Can Polarised Light Microscopy Be Used As A Non-Invasive Tool For Selecting The Optimal Spermatozoa For ICSI?

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Aim:

To investigate PLM as a reproducible method in the assessment of sperm head retardance, and observing PLM within a clinical setting to select an optimal sperm for ICSI.

Methods:

1. PLM images from 368 spermatozoa were assessed to establish intra and inter-observer variation.
2. A range and frequency of head retardance was developed from pooled samples and then analysed further comparing normal/abnormal head morphology.
3. Normal sperm selected for ICSI (WHO 5th edition), were imaged prior to injection. Images were analysed later-blinded to the ICSI outcome. Single embryo culture and elective single blastocyst transfer (eSET) were undertaken. PLM retardance was compared with fertilisation, cleavage, blastulation, utilisation and clinical pregnancy rates using independent sample T tests, Pearson's correlation coefficients, ROC curves and X^2 tests.

Results:

1. The intra/inter-observer variation revealed our method to be reproducible by an Intra-class correlation coefficient and Pearson's co-efficient ($P<0.05$).
2. The pooled sperm sample had mean retardance 0.97 +/-0.27nm, range 0.49-1.89nm. When comparing the range with head morphology it was found that retardance significantly decreases with normality ($P<0.05$).
3. 63 fresh and 38 frozen cases were analysed. 486 oocytes were injected and 364 fertilised (74.9%). 101 cumulative fresh and frozen single blastocysts were transferred (eSET) to produce 34 clinical pregnancies, 33.7% per Tx, 54.0% per case (fresh and frozen per OPU). There were no significant differences ($P>0.05$) in sperm retardance when comparing normally fertilised oocytes against abnormal/nil fertilisation. Good quality embryos had lower retardance ($P<0.05$) where ≤0.91nm was optimum. There were no significant differences ($P>0.05$) in retardance when comparing blastulation and failed blastulation, however utilised embryos had lower retardance compared to discarded embryos ($P<0.05$). Embryos with lower retardance were more successful ($P<0.05$) at clinical pregnancies where ≤0.91nm was optimum.

Conclusion:

Measuring head retardance is a reproducible non-invasive method of assessing sperm. Results suggest head retardance ≤0.91nm are more likely to result in a clinical pregnancy. Selecting sperm with lower head retardance may result in better embryo quality and higher clinical pregnancy rates.

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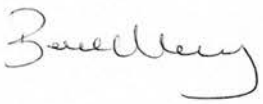

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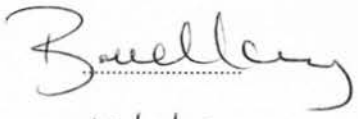
Signed 

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ABSTRACT

This thesis questions a new method to assess spermatozoa using polarised light microscopy (PLM) to measure sperm head retardance. This method was tested for accuracy then observed in sperm selected for Intra Cytoplasmic Sperm Injection (ICSI) and what the clinical outcomes were.

Literature review revealed limited studies assessing PLM in spermatozoa and their outcomes. It was found that sperm have total head and partial head birefringence whereby selecting sperm with partial birefringence is optimal for increased clinical pregnancy rates. It was also noted that DNA damage is linked to increased head retardance when sperm were subjected to artificial means of damage using ultra violet light. Validation of their methods in the literature was not clear, or not useful within this thesis. The value of PLM as a selection tool remains controversial, however this thesis aims to challenge this controversy with significant data using an observational approach to perhaps use PLM in a clinical setting.

METHOD

PLM (Oosight ™) images from 368 spermatozoa were assessed by two observers on two occasions blinded to the previous result to establish intra and inter observer variation. A range and frequency of head retardance was developed from pooled samples and then analysed further comparing normal and abnormal head morphology. Sperm assessed as being normal according to the World Health Organisation (WHO) 5th Edition and selected for ICSI were imaged prior to injection. Images were later analysed and measured blinded to the ICSI outcome. Single embryo culture and elective single blastocyst transfer (eSET) were undertaken. PLM retardance was compared with embryo fertilisation, cleavage, blastulation, utilisation and clinical pregnancy rates using independent sample T tests, Pearson's correlation coefficients and X^2 tests.

RESULTS

The intra/inter observer variation revealed our method to be reproducible. The intra variance being 3.3% and inter variance 4.4%. An intra class correlation coefficient report and Pearson's co-efficient revealed our method to being reproducible ($P<0.05$).

A range of head retardance was produced (0.49-1.89 nm) from the pooled samples with mean 0.97 +/- 0.27 nm. When comparing the range with head morphology it was found that retardance significantly decreases with normality ($P<0.05$).

63 fresh ICSI cases were analysed where 486 oocytes were injected and 364 fertilised (74.9%). 101 cumulative fresh and subsequent frozen single blastocysts were transferred to produce 34 clinical pregnancies (33.7% per Tx, 54.0% per case (fresh and frozen per OPU). The fresh clinical pregnancy rate was 31.8% (n=63) and the frozen clinical pregnancy rate was 36.8% (n=38). Independent samples T test's revealed no significant differences ($P>0.05$) in sperm retardance when comparing normally fertilised oocytes against either abnormal fertilisation or nil fertilisation. Normal cleavage embryos on day 3 (6-10 cells A or B grade), resulted from sperm with significantly ($P<0.05$) lower sperm head retardance compared to abnormal cleavage on day 3.

When embryo growth was extended to blastocyst culture, the rates of blastulation did not appear to be significant ($P>0.05$). Embryos selected for transfer and cryopreservation had a significantly ($P<0.05$) lower sperm retardance compared to embryos that were discarded due to poor quality. Sperm retardance was significantly lower ($P<0.05$) in blastocysts that resulted in clinical pregnancies.

A X^2 test comparing clinical pregnancies with sperm retardance ≤ 0.91 nm to sperm >0.91 nm was statistically significant ($P<0.05$). No clinical pregnancies occurred <0.56 nm.

CONCLUSION

In conclusion, PLM head retardance is a reproducible non-invasive method for assessing sperm. Our results suggest sperm with head retardance ≥0.56 - ≤0.91 nm is more likely to result in a clinical pregnancy. In a prospective manner, selecting sperm with retardance between these ranges may result in a greater number of good quality embryos and higher clinical pregnancy rates.

PRIZES AWARDED:

1. Best Poster Presentation.

Can Polarised Light Microscopy Be Used As A Non-Invasive Tool For Selecting The Optimal Sperm For ICSI?

14th World Congress on Human Reproduction/ Fertility Society of Australia 2011 Conference, Melbourne.

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SUMMARY OF ABBREVIATIONS AND SYMBOLS

ART	Assisted Reproductive Technology
ATP	Adenosine triphosphate
CG	Cortical Granules
DEG	Degenerate
DNA	Deoxyribonucleic Acid
eSET	Elective Single Embryo Transfer
FH	Foetal Heart
FISH	Fluorescence In-Situ Hybridization
FSH	Follicle Stimulation Hormone
GIFT	Gamete Intra-Fallopian Transfer
HA	Hyaluronic Acid
HCG	Human Chorionic Gonadotropin
HOS	Hypo-Osmotic Swelling
ICSI	Intra Cytoplasmic Sperm Injection
IVF	In Vitro Fertilisation
LH	Luteinizing Hormone
MESA	Microsurgical Epididymal Sperm Aspiration
MSOME	Motile Sperm Organelle Morphology Examination
MT	Microtubules
MI	Metaphase 1

MII	Metaphase 2
NEOF	No Evidence Of Fertilisation
OATS	Oligoasthenoteratozoospermia
OHSS	Ovarian Hyper Stimulation Syndrome
OPU	Oocyte Pick-Up
PLM	Polarised Light Microscopy
PVP	Polyvinylpyrrolidone
PVS	Perivitelline Space
ROS	Reactive Oxygen Species
SCSA	Sperm Chromatin Structure Assay
TESE	Testicular Sperm Extraction
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin End-Labelling
Tx	Transfer
ZP	Zona Pellucida
1PN	1 Pro-nuclei
2PN	2 Pro-nuclei (normal fertilisation)
3PN	3 Pro-nuclei

ETHICS APPROVAL

All studies applied in this thesis were approved by the IVF Australia Research and Development Committee and had ethical consent from a duly formed NHMRC ethics committee, before commencement within their laboratory.

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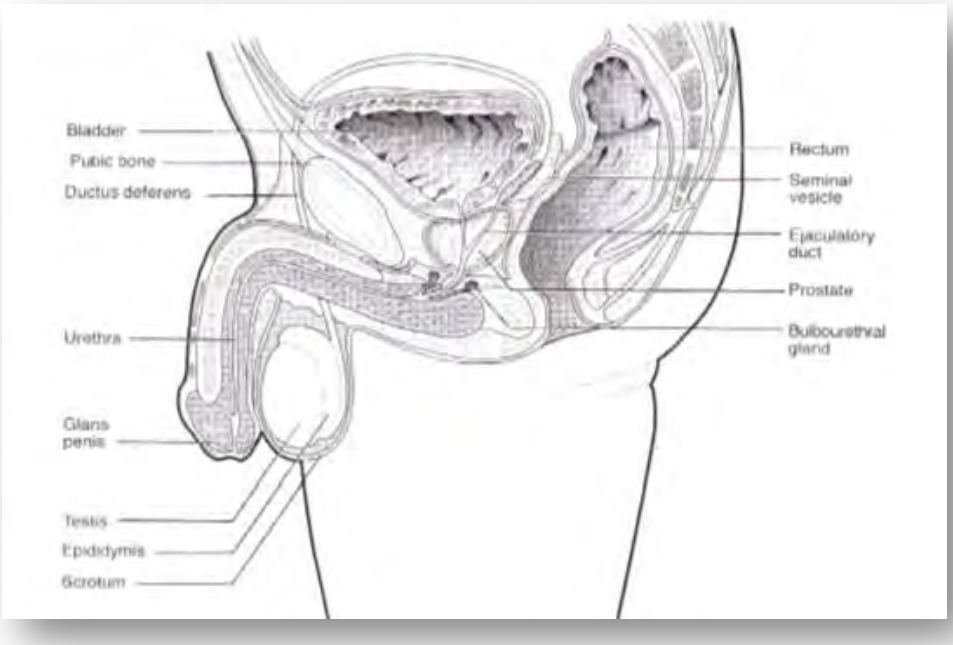
This thesis explores a new approach to determining the optimum spermatozoa for ICSI using polarised light microscopy with the goal of improving pregnancy success rates of IVF

HUMAN SPERMATOGENESIS AND PHYSIOLOGY

Spermatogenesis and Maturation

The male reproductive system is designed to produce as many gametes as possible in order to swim, seek and fertilise an ovum within the female reproductive tract (Figure 1). In order to do this, spermatogenesis, being the production of sperm, occurs within the testis then stored within the epididymis to enable maturity. The semen is then delivered into the female vagina via the penis through the mechanism of ejaculation.

Figure 1: Male reproductive system (Nicholson and Assinder, 2010).



Spermatogenesis and maturation is an ongoing process throughout a male's life, whereas females who are born with a set number of gametes, gradually decline over the years to ultimately end with virtually none at menopause. More recently however, growing studies show that the fertility potential of males

also decreases with age (Sherman, 1991). Male adults of reproductive age, have the ability to produce as many as 45-207 million sperm a day (Hill M, 2009). Spermatogenesis is hormonally regulated by a hypothalamic– pituitary testis axis, as well as cellular regulation within each testis.

The testis is predominantly composed of spermatogenesis coiled seminiferous tubules. These tubules contain two sections; the outer epithelium containing sertoli cells where developing spermatids are produced and the fluid filled lumen. The elongated sertoli cells house a limited number of developing germ cells during their development, providing nutrition and support (Nicholson and Assinder, 2010). The amount of germ cells that a testis produces is dependent on the concentration of sertoli cells.

Figure 2: Human male testicle (Nicholson and Assinder, 2010).

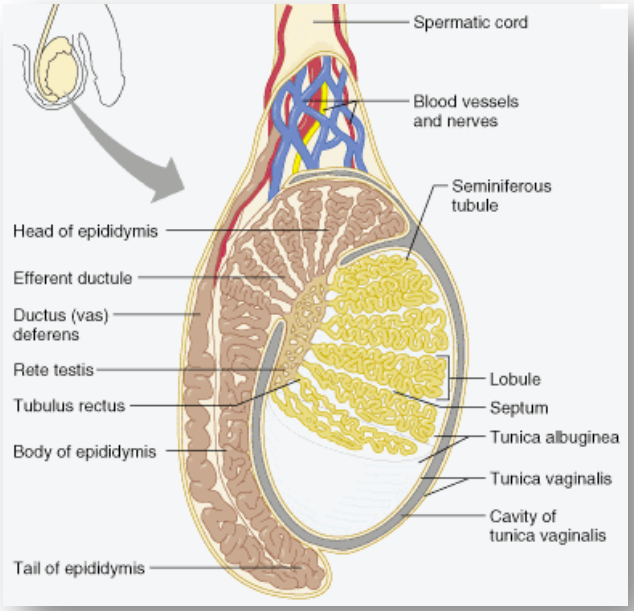
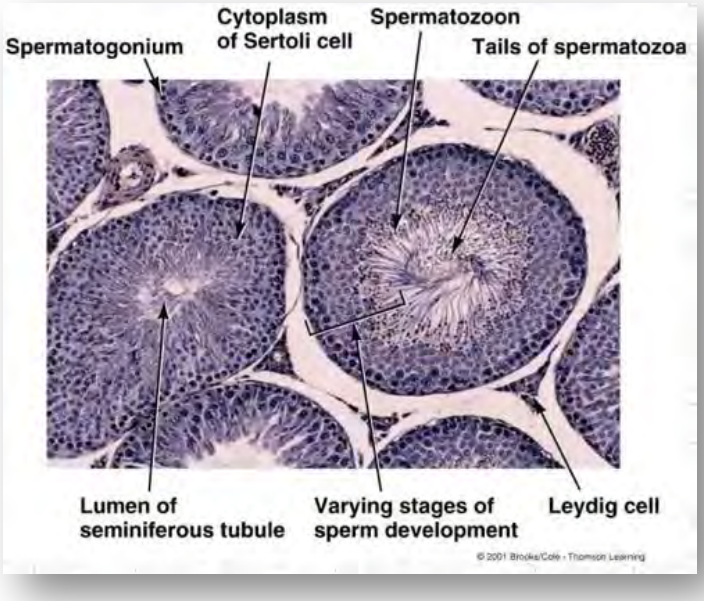
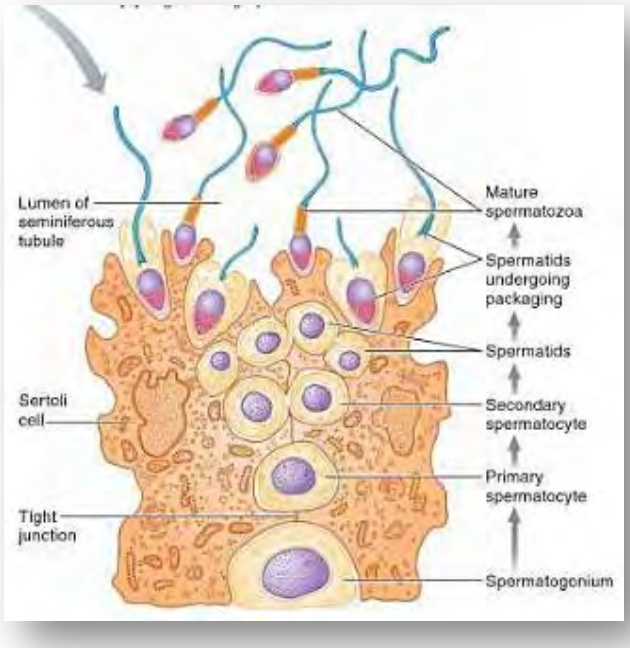


Figure 3: Cell structure within human seminiferous tubules (Sherwood, 2010).



It is within the spermatogonium stem cells, situated between the dormant sertoli cells that the first stage of spermatogenesis takes place (Figure 4). This is known as mitotic proliferation.

Figure 4: Spermatogenesis (Sherwood, 2010).



After two mitotic divisions of spermatogonia, the daughter cells remain bound together forming a string like apparatus of cloned cells. Then, the second stage of spermatogenesis begins, meiosis, a fundamental process to allow sexual reproduction (Nicholson and Assinder, 2010).

At this stage, each cell (primary spermatocyte) contains forty-six chromosomes. The diploid primary spermatocyte undergoes two cycles of meiosis to reduce the number of chromosomes by half, whilst producing four secondary spermatocytes. These newly formed haploid cells, containing twenty-three chromosomes, are now referred to as spermatids. Not all spermatids will continue to mature into spermatozoa, as human males are more susceptible to cell loss, particularly during meiosis (Nicholson and Assinder, 2010).

The final stage of spermatogenesis takes place within sertoli cells and is named late phase meiosis or spermiogenesis. This is a process where round spermatids undergo differentiation to form elongated spermatozoa, which generally takes twenty-two days to complete in man (Gupta, 2005). These events to form the head, neck and tail include acrosome and flagellum development, as well as the condensation of the nucleus and cytoplasm loss. Spermatogenesis occurs over a series of phases: the golgi phase, cap phase, acrosome phase, and the maturation phase.

The golgi phase requires the development of tiny carbohydrate rich granules within the membrane vesicle of the golgi apparatus. The granules and vesicle begin to fuse together to form the acrosomal vesicle, where polarity of the cell begins to form. A thickened mid piece begins to form and the mitochondria cluster together to form an axenome of a nine plus two arrangement within the flagellum. The DNA within the cell's nucleus begins to condense into a package known as chromatin.

The cap phase is the development of the anterior acrosomal region of the spermatid product of the acrosomal vesicle, which flattens and spreads (Dudek, 2011). The complete formation of the acrosome occurs within the acrosome phase, as well as the elongation of the condensed nucleus.

Finally maturation of the spermatid begins to take place. Once the tail has formed, the spermatid is released from its excess cytoplasm, leaving behind its residual body attached to the sertoli cell on the epithelium. The released spermatids now move within the fluid filled lumen, a process otherwise referred to as spermiation. The developing spermatids at this stage are not motile and thus rely on ciliated cells, fluid and the contracting smooth muscle activity of the tubules to mobilise them toward the epididymis for further maturation.

The epididymis is a structure that is capable of altering its environment such as pH, protein, ionic and carbohydrate concentration to suit the needs of the maturing spermatid. Within the epididymis, maturation of the flagellum introduces motility, allowing the mature spermatozoa to seek and fertilise oocytes. Other roles of the epididymis include protection, concentration, storage and transport of spermatozoa.

The epididymis lays adjacent to the posterior surface of the testis and is partitioned into two sections, the lumen and the epithelium. The epididymis is regulated mainly by androgens, but more recent research has found retinoid, oestrogen and other growth factors contributing to its regulation (Robaire and Hinton, 2002).
Many types of cells are located within the epithelium, each having individual functions. The most abundant cells found are principal cells, contributing to 65-80% of the epithelium (Robaire and Hinton, 2002). Other cell types include basal, narrow, clear and halo (Nicholson and Assinder, 2010).

Spermatids have no "biosynthetic capability", and so rely on excreted biocatalysts (enzymes and transfer proteins) within the lumen to complete maturation. These proteins that are excreted from the epithelium are absorbed through the head allowing mature spermatozoa to recognise an oocyte and allow binding to its zona pellucida (Amman and Hammerstedt, 1993). Once absorption of proteins has completed, the storage of mature spermatozoa takes place within the cauda and ampulla of the epididymis.

Finally, studies have discovered a protective mechanism of maturing spermatozoa by the epididymis. Gamma glutamyl trans peptidases are enzymes that are secreted from cell surfaces have been reported to protect

sperm from reactive oxygen species (Robaire and Hinton, 2002). Tight junction complexes found in-between epididymal epithelial cells form a blood-epididymis barrier, protecting developing spermatozoa from the immunological response of the man (Robaire and Hinton, 2002).

Physiology

Van Leeuwenhoek first visualised the human male spermatozoa under a simple microscope in 1677 and described its structure and mobility. With the development of microscopy, the external structures of human spermatozoa were more clearly described using the compound microscope, followed by their internal structure using the electron microscope during the 1950's.

Spermatozoa are an immensely differentiated sex cell, which can be characterised into three sections: the head, principle piece or neck, and tail (DeJonge and Barratt 2006).

The function of the head is to protect, house and deliver haploid paternal DNA after binding, entering and releasing the DNA into the cytoplasm of an oocyte to initiate fertilisation. The head piece of human spermatozoa are oval/spatulate in shape, bilaterally flattened and covered in a plasma membrane that continues to the tip of the tail.

The nucleus of the spermatozoa contains twenty-three highly condensed inactive chromosomes, one half of each chromosome pair. They are kept separate from conjoining neckpiece and tail by a series of "fine cords and striations made up of small membrane particles" known as the posterior ring (Yovich, 1995). Along with DNA, protamine's exist within the nucleus and are made up of small nuclear proteins, mostly amino acids arginine and cysteine. It has been found that humans have two active protamine genes within the spermatozoa, however the location of these genes is unclear. A disruption in the protamine's when the sperm enters an oocyte, reactivates the DNA to replicate (Eddy, 2006).

The nuclear envelope is a double lipid bilayer that acts as a barrier separating cytoplasm of the cell from the DNA, which is continuous through to the basal plate. Unlike nuclear envelopes in other eukaryotic cells, human spermatozoa are unique due to the implanting surface of the tail fossa at the posterior section of the envelope.

The acrosome is defined as a membrane bound vesicle covering the anterior section of the nucleus (Yovich, 1995). It is a flattened membrane vesicle that covers 40 – 70% of the anterior head and is made up of multiple layers these being the outer acrosome membrane, inner acrosome membrane, nuclear envelope and the plasma membrane. The space between the inner acrosome membrane and nuclear envelope is known as the acrosomal matrix or intra-acrosomal space, where specific enzymes are located. Hyaluronidase and acrosin are two main enzymes located in this matrix. Hyaluronidase enables the spermatozoa to digest its way through the oocytes cumulous matrix enabling to reach the zona pellucida, where acrosin allows penetration through the zona. The post acrosomal segment lies posterior to the acrosome, and plays an active role in sperm-egg fusion.

Before spermatozoa undergo the acrosome reaction, capacitation occurs post ejaculation. This is a fundamental process to complete maturation and to allow fertilisation. Capacitation involves a series of controlled changes throughout the entire sperm cell, from the head to tail, membranes and cytoskeletons. These changes begin from the moment spermatozoa interact with cervical mucous where some surface-adsorbed materials are lost, and continues up until it reaches cumulous cells of the oocyte (Lamirande *et.al.,* 1997).

Capacitation was described by Chang and Austin (1951), "A period of time that sperm must reside in the female reproductive tract before they acquire the ability to fertilise oocytes". It is a reversible process (de-capacitation) that involves the net reduction of surface charge, loss of carbohydrates, and removal of peripheral membrane factors.

On the spermatozoa surface lie factors preventing the binding to the surface of an oocyte or block the acrosome reaction from occurring. These ‘blockers’ are

thought to prevent signals from reaching receptors on the sperm surface by altering the plasma membrane lipid bilayer, ion channels, enzymes and stability factors so that it cannot respond. Capacitation is the removal of these blocking factors prior to the acrosome reaction occurring.

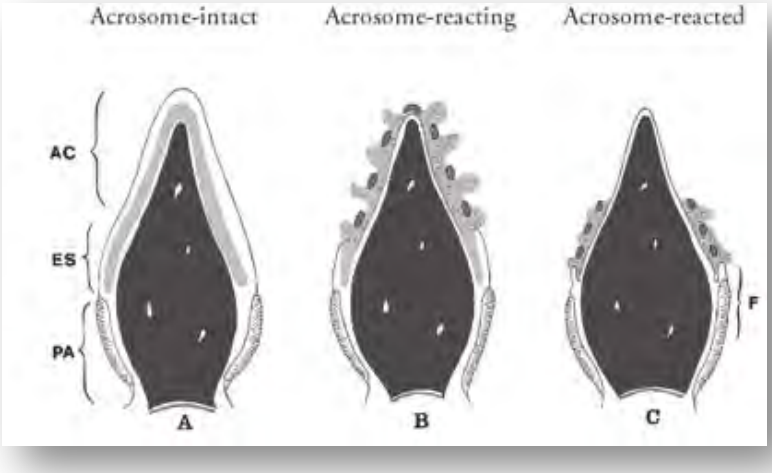
The plasma membrane remains stable by a cholesterol/phospholipid ratio. With a reduction in cholesterol or increase in phospholipids, this ratio becomes unstable resulting in membrane permeability whilst reducing its blocking factors (Zaneveld *et.al.,* 1991).

It has been noted that mitochondria found in the axoneme of the flagellum are packed tightly prior to capacitation. Mitochondria, being energy producing organelles are highly dynamic and have the "ability to change shape and size depending on their metabolic state" (Vorup-Jensen *et.al.,* 1999). X- ray microscopy has recently illustrated the distension of the mitochondria and loose packing around the axoneme post capacitation. This change in state of the mitochondria is thought to be due to the hyper activation of spermatozoa that occurs after capacitation.

Hyper activation of spermatozoa, usually seen as a vigorous whiplash movement, is an essential event occurring at the site of fertilisation. This high amplitude, asymmetrical movement of the flagellum has been suggested to occur due to an influx of calcium within the flagellum and enables the penetration through the cumulus matrix and zona pellucida.

In conjunction with hyper activation, a series of chemical changes begin within the acrosome allowing the fusion of the spermatozoa to the oocytes cell membrane, otherwise known as the acrosome reaction. The acrosome reaction has been defined as "the morphological manifestation of sperm capacitation where sperm acquire the ability to penetrate the egg vestments, cumulus oophorus and the zona pellucida aided by hyper-activated motility" (Sathananthan, 2010) as illustrated in Figure 5.

Figure 5: Acrosome reaction (Sathananthan, 2010).



The acrosome reaction occurs over two stages. The first stage is where the spermatozoa acrosome cap reacts and is known as the physiological acrosome reaction. The second stage is the reaction of the equatorial segment within the acrosome initiated by glycoproteins on the surface of the zona pellucida (ZP). There are three types of glycoproteins, ZP1, ZP2 and ZP3. Oocyte zonae contain an estimated one billion copies of ZP3, and it is this specific glycoprotein that initiates spermatozoa acrosome reaction, the release of hyaluronidase and acrosin enzymes (Plachot and Mandelbaum, 1990).

These enzymes are released after multiple fusions between the plasma and outer acrosome membranes occur, after proteins on the sperm membrane have been activated. These proteins cause the structural ability of the acrosome cytoskeleton to fail, causing the outer membrane to come into contact with the plasma membrane.

Once contact has occurred, fusion proteins manifest within the plasma membrane allowing 'channels' to develop, opening a gateway for enzymes to be released from the acrosome (Zaneveld *et.al.,* 1991).

It has been noted that up to 60% of the spermatozoa acrosome has reacted at the sight of the ZP, and to enable the fusion of sperm and oocyte, spermatozoa need to complete this reaction to penetrate the inner ZP (Sathananthan, 2010). Once the acrosome reaction has completed, the spermatozoon plasma membrane (anterior to the post acrosome segment) fuses to the oolemma. The sperm nucleus and its genetic contents begin to expand within the ooplasm, eventually forming the male haploid pro-nucleus (n=23). Simultaneously, the oocyte completes its second meiotic division activated by sperm-egg fusion, exemplified by the release of the second polar body into the PVS containing the excess maternal chromosomes.

