2_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |      -3.48   .9594203    -3.63   0.000    -5.360429   -1.599571
------------------------------------------------------------------------------
```

c) 60 minute incubation

```
. lincom _Imethod1_2 +_ItimXmet_3_2
( 1)  [mot]_Imethod1_2 + [mot]_ItimXmet_3_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -7.063333   1.273402    -5.55   0.000    -9.559155   -4.567512
------------------------------------------------------------------------------
```

3. Contrast between control (Sperm Washing) and SpermSlow

a) 5 minute incubation

```
. lincom _Imethod1_3
( 1)  [mot]_Imethod1_3 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.8533336   .7532272    -1.13   0.257    -2.329632    .6229647
------------------------------------------------------------------------------
```

b) 30 minute incubation

```
. lincom _Imethod1_3+ _ItimXmet_2_3
( 1)  [mot]_Imethod1_3 + [mot]_ItimXmet_2_3 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -3.023333   .8428713    -3.59   0.000    -4.675331   -1.371336
------------------------------------------------------------------------------
```

c) 60 minute incubation

```
. lincom _Imethod1_3+ _ItimXmet_3_3
( 1)  [mot]_Imethod1_3 + [mot]_ItimXmet_3_3 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -4.942222   1.127948    -4.38   0.000     -7.15296   -2.731484
------------------------------------------------------------------------------
```

4. Equivalence between two active mediums

a) 5 minute incubation

```
. lincom _Imethod_2
( 1)  [mot]_Imethod_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.0111114   .7014137    -0.02   0.987    -1.385857    1.363634
------------------------------------------------------------------------------
```

a) 30 minute incubation

```
. lincom _Imethod_2+ _ItimXmet_2_2
( 1)  [mot]_Imethod_2 + [mot]_ItimXmet_2_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   .4566667   .9059025     0.50   0.614     -1.31887    2.232203
------------------------------------------------------------------------------
```

a) 60 minute incubation

```
. lincom _Imethod_2+ _ItimXmet_3_2
( 1)  [mot]_Imethod_2 + [mot]_ItimXmet_3_2 = 0
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   2.121111   1.152814     1.84   0.066    -.1383627    4.380585
------------------------------------------------------------------------------
```

CASA Analysis – Progressive sperm motility

```
by time, sort : tabstat prog , statistics( mean sd min p25 p50 p75 max    ) by(method) varwidth(8)
columns(statistics) nototal
-------------------------------------------------------------------------------------------
method variable |      mean        sd       min       p25       p50       p75       max
----------------+--------------------------------------------------------------------------
->time = 5
PVP         prog |  51.40111  18.53655      12.4        34      54.7        66      89.5
SPS         prog |  53.86333  18.09419      11.5      39.7      57.9      69.3      82.2
SPW         prog |  52.11778  17.79094      14.5        40      53.6      66.6      90.1
->time = 30
PVP         prog |  58.65333  13.93976        23      50.4      61.5      68.3      86.3
SPS         prog |  59.16778   14.4915      24.7      50.1     61.05      70.4      85.8
SPW         prog |  60.99556   13.5057      28.3      50.1     62.05        73        85
->time = 60
PVP         prog |  54.47444  13.00238      23.2        46      54.8        64      80.8
SPS         prog |     56.16   14.0835      26.1        46     57.75      67.2      82.6
SPW         prog |  59.43556   14.5128      28.5      47.8     61.15      69.8      88.5
-------------------------------------------------------------------------------------------
```

Models

1. Interaction between time and medium

```
. test _ItimXmet_2_2 _ItimXmet_2_3 _ItimXmet_3_2 _ItimXmet_3_3
( 1)  [prog]_ItimXmet_2_2 = 0
( 2)  [prog]_ItimXmet_2_3 = 0
( 3)  [prog]_ItimXmet_3_2 = 0
( 4)  [prog]_ItimXmet_3_3 = 0
chi2(  4) =   21.50
          Prob > chi2 =    0.0003
```

2. Contrast between control (Sperm Washing) and PVP

a) 5 minute incubation

```
. lincom _Imethod1_2
( 1)  [prog]_Imethod1_2 = 0
------------------------------------------------------------------------------
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.7166667     .99188    -0.72   0.470    -2.660716    1.227382
------------------------------------------------------------------------------
```

b) 30 minute incubation

```
. lincom _Imethod1_2+ _ItimXmet_2_2
( 1)  [prog]_Imethod1_2 + [prog]_ItimXmet_2_2 = 0
------------------------------------------------------------------------------
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -2.342222   1.135542    -2.06   0.039    -4.567844   -.1166008
------------------------------------------------------------------------------
```

c) 60 minute incubation