The remaining chromosomes within the ooplasm form the second haploid pro-nuclei (n=23); this is known as the female pro-nucleus. Together the male and female pronuclei form 46 chromosomes (diploid) within the oocytes ooplasm. Pronuclear association can be seen clearly seen 12-16 hours post gamete fusion with syngamy occurring at approximately 20-24 hours (Plachot and Mandelbaum, 1990; Sathananthan, 2010). Syngamy can be defined as the breakdown of the pronuclear envelope, allowing the fusion of maternal and paternal DNA.

One form of abnormal fertilisation is polyspermy. This is the event where more than one spermatozoon penetrates the ZP yielding in the formation of excess genetic material. One way the oocyte attempts to prevent polyspermy from occurring is through the cortical reaction.

The human oocyte contains cortical granules (CG), which are secretory golgi-derived organelles located within the cortical region. They migrate to the oocyte cortex during meiotic maturation. CG contains different enzymes such as proteinases and glycosidases that modify and remove sperm receptors from the extracellular investments of eggs upon fertilisation (Ducibella, 1996; Ghetler *et.al*. 1998).

Once gamete fusion has occurred in conjunction with an influx of calcium, exocytosis and release of the CG into the PVS takes place. This is known as the cortical reaction.

The CG form a layer beneath the PVS, and with the release of specific enzymes and interaction with glycoproteins on the ZP, the protein structure of the ZP is altered to function as a polyspermic block (Wang *et.al.*, 1997; Sathananthan 2010). This process of chemical hardening of the ZP is known as the zona reaction.

The spermatozoa neck region is located posterior to the head and is structurally different to that of the mid piece that is positioned directly beneath. The major difference is the organisation of microtubules (MT), which comprise of α-tubulin and β-tubulin.

The neck region consists of a proximal centriole comprising of a 9 + 0 arrangement of MT, whilst the mid piece contains a 9 + 2 arrangement. Seen within a transverse section, the 9 + 2 arrangement is characteristic of cilia and flagella, and is otherwise referred to as the central axoneme. The spermatozoon mid-piece contains spirals of mitochondrial organelles, providing ATP as the source of energy for motility.

The tail composed of cysteine proteins is the most posterior segment of the spermatozoa and consists of a principal piece and an end piece (Eddy, 2006). The principal piece can be defined as "the segment of the mammalian sperm flagellum which is enclosed by the fibrous sheath" (Fawcett, 1970). The fibrous sheath that surrounds the principal piece is composed of differing thickness of "filamentous subunits oriented circumferentially" (Fawcett, 1970). The fibrous sheath extends the length of the principal piece, attaching itself to axonemal doublets 3 and 8, terminating at the beginning of the spermatozoon end piece (Eddy, 2006).

The original function of the fibrous sheath was thought to act as an external cytoskeletal support, but with increasing evidence its function is to promote elastic properties or restraint to aid the flagella beat during motility. The fibrous sheath is also involved with signal transduction, energy production and processes leading to fertilisation by acting as a docking station for materials such as proteins and enzymes (Eddy, 2006).

Infertility and Causes

The definition of infertility is the inability to conceive or carry a pregnancy with regular unprotected intercourse after a period of 12 months. Today, one in six couples will have trouble trying to conceive a child. It is estimated that 50% of these couples having difficulty will seek medical advice (Herbert *et. al.*, 2009).

There are a range of factors contributing to infertility in both male and females, which include anatomical, genetic, hormonal and lifestyle factors.

Female factor infertility makes up for 35-40% of infertility. Some causes for female infertility include endometriosis, tubal disease, anovulation, fibroids and polycystic ovarian syndrome (PCO/S). Some lifestyle factors that may contribute to female infertility include obesity, with increased body max index, increased levels of alcohol consumption, recreational drugs and smoking.

Male factor infertility makes up for 35-40% of infertility. Some of these factors are anatomical such as abnormal or absent vas deferens, poor spermatogenesis with reduced sperm concentration, poor sperm motility and abnormal morphology. Other factors also include auto immune responses against sperm causing anti-sperm antibodies, erectile dysfunction and retro-ejaculation. Some lifestyle factors include increased levels of alcohol consumption, recreational drugs and smoking.

The remaining 20-30% of couples who experience infertility has a combination of both male and female factors or who have unexplained causes otherwise referred to as idiopathic (Strachan and Walker, 2010).

Female fertility declines with age and is accompanied with an increase of miscarriage risk. The late onset of 'trying' to conceive in today's society is major cause for infertility. Studies have shown that male fertility also decreases with age, with the percentage of damaged and genetically abnormal sperm increasing (Singh *et.al.,* 2003; Wyrobek *et.al.,* 2006).

Assisted Reproductive Technology

History

Assisted reproductive technologies (ART) like many other medical advances have been introduced and rapidly developed throughout the 20th century.

The history of ART began in 1935 when the rabbit's follicular oocytes were studied. Pincus and Enzmann showed that oocyte maturation, where the first polar body is extruded, occurs eight hours after copulation. They performed the earliest experiments on in vitro fertilisation (IVF) with success when fertilised ova were transferred back into the fallopian tubes of adult rabbit. There were reports cleavage had taken place, although no pregnancies (Pincus and Enzmann, 1935).

In 1948, Memkin and Rock collected oocytes from women who underwent surgery to remove their reproductive organs (due to various medical conditions). They inseminated these oocytes 29 hours after retrieval, followed by close observation for fertilisation. It was noted some oocytes did cleave into two and three cells, however, division failed to progress further (Memkin and Rock 1948). This was significant in the history of IVF as this was the first known attempt of fertilising human gametes. Modern knowledge suggests that the cause for the low fertilisation and cleavage rate could have been due to the incorrect timing of insemination.

In 1950, Wharton studied rabbits' pre-ovulatory oocytes by stimulating their ovaries with three separate gonadotropins, Synapoidin, Serogan and Gonan. To further his knowledge in embryology, his aim was to discover the optimum gonadotropin to produce abundant oocytes, in which he succeeded. In his experiments, Wharton also attempted to fertilise human oocytes although he was unsuccessful. The proposed reason for his failed attempt of fertilisation was the prolonged age of the oocytes from retrieval to insemination, and their culture in saline (Wharton B. 1950).

In 1959 Chang developed a reproducible method to fertilise mammalian oocytes using the rabbit, and was successful in producing the first live young

after transferring these fertilised oocytes back into a doe recipient (Chang, 1959).

In 1961 Palmer proclaimed to be able to view the human ovary via a new surgical procedure named laparoscopy. He ended one of his presentations with "this technique should be of great interest for the study of the physiology of ovulation, and should lead to a new way of treating sterility due to tubal occlusion. It should be of great interest in human embryology and genetics and help to obtain embryological material after in vitro fertilisation"(Grzegorz and Litynski, 1997; Palmer and Cohen, 1965). It was not long after this time, laparoscopy was routinely used as the method for oocyte retrievals.

It was understood that within animals, oocyte maturation needed to occur before fertilisation could take place, it was also understood that follicular or pre-ovulatory oocytes had a higher fertilisation potential than oocytes that had been retrieved after they had been ovulated.

In 1967, Barros and Austin used this knowledge to further study hamster fertilisation in vitro. Their study supported the theory that oocytes with the largest fertilisation potential were those retrieved one to two hours pre ovulation. Barros and Austin tested the theory that hamster spermatozoa can undergo capacitation in vitro, shortly followed by the acrosome reaction. They hypothesised that an agent within the pre-ovulatory follicle, not from oocytes, triggered capacitation and the acrosome reaction (Barros and Austin 1967).

For various medical conditions, twelve women underwent hysterectomies in London. Leading up to surgery, they were injected with follicle stimulation hormones (FSH) for six consecutive days, (day four to nine of cycle) followed by a human chorionic gonadotropin (HCG) injection on day nine (6000 I.U). Between 24-45 hours later, laparotomy occurred and all follicles were drained and oocytes were obtained. Not all oocytes retrieved were mature, but they were retained in culture. This was the first linked discovery that like other mammalian animals, human oocytes also have the potential to mature in vitro (Jagiello *et.al*., 1968). At the same time, Whittingham reported mice embryos, created in vitro, growing further than two to three cells. Embryos grew to blastocyst stage, typically seen five days post insemination. This opened up a

new realm where under specific scientific and culture conditions, mammalian blastocysts had the capacity to develop in vitro (Whittingham, 1968).

Two of ART's greatest pioneers Edwards and Steptoe, along with their colleague Bavister, closely repeated the experiment Barros and Austin completed in 1967 but on humans. This was the first published experiment showing that human in vitro fertilisation was achievable, and that human oocytes can be fertilised in conditions similar to those found most suitable for the fertilisation of hamster eggs in vitro (Edwards *et.al.,* 1969).

In 1973, Carl Wood and John Leeton reported the first transfer of an eight-cell embryo into the uterus of a thirty-six year old woman. The patients' cause for infertility was tubal occlusion. A rise in HCG after the embryo transfer could not be explained any other than implantation, which was then followed by an early miscarriage (Wood *et.al.,* 1973).

Three years later in 1976, Steptoe and Edwards also attempted a clinical pregnancy in vitro. They transferred an embryo that was transitioning from morula to early blastocyst into a thirty-five year old woman. This attempt was unsuccessful, as it resulted in an ectopic pregnancy (Steptoe and Edwards, 1976). They were however finally successful, with the birth of Louise Brown on July 25th, 1978 (Steptoe and Edwards, 1978).

In July 1982, Jones reported a specifically designed 'Vital Initiation of Pregnancy' (VIP) protocol, which illustrated how their clinic was producing clinical pregnancies. They spoke of using stimulated cycles and ultrasound to predict the time of ovulation and oocyte aspiration. They described their insemination procedures and use of culture control (Jones *et.al.,* 1982).

With the increased need to improve the success of ART, there was an influx of new techniques during the 1980's. Lenz first described the improved method of oocyte collection via a foot controlled use of an ultrasound guided probe and needle. Compared to laparoscopy, their new technique was surgically simpler, more efficient in the amount of time it took to perform, and allowed patients to be discharged within the same day (Lenz *et.al.,* 1981; Tan *et.al.,* 1990).

In 1981, Leeton demonstrated that pregnancies could be achieved using the clomid citrate – HCG Protocol. This follicle stimulating protocol was introduced due to inadequacies of LH sampling. These inadequacies of sampling included the uncertainty of the established LH surge, the chance of ovulation prior to oocyte collection, round the clock testing and the inaccuracy of some LH urine kits. The HCG trigger allowed the control of ovulation, where the injection that was given thirty-six hours prior to oocyte collection (Leeton *et.al.,* 1981). This was an important change in ART, as it allowed more control of a patient's cycle, and produced higher success rates.

In 1983, the world's first reported donor cycle was attempted at Monash University. A forty-two year old woman donated an oocyte to a thirty-eight year old infertile woman. The oocyte that was donated was fertilised using donor spermatozoa. Unfortunately, the pregnancy failed at ten weeks gestation. The donation of gametes opened up many debates regarding the "ethical, legal and psychological implications of using donor material to achieve a pregnancy", some of which are still being discussed today (Trounson *et.al.,* 1983).

Gamete intra-fallopian transfer (GIFT) was recognised as an innovative technique that proved to be successful when in 1984, Asch reported the first clinical pregnancy. The methodology of GIFT firstly involved semen preparation, to allow a final concentration of 100,000 per 25 uL of rapidly motile spermatozoa. Oocyte collection then proceeded via laparoscopy, at which all follicles were drained and oocytes were assessed for their maturity. The prepared spermatozoa sample was then loaded through the transfer catheter, followed by the mature oocytes into the fallopian tubes. Asch reported GIFT as a useful ART technique for female patients whose fallopian tubes are patent, particularly in unexplained infertility between the couple (Asch *et.al.,* 1984).

The ART techniques used up until 1984 mainly assisted patients whose reason for infertility was female factor and/or minor male factor. After 1984, new techniques began to target severe male factor infertility, where one or more seminal parameters were poor.

One such new advancement was to aspirate spermatozoa from the epididymis. Microsurgical Epididymal Sperm Aspiration (MESA) was mainly used for males that presented with obstructive azoospermia, having absent or non-patent vas deferens. The first pregnancy using this technique with IVF occurred in 1985 (Temple-Smith *et.al.,* 1985).

A technique to evaluate the DNA integrity of spermatozoon was introduced in 1984. By using Acridine Orange (AO) with a fluorescence microscope, spermatozoon heads were assessed for their fertility potential. Under the fluorescence microscope, the spermatozoon with double stranded normal (native) DNA were one colour, while those with single stranded, denatured, abnormal DNA were another colour. This new technique was advantageous as it allowed scientists to estimate a new "effective sperm count". The fertile sperm count was calculated by adjusting the raw concentration of spermatozoon against the percentage of normal DNA sperm. Tajada estimated an effective sperm count cut-off of 50 million per millilitre for any fertility potential (Tajada *et.al.,* 1984). This test was developed further with the combined use of AO, DNA probe and a flow cytometer and was named SCSA (Sperm Chromatin Structure Assay) (Evenson *et.al.,* 1991). This test works by measuring the amount of DNA fragmentation from thousands of sperm after the DNA has been denatured with acid *in situ.* It is a very reliable test that has become a significant indicator for male fertility, especially when all other seminal parameters may be normal.

Trounson *et.al.,* (1987) reported a technique named subzonal insemination (SUZI) to assist severe male factor infertility. This technique involved microinjecting a single capacitated sperm under the zona pellucida of a mature oocyte's perivitelline space (PVS). With the injected spermatozoa having direct contact with the oocytes oolemma, fertilisation and cleavage could be achieved. The first pregnancy obtained via SUZI was announced in 1988. Figure 6: Subzonal insemination- the injection of spermatozoa within the PVS.

Figure 6: Subzonal insemination (Bourne, 2010).

Partial Zona Dissection (PZD) was announced in 1989 as a new technique in which small holes were mechanically drilled through the oocytes zona pellucida (Cohen *et.al.,* 1989). Acidic Tyrode's solution using micromanipulation was used to drill small holes, allowing spermatozoa to travel through the zona to enhance fertilisation potential. PZD was useful where semen samples had reduced motility. It was not beneficial for use in semen samples with reduced concentration, morphology or with autoimmunity factors. Figure 7: Partial Zona Dissection

Figure 7: Partial Zona Dissection (Bourne, 2010).

In 1990, a Californian IVF clinic developed a new method for semen preparation for samples with severely reduced concentration and motility. The advantage of the new mini-percoll method over other semen preparations was the improved recovery of morphologically normal, motile sperm in a cleaner final sample. The improved recovery of good quality spermatozoon was due to the reduced volume of each gradient layer. The mini-percoll method resulted in improved fertilisation and pregnancy rates, whilst having no negative impact on miscarriage rates (Ord *et.al.,* 1990).

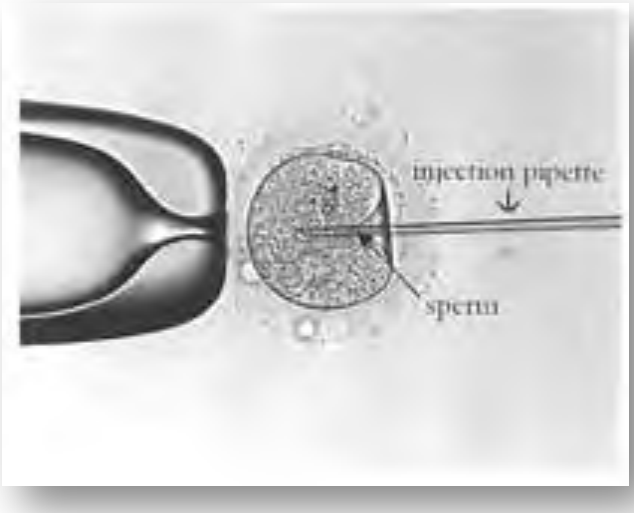
In 1992, assisted hatching was tested using human embryos to aid implantation success. The cleavage stage embryo was hatched using acidic Tyrode's solution to thin an area of the zona pellucida. Cohen reported in their randomized trial on 330 patients, that embryos with thick (>15µm) zona had a significantly higher implantation rate if they were hatched with assistance. If assisted hatching was used on thin (<13µm) zona, the procedure put the embryo at risk of damage. Cohen concluded from this study that assisted hatching was advantageous to embryos in which the woman was older than thirty-eight years (Cohen *et.al.,* 1992).

The face of ART changed in 1992, with the announcement of the first live birth generated by Intra-cytoplasmic Sperm Injection (ICSI). ICSI was developed to enhance fertilisation capability for males diagnosed with severe infertility (Palermo *et.al.,* 1992).

In the original reports ICSI was performed after spermatozoa were prepared one day prior to oocyte collection. The spermatozoa were cultured in T6 medium overnight, followed by exposure to an electrical field. This was done to increase the percentage of acrosome reacted spermatozoon. This practice however no longer occurs. Once oocytes were collected, assessment for maturity took place after they were stripped from their coronal and cumulus cells by submerging them into hyaluronidase for up to one minute. Mature oocytes that had expelled their first polar body were microinjected by using handmade micro injector and holding pipettes. By holding the oocyte with negative pressure, a single immobilised spermatozoon was injected into the ooplasm. Fertilisation was assessed sixteen to eighteen hours post injection followed by transfer of up to three embryos forty-eight hours later. Four

couples, whose reason for infertility was severe male factor, underwent ICSI resulting in three successful live births. The impact ICSI would potentially have on the future of assisted reproductive technology has hypothesised the need to further understand the negative impacts ICSI might have, especially damage to ooplasmic structures. Figure 8: Intra Cytoplasmic Sperm Injection

Figure 8: Intra Cytoplasmic Sperm Injection (Bourne, 2010).



A larger study reported in 1995 involving 296 ICSI cycles, showed the effectiveness of this revolutionary technique. This study also assessed the effectiveness of ICSI on different types of severe male factor infertility and the impact it may have had on frozen embryos. Spermatozoa in this study were obtained from the epididymis or testis of patients, due to autoimmunity, morphological and genetic disorders of male patients. With healthy live births gained from this study, ICSI clearly demonstrated its ability in cases that allow conception, where previously they were regarded as impossible. This study showed little impact of ICSI on frozen embryos, suggesting that microinjection had not compromised the ability of embryos to survive freezing and thawing (Bourne *et.al.,* 1995).

Many studies have focussed on children conceived via ICSI. The injection of an abnormal morphological spermatozoon when no normal forms could be found raised concerns of potential negative implications, since ICSI doesn't allow for natural selection of the fertilising spermatozoon. ICSI potentially could increase

the chances of genetic abnormalities in offspring. Another concern was for males with bilateral absence of vas deferens who underwent ICSI. There was evidence that some males, who expressed this defect, carried a mutated Cystic Fibrosis gene that could potentially be genetically passed onto their offspring (Pierce and DeJonge, 1995).

A study in 1996 of 423 children born after ICSI, suggested that ICSI caused no increase to genetic abnormalities compared to natural conception and IVF population registries (Bonduelle *et.al.,* 1996). The European Society of Human Reproduction and Embryology, together with the international working group for registrars, released a report in 1997 indicating a 2.7% incidence of offspring malformation that were conceived via ICSI in the births of 3325 babies. This report showed comparable malformation percentages to those conceived via natural conception. However, there was an increase of 2% for sex chromosomal abnormalities in babies conceived via ICSI compared to natural conception. This increase was potentially caused by the inheritance of paternally derived chromosomal abnormalities (Mansour, 1998).

Ethical and moral issues of ICSI arose. Counselling was encouraged for patients having ICSI to ensure they understood the potential risk of transmitting sex chromosomal abnormality and risk of transmitting fertility problems to their offspring (Pierce and DeJonge, 1995).

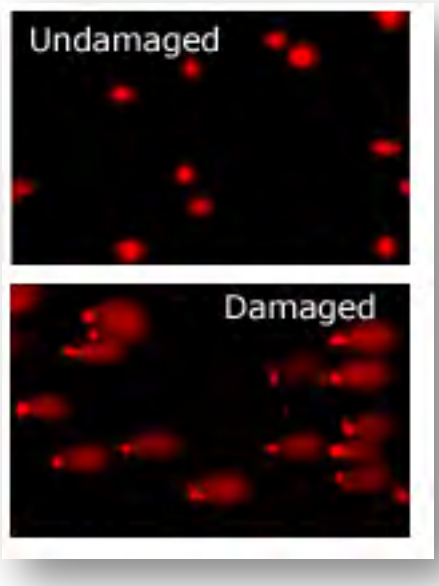
Male patients diagnosed with azoospermia but who had spermatogenesis, could now take advantage of ART with the introduction of microsurgical aspiration of epididymal spermatozoa (MESA) and testicular sperm extraction (TESE). In many reported cases of MESA, the sperm aspirated had the concentration and motility capable for conventional IVF, although experiments from Silber showed that ICSI was more favourable with increased fertilisation and pregnancy rates (Silber *et.al.,* 1994). In 1995, studying a group of fifteen couples where males were diagnosed with non-obstructive azoospermia, TESE was performed with sperm being recovered for thirteen of these men, and producing a live birth rate of 25% (Devroey *et.al.,* 1995). Without the introduction of surgical sperm aspiration, the ability to reproduce for these couples and thousands more across the world would have been unachievable.

With more clinics using TESE for patients with severe oligoasthenozoospermia, a method was needed to distinguish the viability of these spermatozoa for ICSI. To that date, the method to assess viability had been using cytoplasmic stains such as eosin-nigrosin, but it was unclear if by using these stained spermatozoa to inject into an oocyte, it could affect ICSI success. In 1996, spermatozoa were tested for viability using the new hypo-osmotic swelling test (HOS). When the spermatozoa were subjected to the HOS solution (sodium citrate, fructose and distilled water), the tails of the spermatozoon swell to indicate viability. This hydrophobic sensitivity of the tails showed viability as their osmotic regularity systems were in place and as well as having functioning membrane integrity (Esteves *et.al.,* 1996). By conducting the HOS test at the time of ICSI, selection of a viable sperm was achieved.

Knowing that sperm DNA damage is associated with poor fertilisation results, poor embryo quality and risk of miscarriage, new techniques were developed for it's testing.

The COMET assay, otherwise known as Single Cell Gel Electrophoresis (SCGE), was reported in 1996. (Figure 9) SCGE works by reflecting damage as strand breaks in the DNA. SCGE results are visualised under fluorescence light, where cells with increased DNA damage display an increased migration of the DNA from the nucleus (comet head) towards the anode (tail)(Gutiérrez *et.al.,* 1998). The main outcomes of this study demonstrated the reproducibility of SCGE to assess sperm DNA damage and the sensitivity of the test on sperm cells. To provide the percentage of DNA damage in a total sperm population, this study showed that fifty sperm was sufficient to provide this evaluation. This relatively quick sample testing, ease of application and low cost technique, allowed a new reliable testing method to assess sperm DNA damage for patients (Hughes *et.al.,* 1997).

Figure 9: COMET assay using undamaged and damaged sperm (Duty *et. al.,* 2003).



In 1997, a Canadian group reported the incidence "of DNA fragmentation, its correlation with semen analysis parameters and fertilisation rates" in 298 semen samples using a new technique (Sun *et.al.,* 1997). After undergoing swim up preparation to select the optimum spermatozoon, a TUNEL (Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin End-Labelling) assay was performed. Results showed that samples containing poor seminal parameters were more likely to have increased DNA fragmentation whilst producing poorer quality embryos. It was also found that spermatozoon with higher percentages of DNA fragmentation were more likely to result in failed fertilisation, making these men candidates for ICSI (Sun *et.al.,* 1997).

In the mid 90's, assisted reproduction success rates were slowly increasing in conjunction with associated changes in culture conditions and the development of new tests and laboratory techniques. Up until 1998, embryo transfers occurred mainly at cleavage stage of between four to eight cells, with implantation rates being between five and thirty percent (Gardner *et.al.,* 1998). With implantation rates being low, the popular approach was transferring more than one embryo to increase the pregnancy rate. As a result of this, the incidence of multiple pregnancies with its associated risks such as preterm delivery, low birth weight, foetal death and cerebral palsy increased.

David Gardner *et.al.,*(1998) reported a randomised trial on blastocyst culture and transfer. They found an equivalent pregnancy rate when single blastocysts were transferred compared to multiple cleavage stage embryos. However, the implantation rate was significantly higher when a blastocyst was transferred. "It is therefore possible to attain high pregnancy rates without having to transfer three or more embryos." However, it was not until 2004 when there was a push for eSET, which the majority of clinics within Australia now comply (Gardner *et.al.,* 2004).

In 2004, Carrell discovered a link between abnormal seminal parameters and sperm chromosome aneuploidy. Using routine semen analysis and Fluorescence In-Situ Hybridization (FISH), they showed that patients who had moderate male factor, oligoasthenoteratozoospermia or severe ultrastructure defects such as round heads, had a higher aneuploidy rate compared to samples with normal seminal parameters. In particular the sperm chromosome aneuploidy rate was much higher in patients with ultrastructure defects. Carrell questioned the potential of these men passing on some aneuploidy to offspring, especially sex chromosome aneuploidy given the ability to use ICSI to procreate (Carrell *et.al.,* 2004).

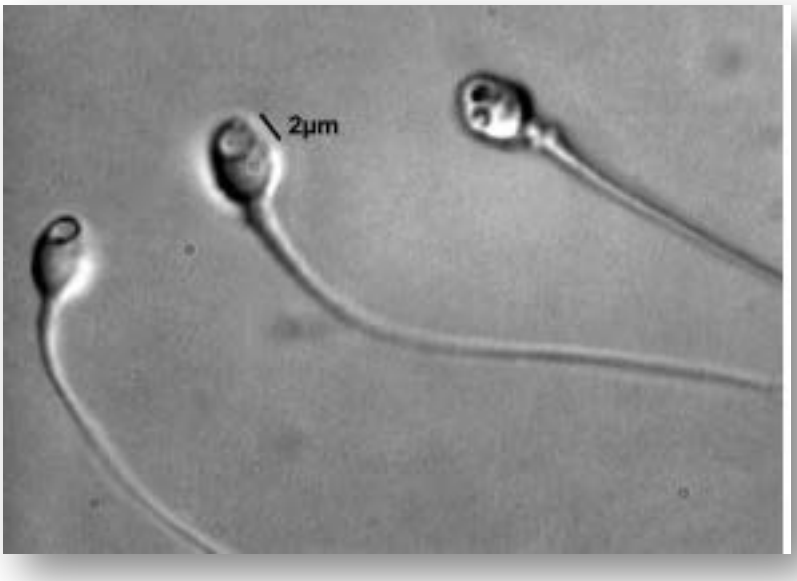
With reports stating that more than fifty percent of embryos created have aneuploidy, pre-implantation genetic diagnosis was being used to select a chromosomally normal embryo for transfer to increase the chances of a normal live birth (Mahmood *et.al.,* 2000). After biopsy of one or two cells from each viable embryo on day three, the cells were then tested using FISH or polymerase chain reaction for their genetic makeup (Harper and Bui, 2002).

In 2006 a new technique was developed to identify a normal morphological spermatozoon to inject during ICSI. This technique might also minimise the selection of a chromosomally abnormal sperm. High-Magnification Motile Sperm Organellar Morphology Examination (MSOME) ICSI allows examination of spermatozoon at a magnification of >6000-7000 compared to conventional ICSI at x400. With such high magnification, structures such as intranuclear vacuoles can be seen. Hazoult *et.al.,* (2006) discovered the pregnancy and implantation rates were significantly higher when MSOME was used compared to conventional ICSI. Another study has shown that injecting a vacuolated

sperm reduces pregnancy rates and increases early miscarriages (Berkovitz *et.al.,* 2006). See figure 10 for vacuolated heads in spermatozoa.

MSOME was reported to be an advantageous new technology for patients with repeated ICSI failure, high sperm DNA fragmentation and poor sperm morphology.

Figure 10: MSOME High-powered magnification showing vacuoles within the head of spermatozoa (Oliveira *et.al.,* 2010).



Ainsworth *et.al.,* (2007) reported the first successful pregnancy and normal birth after selecting a spermatozoon using an electrophoretic (COMET) method, and then injecting it with ICSI. This technique allowed for a rapid and reliable isolation of spermatozoon possessing minimal DNA damage as well as normal motility, morphology and viability. The modified COMET method was tested on a range of semen samples from slow frozen, vitrified and testicular biopsies. High-quality spermatozoa were effectively isolated showing the versatility of the new technique. Ainsworth believed this new technique to be a convenient method in avoiding DNA damaged spermatozoa, and improving ART outcome.

PICSI™ (Pre-treated Dish ICSI) was released into the ART market in 2007. From belief that mature spermatozoa bind to Hyaluronic Acid (HA) within oocytes

cumulous matrix, a synthetic HA is used within an ICSI dish to attract and select the best quality spermatozoa for ICSI. Using these PICSI™ dishes, it has been stated, "clinical pregnancies were significantly improved when the PICSI™ procedure was implemented" (Nasr-Esfahani and Deemeh, 2012). Huszar, who developed PICSI™, found that sperm that bound to HA had reduced DNA damage and aneuploidy. In 2007, a study of 240 patients was performed to compare ICSI and PICSI™. Results showed a statistically significant improvement in fertilisation and embryo quality using HA bound spermatozoa, with an increased percentage in pregnancies and reduction in miscarriages (Worrilow *et.al.,* 2007). Razavi *et.al.,* (2008) also reported similar results, concluding that HA bound sperm selected for ICSI may improve success rates. HA bound sperm or PICSI™ may be a useful technique to use for men who have poor sperm quality (except for men with oligozoospermic samples) to enhance embryo quality derived from the paternal effect within embryo development.

Knowing that HA bound sperm and sperm without vacuoles have improved DNA integrity and overall quality, Peterson *et.al.,* (2010) experimented on human spermatozoa using both the HA binding assay and MSOME techniques. Assessing each spermatozoon with MSOME, they found that spermatozoa that were HA bound did not have a significant different morphology compared to spermatozoa that were not bound. They concluded from this study that HA binding assays are not an efficient method in selecting the optimum spermatozoa for ICSI based on morphological assessment using MSOME.

Success Rates

The success of IVF has steadily increased since its introduction mainly due to improved culture systems, laboratory technology and stimulation protocols.

The most recent success rates were released in November 2012 for all cycles undergone from 2010 within Australian and New Zealand. It was calculated that a total of 61,774 treatment cycles were completed with 23.9% resulting in a clinical pregnancy and 18.1% resulting in a live birth. As a result in 2010, 12,056

babies were born following ART treatment. The average age of women undergoing ART treatment in 2010 was thirty-six years (Wang *et.al.,* 2010).

The success of IVF is greatly dependent on the individual age of the woman being treated. Fertility of women begins to dramatically decline from the age of thirty-five, and history shows that women who are at thirty-eight years of age or less have higher chance of a clinical pregnancy using assisted reproductive technologies. This is particularly related to reduction in oocyte quality. Male factor does not appear to be a significant contributor to reduced ART outcomes (Wang *et.al.,* 2010) with current techniques although this still remains controversial.

Complications

IVF, which has many benefits, also has its downsides. Some of these negative aspects of assisted reproductive technology include financial cost, complications associated with multiple gestations, ovarian hyper stimulation syndrome (OHSS) and in extreme cases the rare possibility of death.

For patients who undergo ART, their emotional and financial investments are very high. The financial cost for IVF in Australia is not cheap, but compared to other countries around the world, the out of pocket investment per cycle is not as expensive due to the Australian government Medicare rebate. The expense of IVF is related to the skilled labour required that includes doctors, scientists, nurses, counsellors and running of a controlled laboratory environment. Expensive laboratory equipment such as dissecting and inverted microscopes, culture media, diagnostic assays, consumables, computers and their software are a necessity for a successful embryology laboratory.

OHSS became a complication associated with ART since the introduction of gonadotropin stimulation. Although artificially stimulating the ovary to increase the cohort of oocytes and induce ovulation was a major breakthrough in ART's success, it has also produced this syndrome. OHSS is the result of "capillary hyper permeability mediated by ovarian-derived vasoactive substances" (Abramov *et.al.,* 1999). Several effects that include fluid "leakage from

capillaries, pleural and pericardial effusions, reduced effective blood volume, oliguria, electrolyte imbalance, haemoconcentration, thromboembolic phenomena and occasionally death" express the syndrome (Abramov *et.al.,* 1999).

The major signs of OHSS are abdominal bloating and tenderness, weight gain, shortness of breath and reduced voiding of urine. Within Australia and New Zealand, patients and clinicians report cases of OHSS where hospitalisation is required. In 2010, 206 cases of OHSS were admitted to hospital. The incidence of severe OHSS occurs in 0.6% of cases with oocyte retrieval. The risk of OHSS increases when quantities of oocytes increase (Figure 11).

Figure 11: Number of cycles with OPU performed and OHSS by number of oocytes collected, Australia and New Zealand, 2010 (Wang *et.al.,* 2010).

	Number of oocytes collected						
	None	1–4	5–9	10–14	15–19	≥ 20	All
Cycles with OHSS	0	2	16	45	52	89	204
Cycles with OPU	608	8,157	12,464	7,954	3,508	2,219	34,910
OHSS per OPU cycle (per cent)	*0.0*	*0.0*	*0.1*	*0.6*	*1.5*	*4.0*	*0.6*

Death associated to OHSS and other ART complications are very rare. Other problems in ART that can become life threatening include complications of surgical procedures, infection, allergic reactions and some reports within Australia of patients committing suicide who failed to conceive after ART (Venn *et.al.,* 2001).

A study was conducted in the Netherlands to observe ART related maternal deaths between 1984-2008. In this time frame, six deaths were reported that were directly related to IVF treatment, and seventeen IVF pregnancy maternal deaths (Braat *et.al.,* 2010). There were three deaths that were a direct result of OHSS. These patients suffered from secondary effects such as respiratory distress and cerebrovascular thrombosis. Sepsis was the cause of two deaths in relation to IVF treatment, who had recently undergone oocyte retrieval. The remaining death was due to a dosage error of anaesthetic prior to oocyte

retrieval. In relation to IVF pregnancy related deaths, the causes include pre-eclampsia, sepsis, vascular dissection, liver failure, pulmonary embolism, meningitis, suicide, and amniotic fluid embolism (Braat *et.al.,* 2010).

This study and others like it have compared mortality rate of IVF patients to the general population and found the mortality rate to be lower in the cohort of IVF patients. This is reassuring to know that ART treatment is not permitting an increased mortality risk to its patients, although there are risks to be addressed.

Miscarriage and Spermatozoa

A miscarriage or early pregnancy loss is defined as a pregnancy that ceases to progress with a gestation of ≤ 20 weeks or birth weight ≤400 grams (Wang *et.al.,* 2010). In the human population, an early pregnancy loss is common, occurring in 25% of pregnancies usually before a gestation of 12 weeks.

Of the 14,733 clinical pregnancies reported in the recent release (November 2012) of Assisted Reproductive Technology in Australian and New Zealand 2010, there was an estimated 21.7% that resulted in an early pregnancy loss. It was noted that the causes of these miscarriages were spontaneous, foetal reduction, termination and ectopic.

The IVF miscarriage rate is comparable to the risk of miscarriage of naturally occurring pregnancies. However, some studies have shown that pregnancies conceived through IVF are at a higher risk. Thoughts around this conclude IVF procedures not being the cause for the increased risk, but more so the underlying reasons for patients using fertility treatment. The increased age of both male and female partners and their underlying health problems are some examples of these.

Focusing on the male reproductive system, miscarriage in IVF has been highly correlated to poor sperm DNA (fragmentation) and morphology (shape and vacuoles). Measuring sperm DNA damage is a useful biomarker in assessing fertility, as it has been associated to poor embryo quality and higher miscarriage rates. One study concluded that some IVF patients who suffer recurrent miscarriages have significantly increased percentage of sperm DNA fragmentation compared to men from the general population (Carrell *et.al.,* 2003).

Oxidative stress has shown to be the major cause for sperm DNA damage, as sperm have a low tolerance to reactive oxygen species (ROS). ROS are highly active molecules that are by-products of oxygen metabolism. Spermatozoa are capable of producing ROS at low, steady levels that are significant in the signal transduction pathways that control capacitation. However, if the physiological rate of ROS increases due to environmental stresses such as

cigarette smoking, alcohol consumption and an increase male age, oxidative stress occurs (Aitken and Baker, 2005; Cocuzza *et.al.,* 2008*).*

Oxidative stress impacts spermatozoa, their normality to function and DNA integrity of their mitochondrial and nuclear DNA (Aitken and Baker, 2004). It has been reported that sperm with DNA damage caused from oxidative stress have the ability to fertilise and form embryos, but subsequently fail to implant or result in early pregnancy loss (Robinson *et.al.,* 2012).

POLARISED LIGHT MICROSCOPY

History and its Role in Assisted Reproductive Technologies.

Christian Huygens first described polarised light in 1690. It was later described by Shurcliff (1962) as one of 'nature's ultimates'. Shurcliff described polarised light as a slender, monochromatic polarised ray, which can be altered by passage through objects of different density, with no loss in power and exudes ultimate speed yet delicacy. Many fields have benefited from its use in imaging such as biological, optical, chemistry and mineralogy.

Polarised light enables structures with molecular order to be observed (birefringence) when a single ray of polarised light is refracted into 2 polarised rays travelling at different speeds. The difference between these phases is otherwise referred to as retardance (Gianaroli *et.al.,* 2010). Biologically, polarised light has been extremely advantageous, as it has allowed scientists to explore sub microscopic molecular order dynamically and non-destructively in samples that can generally be kept in native environmental conditions (Oldenbourg, 1996).

In 1981, Hiramoto *et.al.,* conducted a study to observe spindles within sea urchin oocytes. They used the polarised light concurrently with an electron microscope to distinguish the role and distribution of microtubules during mitosis. When Hiramoto was completing their study, they calculated the retardance of the microtubules by the use of a hand written equation. More recently, however, with the development of computers and their software, calculating retardance by hand has become a way of the past.

In the mid 1990's, the revolutionary change to the way polarised light was used within IVF laboratories occurred, with commercialisation of the PolScope; polarised light microscopy (PLM) by Oldenbourg and his team at Marine Biological Laboratory in Woods Hole, Massachusetts (Oldenbourg, 1996).

They proposed two main changes to the traditional polarised microscope to overcome certain limitations. These new modifications include two electro-

optical modulators instead of a single compensator and that the specimen in view is 'illuminated with nearly circularly polarised light' allowing for specimens birefringence regardless of orientation and magnitude with the help of image processing software. Therefore no more hand written equations were needed to calculate retardance (Oldenbourg, 1996; Oldenbourg and Mei, 1995).

Within a year, Keefe revolutionised the polarised microscope into the modern PLM, tested by imaging hamster zona pellucida multi-laminar structures. This experiment showed with the concurrent use of the electron microscope that birefringence is a useful yet non-invasive tool in distinguishing macro-molecular organisation within the oocyte (Keefe *et.al.,* 1997).

PLM was then used on mice oocytes, knowing that meiotic spindles can be visualised in living oocytes without the need to fix, stain or label (Keefe *et.al.,* 2000). With this knowledge, observations with PLM of living mice oocytes occurred before and after parthenogenetically activating them with calcium ionophore and strontium. It was observed that meiotic spindle retardance increased dramatically after activating the oocytes, and continued to be increased throughout spindle rotation and second polar body extrusion. From this study, it was concluded that PLM was a non-invasive procedure that could be a useful marker for selecting high-quality oocytes in IVF and improve the efficiency of assisted fertilisation.

Cooke *et.al.,*(2003) identified that human oocytes cleave from the animal-vegetable pole, which is not identified by the polar body as in other species, but by the spindle itself. They showed that human embryos that start cleaving from this animal-vegetable pole have better morphology (Cooke *et.al.,* 2003).

Over the next coming years, many studies were undertaken using the PLM imaging the human meiotic spindle, and the biological foundations that occur before and after fertilisation.

In 2008, a team from Brazil wrote a paper; Spindle Imaging: A Marker for Embryo Development and Implantation, whose results paralleled other similar studies showing that a higher fertilisation rate and embryonic development was achieved when spindles could be detected (Madaschi *et.al.,* 2008). It had

become evident that meiotic spindles lose their birefringence in certain environmental conditions such as temperature and pH, preventing them being detected with PLM. This is caused by "temporary depolymerisation of microtubules" (Zenzes *et.al.,* 2001; Pickering *et.al.,* 1990).

With the link between the presence of the meiotic spindle and increased fertilisation and embryo development, Madaschi also observed an increased pregnancy and implantation rate, suggesting that spindle visualisation has a predictive value for embryo selection.

This theory was analysed further in 2009 by (Kilani *et.al.,* 2009) who studied PLM to discover any non-invasive markers within the human oocyte that can predict pregnancy outcome. With the increasing trend of single embryo transfers, there was a need to define parameters of gametes and embryos, which would lead to maximum success rates in IVF. This study concluded that a normal shaped spindle was a significant marker for achieving a pregnancy, where 100% of the pregnancies in this study originated from normal shaped spindles.

Kilani *et.al.,* (2011) continued their research on the human oocyte and the dynamic meiotic spindle, and proved Raju's theory (Raju *et.al.,* 2007) that the spindle retardance is an indicator of spindle integrity. In time-phased observation, they showed that the meiotic spindle density changes over time, which parallels the maturity of the oocyte post collection, before spindles start to disaggregate. It was found that the spindle density or retardance was highest at 39-40.5 hours post hCG trigger before the meiotic spindle begins to break down and oocyte quality decreases.

This link between spindle retardance and optimum time for intra-cytoplasmic sperm injection post hCG has allowed scientists to optimise PLM in the benefit of increasing clinical pregnancy rates (Kilani *et.al.,* 2011).

Over the last two decades, PLM has allowed scientists to gain much information in the area of assisted reproduction and the biological makeup and function of the human oocyte.

Applying Polarised Light Microscopy to sperm

Whilst PLM has been used to visualise items with ordered structure by its size, sperm has not been widely imaged. With improvements of PLM (from PolScope to Oosight™) and an improvement in resolution and control of annotation, the structure of the sperm condensed chromatin can now be studied.

It is only recently however, that the PLM has been used on the human spermatozoa in the attempt to gain more information and insight into their function within human reproduction much like its counterpart; the human oocyte.

In addition to the overall parameters of a semen analysis, other abnormalities at an individual sperm level can occur. It has been found if sperm are subjected to reactive oxygen species (ROS) at high proportions, strand breaks occur within the DNA, otherwise referred to as DNA fragmentation. Other identifications of high DNA fragmentation are associated with poor embryo quality, decreased implantation rates, and increased risks of spontaneous miscarriage. Some indicators of high levels of DNA fragmentation include nuclear vacuoles and poor morphology. With this in mind, new techniques to test the quality of spermatozoa selected during ICSI are being studied.

An oral presentation in 2008 at the annual ESHRE conference introduced a potential method to select an individual sperm for ICSI based on sperm head retardance (measurement of birefringence). They hypothesised that sperm head should exhibit high retardance based on their organised structure comprised within the acrosome. They positively correlated sperm DNA damage to retardance using the PolScope. Their results concluded that with increased DNA damage, the level of retardance dramatically increases (Keefe *et.al.,* 2008). Since then, PLM has proven to be an innovative tool to visualise sperm under polarised optics, as a reflection of their protoplasmic structure.

In 2008, Gianaroli *et.al.,* (2008) stated that the head of sperm were birefringent due to anisotropic structures within the mature nucleus and acrosome. The birefringence within the mature sperm nucleus is created by molecular order within nucleoprotein filaments. Likewise, the acrosome has birefringence due

to protein filaments oriented longitudinally. The purpose of their study was to assess any correlation linking morphology with birefringence. They also applied PLM with ICSI by selecting sperm for ICSI that had head birefringence (study group) and comparing results against the control group that did not use PLM. Their results showed that sperm with normal morphology had a higher proportion of birefringent sperm compared to sperm with abnormal morphology regardless of progressive motility. When comparing the study group to the control group, there were no significant differences between fertilisation and cleavage rates. They did note a higher frequency of high quality embryos on day 3 in the study group compared to the control ($P< 0.025$). It was found that the implantation and ongoing pregnancy rates were significantly higher amongst the study group compared to the control ($P<0.05$ and $P<0.025$ respectively). They concluded by stating that birefringence may reflect the health of spermatozoa, and by selecting sperm with birefringence it would indicate a normal sperm structure as demonstrated by transmission electron microscopy.

In 2009, Gianaroli *et.al.,* presented their work to the 25th annual ESHRE meeting showing that sperm head birefringence can be evaluated as total birefringence and partial birefringence. The difference between the two is determinant on whether the sperm have undergone the acrosome reaction. Sperm that have not completed the acrosome reaction have the total acrosome intact and therefore have complete molecular order within the acrosome. This would be characterised by total head birefringence. When the sperm has completed the acrosome reaction, the acrosome is no longer intact and therefore molecular order is reduced, creating partial head birefringence and reduced retardance (See Figure 12).

Figure 12: Image of sperm head birefringence observed at x2500 magnification (Peterson *et.al.,* 2011).

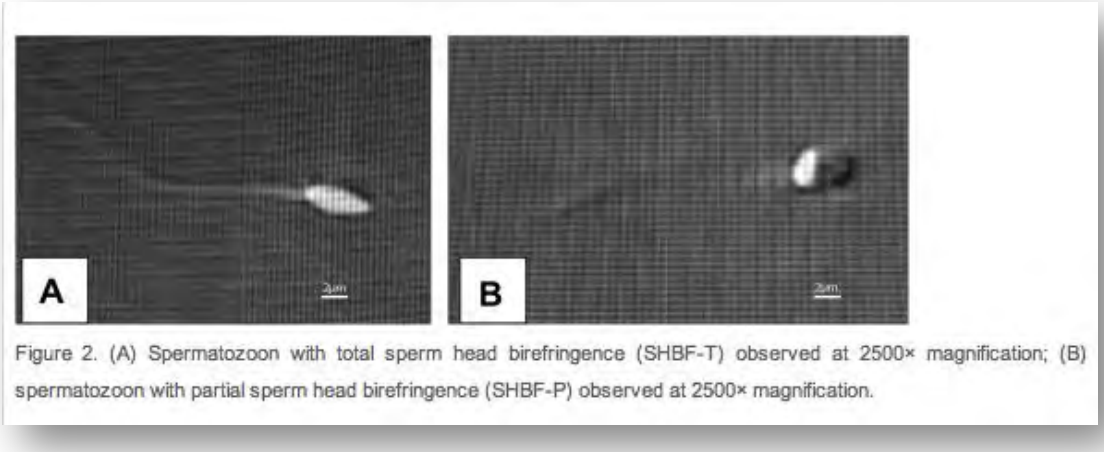


Figure 2. (A) Spermatozoon with total sperm head birefringence (SHBF-T) observed at 2500× magnification; (B) spermatozoon with partial sperm head birefringence (SHBF-P) observed at 2500× magnification.

Their study demonstrated that sperm with normal morphology had 58.8% total head birefringence, 29.2% partial head birefringence and the remaining 12% showed no evidence of birefringence. Comparing these results to sperm with abnormal morphology, 43% had total head birefringence, 35% had partial birefringence and 22% showed no evidence of birefringence. They concluded their study by stating that wherever possible select a sperm with normal morphology for injection.

Crippa *et.al.,* 2009 evaluated a possible correlation between sperm with positive DNA fragmentation and their birefringent properties. It was concluded that there is a higher incidence of DNA fragmentation in sperm without birefringence, and that by selecting a sperm for ICSI with birefringence increases the chance of selecting a sperm with DNA integrity. The question is, what is the optimal retardance range to choose?

In 2010, Gianaroli *et.al.* released another study comparing clinical outcome of ICSI where sperm selected were based on partial head birefringence versus total head birefringence. Their results showed little significant difference between fertilisation, cleavage and embryo quality. Clinical pregnancies were significantly higher ($P<0.025$) amongst the group where acrosome reacted

spermatozoa were selected for ICSI compared to non-reacted, (54.5% vs. 14% respectively.) They concluded that where possible, sperm selected for ICSI should be based on partial head birefringence to increase pregnancy rates of patients with severe male factor infertility.

Peterson *et.al,* (2011) conducted a study to distinguish a relationship between DNA fragmentation and sperm head birefringence. Using the TUNEL assay, their results show that sperm with total head birefringence present with significantly higher proportions of DNA fragmentation compared to sperm with partial head birefringence. Interestingly, the result from this study is opposite to the results by Crippa *et.al.,* 2009. It must be noted that Crippa did not distinguish sperm head birefringence as total and partial, but grouped them as birefringent versus non birefringent.

In 2012, Magli *et.al.,* correlated patterns of sperm head birefringence in relation to motility and morphology. It was found that head birefringence was optimum in sperm with normal morphology and motility. The proportion of total head birefringence significantly increased in the presence of motility (59%) compared to immotile sperm (45%). Compared to morphologically abnormal sperm, there were no significant differences of total or partial head birefringence between motile or immotile sperm. They concluded their study by stating that the distribution of birefringence in the sperm head is significantly different when comparing morphology and motility. Morphology is a more defiant factor in head birefringence than motility.

Introduction and hypothesis

The literature reveals an importance to further our understanding and selection of an optimal spermatozoon for use within ICSI, to increase the potential of a human embryo to form a viable pregnancy.

Prior to MSOME, there have been no developments to measure optimum integrity of a single spermatozoon at the time of ICSI. We have solely relied upon morphology and motility as the selection criteria despite knowing that sperm DNA has a massive effect on the success of an IVF cycle.

We have the ability to measure the percentage of DNA damage within the patients' semen sample (SCSA, TUNEL), but they are invasive fertility tests performed in the work up prior to an IVF cycle. Even with this added information of percentage of DNA fragmentation within a sample, scientists cannot select the sperm with zero DNA damage at the time of ICSI.

It is my belief that a reliable test needs to be developed to allow scientists to gain intricate information about a single spermatozoon prior to its injection.

PLM has been identified as a highly beneficial non-invasive tool to measure oocyte integrity and to increase IVF pregnancy rates (Kilani *et.al.,* 2011). PLM has also recently been identified as a tool to help distinguish optimal spermatozoa (Peterson *et.al.,* 2011). PLM has allowed scientists to determine the optimum spermatozoa whose birefringence is referred to as 'partial', or sperm that has undergone the acrosome reaction rendering them mature.

The measurement of their birefringence is important, as it has also been noted that a higher retardance is correlated with DNA damage. But what is the optimal range of retardance signalling the optimum sperm to use for injection? We hypothesise the following outcomes-

1. PLM is a reproducible and accurate method of assessing spermatozoa head retardance.

2. There is a range of retardance within human spermatozoa which differs between normal and abnormal head morphology.

3. Retardance of spermatozoa head allows the identification and selection of the optimum sperm to inject into an oocyte.

Optics and software for obtaining images

The following is needed:

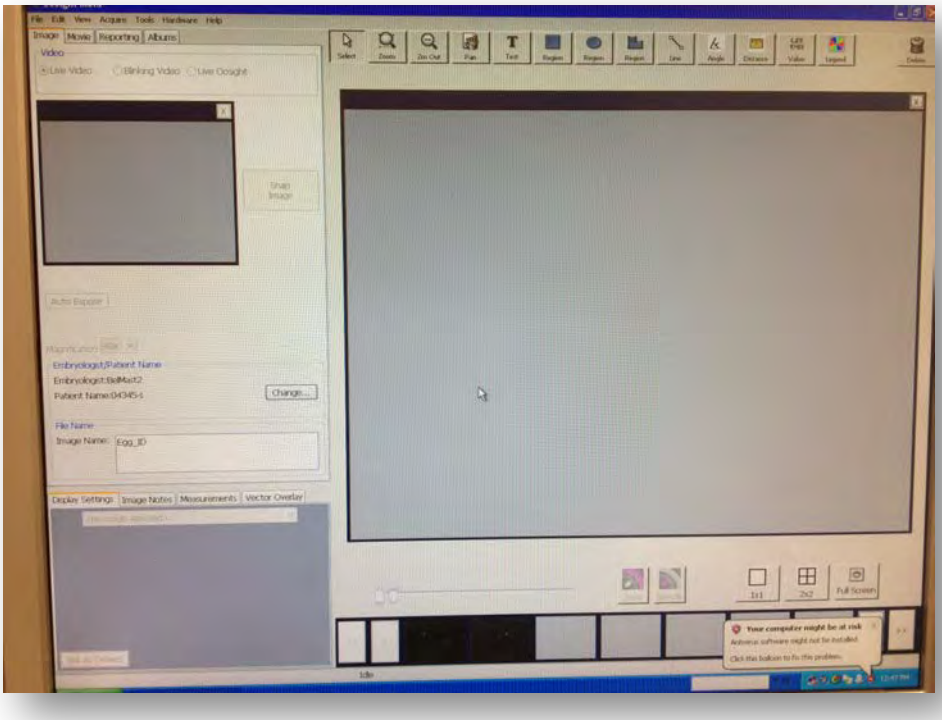
- CRI Oosight software,
- CRI camera and 546 nm filter,
- Olympus IX-70 with Nomarski DIC optics,
- Heated stage without interference from glass/plastic stage warmer,
- Glass bottomed dishes (FluoroDish™ by World precision instruments Inc.),
- Monitor (i.e. 22 inch).

The image below illustrates the setup of the polarised inverted microscope for this study.

Figure 13: ICSI machine with CRi Oosight setup.

- Once the computer attached to the inverted microscope is switched on, the main menu contains the software link for Oosight™. This needs to be opened before the setup can begin. Figure 14 illustrates the 'opening page' once the Oosight™ programme is switched on.