```
. lincom _Imethod1_2 +_ItimXmet_3_2
( 1)  [prog]_Imethod1_2 + [prog]_ItimXmet_3_2 = 0
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -4.961111   1.052442    -4.71   0.000     -7.02386   -2.898362
------------------------------------------------------------------------------
```

3. Contrast between control (Sperm Washing) and SpermSlow

a) 5 minute incubation

```
. lincom _Imethod1_3
( 1)  [prog]_Imethod1_3 = 0
------------------------------------------------------------------------------
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   1.745556     1.1155     1.56   0.118     -.440784    3.931895
------------------------------------------------------------------------------
```

b) 30 minute incubation

```
. lincom _Imethod1_3+ _ItimXmet_2_3
( 1)  [prog]_Imethod1_3 + [prog]_ItimXmet_2_3 = 0
------------------------------------------------------------------------------
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -1.827778   .9093028    -2.01   0.044    -3.609978   -.0455771
------------------------------------------------------------------------------
```

c) 60 minute incubation

```
. lincom _Imethod1_3+ _ItimXmet_3_3
( 1)  [prog]_Imethod1_3 + [prog]_ItimXmet_3_3 = 0
------------------------------------------------------------------------------
prog |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -3.275556   1.075053    -3.05   0.002     -5.38262   -1.168491
------------------------------------------------------------------------------
```

4. Equivalence between two active mediums

a) 5 minute incubation

```
. lincom _Imethod_2
( 1)  [mot]_Imethod_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.0111114   .7014137    -0.02   0.987    -1.385857    1.363634
------------------------------------------------------------------------------
```

b) 30 minute incubation

```
. lincom _Imethod_2+ _ItimXmet_2_2
( 1)  [mot]_Imethod_2 + [mot]_ItimXmet_2_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   .4566667   .9059025     0.50   0.614     -1.31887    2.232203
------------------------------------------------------------------------------
```

c) 60 minute incubation