Figure 14: Oosight software opening image

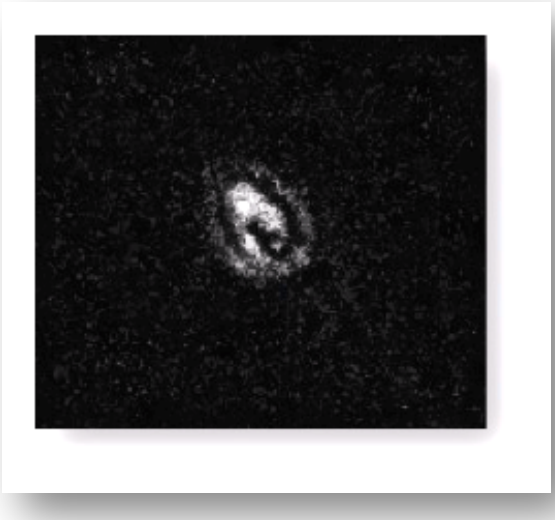


- The lighting on the inverted microscope needs to be changed so that no condensers, other than the polarised optics, are obscuring the light.
- The software needs to prime the optics so that the light is at its optimum depending on what objective you use to take an image. In this study, the objective is set to x40. Therefore, a background image at an objective x40 is needed before the software is usable.
- To do this, place a pre-prepared glass bottomed ICSI dish on the microscope stage. Click on 'Acquire' in the left menu. From the pull down menu, select 'Background'.
- The programme will ask you to move any objects in the dish out of view so that there is a 'clean' image. Simply do this by moving the stage so that there is only clean media in the field of view.
- The programme will take a background image, and optimise the light for further imaging. Once this is complete, the software is ready to use.

Taking an image of a sperm

- Without the polarised optics in place, use white light to immobilise a sperm with the microinjecting pipette by striking its tail.
- To allow the ovoid view of the headpiece to be seen, roll the sperm with the injection pipette so that one of the bilaterally flattened sides of the headpiece is lying flat against the ICSI dish.
- Move the polarised condenser in place to allow green polarised light to shine through.
- Position the sperm so that the head is vertical or horizontal. This needs to be accurate, as the software region tool to measure retardance of an area doesn't allow for diagonal objects to be measured precisely.
- Click on the icon "Take image". Figure 15: Polarised image of an immobilised spermatozoa.

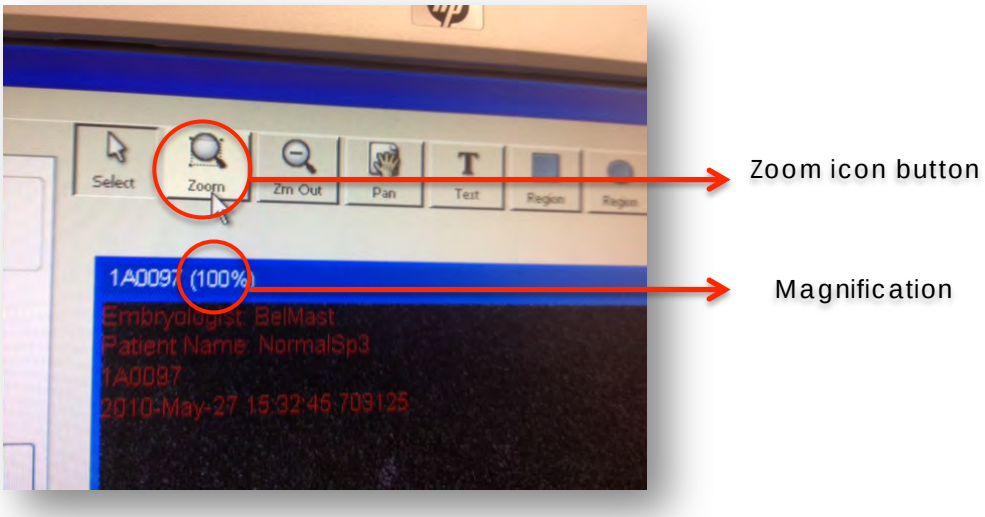
Figure 15: Polarised image of an immobilised spermatozoa



- Images that have been taken will line up on the bottom of the screen. Double click on the image that you want to analyse.
- To analyse the sperm, the image needs to be enlarged to 200% to get accurate detail of the headpiece. To do this, click on 'zoom'. The zoom

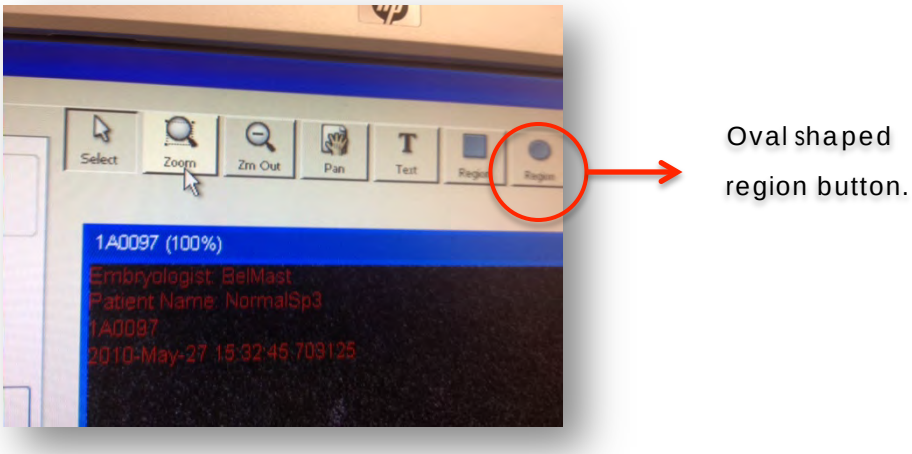
tool will then be your mouse cursor. Click on the headpiece of the sperm until 200% magnification is reached (Figure 16).

Figure 16: How to zoom an image using Oosight software.



- Once the image is zoomed to 200%, the headpiece can be traced accurately with the area dissection tool. With your mouse cursor, click on "Region" that symbolises an oval shape (Figure 17).

Figure 17: Location of region tool.

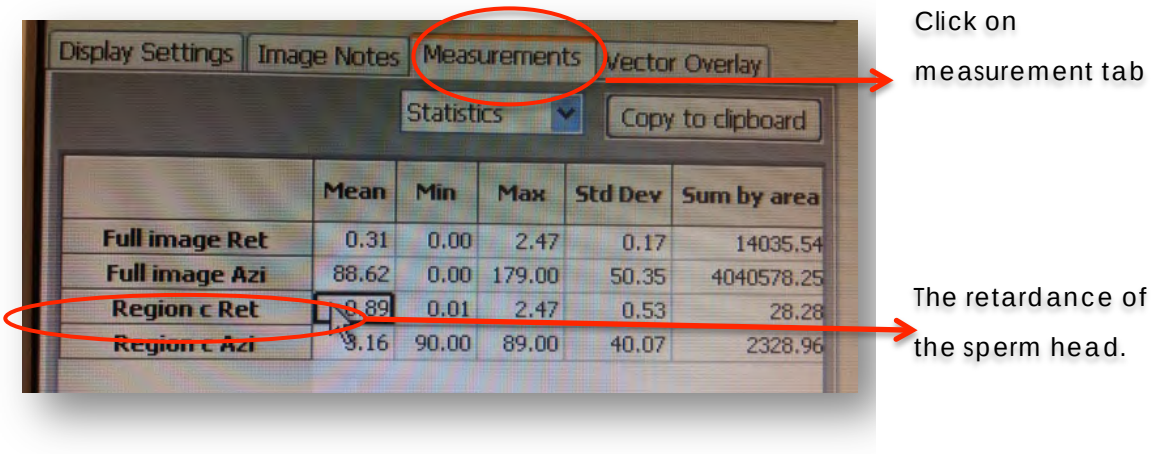


- Use this tool to draw the shape of the head for which the area will be measured for its retardance. You will need to start at the top or bottom and drag the cursor to the opposite end (Figure 18).

Figure 18: Dissecting the region to be measured for retardance.

- Once the area has been highlighted for measurement, the Oosight™ software will automatically measure the attributes. Click on "measurements" to open up the results tab. The result for mean "Region C Ret" is the retardance of the sperm head. For measurement tab and 'Region C Ret' location, refer to Figure 19.

Figure 19: Where to find the calculated retardance using Oosight software.



EXPERIMENT 1 - REPRODUCIBILITY

Aim

1. To determine if retardance is a reproducible tool to assess single spermatozoon independent of head morphology.

2. To determine if retardance is a reproducible tool to assess single spermatozoa within normal and abnormal head morphology.

Methods and Materials

To assess the reproducibility and validity of this observer study, an inter and intra correlation was used. This style of reliability estimate assesses the observers' ability to produce consistent estimates of the same question at hand. In general, one or more observer/s estimates the retardance of the sperm head, where the results of these observer/s are measured against each other to produce the reliability estimate.

In this experiment, spermatozoa were assessed by two observers and blinded to the previous observers result to establish an intra and inter observer variation.

The first study compared the observers' variance of 368 images across a pooled sample of varying qualities of individual spermatozoa. This included random types of morphology as defined by WHO 5th edition. The second study compared the observers' variance of 368 images of normal and abnormal head morphology (WHO 5th edition). Refer to "Optics and software for obtaining images, page 41 on the method to measure sperm head retardance.

An intra class correlation coefficient statistical test (ICC) was used to measure the intra and inter variance. A two way mixed model with consistency type was chosen for reliability. The Pearson's correlation coefficient was used to determine a P value for significance. The intra variance was statistically achieved by comparing the original measurements against the secondary measurements of the original observer. The inter variance was statistically

measured by comparing the mean of the original observer's two measurements against the secondary observer's measurements. Intra and inter variance was also calculated as a percentage by comparing the results that were the same, and dividing this by the sample number.

A Bland-Altman Plot was used to visualise the difference of the two measurements, where the average difference of the measurements should be close to zero. The upper and lower control limits were also applied.

IBM SPSS statistics, version 19 was used to calculate statistical measurements.

Results

Reproducibility in the pooled sample

A total of 368 spermatozoa were randomly selected for measurement for testing reliability.

Table 1 represents the intra and inter observer variation of varying qualities of sperm.

Table 1: Intra/inter observer variance by the use of an intra class correlation coefficient.

	Cronbach's Alpha[a]	95% Confidence Interval	ICC Value[b]	P Value r= Pearson's correlation[c]
Intra Variance	0.981	(0.955-0.970)	0.963	<0.0001* r= 0.963
Inter Variance	0.988	(0.971-0.980)	0.988	<0.0001* r= 0.976

a. The level of internal consistency. A result above 0.70 is considered acceptable.

b. ICC is a number between 0 and 1. A value >0.90 is considered to be a good agreement of a correlation.

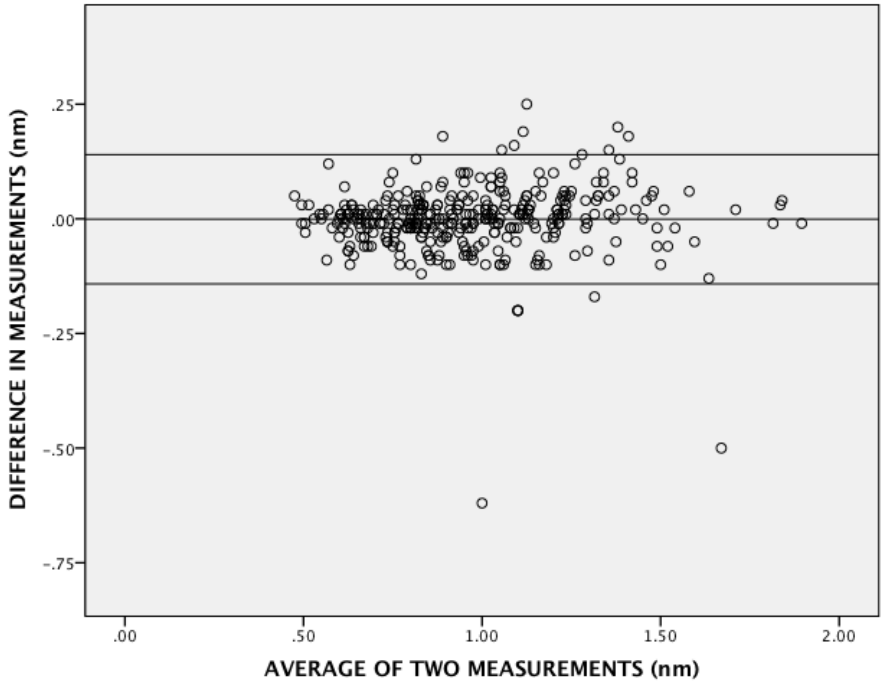
c. As the r value approaches 1, the stronger a correlation.

* Significance if P<0.05.

As a percentage, the intra observer variation was 3.3% and the inter observer variation was 4.4%.

The Bland-Altman Plot (Bland and Altman, 1983) below represents the 368 sperm that were measured for intra and inter variance. With the majority of the measurements being close to zero with mean bias of -0.0008, standard deviation of 0.07 and coefficient of reproducibility of 0.14, it can be said that measuring sperm head retardance using Oosight™ is a reproducible technique (Figure 20).

Figure 20: Bland-Altman plot- the difference between two observers (Bland and Altman, 1986)



The intra and inter observers' were compared for the mean, standard deviation, minimum, and maximum values. The mean pooled head retardance of the original observer was 0.969 nm with standard deviation of 0.275. The secondary observer's mean retardance was 0.968 nm with standard deviation of 0.266. The minimum values for the original and secondary observers respectively were 0.47-2.08 nm and 0.45-1.92 nm (Table 2).

Table 2: Frequency data of intra and inter observer measurements.

	Intra observer	Inter observer
N	368	368
Mean	0.969	0.968
St.Dev.	0.275	0.266
Minimum (nm)	0.47	0.45
Maximum (nm)	2.08	1.92

Reproducibility within normal head morphology

Of the 368 sperm, 195 (53%) were categorised with having normal head morphology.

The ICC report and Pearson's correlation was conducted (Table 3).

Table 3: Normal head morphology intra and inter observer variation.

N=195	Cronbach's Alpha[a]	95% Confidence Interval	ICC Value[b]	P Value R= Pearson's correlation[c]
Intra Variance	0.977	(0.942-0.967)	0.956	<0.0001* r = 0.957
Inter Variance	0.988	(0.970-0.983)	0.977	<0.0001* r = 0.977

a. The level of internal consistency. A result above 0.70 is considered acceptable.

b. ICC is a number between 0 and 1. A value >0.90 is considered to be a good agreement of a correlation.

c. As the r value approaches 1, the stronger a correlation.

* Significance if P<0.05.

As a percentage, the intra observer variation was 3.0%. The inter observer variation was 4.1%.

These results suggest that measuring retardance on sperm classified with normal head morphology is a reproducible and reliable technique.

Reproducibility within abnormal head morphology

Of the 368 sperm, 173 (47%) were categorised with having abnormal head morphology.

The ICC report and Pearson's correlation was conducted (Table 4).

Table 4: Abnormal head morphology intra and inter observer variation.

N=173	Cronbach's Alpha[a]	95% Confidence Interval	ICC Value[b]	P Value R= Pearson's correlation[c]
Intra Variance	0.985	(0.960-0.978)	0.971	<0.0001* r = 0.971
Inter Variance	0.987	(0.966-0.981)	0.975	<0.0001* r = 0.975

a. The level of internal consistency. A result above 0.70 is considered acceptable.

b. ICC is a number between 0 and 1. A value >0.90 is considered to be a good agreement of a correlation.

c. As the r value approaches 1, the stronger a correlation.

* Significance if P<0.05.

As a percentage, the intra observer variation was 3.5%. The inter observer variation was 4.6%.

These results suggest that measuring retardance on sperm classified with abnormal head morphology is a reproducible and reliable technique.

Discussion

For this study, a new methodology using PLM was developed in order to accurately image and measure sperm head retardance with use of conventional light microscope magnification. Magnification of x2500 had been used to image head birefringence, total and partial (Peterson *et. al.,* 2011). It was thought before conducting this study that a microscopic power of x400 would be sufficient to measure birefringence with the use of Oosight™ software, much like measuring meiotic spindles in oocytes at x400 (Kilani *et.al.,* 2010).

It was found that once a sperm was immobilised and an image was taken to measure its birefringence, the retardance differed in retrospect to what position the sperm was lying due to its spatulate shape and bilaterally flattened sides. If the sperm were lying on its side the retardance reading was higher than if the sperm were lying flat against the glass-bottomed dish. Once this was recognised, it was clear that the user measuring for sperm head retardance needed to be accurate and consistent in positioning the sperm before an image and measurement was taken. Once the sperm were immobilised, repeated images on 10 sperm confirmed no change in the measurements.

The second factor that affected the measurement of birefringence at x400 magnification was where to measure within the sperm head using the region tool on the Oosight™ imaging software. It was found that if the user did not accurately draw the oval shape correctly across the sperm head with the region tool, retardance results were obscured. To prevent this from happening, it was found that accurate results were obtained by zooming in on the sperm head with the Oosight™ software so that defined edges were more clear. The magnification that was used to create clear boundaries for accurate measurement was 200%. The zoom tool on Oosight™ enables a magnification greater than 200%, but it was found this was not needed, as the image of head boundaries became more pixelated.

For a test to be useful on an individual patient, the user needs to be confident that reproducibility is real and not subject to artefact of user error. Before applying the test, we needed to know if it was reliable and accurate. To do

that, we established an intra/inter observer correlation (or variance). This test shows that the observation from one user is consistent and controlled with minimal variation. If the observer's variation is small, then the user can be satisfied that the test was reliable and accurate.

In experiment one, two aims were being investigated. The first was to determine if our method for measuring sperm head retardance was a reproducible technique independent on morphological factors, and the second aim was to determine if reproducibility was consistent within normal and abnormal head morphology. The two sets of results were validated for accuracy using an intra class correlation coefficient (ICC) and Pearson's correlation coefficient. A Bland-Altman Plot was also used to illustrate any variance between observers when testing retardance independent on morphological factors.

To our knowledge of the previous studies observing retardance or birefringence on sperm, their described methods have not illustrated repeated experiments to determine their reproducibility.

An intra observer variation is when the primary results of one tester are blinded and compared to a secondary set of results by the same tester. Within this experiment, our intra observer variance when measuring 368 sperm of varying quality concluded that our testing method is reliable and reproducible. The ICC resulted in a value of 0.963 where a value >0.90 is considered to be a good agreement of a correlation. A high result of r= 0.963, where r has its strongest correlation at 1, when a Pearson's correlation coefficient was used shows that the original tester had uniform results between the two blinded samples which was confirmed with a statistically significant value P (2) 0.0001<0.05.

When a secondary tester compared their results to the mean of the original tester's two sample results, the same conclusion was found, that our methods for testing head retardance in spermatozoa is a reliable technique. The ICC for the secondary observer was 0.988 with a correlation coefficient r= 0.976, P (2) 0.0001<0.05. Our methods used to measure sperm head retardance were reliable according to the Bland-Altman test. The Bland-Altman test also illustrated that in a couple of samples, the results between users were different

by 50%. A reason for this wide variation could be due to the shape of the sperm head, and the measurement tool being a perfect oval shape.

175 sperm with normal head morphology were measured for retardance. An intra and inter variance using an ICC was 0.956 and 0.977 respectively and Pearson's r value 0.967 and 0.977 respectively. The original tester had uniform results between the two samples as well as the secondary tester's results to the original mean results. These results were confirmed with a statistically significant value P (2) 0.0001<0.05.

Another way of observing the intra and inter variance was by a percentage. Our data showed that the first observer presented with the same result 97.0% of the time compared to the secondary observer with 95.9%. The possible reason why the secondary tester had a lower percentage is explained below. These results show that our methods for measuring sperm head retardance in sperm with normal head morphology are a reliable and accurate tool.

Our final question within this experiment was to determine if our testing method is also reliable and accurate for sperm with abnormal head morphology as indicated by WHO 5th edition. An intra and inter variance using an ICC was 0.971 and 0.975 respectively and Pearson's r value 0.971 and 0.975 respectively. The original tester had uniform results between the two samples as well as the secondary tester's results to the original mean results. These results were confirmed with a statistically significant value P (2) 0.0001<0.05. Our data showed that the first observer presented with the same result 96.5% of the time compared to the secondary observer with 95.4%.

It became obvious despite a small variance that the first and secondary testers were not as accurate when measuring abnormal head morphology compared to normal head morphology as depicted by the percentage of variance. This small variance may be due to a limitation of the Oosight™ software for measuring a region. The measuring tool is a click and drag system that creates a perfect oval although the size of the oval is variable. The downside of the tool is that not all headpieces of human spermatozoa are perfect oval shapes.

Knowing that our methodology is reliable and reproducible, we could further our investigation to distinguish the range of head retardance within human spermatozoa.

In summary, we assessed the reproducibility and reliability of a new method to measure retardance of individual sperm heads using PLM at conventional magnification (x400), and determined that:

1. Imaging and measuring sperm head retardance is reproducible and accurate utilising PLM (Oocyte™) across all sperm quality types.

EXPERIMENT 2 – RANGE OF RETARDANCE WITHIN HUMAN SPERMATOZOA

Aim

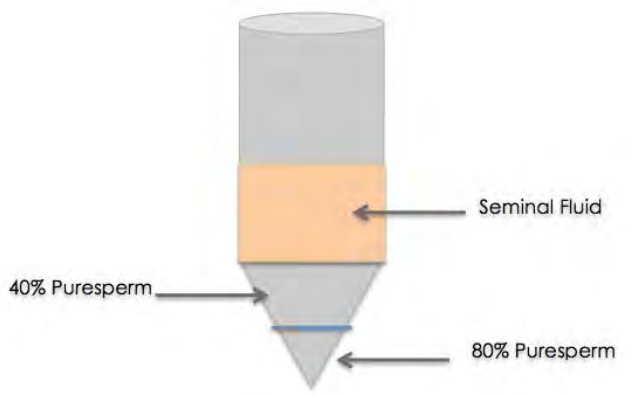
1. To distinguish a range of retardance within human spermatozoa.
2. To determine if the range and frequency of retardance differs between normal and abnormal head morphology.

Methods and Materials

This experiment has two studies. The first was to determine a range of retardance within a pooled sample of eight ejaculates of human spermatozoa with varying types of morphology and as determined by WHO 5th edition. The second part of this experiment was to determine if the range of retardance differs when normal head morphology was compared to abnormal head morphology.

Before each study commenced, spermatozoa were removed from the seminal fluid and washed using COOK Sydney IVF gamete buffer (COOK Sydney IVF Limited, National Technology Park, Ireland, UK). This was done by placing a maximum of 2 ml of semen over a density gradient of 80% and 40% puresperm (Nidacon, Molndal, Sweden)(Figure 21). This solution allowed the separation of spermatozoa from seminal fluid, whilst providing optimum conditions for sperm motility, survival time, and fertilisation capability.

Figure 21: Semen Preparation.



Belinda Vermey, Masters Thesis. Faculty of Medicine, University of New South Wales.

The puresperm gradient was centrifuged for 10 minutes at 300g. The sperm pellet was then removed and placed into a clean test tube and washed with 2-3 ml of gamete buffer. The sperm was then centrifuged again for 5 minutes at 300g. Once complete, the suspension was removed leaving 0.2-0.5 ml of the final sperm preparation in the tube.

A range of sperm head retardance was measured by observing the eight pooled semen samples. A descriptive analysis of the results was conducted and graphed. Once the frequency and distribution of retardance of the pooled semen samples were observed, retardance was compared between normal and abnormal head morphology. Morphology was determined by using the WHO 5th edition where abnormal sperm head is tapered, pyriform, round, amorphous, vacuolated or with small or no acrosome. Normality of sperm head can be seen under x400 magnification without needing to stain. The range and frequency was then calculated within each.

A ROC curve was used to compare retardance between sperm with normal and abnormal head morphology. The ROC curve was used to evaluate the quality or performance of our test. An independent samples T Test was used to compare means between the two groups.

Results

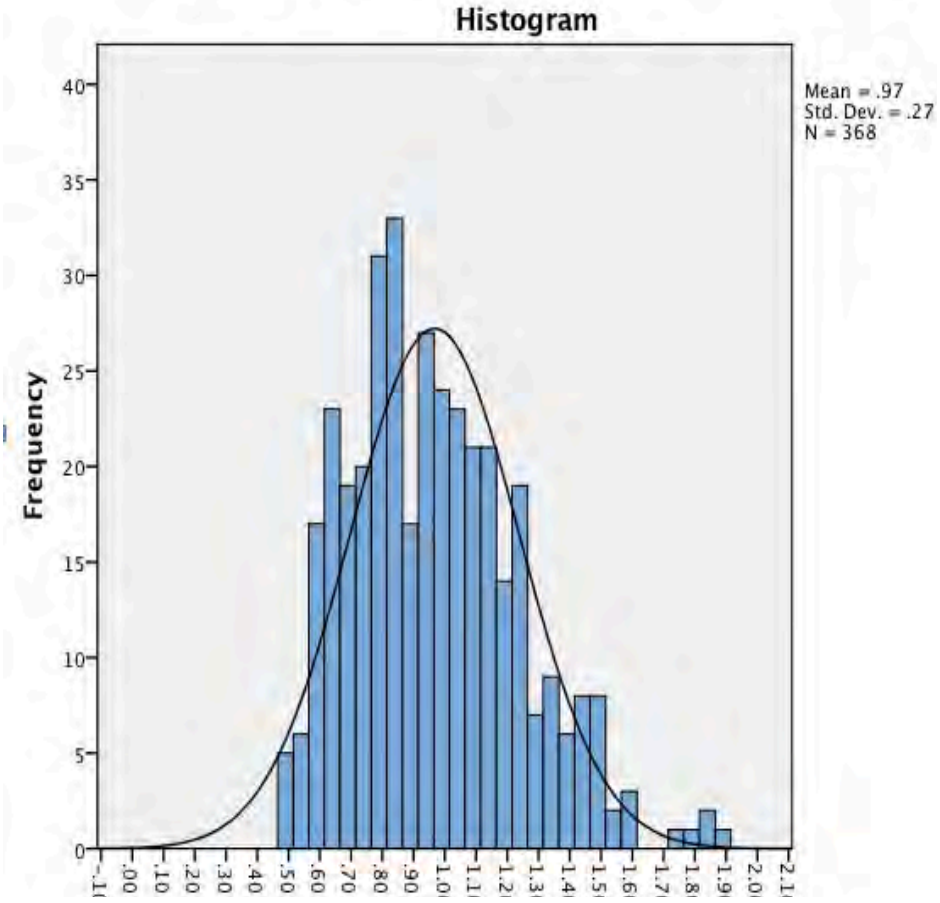
A total of eight semen samples were used to measure the range of retardance within an ejaculated sample (Table 5). The average age was 37.6. The eight reports for these analyses can be found within index (page 112).

Table 5: Semen analysis results.

Sample #	Date of sample	Male age	Concentration (m/ml)	Motility (A+B %)	Normal Morphology (%)
1	18/3/10	25	79.3	65	11
2	23/3/10	34	223.0	74	0
3	1/4/10	40	19.75	80	9
4	6/4/10	59	50.2	45	11
5	15/4/10	42	43.9	65	7
6	11/5/10	36	77.4	67	31
7	18/5/10	30	76.0	53	3
8	27/6/10	35	203.0	70	12

368 sperm were measured for their retardance. The histogram below represents head retardance and their frequency in human spermatozoa (Figure 22).

Figure 22: Range and frequency of retardance within human spermatozoa.



The mean head retardance of 368 sperm was 0.97 nm with standard deviation of 0.27 nm. This most frequent head retardance was between 0.80 nm -0.85 nm. The minimum and maximum values measured were 0.49-1.89 nm respectively. 54.5% of sperm had head retardance between 0.7-1.1 nm. The table below is the statistical analysis of the pooled samples (Table 6).

Table 6: Descriptive analysis of head retardance in human spermatozoa.

Statistics

		sperm_ret	head_morph
N	Valid	368	368
	Missing	0	0
Mean		.9674	
Median		.9300	
Std. Deviation		.26967	
Skewness		.648	
Std. Error of Skewness		.127	
Minimum		.49	
Maximum		1.89	

Normal Head Morphology

During the measurement of retardance, the sperm were assessed for normality. 195/368 sperm (53%) were categorised as having normal head morphology. The mean head retardance was 0.93 nm with standard deviation 0.27 nm. The minimum and maximum range was 0.49-1.86 nm respectively (Table 7).

Table 7: Descriptive analysis comparing normal to abnormal head morphology and retardance.

Statistics

Normal_morphology

N	Valid	195
	Missing	173
Mean		.9323
Median		.8800
Std. Deviation		.26714
Minimum		.49
Maximum		1.86

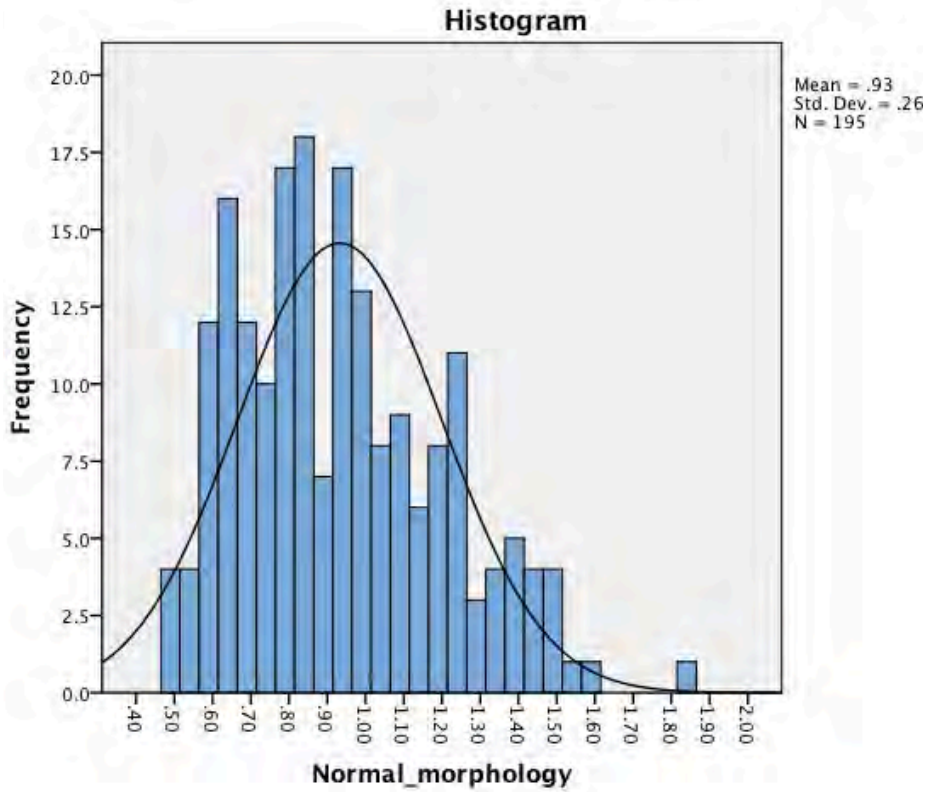
Statistics

Abnormal_morphology

N	Valid	173
	Missing	195
Mean		1.0070
Median		1.0000
Std. Deviation		.26779
Minimum		.51
Maximum		1.89

The histogram below represents head retardance and its frequency in 195 sperm with normal head morphology. The most frequent head retardance was between 0.79 -0.85 nm (Figure 23).

Figure 23: Range and frequency of retardance within normal head morphology.



Abnormal Head Morphology

During measurement of retardance, the sperm were assessed for normality. 173/368 (47%) sperm were categorised as having abnormal head morphology. The mean head retardance was 1.00 nm with standard deviation 0.27 nm. The minimum and maximum range was 0.51-1.89 nm respectively (Table 8).

Table 8: Descriptive analysis for abnormal head morphology and retardance.

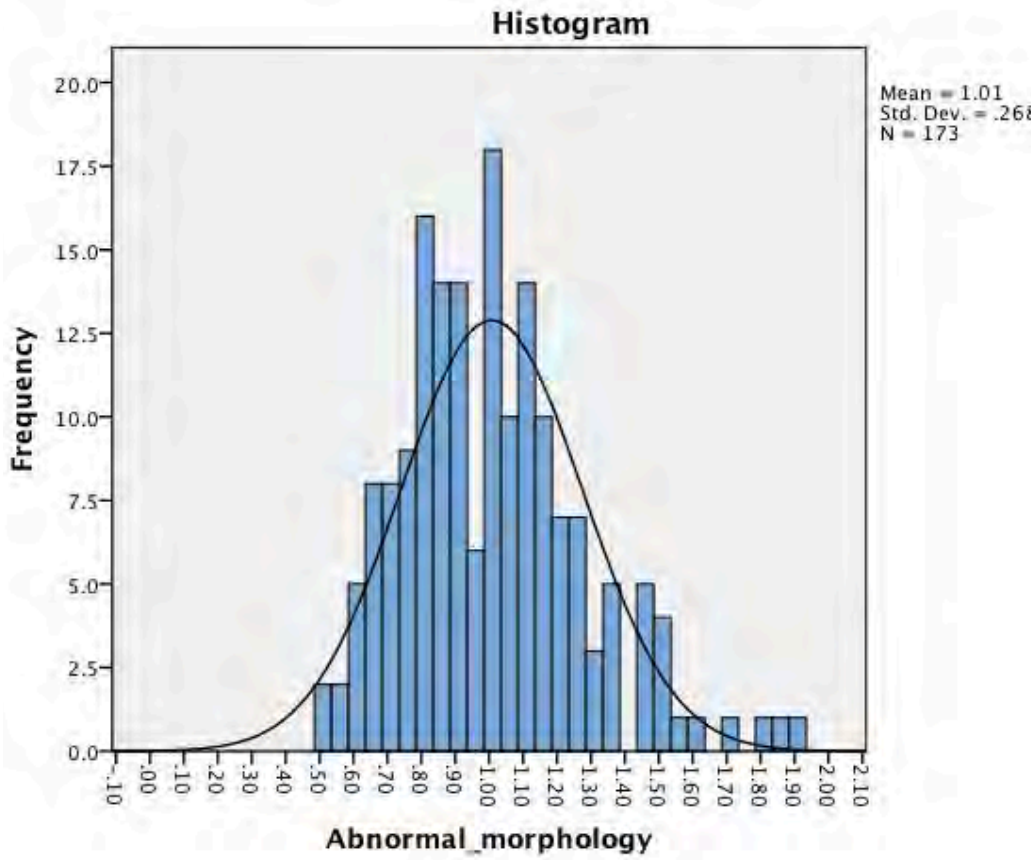
Statistics

Abnormal_morphology

N	Valid	173
	Missing	195
Mean		1.0070
Median		1.0000
Std. Deviation		.26779
Minimum		.51
Maximum		1.89

The histogram below represents head retardance and its frequency in 173 sperm with abnormal head morphology. The most frequent head retardance was between 1.0 -1.05 nm (Figure 24).

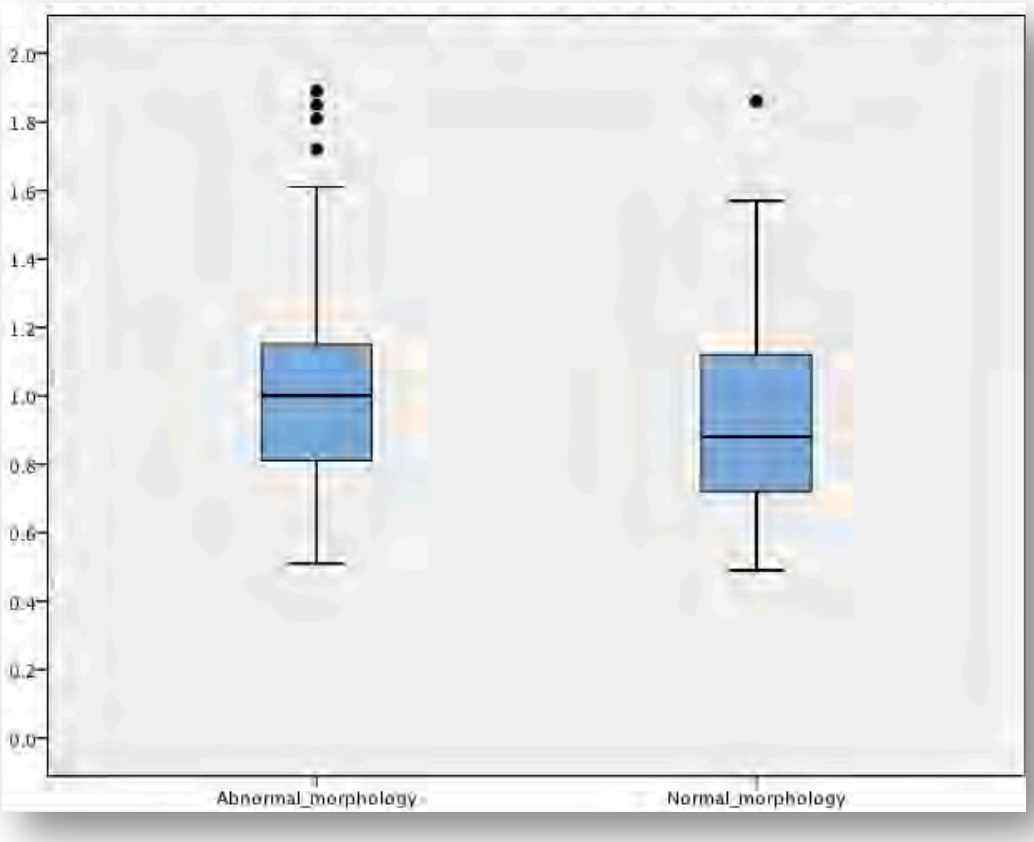
Figure 24: Range and frequency of retardance within abnormal head morphology



Comparing normal and abnormal head morphology and range of retardance

The box plot in Figure 25 compares the two variables normal and abnormal head morphology. The plot shows that the ranges of head retardance when comparing head morphology are similar to each other.

Figure 25: Box plot comparing normal and abnormal morphology and retardance.



An independent samples T test is was carried out to test significance between the means of both variables, normal and abnormal head morphology (Table 9).

Table 9: Independent sample's T Test comparing sperm retardance with normal and abnormal head morphology.

Group Statistics

	head_morph	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	normal	195	.9323	.26714	.01913
	abnormal	173	1.0070	.26779	.02036

Independent Samples Test

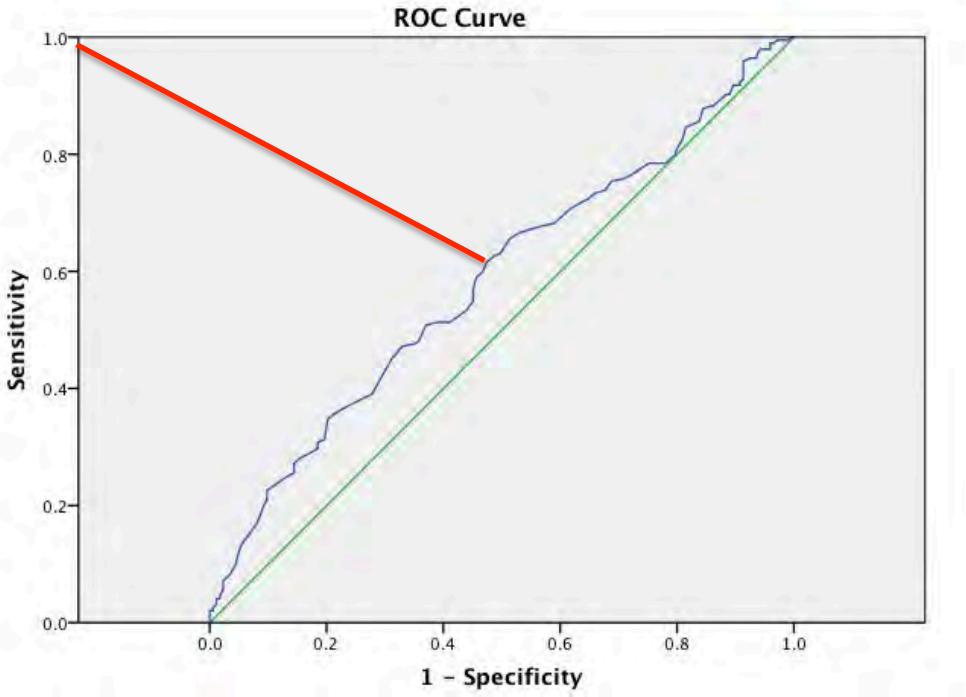
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	.267	.606	-2.674	366	.008	-.07469	.02793	-.12962	-.01976
	Equal variances not assumed			-2.673	360.586	.008	-.07469	.02794	-.12963	-.01975

Levene's test for equality of variances had a significance >0.05. This allows us to interpret our data as having equal variance. The independent samples T-test had a significance level P (2) 0.008 < 0.05.

Therefore, it can be said that head retardance between sperm with normal head morphology and abnormal head morphology are significantly different from each other. Sperm with normal head morphology have a significantly lower mean retardance.

A ROC curve was conducted to evaluate the quality or performance of our testing methods (Figure 26). It works by plotting the true positive rate (sensitivity) against the false positive rate (specificity) for different cut points. A testing method is considered poor if the ROC curve follows the green diagonal line, and more reliable the closer it becomes to the top left corner (0,1). The point on the curve closest to (0,1) is considered to be the cut off value for the test as indicated by the output for the co-ordinates of the curve (appendix page 127).

Figure 26: Receiver Operating Characteristic curve comparing retardance within normal and abnormal head morphology.



Area Under the Curve

Test Result Variable(s):sperm_ret

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.583	.030	.006	.525	.641

Probability of the test (asymptotic significance) was P (2) 0.006 < 0.05, therefore we can conclude that sperm head morphology is an indicator for sperm head

retardance and is a reliable testing method. The ROC curve demonstrated that the point on the curve closest to the top left corner (0,1) was at specificity 0.487 and sensitivity 0.607. Using the coordinates of the curve output, 0.91 nm was the cut off value (appendix page 127). Sperm with a head retardance ≤0.91 nm are more likely to have normal head morphology.

Discussion

This experiment aimed to establish a normal range of sperm head retardance within human spermatozoa. We also sought to establish if the retardance range and frequency differed when comparing abnormal and normal head morphology.

Literature review revealed no previous papers relating directly to our experiment to test for the range or frequency of sperm head retardance within human spermatozoa. Keefe *et.al.,* (2008) was the only study that used PLM to measure retardance in 51 sperm under high magnification before and after subjecting them to DNA damage using ultraviolet light. His primary goal was to observe the effect of DNA fragmentation on retardance, which it did. Their results had sperm retardance before being damaged in the range of 0.26-0.41 nm, and never having retardance of zero.

Stemming from this study, we assumed that there was always going to be some degree of retardance as noted that sperm head are birefringent due to molecular order within protein filaments within the acrosome and nucleoprotein filaments within the nucleus (Gianaroli *et.al.,* 2008). It was hypothesised that retardance does not begin at zero as molecular order from the nucleus of the sperm would still exist after the sperm have lost some molecular order once completing the acrosome reaction (Gianaroli *et.al.,* 2009).

Our first aim was to distinguish the range of head retardance within human spermatozoa. Once a methodology for measuring retardance was tested for user ability and reproducibility it was applied to a random sample of 368 sperm of varying qualities. This pooled sample from eight separate ejaculates comprised of measurements from normal and abnormal morphology, as per WHO 5th edition. The range of male age from the pooled samples ranged from 25 -59 years with mean age 37.6 years. Men from an array of ages were included due to a possible decline in male fertility with increasing age. The literature states that male age is a factor for infertility due to the increase in the occurrence of DNA damage caused by ROS (Cocuzza *et.al.,* 2008).

Our results from measuring the sperm from the pooled results had a mean head retardance of 0.97 +/- 0.27 nm. The minimum and maximum values measured were 0.49-1.89 nm respectively with the most frequent retardance being 0.84 nm. Our results differ significantly to those of Keefe *et.al.,* (2008). One of the main differences between our methods is that Keefe *et.al.,* incorporated high magnification to visualise and measure sperm heads for their retardance. It would be interesting to see if by using our methods with high magnification, our retardance ranges would be similar to their study. However at this stage we believe that conventional ICSI magnification is sufficient to gain information on the optimum sperm based on their retardance given the ability to measure an optimal spindle retardance of an oocyte at x400 magnification (Kilani *et.al.,* 2009).

As confirmed by transmission electron microscopy, it has been observed that birefringence represented by total and partial head birefringence reflects the normality of highly organised structures, the acrosome and nucleus. Gianaroli *et.al.,* (2009) and Magli *et.al.,* (2012) have demonstrated that birefringence of a spermatozoa is directly correlated to morphological parameters where normal head morphology have an increased percentage of partial head birefringence, where 96% of sperm with this birefringence are acrosome reacted. A loss in molecular order once the acrosome has reacted causes a decrease in head birefringence, and we hypothesise that the retardance would also be reduced.

The second aim of this experiment was to determine if the range and frequency of retardance differs between normal and abnormal head morphology.

Our descriptive analysis of normal head morphology had a mean retardance of 0.93 +/- 0.27 nm with range being 0.49-1.86 nm compared to abnormal head morphology with mean retardance of 1.00 +/- 0.27 nm and range being 0.51-1.89 nm. It was clear on the histograms (Figures 25 and 27) that although the mean and range were similar, the frequency and mode was visually different. The most frequent retardance for sperm with normal and abnormal head morphology respectively were 0.8 nm and 1.00 nm. Comparing the normality curve on the range and frequency histograms, it was apparent that the curve

was at a lower spectrum on the graph with normal morphology compared to abnormal morphology that may indicate a difference in the retardance results between the two groups. Statistical analysis using an independent samples T test confirmed this observation with a highly significant difference of retardance when comparing head morphology. Sperm with normal head morphology had lower retardance compared to sperm with abnormal head morphology. These results support the conclusions of previous studies by Gianaroli *et.al.*, (2009) and Magli *et.al.*, (2012) that slow sperm with normal morphology have an increased proportion of sperm with partial head birefringence that is characteristic of a reduced retardance measurement.

It was important to evaluate if sperm retardance was correlated to morphology normality and acrosome reactivity due to previous studies showing the association of better quality of embryos from sperm that are acrosome reacted as well as increased ongoing clinical pregnancies (Mansour *et.al.,* 2008*).* Studies by Crippa *et.al.,* (2009) and Peterson *et.al.,* (2011) have shown that sperm DNA fragmentation is associated to the type of head birefringence, where sperm with full head birefringence have a higher proportion of DNA fragmentation (abnormality in nuclear remodelling and chromatin packaging during spermatogenesis) causing a decrease in embryo quality and ongoing clinical pregnancies. If this is the case, it can be assumed that high retardance is also linked to sperm with full head birefringence and an increase in the proportion of DNA fragmentation due to the increased proportion of molecular order from the intact acrosome. Keefe *et.al.,* (2008) have shown in their small study that sperm head retardance significantly increased after sperm were subjected to DNA damage using UV light.

On the other end of the spectrum, Gianaroli *et.al.*, (2009) suggested that in 12% of cases, sperm with normal morphology are devoid of birefringence that demonstrates abnormalities within their protoplasmic structure. This was the only study from the literature stating that no birefringence was seen from a sperm, so we question if this could be correct? It could be assumed however, that a sperm with minimal birefringence paralleled to very low retardance levels may also be correlated to DNA fragmentation.

We now hypothesise that sperm retardance must have an optimal cut off value, or even possibly a range that predicts the health of its protoplasmic structures that would be demonstrated by embryo quality and clinical pregnancies. Observing the retardance from sperm selected by normal morphology and motility during ICSI tested this within experiment 3.

In summary, in this experiment we measured human sperm head retardance to distinguish the range and frequencies and concluded that:

1. The range of head retardance in an ejaculate is 0.49-1.89 nm with the most frequent retardance being 0.84 nm.

2. Sperm with normal head morphology have significantly lower retardance compared to abnormal head morphology.

3. Sperm with head retardance ≤0.91 nm are more likely to have normal head morphology.

EXPERIMENT 3 – CLINICAL EVALUATION OF RETARDANCE

Aim

To assess a value or range of sperm head retardance in the selection of the optimum sperm for ICSI.

Methods and Materials

Patients Inclusion and Exclusion Criteria

This study used inclusion and exclusion criteria to ensure patient groups were reproducible and controlled.

Inclusion criteria were:

1. *Women ≤38 years old at OPU.*

   Woman's age was used as an inclusion to reduce the incidence of age being a factor for infertility. The success of IVF/ICSI is greatest for women whom are 38 years or less at the time of egg collection.

2. *Women whom had between ≥5 and ≤15 mature oocytes.*

   When less than five mature oocytes are used for ICSI, the risk of the cycle being poor is elevated due to failed fertilisation or poor embryo growth emanating a possible early transfer at cleavage stage or cancelled transfer on day 5. When more than 15 mature oocytes are retrieved, the quality of these oocytes may be poor due to known embryo development reductions in potential OHSS patients and a reduction in patients having an embryo transfer. This range also eliminates potential PCO/S patients.

3. *ICSI as the chosen method of insemination.*

   The reason for ICSI is to enable an image of the selected sperm to be taken via Oosight™ prior to injection.

4. *Blastocyst culture.*

   Blastocyst culture was used to ensure maximum embryo development was seen.

5. *Elective sET.*

   When one embryo is transferred, there is an ability to know which embryo resulted in a clinical pregnancy. This allowed us to know the outcomes from each sperm and its retardance.

Exclusion criteria were:

1. *Use of donor gametes.*

   No donor gametes were analysed, as we wanted to gain information about the couple at hand. This allowed us to know the patients full reproductive history and to eliminate other factors that may prevent independence.

2. *Use of frozen gametes (excludes frozen embryos).*

   It is unknown at this stage if cryopreservation of sperm alters the retardance value. Cryopreserved oocytes were not used as their optimal injection timing may have been exceeded.

3. *Use of Oosight™ for the analysis of oocytes.*

   Oocyte spindle presence was not assessed.

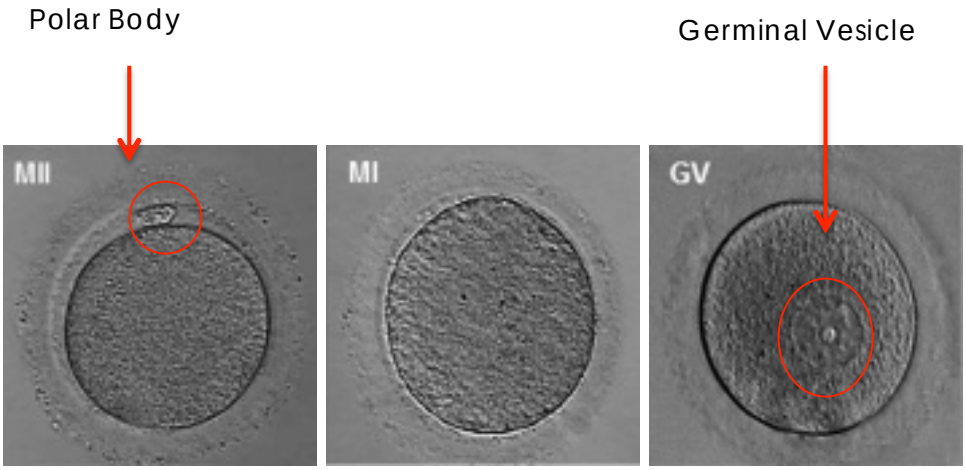
Preparation for Egg Collection and ICSI

Patients selected for this study that met the criteria and were being treated with ICSI were stimulated according to IVF Australia protocols. Patients were administered with Puregon FSH (Merck Sharp & Dohme, Australia), at a dosage between 150-300 I.U per day. Patients were monitored with regular blood tests and ultrasounds to measure estrodial levels and follicular growth. Egg collection under sedation was scheduled 36 hours post trigger injection of Pregnyl 10,000 I.U or Ovidrel 250 μg when follicles were mature.

Once oocytes were collected, they were taken to the laboratory where they were denuded of their cumulous and coronal cells. Oocytes were assessed for maturity.

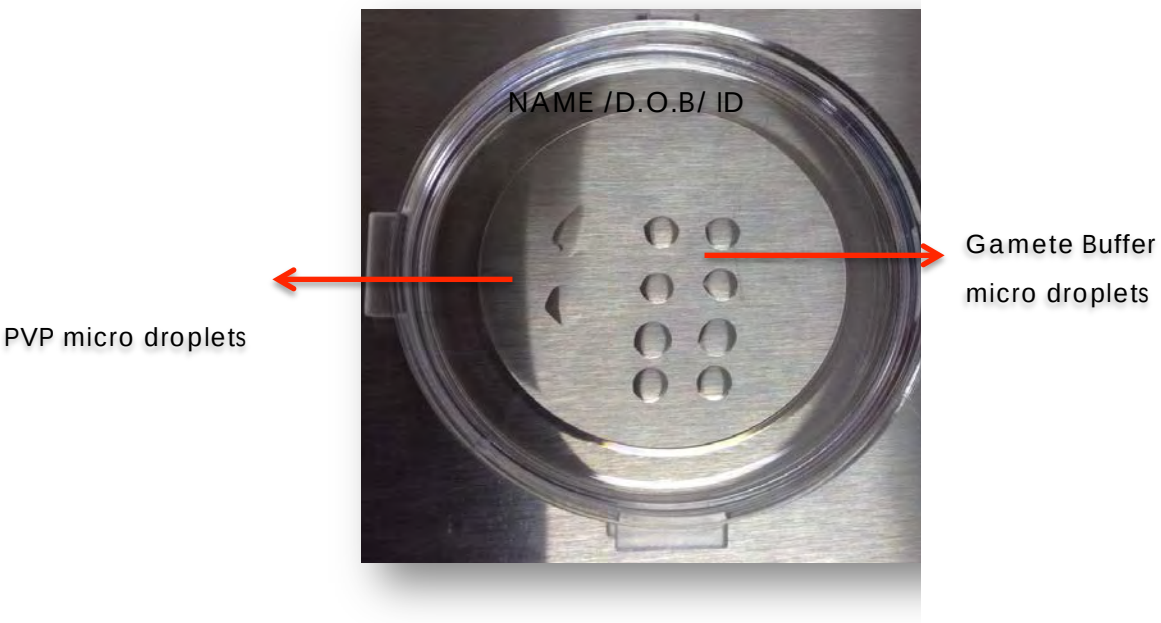
Metaphase I and Metaphase II oocytes were transferred into a culture dish containing COOK Sydney IVF cleavage medium (COOK Sydney IVF Limited, National Technology Park, Ireland, UK) that had been equilibrated overnight. Metaphase I and II oocytes were kept separately. Immature oocytes containing germinal vesicles were not injected. See images below for stages of oocyte maturity (Figure 27).