```
. lincom _Imethod_2+ _ItimXmet_3_2
( 1)  [mot]_Imethod_2 + [mot]_ItimXmet_3_2 = 0
------------------------------------------------------------------------------
mot |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   2.121111   1.152814     1.84   0.066    -.1383627    4.380585
------------------------------------------------------------------------------
```

ADDENDUM XII Holding solutions' specifications (from product leaflet)

Table 5.3 Table of PVP and SpermSlow™ holding solutions' specifications.

	PVP	SpermSlow™
Usage	For slowing down the movement of spermatozoa for ICSI.	For slowing down the movement of the sperm to allow for the selection of the mist mature, viable spermatozoa for ICSI.
Components	polyvinylpyrrolidone, HEPES-HTF, human serum albumin	water, sodium hyaluronate, human serum albumin, sodium chloride, amino acids, glucose, sodium bicarbonate, disodium hydrogen phosphate, porassium chloride, sodium pyruvate, nucleotides, calcium chloride, magnesium sulphate, vitamins, hydrochloric acid, sodium phosphate, calcium lactate, sodium citrate, potassium phosphate, L-malic acid, sodium acetate, human insulin recombinant, synthetic cholesterol, gentamicin sulphate
Appearance	Pink-rose color	Clear, colorless liquid
Odor	Odorless	Odorless
Solubility H_2O	Soluble	Soluble
Storage requirements	Store in original container at 2-8°C, protected from light and oxidizing agents. Do not freeze.	Store in original container at 2-8°C, protected from light. Do not freeze.
Shelf-life	12 months	12 weeks (minimum)
Cost per ml	R 2 538.00	R 6 682.50

Directions for use	1. Remove PVP ready to use solution from storage at 2-8°C and leave at room temperature for 10 minutes. 2. Depending on the number of oocytes for injection, pipette a corresponding number of 10 µl droplets of holding medium onto the bottom of the ICSI dish. 3. In the middle of the same dish place a 5-10 µl droplet of PVP ready to use solution. 4. Cover with pre-equilibrated liquid paraffin and place the dish in a 5-6% CO_2 environment at 37°C for 30 minutes prior to use. 5. Introduce 2 µl of prepared and washed sperm to the droplet of PVP ready to use solution. The PVP will reduce the motility of the sperm and facilitate the capture and loading of a single spermatozoon in the injection pipette.	1. Remove SpermSlow™ and preferred holding medium from storage at 2-8°C and leave at room temperature for 10 minutes. 2. Pipette 2x 10µl of SpermSlow™ onto the bottom of the ICSI dish, which should be kept at 37°C during the whole procedure (1 drop in centre, 1 drop at the rim of the dish). 3. Depending on the number of oocytes used in the ICSI procedure, pipette a corresponding number of 5-10µl droplets of holding medium. 4. Introduce a small amount e.g. 1-5 µl of prepared and washed sperm close to the SpermSlow™ drop in the centre of the ICSI dish. 5. Use the pipette tip to create a junction between the sperm droplet and the SpermSlow™ drop in the centre. 6. Immediately cover the ICSI dish with pre-equilibrated Liquid Paraffin and place it in a 5-6% CO_2 incubator at 37°C for 15 minutes prior to use. 7. Wash/rinse the injection pipette in pre-equilibrated Sperm Preparation Medium. Aspirate 10-20 mm into the holding pipette and 2-5 mm into the injection pipette 8. After 1 minute expel the Sperm Preparation Medium from the injection pipette into the dish with the Sperm Preparation Medium. The injection pipette is now coated to avoid sperm and

		SpermSlow™ adhering to the needle. 9. Place an oocyte in each droplet of holding medium. 10. Aspirate 5-6 mm SpermSlow™ from the SpermSlow™ drop at the rim of the ICSI dish. 11. Carefully select a mature spermatozoon from near the junction between the sperm droplet and the SpermSlow™ drop. Mature spermatozoa will be bound to the Hyaluronate in SpermSlow™. Therefore, look for the spermatozoon with the best morphology, and which has a moving tail but no forward motion (i.e. is 'moving in place'). *Spermatozoa moving freely in the SpermSlow™ drop are immature spermatozoa and should not be selected.*
Incubation requirements	Incubate 5-10 µl droplet of PVP ready to use solution onto the bottom of the ICSI dish, cover with liquid paraffin and place it in a 5-6% CO_2 environment at 37°C for 30 minutes prior to use.	Incubate 2x 10µl of SpermSlow™ onto the bottom of the ICSI dish, cover with liquid paraffin and place it in a 5-6% CO_2 incubator at 37°C for 15 minutes prior to use.

ADDENDUM XIII Chemicals used during experiment

Table 5.4 List of suppliers of the chemicals used in the study.

		Chemical	Supplier	Catalog number
Semen sample preparation and handling		Quinn's Sperm Washing Medium	SAGE, Cooper Surgical, Harrilabs, Paulshof, South Africa	ART-1006
		PVP 7% Ready-to-Use Solution	SAGE, Cooper Surgical, Harrilabs, Paulshof, South Africa	ART-4005-A
		SpermSlow™	Origio, Cooper Surgical, Harrilabs, Paulshof, South Africa	10944000
Experiment A	**Viability stain**	Eosin	Merck, Modderfontein, Gauteng, South Africa	1159350025
		Nigrosin	Merck, Modderfontein, Gauteng, South Africa	1159240025
	CMA_3 stain	Methanol	Merck, Modderfontein, Gauteng, South Africa	8222832500
		Acetic acid	Merck, Modderfontein, Gauteng, South Africa	1990619025
		Citric acid	Sigma-Aldrich, Johannesburg, South Africa	C2404-500G
		Sodium phosphate dibasic	Sigma-Aldrich, Johannesburg, South Africa	S7907-500G

		Chromomycin A_3 from *Streptomyces griseus*	Sigma-Aldrich, Johannesburg, South Africa	C2659 – 10MG
		Ethanol	Merck, Modderfontein, Gauteng, South Africa	1070172511
		Dabco 33-LV	Sigma-Aldrich, Johannesburg, South Africa	290734 – 100ML
		PBS tablets - Calbiochem	Merck, Modderfontein, Gauteng, South Africa	524650
		Formaldehyde solution	Merck, Modderfontein, Gauteng, South Africa	103999
	TUNEL stain	Human Serum Albumin	SAGE, Cooper Surgical, Harrilabs, Paulshof, South Africa	ART-3003
		Triton X-100 detergent - Calbiochem	Merck, Modderfontein, Gauteng, South Africa	648466
		Roche In Situ Cell Death Detection Kit, Fluorescein	Roche Diagnostics, Mannheim, Germany	11684795910
Experiment B	**Transmission Electron Microscopy**	Gluteraldehyde 25% aqueous solution	Merck, Modderfontein, Gauteng, South Africa	354400-500ML