Figure 27: Oocyte maturity (Hill, 2009).



Timing of ICSI occurred between 39-41 hours post trigger injection to allow for optimum oocyte maturity and fertilisation (Kilani *et.al.,* 2009). ICSI was performed within a glass-bottomed tissue culture dish (Fluro-dish, World Precision Instruments, Florida, USA). For each oocyte to be injected for ICSI, a 5 uL droplet of gamete buffer (COOK Sydney IVF Limited, National Technology Park, Ireland, UK) was placed within multiple rows, along with two droplets of 7% PVP (Sage Media, USA), one droplet containing an aliquot of the prepared sperm. The droplets were then covered with warmed culture oil (COOK Sydney IVF Limited, National Technology Park, Ireland, UK). The diagram below illustrates how the ICSI dish is prepared (Figure 28).

Figure 28: ICSI dish preparation.



Sperm Preparation

A fresh semen sample was collected on the day of egg collection and prepared as described in experiment 2 (page 57).

Measuring Retardance

- An inverted ICSI microscope was set up for ICSI. The holding and injection pipettes (The pipette company, South Australia) were placed into their holders and aligned with each other (Palermo *et.al.,* 1992). The PC computer and the monitor were turned on as described in section "Optics and software for obtaining images" (page 41).

- Metaphase I and II oocytes were placed within the prepared ICSI dish once the dish had warmed to 37 degrees Celsius for at least 10 minutes. One oocyte was placed within each micro drop of media.

- Sperm with normal morphology seen under x400 magnification, which had linear rapid motility, were selected.

- An image of the immobilised sperm was taken prior to ICSI (See "Taking an image of a sperm" page 43 for detailed method). The measurement of retardance was not completed now. Only images were taken and saved with the patients surname and ID number as a reference. This was to ensure embryo choice for transfer was by standard morphology. The measurement for sperm retardance was acquired once the patients cycle was complete.

- ICSI was performed (Palermo *et.al.,* 1992).

Fertilisation Assessment

Fertilisation was assessed 18 hours post ICSI.

Normal fertilisation is referred to as the presence of 2 pro-nuclei with 2 polar bodies (2PN).

Abnormal fertilisation in this study is referred to one of the following.

1. No Evidence of Fertilisation (NEOF)
2. 1PN
3. 3PN
4. Degenerate

Cleavage Assessment

Cleavage was assessed three days post ICSI. This study recognised normal embryo cleavage as having between 6-10 cells for the hours of assessment on day three. These embryos were graded as:

- "A" grade embryos have ≤20% fragmentation.
- "B" grade embryos have >20% fragmentation.

This percentage accounts for the total zona volume. Abnormal cleavage in this study referred to embryos <6 cells or >10 cells.

Blastocyst and Utilisation Assessment

Blastocyst and utilisation assessment occurred on day 5 and 6 post ICSI. Normal blastocysts have a distinct trophoblast, Inner Cell Mass (ICM) and blastocoel. There are varying stages of blastocyst development and all are considered normal for day 5 and 6 development. These include:

1. Very early blastocyst (VEBL)
2. Early blastocyst (EBL)
3. Full blastocyst (BL)
4. Expanded blastocyst (XBL)

5. Hatching blastocyst (HGBL)
6. Hatched blastocyst (HBL)

The Gardner's scale grading system is used within this laboratory (Sathananthan *et.al.,* 2010). The grading of an A, B, or C depicts the quality of the ICM and the trophoblast respectively e.g BLAB. This is a blastocyst with an A grade ICM, and B grade trophoblast.

Utilisation is referred to a blastocyst of high quality whose grade resembles any combination of an A or B. These embryos can either be transferred into the patient or cryopreserved. Non-utilised blastocysts, or embryos that have not reached blastocyst, are referred to those whose quality prevents them from being suitable for cryopreservation or transfer. They usually incorporate a C grade. These embryos may also be delayed in development.

Clinical Pregnancy Assessment

A clinical pregnancy is defined by ANZARD as the presence of a gestational sac with or without the presence of a foetal heart. Our clinic performs an ultrasound at seven weeks gestation after the indication of pregnancy with rising bHCG.

Results

Results from 63 separate ICSI injection cycles were included in this study. This included 63 fresh transfers and 38 subsequent frozen transfers. The frozen transfers were included because their data was available from the fresh ICSI cycle. The survival rate post vitrification means none of the blastocysts were damaged, and they were all transferred as single embryos, so the outcomes and sperm parameters used to generate the embryos were still valid. There was sperm data available from these cycles for fertilisation, cleavage, blastulation and clinical pregnancies of the embryo created.

Patient demographics

- Women's mean age in this study was 32.9 years.
- Male's mean age was 35.4 years.
- Number of previous cycles:

First	N= 40 (63.5%)
Second	N= 14 (22.2%)
Third	N= 6 (9.5%)
Fourth	N= 1 (1.6%)
Fifth	N= 3 (3.2%)

Of the 63 fresh cases, ICSI was selected as their treatment for assisted reproduction due to the following demographics:

1. 18 (28.6%) of the men had reduced or borderline sperm concentration.
2. 20 (31.7%) had reduced normal sperm morphology.
3. 11 (17.5%) had reduced sperm motility.
4. 14 were clinician choice (22.2%).

The reasons behind the clinicians' decision to choose ICSI were:

5. 1 (1.6%) due to female age (increased zona thickness).
6. 6 (9.5%) had poor IVF fertilisation previously or previous failed IUI or IVF cycles.
7. 1 (1.6%) had sperm antibodies.

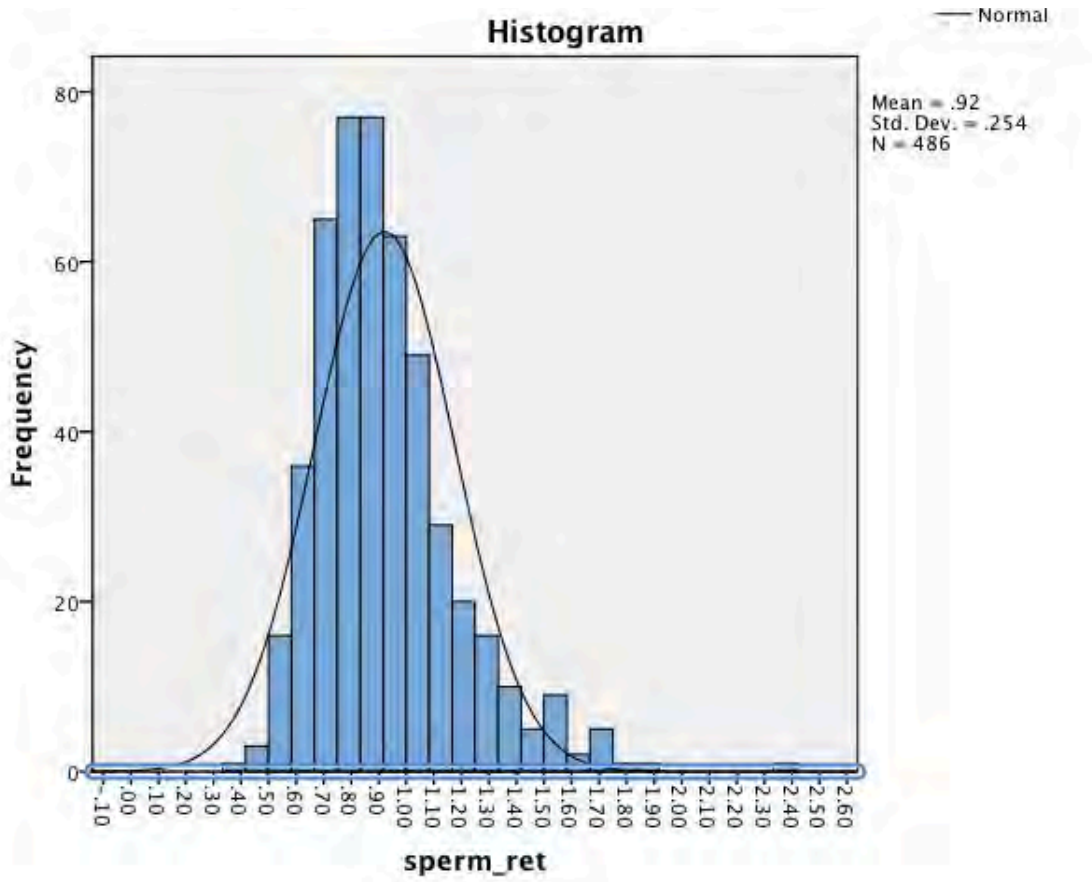
8. 6 (9.5%) had no reason behind choosing ICSI other than personal preference.

No patients in this study were tested for DNA fragmentation.

Range and frequency of head retardance

Figure 29 represents the head retardance of all the sperm utilised during ICSI that were selected based purely on normal motility and morphology. A total of 486 sperm were utilised and measured. Sperm retardance is considered the main variable within this study.

Figure 29: Range and frequency of head retardance from sperm utilised during clinical evaluation.



The mean head retardance of sperm selected for ICSI was 0.92 nm with standard deviation 0.25 nm. The minimum and maximum values respectively were 0.40- 2.38 nm.

Quartiles were analysed:

- 25th percentile =0.75 nm
- 50th percentile =0.87 nm
- 75th percentile =1.05 nm

Below is the descriptive analysis from SPSS of all the sperm utilised during ICSI (Table 10).

Table 10: Descriptive analysis of sperm utilised during ICSI.

Statistics

sperm_ret

N	Valid	486
	Missing	0
Mean		.9208
Median		.8700
Std. Deviation		.25438
Minimum		.40
Maximum		2.38
Percentiles	25	.7475
	50	.8700
	75	1.0500

Comparing retardance from normal morphology in Experiment 2 and sperm utilised during ICSI in Experiment 3

Analysis was conducted to test differences between sperm retardance measured between experiment 2 and 3. It was hypothesised that there would be no differences as both groups only contained sperm with normal morphology (WHO 5th edition).

The mean retardance between experiment 2 and 3 respectively were 0.93 +/- 0.27 nm and 0.92+/- 0.25 nm as per the descriptive analysis (SPSS). Quartiles were comparable with minimum retardance 0.49 and 0.40 nm, 25th percentile 0.72 and 0.75 nm, 50th percentile 0.88 and 0.87 nm, 75th percentile 1.11 and 1.05 nm and maximum values of 1.86 and 2.38 nm (Table 11).

Table 11: Comparing frequency and distribution of normal head morphology between experiment 2 and 3.

Statistics

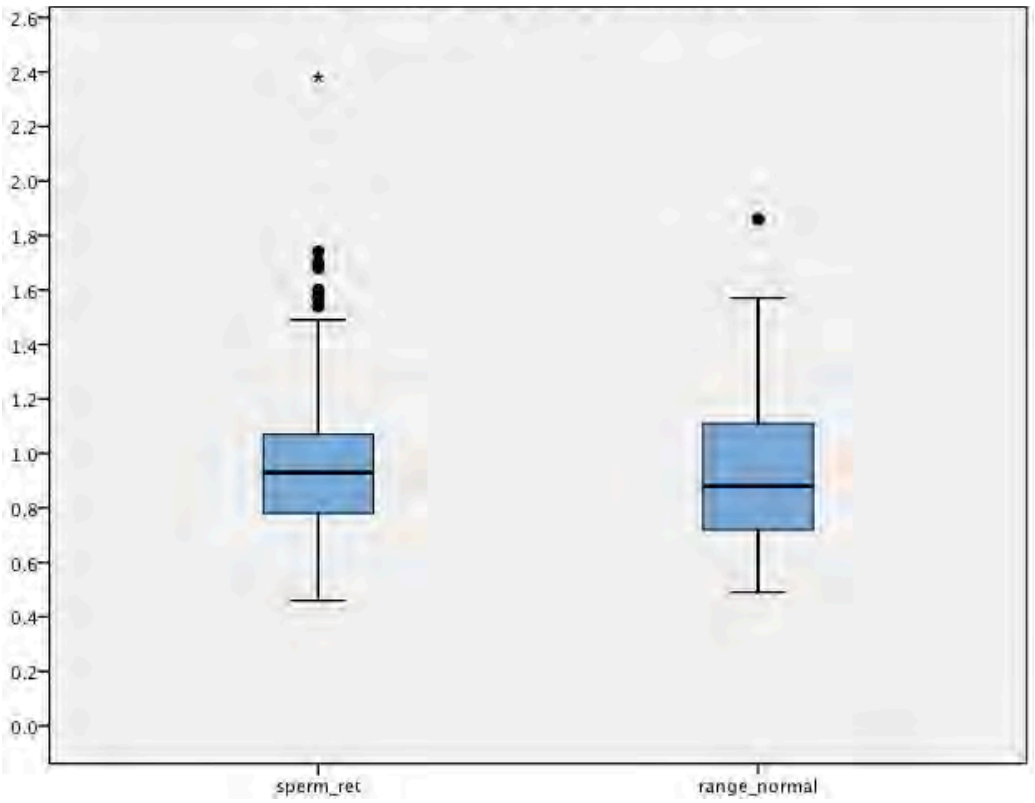
		sperm_ret	range_normal
N	Valid	486	195
	Missing	0	291
Mean		.9208	.9323
Median		.8700	.8800
Std. Deviation		.25438	.26714
Minimum		.40	.49
Maximum		2.38	1.86
Percentiles	25	.7475	.7200
	50	.8700	.8800
	75	1.0500	1.1100

Sperm_ret = Sperm utilised during ICSI for experiment 3.

Range_normal = Retardance of normal head morphology within experiment 2

The box plot of the descriptive analysis is shown below (Figure 30).

Figure 30: Box plot comparing retardance between normal head morphology (Experiment 2) and sperm utilised for ICSI (Experiment 3).



The box plot shows that there is no significant difference in the descriptive statistics of sperm with normal morphology and their head retardance between experiment 2 and 3.

A Pearson's correlation was conducted (SPSS) to test differences between the retardance of normal head morphology in both experiments. A significant result is indicated if P<0.05. As an r value approaches 1, the more significant a correlation.

The Pearson's correlation showed an r value of -0.41 between the samples, which results in a weak correlation. The P value was P (2) 0.568 > 0.05. The results from the analysis showed that there is no significant difference between the two samples with normal head morphology in experiment 2 and 3 (Table 12).

Table 12: Pearson's correlation between sperm utilised during ICSI and sperm with normal head morphology in experiment 2.

Correlations

		sperm_ret	range_normal
sperm_ret	Pearson Correlation	1	-.041
	Sig. (2-tailed)		.568
	N	486	195
range_normal	Pearson Correlation	-.041	1
	Sig. (2-tailed)	.568	
	N	195	195

Fertilisation

A total of 486 oocytes were injected. 364 oocytes fertilised normally (74.9%) with mean head retardance 0.91 +/- 0.25 nm. 122 oocytes fertilised abnormally or did not fertilise (25.1%) with mean head retardance 0.95 +/- 0.27 nm (Table 13).

Table 13: Fertilisation and sperm head retardance.

		Total (n=486)	Percentage (%)	Median (nm)	Mean (nm)	St. Dev
Normal Fertilisation	2PN	364	74.9	0.87	0.91	0.25
Abnormal Fertilisation	NEOF	71	14.6	0.91	0.95	0.27
	1PN	14	2.9			
	3PN	5	1.0			
	DEG	32	6.6			

An independent samples T-test was used to measure for significance between the two groups, normal and abnormal fertilisation (SPSS). Levene's test for equality of variances had a significance P (2) 0.193 > 0.05. This allowed us to interpret our fertilisation data as having equal variance (Table 14).

Table 14: Independent samples test to compare normal and abnormal fertilisation.

Group Statistics

	Fertilisation	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	2PN	364	.9097	.24751	.01297
	1PN_3PN_NEOF_DEG	122	.9540	.27223	.02465

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	1.699	.193	-1.668	484	.096	-.04432	.02656	-.09651	.00787
	Equal variances not assumed			-1.591	192.418	.113	-.04432	.02785	-.09925	.01062

The independent samples T-test had a significance level P (2) 0.096 > 0.05. Therefore there were no significant differences in sperm head retardance between oocytes that fertilised normally and those that did not fertilise normally or failed to fertilise.

Cleavage

A total of 364 oocytes fertilised normally and were cultured through to day 3 (74.9%), when embryos were assessed for cleavage and were graded. If the embryos had between 6-10 cells (A or B grade), they were considered normal. If the embryos had less than 6 cells, they were considered abnormal.

276 embryos were considered normal on day 3 (75.8%) with mean head retardance 0.89 +/- 0.23 nm. 88 embryos were considered abnormal (24.2%) with mean head retardance 0.98 +/- 0.28 nm (Table 15).

Table 15: Embryo cleavage and sperm head retardance

	Total	Median (nm)	Mean (nm)	St. Dev
Normal Cleavage 6-10 cells A/B grade	276	0.85	0.89	0.23
Abnormal Cleavage (<6 cells A/B grade)	88	0.96	0.98	0.28

An independent samples T-test was used to measure for significance between the two groups normal and abnormal cleavage (SPSS). Levene's test for equality of variances had a significance P (2) 0.059 >0.05. This allowed us to interpret our cleavage data as having equal variance (Table 16).

Table 16: Independent samples T-test for cleavage.

Group Statistics

	Cleavage	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	6-10_cell_a/b	276	.8865	.23386	.01408
	abnormal cleavage	88	.9824	.27513	.02933

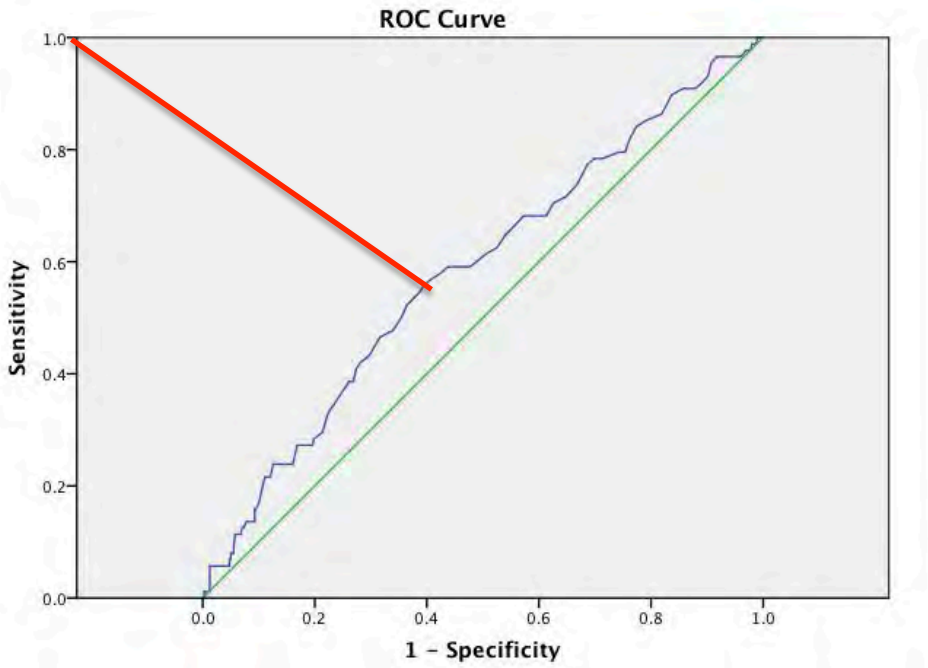
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	3.596	.059	-3.204	362	.001	-.09586	.02992	-.15471	-.03702
	Equal variances not assumed			-2.947	129.524	.004	-.09586	.03253	-.16023	-.03150

The independent samples T-test had a significance level P (2) 0.001 < 0.05. Therefore good quality embryos on day 3 have significantly lower sperm head retardance compared to poor quality embryos.

A receiver's operation curve (ROC) was conducted to evaluate the quality or performance of our testing methods, as well as to identify the optimal threshold value as a useful detector for normal cleavage (Figure 31). It works by plotting the true positive rate (sensitivity) against the false positive rate (specificity) for different cut points. A testing method is considered poor if the ROC curve follows the green diagonal line, and more reliable the closer it becomes to the top left corner (0,1). The point on the curve closest to (0,1) is considered to be the cut off value for the test as indicated by the output for the co-ordinates of the curve (appendix page 130).

Figure 31: ROC curve comparing retardance between normal and abnormal cleavage



Area Under the Curve

Test Result Variable(s):sperm_ret

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.584	.034	.014	.517	.651

The test result variable(s): sperm_ret has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

The probability of the test (asymptotic significance) was P (2) 0.014< 0.05, therefore we can conclude that a lower sperm head retardance is an indicator for normal cleavage and is a reliable testing method. The ROC curve demonstrated that the point on the curve closest to the top left corner (0,1) was at specificity 0.4 and sensitivity 0.57. Using the coordinates of the curve output, 0.91 nm was the cut off value (appendix page 130). When retardance is greater than 0.91 nm, embryo quality decreases.

A chi-square analysis was conducted to test ≤0.91 nm as a cut off value for increased embryo quality. Embryos that incorporated sperm head retardance ≤0.91 nm had 82% being good quality on day 3 compared to 67% if the embryos incorporated head retardance >0.91 nm. Pearson Chi-Squared had a value of 10.41 that was significant P (2) 0.001< 0.05 (Table 17).

Table 17: Chi square analysis of cut off value for cleavage.

Cleavage * cutoff_0.91nm Crosstabulation

Count

		cutoff_0.91nm		
		ret>0.91nm	ret<0.91nm	Total
Cleavage	6-10_cell_a/b	103	173	276
	abnormal cleavage	50	38	88
Total		153	211	364

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	10.413[a]	1	.001		
Continuity Correction[b]	9.628	1	.002		
Likelihood Ratio	10.309	1	.001		
Fisher's Exact Test				.002	.001
N of Valid Cases	364				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 36.99.
b. Computed only for a 2x2 table

Blastulation

A total of 364 embryos were grown to day 5 or 6 for blastocyst development. On day 5 and 6, embryos were classified as either having developed blastulation or non-developed blastulation. Developed blastulation was any embryo that had a defined cavity or blastocoel (very early blastocyst through to hatched blastocyst).

235 embryos developed blastulation on day 5 and/or 6 (64.6%) with mean head retardance 0.90 +/- 0.24 nm. 129 embryos failed to develop blastulation (35.4%) with mean head retardance 0.93 +/- 0.26 nm (Table 18).

Table 18: Blastulation development and sperm head retardance

	Total	Median (nm)	Mean (nm)	St. Dev
Developed Blastulation	235	0.87	0.90	0.24
Non-developed Blastulation	129	0.86	0.93	0.26

Independent samples T-test was used to measure for significance between the two groups, developed and non-developed blastulation (SPSS). Levene's test for equality of variances had a significance P (2) 0.063 >0.05. This allowed us to interpret our blastulation data as having equal variance (Table 19).

Table 19: Independent samples T test for blastulation.

Group Statistics

	Blastulation	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	Blastulation	235	.8998	.24196	.01578
	Failed blastulation	129	.9277	.25731	.02265

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	3.469	.063	-1.027	362	.305	-.02784	.02712	-.08118	.02549
	Equal variances not assumed			-1.008	250.174	.314	-.02784	.02761	-.08222	.02653

The independent samples T-test had a significance level P (2) 0.31 > 0.05. Therefore there was no significant difference in sperm head retardance between embryos that formed a blastocyst and from those that did not.

Utilisation

A total of 364 embryos were grown to day 5 and 6 for blastulation development. The embryos were assessed for developmental quality and utilisation ability. Embryos were utilised if they were transferred (TX) or cryopreserved (FZ). Embryos were discarded if they failed to develop blastulation, or if the quality of the blastocyst was poor (C grade).

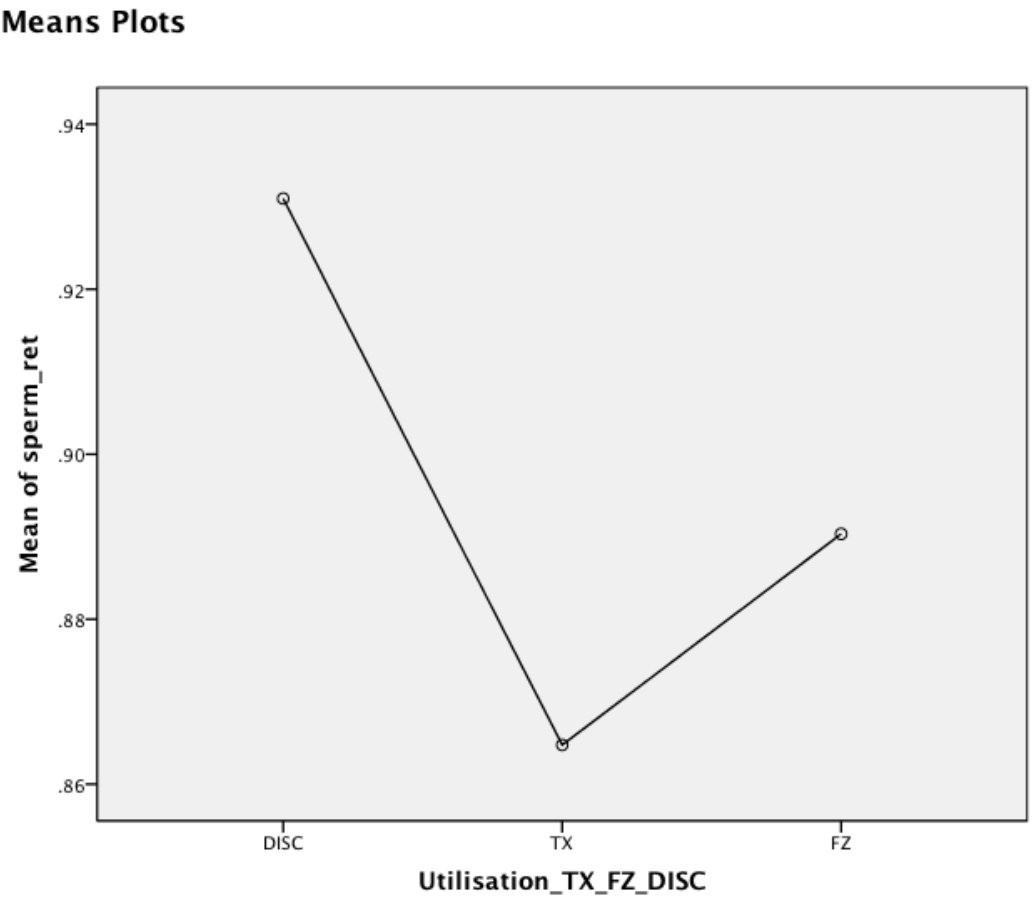
151 embryos were utilised on day 5 and/or 6 (41.5%) with mean head retardance 0.88 +/- 0.20 nm. 213 embryos were discarded, or not utilised (58.5%) with mean head retardance 0.93 +/- 0.27 nm (Table 20).

Table 20: Embryo utilisation and sperm head retardance.

	Total	Median (nm)	Mean	St. Dev
Discarded	213	0.88	0.93	0.27
Utilised (TX or FZ)	151	0.86	0.88	0.20

A means plot was used (SPSS) to visualise the mean head retardance of embryos transferred, cryopreserved or discarded (Figure 32).

Figure 32: Means plot of utilisation.



The means plot illustrates the mean difference of retardance with discarded embryos having the highest retardance of 0.93+/- 0.27 nm, transferred embryos having the lowest retardance of 0.86 +/- 0.27 nm and cryopreserved embryos having a middle retardance of 0.89 +/- 0.20 nm.

An independent samples T-test was used to measure for significance between discarded and utilised embryos on day 5 and/or 6 (SPSS). Levene's test for equality of variances had a significance P (2) 0.026 < 0.05. This allowed us to interpret our blastulation data as having unequal variance (Table 21).

The independent samples T-test had a significance level P (2) 0.04 < 0.05. Therefore good quality embryos that are utilised in a transfer or for cryopreservation have a significantly lower sperm head retardance compared to poor quality embryos that were discarded.

Table 21: Independent samples T test for utilisation.

Group Statistics

	Utilisation	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	Discarded	213	.9310	.27224	.01865
	Utilised	151	.8797	.20484	.01667

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	4.964	.026	1.956	362	.051	.05132	.02623	-.00026	.10290
	Equal variances not assumed			2.051	360.694	.041	.05132	.02502	.00212	.10051

Clinical Pregnancy

101 cumulative fresh (n=63) and subsequent frozen (n=38) single blastocysts were transferred (eSET) to produce 34 clinical pregnancies (33.7% per Tx, 53.9% per case (fresh and Frozen per OPU). Fresh clinical pregnancy rate was 31.7% and frozen clinical pregnancy rate was 36.8% (n=38).

34 embryos formed clinical pregnancies with mean head retardance 0.81 +/- 0.16 nm. 67 embryos did not form clinical pregnancies with mean head retardance 0.91 +/- 0.24 nm (Table 22).

Table 22: Clinical pregnancy rate and sperm head retardance.

	Total (N=101)	Median (nm)	Mean (nm)	St. Dev	Min (nm)	Max (nm)
Clinical Pregnancy (Sac +/- FH)	34	0.77	0.81	0.16	0.56	1.17
No Clinical Pregnancy (-ve or +ve BhCG, no sac)	67	0.87	0.91	0.24	0.51	1.56

An independent samples T-test was used to measure for significance between the two groups clinical pregnancy and non-clinical pregnancy (SPSS). Levene's test for equality of variances had a significance P (2) 0.026 < 0.05. This allowed us to interpret our cleavage data as having unequal variance (Table 23).

Table 23: Independent sample T test for clinical pregnancies.

Group Statistics

	Clin_preg	N	Mean	Std. Deviation	Std. Error Mean
sperm_ret	NO	67	.9148	.23983	.02930
	YES	34	.8091	.16301	.02796

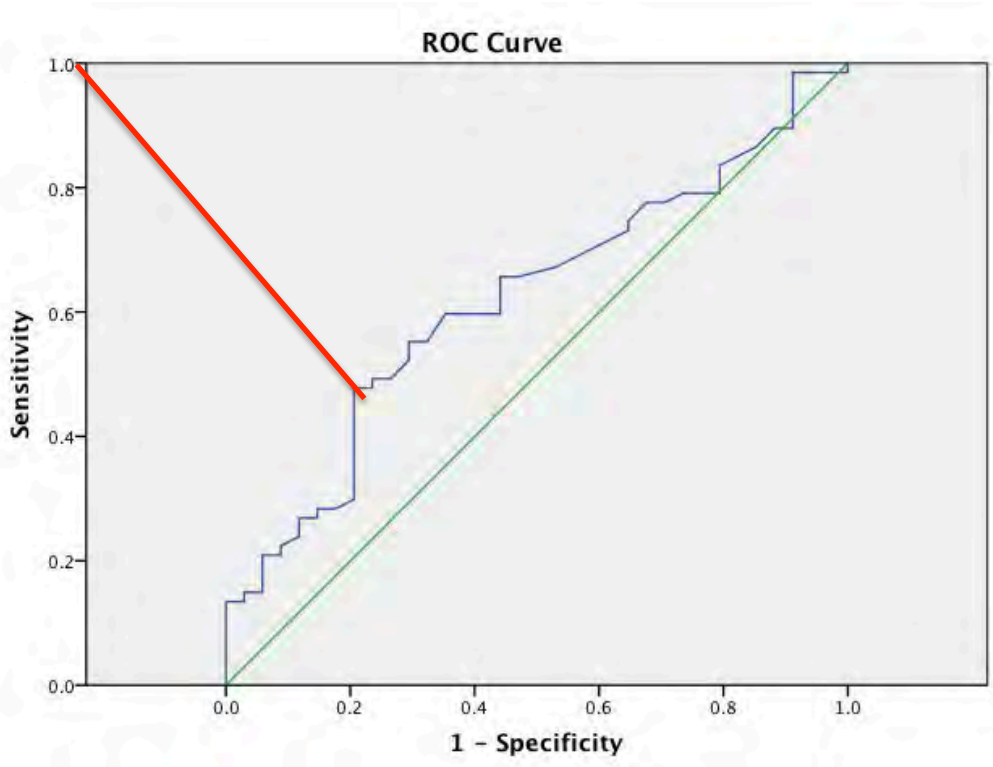
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
sperm_ret	Equal variances assumed	5.132	.026	2.310	99	.023	.10566	.04575	.01488	.19643
	Equal variances not assumed			2.609	90.636	.011	.10566	.04050	.02521	.18611

The independent samples T-test had a significance level P (2) 0.011 < 0.05. Therefore embryos that form clinical pregnancies are derived from sperm with significantly lower sperm head retardance as compared to embryos that do not form clinical pregnancies.

A receiver's operation curve (ROC) was conducted to evaluate the quality or performance of our testing methods as well as to identify the optimal threshold value as a useful detector for clinical pregnancies (Figure 33). It works by plotting the true positive rate (sensitivity) against the false positive rate (specificity) for different cut points. A testing method is considered poor if the ROC curve follows the green diagonal line, and more reliable the closer it becomes to the top left corner (0,1). The point on the curve closest to (0,1) is considered to be the cut off value for the test as indicated by the output for the co-ordinates of the curve (appendix page 133).

Figure 33: ROC curve comparing retardance between clinical and non-clinical pregnancies.



Area Under the Curve

Test Result Variable(s):sperm_ret

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.620	.057	.049	.509	.732

The test result variable(s): sperm_ret has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

The probability of the test (asymptotic significance) was P (2) 0.049 < 0.05, therefore we can conclude that a lower sperm head retardance is an indicator for clinical pregnancies and is a reliable testing method. The ROC curve demonstrated that the point on the curve closest to sensitivity (1) was at specificity 0.23 and sensitivity 0.48. Using the coordinates of the curve, 0.91 nm was the cut off value (appendix page 133). When retardance is greater than 0.91 nm, the likelihood of a clinical pregnancy decreases.

A chi-square analysis was conducted to test ≤0.91 nm as a cut off value for clinical pregnancies. 79.4% (n=27) of embryos that incorporated sperm head retardance ≤0.91 nm formed clinical pregnancies compared to 20.6% (n=7) if the embryos incorporated a head retardance >0.91 nm. Pearson Chi-Squared had a value of 7.03 that was significant P (2) 0.008< 0.05 (Table 24).

Of the 101 embryos transferred, 39 embryos had head retardance >0.91 nm with a clinical pregnancy rate of 18.1%. 62 embryos had head retardance ≤0.91 nm with a clinical pregnancy rate of 44.0%.

Table 24: Chi square analysis of cut off value of 0.91 nm for clinical pregnancies.

cutoff_0.91nm * Clin_preg Crosstabulation

Count

		Clin_preg		
		NO	YES	Total
cutoff_0.91nm	ret>0.91nm	32	7	39
	ret<0.91nm	35	27	62
Total		67	34	101

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.026[a]	1	.008		
Continuity Correction[b]	5.926	1	.015		
Likelihood Ratio	7.410	1	.006		
Fisher's Exact Test				.010	.007
Linear-by-Linear Association	6.956	1	.008		
N of Valid Cases	101				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.13.
b. Computed only for a 2x2 table

The Fisher's exact test to compare clinical pregnancies with sperm head retardance ≤0.91 nm and >0.91 nm was statistically significant P (2) 0.01< 0.05.

The results illustrate that a sperm with head retardance of 0.91 nm or less form a larger percentage of clinical pregnancies. There were no reported clinical pregnancies with head retardance <0.56 nm.

Demographic summary of ≥0.56 nm -≤0.91 nm range within Experiment 3

- 263 of 486 (54.1%) had sperm head retardance within this range.
- 203 of 364 (55.8%) fertilised eggs had sperm retardance within this range.
- 167 of 276 (60.5%) good quality embryos on day 3 had retardance within this range.
- 135 of 235 (57.4%) blastocysts had retardance within this range.
- 90 of 151 (59.6%) embryos utilised had retardance within this range.
- Of 63 fresh transfers 42 (66.7%) had retardance within this range.
- Of 34 clinical pregnancies, 27 (79.4%) had retardance within this range.

Discussion

In this study we have looked at clinical pregnancy rates in a group of patients in whom we have measured retardance of the sperm head after being utilised for ICSI. No previous studies have used this approach. Gianaroli *et.al.,* have looked at clinical pregnancy rates in relation to full and partial head birefringence examined by high magnification, whilst not culturing embryos past day 3 (Gianaroli *et. al.,* 2008; Gianaroli *et.al.,* 2010). Our study has the advantage of requiring only conventional magnification. An attempt to confirm our results and theories in light of different testing methods is being used.

The aim of this experiment was to assess a value or range of sperm head retardance in the selection of the optimum sperm for ICSI. The majority of patients (79.4%) were treated with ICSI due to male factor infertility oligospermia, asthenospermia, teratospermia, or the presence of significant sperm antibodies. Within the laboratory where testing took place up to 80% of its patients are treated with ICSI, not only for male factor infertility but also due to clinician choice (i.e unexplained infertility or where IUI has failed). Most of the patients (85.7%) selected for our study were undergoing their first or second attempt of a fresh cycle. Our inclusion and exclusion criteria was used to best eliminate factors that may affect the outcome of a cycle due to female infertility such as female age, PCO/S and premature ovarian failure as to primarily focus on male factor as an independent variable.

It was shown from the pooled results of Experiment 2, that sperm with normal head morphology have a vast range of head retardance. We compared our range of retardance and their frequency from these normal sperm within Experiment 2 with the pooled results of ejaculates from the 63 men used for our clinical cases in Experiment 3. The groups were comparable in male age and both only incorporate normal head morphology. The only difference between the two groups was the number of sperm measured within each, 195 and 486 respectively.

Descriptive analysis was performed on the two groups and compared using a Pearson's coefficient of correlation using statistical package SPSS.

The descriptive statistics of the two groups showed that the quartiles of the two groups were very similar to each other, which portrays the range and frequency as shown in the box plot (Figure 30). Comparing Experiment 2 and 3 with their quartiles, minimum was 0.49 and 0.40 nm, 25th percentile (0.72 nm and 0.75 nm), 50th percentile (0.88 nm and 0.87 nm), 75th percentile (1.11 nm and 1.05 nm), and maximum (1.86 nm and 2.38 nm). The mean sperm retardance in Experiment 2 compared to 3 was 0.93+/- 0.27 nm and 0.92+/-0.25 nm. When we tested for correlation, results showed that there were no significant differences between the two groups with normal head morphology, further supporting our conclusions for Experiment 2.

Knowing that 96% of normal head morphology have partial birefringence, therefore acrosome reacted, we can strongly assume that the majority of the normal sperm utilised for ICSI in our experiment were also acrosome reacted Magli *et. al.,* (2012). When comparing the retardance of sperm with evidence for fertilisation 18 hours post ICSI compared to abnormal or nil fertilisation, there were no significant differences (P>0.05). This result parallels previous clinical studies testing birefringence on fertilisation, which also resulted with no effect on fertilisation capabilities (Magli *et. al.,* 2012). Knowing the retardance range of these sperm utilised during ICSI, it would be a given that a percentage of these would have abnormalities such as DNA damage or non reacted acrosomes, although at what range is still unclear. But it is known from these fertilisation results the capability ICSI has to achieve fertilisation success with potentially poor sperm quality. The effect of poor sperm quality on pregnancy outcome appears to be a late paternal effect.

It has been shown that the late paternal effect on embryos begins between 4-8 cells (Tesarik *et.al.,* 2004). Hazoult *et.al.,*(2006) incorporated MSOME, selecting sperm without vacuoles (an indicator of DNA fragmentation) and compared clinical pregnancy rates to sperm selected without using MSOME. They found no significant differences between fertilisation success or embryo quality on day 2, although on day 3 when embryo transfer took place of a normal cleavage, good quality embryo, there was a significant increase to clinical pregnancies from the sperm injected utilising MSOME.

Keefe *et.al* (2008) have shown DNA fragmentation is reflected by increased retardance. In our study, we have not tested for DNA fragmentation, but it may be that our high retardance group and lower clinical pregnancy rates may be due to increased molecular order within the acrosome and nucleus. It could be suggestive that these sperm with high retardance have not completed the acrosome reaction therefore reducing embryo quality and clinical pregnancy rates.

Our results confirm those of Hazoult *et.al.,* (2006*)* by observing better embryo quality and development within day 3 embryos that were injected with lower sperm head retardance. Normal cleavage embryos had significantly lower mean sperm retardance 0.89 +/- 0.23 nm compared to abnormal cleavage 0.98 +/- 0.28 nm (P (2) 0.001 < 0.05). We could hypothesise from this that the late paternal effect may be having an impact on embryo development creating a higher proportion of poor quality embryos by day 3. Further statistical analysis using a ROC curve and chi-square analyses found a cut off value of ≤0.91 nm as an indicator for better embryo quality and development on day 3.

Blastulation and utilisation was assessed on day 5 and 6 of culture. From the literature review, there have been no previous studies looking at retardance or birefringence of sperm on the effect of embryo development past day 3. We could hypothesise however, that blastulation rates would increase with lower head retardance due to the late paternal effect on embryo quality. Our results did not show this. Embryos that developed a cavity and formed blastulation had mean retardance of 0.90 +/- 0.24 nm compared to failed blastulation 0.93 +/- 0.26 nm (P (2) 0.31 > 0.05). If we took into account the cut off value for improved embryo quality of ≤0.91 nm found within cleavage embryos, then we could potentially postulate that blastulation increases with lower retardance given the mean of 0.90 +/- 0.24 nm. However, because it was statistically insignificant we cannot make that clear comparison. It must be made apparent that blastulation within this study incorporated poor and good quality blastocysts (grade A, B and C), therefore was not a good indicator for quality.

Blastocyst utilisation, however, was a good estimator for blastocyst quality as only the top quality embryos on day 5 or day 6 (grade A and B) were

cryopreserved or transferred. As previously stated, we hypothesised that a higher proportion of blastocysts would be utilised with incorporation of lower head retardance due to the late paternal effect on embryo quality. Our results showed this to be true. It was found that embryos utilised had significantly lower mean sperm retardance compared to those discarded due to poor quality 0.88 +/- 0.20 nm and 0.93 +/- 0.27 nm respectively (P (2) 0.04 < 0.05). The mean for utilised blastocysts was again below the cut off value for improved embryo quality of ≤0.91 nm also found within cleavage embryos.

When comparing our clinical pregnancies of a day 5 blastocyst to day 3 cleavage embryos in similar studies testing sperm birefringence and MSOME, our results were comparable (Gianaroli *et. al.,* 2008; Gianaroli *et.al.,* 2010; Hazoult *et.al.,* 2006). All the studies have indicated that clinical pregnancies are increased if a sperm is selected that has partial head birefringence and if sperm are selected using MSOME to reduce DNA fragmentation. The sperm with partial head birefringence being defined as acrosome reacted (as shown with transmission electron microscopy) would also have reduced retardance due to the loss in molecular order of the acrosome. So we can assume that lower sperm head retardance would also be seen for increased clinical pregnancies. We would also hypothesise that sperm with reduced retardance have a lower incidence of DNA fragmentation as seen from Keefe et.al (2008), Hazoult *et.al.,* (2006) and Peterson *et.al.,* (2011).

Our results show that clinical pregnancies (sac with or without the presence of a foetal heartbeat) significantly increased with the reduction of sperm head retardance. The mean sperm retardance of clinical pregnancies was significantly lower 0.81 +/- 0.16 nm compared to no-clinical pregnancies 0.91 +/- 0.24 nm (P (2) 0.011 < 0.05). Further statistical analysis using a ROC curve and chi-square analyses found a cut off value of ≤0.91 nm as an indicator for increased clinical pregnancies, which is the same cut off value seen for better quality embryos on day 3. Of the 101 embryos transferred within this study, 39 embryos had sperm retardance >0.91 nm with a clinical pregnancy rate of 18.1% compared to 62 embryos that had sperm retardance ≤0.91 nm and clinical pregnancy rate of 44.0%. Of the 34 clinical pregnancies in this study, 79.4% (n=27) of embryos incorporated sperm head retardance ≤0.91 nm

compared to 20.6% (n=7) of the embryos with head retardance >0.91 nm (P (2) 0.008< 0.05). If, in the future, all oocytes were injected with sperm head retardance ≤0.91 nm, would clinical pregnancies dramatically increase?

It is also interesting to note, that there were no clinical pregnancies when sperm retardance was <0.56 nm. Within this experiment, 0.40 nm was the minimum retardance measured from a sperm with normal morphology and motility injected into an oocyte. Our selection criteria on morphology means we could be routinely selecting sperm for ICSI with retardance between 0.40 nm and 0.56 nm that do not develop into clinical pregnancies as seen from this study, although this percentage is very small (2.3%). Perhaps the reason for these normal looking sperm not progressing into clinical pregnancies is due to abnormalities within their protoplasmic structure or poor DNA packaging, as seen within 12% of sperm with no birefringence in a study conducted by Gianaroli *et al., (*2009). Crippa *et.al.,* (2009) found that sperm without birefringent heads compared to sperm with birefringent heads had an increased incidence of DNA fragmentation. We question however, how a sperm would have no birefringence at all as molecular order should still exist in nucleoprotein filaments once the acrosome reaction is complete? Our results did not reflect this as did Keefe *et.al.,* (2008).

Our results from Experiment 3 show that there is an optimum cut off and range that best selects an individual sperm that could increase embryo quality, development and clinical pregnancy rates, that being ≥0.56 nm -≤0.91 nm. If this is true, only 54.1% of oocytes utilised for ICSI in this experiment comprised of sperm within this range, potentially eliminating a large number (45.9%) of oocytes having the ability to develop into good quality embryos or clinical pregnancies. If we repeated our experimental process within a randomized trial to incorporate only sperm with head retardance between ≥0.56 nm -≤0.91 nm would we see a dramatic difference to ART outcome for patients with male factor infertility? If so, we question the need for extended blastocyst culture if we know the fate of an embryo on day 3 once the paternal genome is being expressed.

It was noted however that in this clinical setting, the overall clinical pregnancy rate was slightly lower than our background rate (33.7% per Tx). Possible

reasons behind the decreased rate could be due to the extra time needed to position the sperm to enable an accurate image to be obtained of its retardance. The extra time needed to change optics and position/image the sperm took an extra 30-45 seconds per oocyte on average compared to conventional ICSI. This short time lag is unlikely to impact on the embryo. However, this time issue in terms of embryology manpower could possibly apply in cases with large number of oocytes. In this study, given the slightly longer time taken to position/image the sperm than conventional ICSI, the optimal timing for injection had routinely been practiced to ensure optimal fertilisation, so this was not a factor (Kilani *et.al.,* 2009).

In summary, in this experiment we assessed sperm retardance using PLM on sperm with normal morphology utilised with ICSI, and followed the embryos through to blastocyst formation, determining that:

1. There was an increase in embryo quality and development on day 3 when sperm utilised for ICSI had retardance ≤0.91 nm. High retardance of the sperm head may be caused by the inactivation or incomplete acrosome reaction creating increased molecular order.

2. No clinical pregnancies formed when sperm had head retardance <0.56 nm. This could be due to poor DNA packaging.

3. We therefore would expect that a mid range of retardance would be the optimal sperm. There was an increase in clinical pregnancy rates when sperm utilised for ICSI had retardance within the range ≥0.56 nm - ≤0.91 nm.

CONCLUSION

Maximising success in ART is the goal of patients, clinicians, and scientists. Sperm obviously are vital for the process. Identification of the best spermatozoa for micromanipulation with ICSI should result in improved pregnancy rates. This thesis examines the possibility of using PLM on sperm as a modality to assist in sperm selection.

The following outcomes were achieved from this thesis:

Experiment 1 –Reproducibility: This experiment was conducted to distinguish if conventional microscopy magnification utilising PLM Oosight™ would be a useful and reliable tool to measure sperm retardance, and if reproducibility was consistent with varying qualities of sperm. The outcome showed that utilising PLM on sperm head to measure retardance is a reproducible and reliable technique across all quality types of spermatozoa ($P(2) =<0.0001$). Although still reproducible, there was a variance between observer's results when measuring sperm with abnormal morphology. This small variance may be due to a limitation of the Oosight™ software for measuring a region. The region measuring tool is a click and drag system that creates a perfect oval although the size of the oval is variable. The downside of the tool is that not all headpieces of human spermatozoa are perfect oval shapes.

Experiment 2 – Range of retardance within human spermatozoa: This experiment was conducted to determine the range within varying qualities of sperm and to determine if the range and frequency differed depending on head normality. The range was identified as 0.49 nm – 1.89 nm. It was found that sperm with normal head morphology have lower mean retardance (0.93 +/- 0.27 nm) compared to sperm with abnormal head morphology (1.00 +/- 0.27 nm) $P(2)= 0.008$. Therefore, sperm head retardance decreases with sperm normality.

Experiment 3- Clinical evaluation of retardance: This experiment was conducted to assess a value or range of sperm head retardance in the selection of the optimum sperm for ICSI. This experiment was novel as

retardance was measured using conventional microscopy magnification as well as incorporating blastocyst culture. It was concluded that utilising PLM on individual spermatozoa at ICSI could be a reliable technique to increase embryo quality and clinical pregnancy rates. This could be achievable by selecting sperm with head retardance ≥0.56 nm -≤0.91 nm to inject within an oocyte (P (2)= 0.01). By selecting optimum sperm for ICSI within this range, it would be expected that there would be an increased surplus of better quality embryos. Sperm outside of this range may have poor DNA integrity, intact acrosomes or incomplete acrosome reactions.

The numerous findings from this thesis confirm that polarised light microscopy has the potential to be a useful tool to non-invasively measure sperm head retardance and select an optimal sperm for ICSI. In lieu of the limited studies using this technique on the spermatozoon, we believe the outcomes resulting from this work will be useful for clinical practice and for future research.

This non-invasive technique to select optimal spermatozoon may benefit patients who have male factor infertility and who need a technology to select normal, healthy sperm at the time of ICSI. It may also be advantageous for couples who have had repeated ART failure, poor embryo quality, or recurrent miscarriages.

FUTURE DEVELOPMENT AND RESEARCH

New questions have arisen whilst conducting this research that would be ideal to develop and study in the future. These questions are:

1. What are the structural features of the sperm that retardance and birefringence are reflecting?

2. Utilising confocal microscopy to further define sperm head morphology, could there be a relationship with their retardance?

3. Is there a relationship between sperm head retardance and level of DNA fragmentation within single spermatozoa using staining techniques to assess oxidative damage to the DNA?

4. Is the percentage of DNA fragmentation shown by SCSA in an ejaculate reflected in a similar percentage of sperm with high retardance?

5. Is there a relationship between sperm head retardance and morphology of sperm heads utilising MSOME?

6. What range of retardance are sperm that bind to HA within PICSI™ dishes? Is there a direct link to mature sperm that bind to HA and the optimal range (≥0.56 nm -≤0.91 nm) of retardance from this study?

7. It would be useful to test the true impact of our outcomes by a randomized trial injecting sperm within the optimal range (≥0.56 nm - ≤0.91 nm) and sperm selected using no PLM assessment.

8. If this range is a useful marker for optimal sperm, embryo quality and increased clinical pregnancies, is there a need for extended blastocyst culture?

APPENDIX

SAMPLE	1
Date:	18.3.10
IVFA#	44566-E
Age	25

CONC m/ml:	79.3
MOTILITY %:	
A	38
B	27
C	8
D	28

MORPHOLOGY %	
Normal	11
Head	75
Midpiece	11
Tail	3

Sperm	Image #	Morphology	Retardance	Intra-variation	Inter-variation
1	.04-05	Tapered	0.96	0.98	0.98
2	.06-07	Tapered	0.89	0.86	0.86
3	.08-09	Tapered	0.98	0.98	0.94
4	.11-10	Round	1.13	1.1	0.98
5	.12-14	Round	1.19	1.19	1.21
6	.15-16	Tapered	0.86	0.87	0.91
7	.17-19	Tapered	0.86	0.94	0.96
8	.20-22	Tapered	0.91	0.9	0.92
9	.24-26	Normal	1.31	1.28	1.27
10	.28-29	Tapered	0.98	0.94	1.03
11	.30-31	Normal	0.95	0.93	0.97
12	.32-33	Pyriform	1.17	1.21	1.01
13	.34-35	Round	1.07	0.97	0.98
14	.36-37	Tapered	0.83	0.89	0.85
15	.38-39	Small	0.81	0.85	0.9
16	.40-41	Tapered	0.6	0.6	0.68
17	42-43	Pyriform	1.21	1.19	1.13
18	44-45	Tapered	1.15	1.05	1.1
19	46-47	Normal	0.98	0.97	0.97
20	48-49	Normal	0.79	0.79	0.8
21	50-51	Small	0.93	0.94	0.95
22	52-53	Tapered	1.14	1.06	1.12
23	54-55	Tapered	1.02	1.07	1
24	56-57	Tapered	1.04	1.01	1.05
25	58-59	Small	0.85	0.85	0.95

26	60-61	Pyriform	0.9	0.92	0.91
27	62-63	Tapered	1.14	1.15	1.15
28	64-65	Tapered	1.01	0.89	0.91
29	66-67	Tapered	1.04	0.79	0.95
30	68-69	Neck	0.61	0.66	0.64
31	70-71	Tapered	1.12	1.08	1.1
32	72-73	Pyriform	0.84	0.84	0.85
33	74-75	Tapered	0.53	0.52	0.5
34	76-77	Normal	0.79	0.73	0.74
35	78-79	Normal	1	0.98	1.1
36	80-81	Normal	0.94	0.89	0.89
37	82-83	Normal	1.14	1.21	1.12
38	83-84	Pyriform	1.03	1	1
39	86-87	Neck	0.92	0.94	0.97
40	88-89	Pyriform	1.49	1.39	1.55
41	90-91	Tapered	1.32	1.42	1.2
42	92-93	Normal	0.57	0.64	0.59
43	94-95	Round	1.1	1.09	1.08
44	96-97	Tapered	1.21	1.13	1.11
45	98-99	Normal	0.88	0.86	0.88
46	100-01	Tapered	0.63	0.74	0.51
47	102-03	Small	1.15	1.1	1.12
48	104-05	Tapered	0.81	0.77	0.82
49	106-07	Normal	0.59	0.68	0.66
50	108-09	Normal	0.69	0.65	0.7

SAMPLE	2
Date:	23/03/10
IVFA#	34394-E
Age	34

CONC m/ml:	223
MOTILITY %:	
A	41
B	33
C	0
D	26

MORPHOLOGY %	
Normal	0
Head	0
Midpiece	0
Tail	0

Sperm	Image #	Morphology	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Tapered	1.1	1.11	1.2
2	02-03.	Pyriform	0.89	0.8	0.92
3	04-05.	Small	1	0.91	0.95
4	06-07.	Normal	0.67	0.68	0.65
5	08-09.	Normal	0.63	0.62	0.62
6	10-11.	Tapered	0.6	0.58	0.66
7	12-13.	Neck	0.65	0.62	0.58
8	14-15	Tapered	1.02	1.04	1.01
9	16-17	Round	1.1	1.04	1.01
10	18-19	Normal	0.65	0.64	0.69
11	20-21	Normal	1.14	1.14	1.16
12	22-23	Normal	0.65	0.65	0.69
13	24-25	Pyriform	1.45	1.35	1.45
14	26-27	Normal	0.83	0.8	0.88
15	28-29	Tapered	1.12	1.16	1.11
16	30-31	Normal	0.81	0.76	0.89
17	32-33	Tapered	0.96	1.04	1.02
18	34-35	Large	0.65	0.6	0.62
19	36=37	Pyriform	0.81	0.81	0.79
20	38-39	Neck	1.03	0.95	0.99
21	40-41	Tapered	1.5	1.52	1.32
22	42-43	Pyriform	1.02	1.09	1.05
23	44-45	Pyriform	0.92	0.9	0.91
24	52-53	Large	0.56	0.55	0.55
25	50-51	Tapered	0.65	0.66	0.67
26	46-47	Round	0.8	0.76	0.81
27	48-49	Normal	1.48	1.38	1.44
28	54-55	Normal	0.88	0.76	0.81
29	56-57	Large	0.71	0.72	0.7

30	58-59	Normal	0.96	0.9	0.95
31	60-61	Normal	1.25	1.22	1.2
32	62-63	Normal	1.22	1.16	1.21
33	64-65	Pyriform	1.23	1.13	1.22
34	66-67	Neck	1.12	1.15	1.11
35	68-69	Normal	1.11	1.1	1.1
36	70-71	Tapered	1.28	1.27	1.22
37	72-73	Small	0.8	0.72	0.8
38	74-75	Small	1.14	1.16	1.12
39	76-77	Tapered	0.72	0.69	0.7
40	78-79	Pyriform	0.95	0.85	0.92
41	80-81	Neck	0.87	0.93	0.91
42	82-83	Tapered	0.79	0.76	0.77
43	84-85	Normal	0.67	0.74	0.69
44	86-87	Pyriform	1.35	1.41	1.3
45	88-89	Tapered	0.78	0.85	0.8
46	90-91	Tapered	1.26	1.16	1.21
47	92-94	Neck	0.84	0.89	0.85
48	97-98	Normal	0.76	0.71	0.77
49	100-101	Round	1.02	1	1.11
50	102-104	Normal	1.07	1.12	1.12

SAMPLE	3
Date:	1/04/10
IVFA#	41609-E
Age	40

CONC m/ml:	19.75
MOTILITY %:	
A	70
B	10
C	16
D	6

MORPHOLOGY %	
Normal	9
Head	88
Midpiece	2
Tail	1

Sperm	Image #	Morphology	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Pyriform	1.36	1.3	1.35
2	02-03.	Tapered	1.24	1.14	1.22
3	04-05.	Pyriform	0.76	0.7	0.75
4	06-07.	Pyriform	0.86	0.93	0.89
5	08-09.	Pyriform	0.88	0.89	0.75
6	10-11.	Small	0.74	0.75	0.8
7	12-13.	Pyriform	1.48	1.47	1.28
8	14-15.	Tapered	0.75	0.76	0.77
9	16-17.	Normal	1.02	1.01	1.02
10	18-19.	Pyriform	1.38	1.31	1.3
11	20-21.	Large	0.51	0.47	0.48
12	22-23.	Tapered	0.89	0.84	0.86
13	24-25.	Pyriform	1.09	1.03	1.03
14	26-27.	Normal	1.21	1.26	1.02
15	28-29.	Tapered	1.26	1.26	1.2
16	30-31	Normal	0.85	0.81	0.82
17	32-33	Pyriform	1.13	1.11	1.13
18	34-35	Normal	0.86	0.84	0.85
19	36-37	Normal	0.77	0.74	0.74
20	38-39	Coiled tail	0.91	0.92	0.99
21	40-41	Tapered	0.83	0.8	0.81
22	42-43	Normal	0.71	0.73	0.75
23	44-45	Normal	1.03	1.13	1.04
24	46-47	Normal	0.83	0.84	0.85
25	48-49	Tapered	0.88	0.9	0.9
26	50-51	Normal	0.84	0.94	0.92
27	52-53	Tapered	0.71	0.69	0.72
28	54-55	Small	1.14	1.15	1.12
29	56-57	Normal	1.22	1.17	1.2

30	58-59	Pyriform	1.45	1.55	1.55
31	60-61	Normal	0.96	1.06	1.06
32	62-63	Pyriform	1.05	1.05	1.05
33	64-65	Pyriform	1.07	1.06	1.09
34	66-67	Normal	0.99	0.94	0.99
35	68-69	Pyriform	0.92	1.02	1
36	70-72	Normal	0.61	0.58	0.6
37	73-74	Small	0.74	0.69	0.77
38	75-76	Pyriform	0.86	0.84	0.85
39	78-80	Small	1	0.99	0.9
40	81-82	Tapered	0.61	0.61	0.62
41	83-84	Normal	1.32	1.3	1.31
42	85	Normal			
43	86-87	Round	1.13	1.09	1.12
44	88-89	Pyriform	0.8	0.79	0.8
45	90-91	Pyriform	1.11	1.08	1.1
46	92-93	Pyriform	1.31	1.37	1.4
47	94-95	Normal	0.94	0.91	0.92
48	96-97	Tapered	0.71	0.76	0.76
49	98-99	Tapered	0.91	0.81	0.92
50	100-101.	Tapered	0.78	0.69	0.7

SAMPLE	4
Date:	6/04/10
IVFA#	44641-E
Age	59

CONC m/ml:	50.2
MOTILITY %:	
A	25
B	20
C	23
D	33

MORPHOLOGY %	
Normal	11
Head	85
Midpiece	3
Tail	0

Sperm	Image #	Morphology	Retardance	Intra-Variation	Inter-Variation
1	00-02.	Small	0.66	0.78	0.72
2	03-05.	Normal	0.88	0.89	0.92
3	06-07.	Small	1.24	1.14	1.2
4	08-09.	Pyriform	1.23	1.24	1.4
5	10-11.	Pyriform	0.81	0.88	0.89
6	12-13.	Pyriform	1.29	1.24	1.29
7	14-15	Small	1.19	1.13	1.14
8	16-17-19	Normal	1.42	2.08	1.92
9	20-21	Pyriform	1.11	1.11	1.1
10	22-23	Tapered	0.82	0.82	0.76
11	24-25	Tapered	0.72	0.73	0.82
12	26-27	Tapered	1.09	1.04	1.04
13	28-29	Normal	0.69	1.13	1.31
14	30-31	Tapered	1.34	1.13	1.3
15	32-33	Tail	1.89	1.87	1.9
16	34-35	Neck	0.99	0.99	0.89
17	36-37	Tapered	1.01	1.01	1.11
18	38-39	Neck	0.71	0.78	0.72
19	40-41	Small	1.46	1.36	1.38
20	42-43	Normal	1.86	1.91	1.82
21	44-45	Normal	0.84	0.74	0.79
22	46-47	Small	1.5	1.55	1.45
23	48-49	Tapered	0.84	0.88	0.8
24	50-51	Pyriform	0.68	0.68	0.67
25	52-53	Small	1.06	0.98	0.99
26	54-55	Tapered	1.25	1.15	1
27	56-57	Tapered	0.92	1	0.9
28	58-59	Pyriform	0.93	1.01	1.01
29	60-61	Round	0.58	0.66	0.68
30	62-63	Normal	0.92	0.94	0.95

31	64-65	Small	1.12	1.14	1.12
32	66-67	Round	1.04	1.05	1.05
33	68-69	Tapered	1.57	1.61	1.7
34	70-71	Tapered	0.71	0.76	0.7
35	72-73	Small	1.08	1.11	1.05
36	74-75	Tapered	0.81	0.9	0.85
37	76-77	Small	1.36	1.36	1.28
38	78-79	Normal	1.04	1.09	1.05
39	80-81	Round	1.53	1.59	1.55
40	82-83	Round	1.16	1.25	1.2
41	84-85	Normal	0.73	0.73	0.78
42	86-87	Normal	0.81	0.84	0.88
43	88-89	Pyriform	1.81	1.86	1.82
44	90-91	Tapered	0.99	1.04	0.91
45	92-93	Normal	0.93	0.87	0.85
46	94-95	Tail	0.85	0.82	0.82
47	96-97	Normal	0.84	0.84	0.81
48	98-99	Round	1.03	1.05	1.02
49	00-01.	Normal	0.74	0.74	0.74
50	02-03.	Round	0.92	0.91	0.98

SAMPLE	5
Date:	15/04/10
IVFA#	44690-E
Age	42

CONC m/ml:	43.9
MOTILITY %:	
A	14
B	51
C	5
D	32

MORPHOLOGY %	
Normal	7
Head	87
Midpiece	5
Tail	1

Sperm	Image #	Morphology	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Normal	0.84	0.8	0.82
2	02-04.	Tapered	0.72	0.76	0.75
3	05-06.	Small	1.02	0.99	1
4	07-08.	Small	1.22	1.2	1.21
5	09-10.	Normal	0.84	0.8	0.82
6	11-12.	Normal	1.09	1.07	1.11
7	13-14	Tapered	0.81	0.81	0.85
8	15-16	Small	1.61	1.54	1.55
9	17-18	Normal	0.66	0.68	0.65
10	19-20	Normal	0.84	0.86	0.8
11	21-22	Pyriform	1.72	1.74	1.7
12	23-24	Pyriform	1	1.11	1.2
13	25-26	Tapered	0.76	0.72	0.77
14	27-28	Small	1.85	1.9	1.82
15	29-30	Pyriform	0.76	0.74	0.71
16	31-32	Small	0.84	0.8	0.81
17	33-34	Tapered	0.97	0.87	0.98
18	35-36	Pyriform	1.27	1.24	1.22
19	37-39	Normal	1.1	1.06	1
20	40-41	Small	1.18	1.18	1.22
21	42-43	Normal	1.25	1.35	1.15
22	44-45	Tapered	0.85	0.81	0.8
23	46-47	Head morph	0.8	0.73	0.7
24	48-49	Small	0.81	0.76	0.81
25	50-51	Small	0.65	0.64	0.65
26	52-53	Small	1.46	1.53	1.52
27	54-55	Round	1.08	1.02	1.02
28	56-57	Normal	1.06	0.97	0.99
29	58-59	Neck	1.02	0.94	1.01

30	60-61	Round	1.07	1.06	1.05
31	62-63	Normal	0.92	0.88	0.92
32	64-65	Tapered	0.67	0.61	0.66
33	66-67	Pyriform	1.03	1.31	1.03
34	68-69	Tapered	0.75	0.9	0.85
35	70-71	Normal	1.18	1.16	1.12
36	72-73	Large	1.15	1.18	1.1
37	74-75	Neck	1.01	1.01	1.01
38	76-77	Tapered	0.82	0.86	0.85
39	78-79	Round	0.64	0.79	0.64

SAMPLE	6
Date:	11/05/10
IVFA#	44884-E
Age	36

CONC m/ml:	77.4
MOTILITY %:	
A	46
B	21
C	4
D	30

MORPHOLOGY %	
Normal	31
Head	57
Midpiece	9
Tail	3

Sperm #	Image #	Motility	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Rapid	0.92	0.83	0.85
2	02-03.	Rapid	0.74	0.69	0.77
3	04-05.	Rapid	0.79	0.83	0.81
4	06-07.	Rapid	0.8	0.76	0.78
5	08-09.	Rapid	0.66	0.64	0.65
6	10-11.	Rapid	1	0.99	0.98
7	12-13.	Rapid	0.59	0.56	0.6
8	14-15	Rapid	0.79	0.77	0.81
9	16-17	Rapid	0.58	0.54	0.56
10	18-19	Rapid	0.71	0.68	0.76
11	20-21	Rapid	0.73	0.83	0.81
12	22-23	Rapid	0.66	0.63	0.66
13	24-25	Rapid	1.29	1.23	1.3
14	26-27	Rapid	0.79	0.82	0.8
15	28-29	Rapid	0.88	0.85	0.86
16	30-31	Rapid	1	1.1	1.2
17	32-33	Rapid	0.61	0.58	0.61
18	34-35	Rapid	0.73	0.71	0.72
19	36-37	Rapid	0.94	0.98	0.92
20	38-39	Rapid	1.01	0.98	1.01
21	40-41	Rapid	1.45	1.38	1.32
22	42-43	Rapid	0.71	0.63	0.68
23	44-45	Rapid	0.76	0.73	0.74
24	46-47	Rapid	1.38	1.32	1.33
25	48-49	Rapid	1.19	1.16	1.2
26	50-51	Rapid	1.11	0.99	1.2
27	52-53	Rapid	0.98	0.97	0.8
28	54-55	Rapid	1.02	1.04	1.05
29	56-57	Rapid	0.7	0.65	0.69
30	58-59	Rapid	1.14	1.09	1.1
31	60-61	Rapid	1.21	1.42	1.22
32	62-63	Rapid	0.72	0.79	0.78

33	64-65	Rapid	1.26	1.32	1.33
34	66-67	Rapid	1.05	0.97	1.09
35	69-70	Rapid	0.64	0.66	0.7
36	71-72	Rapid	0.61	0.61	0.6
37	73-74	Rapid	0.84	0.93	0.81
38	75-76	Rapid	1.25	1.22	1.22
39	77-78	Rapid	1.35	1.33	1.21
40	79-80	Rapid	1.22	1.24	1.3
41	81-82	Rapid	0.52	0.55	0.61
42	83-84	Rapid	0.8	0.78	0.77
43	85-86	Rapid	0.93	1.15	0.89
44	87-88	Rapid	0.63	0.67	0.61
45	89-90	Rapid	0.85	0.83	0.82
46	91-92	Rapid	0.59	0.61	0.66
47	93-94	Rapid	0.83	0.87	0.82
48	95-97	Rapid	1.04	0.99	1.02
49	99-100	Rapid	0.79	0.8	0.81
50	101-102.	Rapid	0.63	0.63	0.6

SAMPLE	7
Date:	18.5.10
IVFA#	44865-E
Age	30

CONC m/ml:	76
MOTILITY %:	
A	21
B	32
C	15
D	33

MORPHOLOGY %	
Normal	3
Head	88
Midpiece	8
Tail	1

Sperm #	Image #	Motility	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Rapid	1.21	1.28	1.21
2	02-03.	Rapid	1.13	1.23	1.23
3	04-05.	Rapid	0.68	0.66	0.69
4	06-07.	Rapid	0.95	0.99	0.96
5	08-09.	Rapid	1.13	1.11	1.1
6	10-11.	Rapid	1.51	1.44	1.45
7	12-13.	Rapid	1.39	1.29	1.29
8	14-15.	Rapid	1.22	1.17	1.2
9	16-17.	Rapid	1.09	1.01	1.01
10	18-19.	Rapid	0.97	1.04	0.98
11	20-21.	Rapid	0.61	0.59	0.6
12	22-23.	Rapid	0.65	0.62	0.62
13	24-25.	Rapid	0.97	0.95	0.96
14	26-27.	Rapid	1.52	1.44	1.5
15	28-29.	Rapid	0.72	0.76	0.73
16	30-31	Rapid	0.65	0.67	0.66
17	32-33	Rapid	1.04	1.04	1.02
18	34-35	Rapid	0.77	0.8	0.89
19	36-37	Rapid	0.8	0.87	0.82
20	38-39	Rapid	0.87	0.87	0.87
21	40-41	Rapid	0.92	0.96	0.99
22	42-43	Rapid	1.21	1.25	1.22
23	44-45	Rapid	1.08	1.15	1.1
24	46-47	Rapid	1	1.19	1.2
25	48-49	Rapid	1.35	1.41	1.4
26	50-51	Rapid	0.95	0.95	0.96
27	52-53	Rapid	0.6	0.6	0.6
28	54-55	Rapid	1.07	1.15	1.09
29	56-57	Rapid	1.12	1.16	1.2
30	58-59	Rapid	0.64	0.7	0.68

SAMPLE	8
Date:	27/06/10
IVFA#	44305-E
Age	35

CONC m/ml:	203
MOTILITY %:	
A	45
B	25
C	1
D	30

MORPHOLOGY %	
Normal	12
Head	79
Midpiece	8
Tail	1

Sperm #	Image #	Motility	Retardance	Intra-Variation	Inter-Variation
1	00-01.	Rapid	1.47	1.37	1.37
2	02-03.	Rapid	0.55	0.54	0.55
3	04-05.	Rapid	0.49	0.48	0.5
4	06-07.	Rapid	0.89	0.88	0.89
5	08-09.	Rapid	0.55	0.53	0.54
6	10-11.	Rapid	0.53	0.52	0.53
7	12-13.	Rapid	0.93	1.04	1.02
8	14-15.	Rapid	0.78	0.84	0.8
9	16-17.	Rapid	1.28	1.28	1.3
10	18-19.	Rapid	0.82	0.72	0.82
11	20-21.	Rapid	1.48	1.55	1.5
12	22-23.	Rapid	0.85	0.85	0.86
13	24-25.	Rapid	0.79	0.8	0.81
14	26-27.	Rapid	1.11	1.15	1.21
15	28-29.	Rapid	0.71	0.71	0.76
16	30-31	Rapid	0.65	0.61	0.63
17	32-33	Rapid	1.19	1.35	1.22
18	34-35	Rapid	1	0.91	1.1
19	36-37	Rapid	0.94	1.04	1.01
20	38-39	Rapid	1.43	1.33	1.28
21	40-41	Rapid	0.67	0.69	0.68
22	42-43	Rapid	0.65	0.68	0.71
23	44-45	Rapid	0.62	0.61	0.62
24	46-47	Rapid	0.88	0.88	0.92
25	48-49	Rapid	1.24	1.28	1.23
26	50-51	Rapid	1.57	1.57	1.62
27	52-53	Rapid	0.81	0.82	0.83
28	54-55	Rapid	1.37	1.32	1.37
29	56-57	Rapid	1.4	1.31	1.38

30	58-59	Rapid	0.58	0.64	0.62
31	60-61	Rapid	1.25	1.21	1.21
32	62-64	Rapid	0.97	0.93	0.92
33	66-67	Rapid	1.2	1.23	1.2
34	68-69	Rapid	0.68	0.72	0.71
35	70-71	Rapid	0.83	0.88	0.92
36	72-73	Rapid	0.6	0.57	0.62
37	74-75	Rapid	0.84	0.86	0.82
38	76-77	Rapid	1.44	1.47	1.42
39	78-79	Rapid	1.35	1.3	1.3
40	80-81	Rapid	0.95	0.9	0.92
41	82-83	Rapid	0.49	0.48	0.52
42	84-85	Rapid	1.01	1.17	1.08
43	86-87	Rapid	1.23	1.28	1.22
44	88-89	Rapid	0.85	0.86	0.87
45	90-91	Rapid	0.5	0.52	0.51
46	92-93	Rapid	0.63	0.64	0.62
47	94-95	Rapid	1.4	1.43	1.34
48	96-97	Rapid	0.81	0.87	0.82
49	98-99	Rapid	0.75	0.74	0.71
50	100-101.	Rapid	0.5	0.5	0.45

- Abnormal and normal morphology results.

  ROC curve co-ordinates for cut-off value.

Coordinates of the Curve

Test Result Variable(s):sperm_ret

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-.5100	1.000	1.000
.4950	1.000	.990
.5050	1.000	.979
.5150	.994	.979
.5250	.994	.974
.5400	.988	.969
.5550	.988	.959
.5650	.983	.959
.5750	.983	.954
.5850	.977	.944
.5950	.977	.928
.6050	.965	.918
.6150	.954	.897
.6250	.954	.892
.6350	.948	.872
.6450	.942	.862
.6550	.919	.831
.6650	.913	.815
.6750	.908	.800
.6850	.902	.790
.6950	.902	.779
.7050	.902	.774
.7150	.873	.754
.7250	.855	.744
.7350	.855	.728
.7450	.844	.718
.7550	.832	.713
.7650	.815	.703
.7750	.815	.692
.7850	.803	.687
.7950	.798	.651
.8050	.775	.636
.8150	.734	.615
.8250	.723	.610

.8350	.711	.590
.8450	.688	.549
.8550	.671	.528
.8650	.647	.523
.8750	.642	.518
.8850	.630	.492
.8950	.613	.487
.9050	.607	.487
.9150	.590	.487
.9250	.561	.467
.9350	.549	.451
.9450	.549	.431
.9550	.543	.410
.9650	.532	.400
.9750	.526	.385
.9850	.514	.374
.9950	.503	.369
1.0050	.486	.344
1.0150	.468	.333
1.0250	.434	.323
1.0350	.410	.318
1.0450	.393	.303
1.0550	.387	.297
1.0650	.382	.292
1.0750	.364	.282
1.0850	.353	.277
1.0950	.341	.267
1.1050	.324	.262
1.1150	.312	.246
1.1250	.289	.241
1.1350	.272	.231
1.1450	.249	.215
1.1550	.231	.215
1.1650	.225	.215
1.1750	.220	.215
1.1850	.214	.210
1.1950	.202	.200
1.2050	.202	.195
1.2150	.191	.174
1.2250	.185	.154
1.2350	.173	.149
1.2450	.162	.144
1.2550	.156	.123

1.2650	.145	.118
1.2750	.139	.118
1.2850	.133	.113
1.3000	.127	.108
1.3150	.121	.103
1.3300	.116	.097
1.3450	.110	.097
1.3550	.104	.082
1.3650	.092	.082
1.3750	.092	.077
1.3850	.087	.072
1.3950	.087	.067
1.4100	.087	.056
1.4250	.087	.051
1.4350	.087	.046
1.4450	.087	.041
1.4550	.075	.036
1.4650	.064	.036
1.4750	.064	.031
1.4850	.058	.021
1.4950	.052	.021
1.5050	.040	.021
1.5150	.040	.015
1.5250	.040	.010
1.5500	.035	.010
1.5900	.029	.005
1.6650	.023	.005
1.7650	.017	.005
1.8300	.012	.005
1.8550	.006	.005
1.8750	.006	.000
2.8900	.000	.000

- Cleavage results.

  ROC curve co-ordinates for cut-off value.

Coordinates of the Curve

Test Result Variable(s):sperm_ret

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-.6000	1.000	1.000
.4300	1.000	.997
.4750	1.000	.995
.5000	1.000	.990
.5150	.989	.987
.5250	.989	.985
.5400	.989	.980
.5550	.977	.977
.5650	.977	.970
.5750	.966	.960
.5850	.966	.957
.5950	.966	.947
.6050	.966	.937
.6150	.966	.930
.6250	.966	.917
.6350	.955	.907
.6450	.932	.902
.6550	.920	.892
.6650	.909	.879
.6750	.909	.857
.6850	.898	.837
.6950	.864	.819
.7050	.852	.791
.7150	.841	.774
.7250	.818	.761
.7350	.795	.754
.7450	.795	.741
.7550	.784	.714
.7650	.784	.698
.7750	.773	.686
.7850	.739	.668
.7950	.716	.648
.8050	.705	.626

.8150	.682	.613
.8250	.682	.588
.8350	.682	.573
.8450	.648	.540
.8550	.625	.525
.8650	.614	.505
.8750	.591	.477
.8850	.591	.447
.8950	.591	.437
.9050	.580	.422
.9150	.568	.405
.9250	.557	.392
.9350	.545	.384
.9450	.523	.362
.9550	.500	.352
.9650	.477	.337
.9750	.466	.314
.9850	.432	.294
.9950	.420	.279
1.0050	.409	.271
1.0150	.386	.266
1.0250	.386	.256
1.0350	.364	.249
1.0450	.341	.234
1.0550	.318	.219
1.0650	.284	.209
1.0750	.273	.193
1.0850	.261	.191
1.0950	.261	.176
1.1050	.261	.163
1.1150	.239	.156
1.1250	.227	.153
1.1350	.227	.141
1.1450	.227	.138
1.1550	.227	.131
1.1650	.227	.126
1.1750	.227	.123
1.1850	.227	.118
1.1950	.205	.116
1.2050	.205	.106
1.2150	.170	.101
1.2250	.159	.098
1.2350	.159	.093

1.2450	.159	.090
1.2550	.136	.090
1.2650	.136	.083
1.2750	.136	.075
1.2850	.125	.070
1.2950	.125	.068
1.3150	.114	.065
1.3350	.114	.060
1.3450	.114	.058
1.3600	.091	.055
1.3750	.080	.055
1.3850	.080	.050
1.3950	.068	.050
1.4050	.068	.048
1.4200	.057	.048
1.4350	.057	.045
1.4500	.057	.043
1.4700	.057	.040
1.4850	.057	.038
1.4950	.057	.035
1.5100	.057	.030
1.5300	.057	.025
1.5500	.057	.020
1.5650	.057	.018
1.5850	.057	.013
1.6300	.045	.013
1.6650	.034	.013
1.6750	.023	.013
1.6900	.011	.013
1.7100	.011	.010
1.7300	.011	.008
1.7500	.011	.005
1.8200	.011	.003
2.1300	.000	.003
3.3800	.000	.000

- Clinical pregnancy results.

  ROC curve co-ordinates for cut-off value.

Coordinates of the Curve

Test Result Variable(s):sperm_ret

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-.4900	1.000	1.000
.5350	.985	1.000
.5650	.985	.941
.5800	.985	.912
.5950	.970	.912
.6050	.940	.912
.6150	.925	.912
.6300	.910	.912
.6450	.896	.912
.6550	.896	.882
.6650	.866	.853
.6750	.836	.794
.6850	.791	.794
.6950	.791	.735
.7050	.776	.706
.7150	.776	.676
.7250	.746	.647
.7350	.731	.647
.7450	.716	.618
.7600	.672	.529
.7750	.657	.471
.7850	.657	.441
.7950	.642	.441
.8050	.627	.441
.8150	.612	.441
.8250	.597	.441
.8350	.597	.353
.8450	.552	.324
.8550	.552	.294
.8650	.522	.294
.8750	.493	.265
.8900	.493	.235
.9050	.478	.235

.9150	.478	.206
.9300	.463	.206
.9450	.448	.206
.9550	.433	.206
.9650	.403	.206
.9750	.373	.206
.9850	.328	.206
.9950	.299	.206
1.0100	.284	.176
1.0250	.284	.147
1.0350	.269	.147
1.0450	.269	.118
1.0550	.239	.118
1.0700	.224	.088
1.0850	.209	.088
1.0950	.209	.059
1.1050	.179	.059
1.1200	.149	.059
1.1400	.149	.029
1.1600	.134	.029
1.1850	.134	.000
1.2050	.119	.000
1.2300	.104	.000
1.2600	.090	.000
1.3200	.075	.000
1.3850	.060	.000
1.4400	.045	.000
1.5000	.030	.000
1.5400	.015	.000
2.5600	.000	.000

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