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The cryopreservation of human semen, and subsequent evaluation of a commercially-available device to isolate motile sperm

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The Cryopreservation of Human Semen, and Subsequent Evaluation of a Commercially-Available Device to Isolate Motile Sperm

This thesis is presented in partial fulfilment of the degree of

Master of Science (Medical Science)

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Abstract

The cryopreservation of human semen is a vital asset in assisted reproductive technologies (ART). Although advances have been in the freezing of sperm, further refinement is both necessary and ongoing. Computer-assisted semen analysis (CASA) has been increasingly utilised in both research and diagnostic however there are a range of variables that must first be controlled in order to produce reliable measurements. Following thawing, sperm must be isolated from both the original seminal plasma and the cryoprotectants; the two most used isolation methods include density gradient centrifugation (DGC) and the swim-up method.

The present thesis sought to investigate the following areas (i) a technical validation phase investigating variables that can influence CASA measurements (ii) the effect of neat glycerol and a commercial cryoprotective medium (CPM) upon sperm motility prior to cryopreservation, and subsequent effects of diluting these samples (iii) the effectiveness of neat glycerol versus a CPM in the post-thaw recovery of motile sperm, and (iv) the effectiveness of DGC, a direct swim-up procedure and a commercial device that utilises the swim-up procedure.

Several variables were identified in the measuring of semen samples in conjunction with CASA software. Firstly the use of a capillary-loading chamber was found to result in decreased levels of total and progressive motility, as well as reduced kinematic parameters when compared to a droplet-loaded configuration. The time between the loading of a sample was found to be stable at the 2 minute time interval, and as such this was set for all measurements in the study. Finally, operator-corrections were

discovered to be crucial in not only accurately measuring sperm concentration, but also sperm motility.

The commercial CPM containing glycerol had the least toxic effect on sperm motility pre-cryopreservation. There was a linear relationship between decreased sperm motility and increased presence of glycerol, as demonstrated by 10% v/v glycerol addition. The further dilution of glycerol-containing semen samples with two common gamete handling media were found to cause a further significant reduction in sperm motility, whereas in contrast seminal plasma was not found to reduce sperm motility in these samples. The CPM was found to have the greatest yield of cryopreserved motile sperm post-thaw when compared to glycerol at both 5 and 10% v/v.

Finally, DGC yielded increased concentrations of sperm post-isolation, but with a reduced level of motility (10.2M/ml and 20% progressive motility respectively), whereas both the swim-up methods had reduced levels of concentration (1.8M/ml for the standard swim-up, and 1.5M/ml for the commercial device) but with increased levels of progressive motility (39.1% and 42.8% respectively for the standard swim-up and commercial device).

In summary, CASA software is able to provide reliable results given the chamber type is controlled and that operator-corrections are applied. Secondly, glycerol has a complex relationship with the cryosurvivability of spermatozoa and the toxic effects it exerts on them. Glycerol toxicity appeared dose-dependent, with decreased sperm motility with increased glycerol presence, both pre and post-cryopreservation/thawing. Finally, the most effective isolation technique for frozen-thawed sperm is dependent on what ART procedure is to be undertaken.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

I. Incorporate without acknowledgment any material previously submitted for a degree or diploma in any institute of higher education;

II. Contain any material previously published or written by another person except where due reference is made in the text of the thesis; or

III. Contain any defamatory material;

Signed: [redacted] August, 2018

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Table of Contents

List of Acronyms

ALH	Amplitude of lateral head displacement (μm)
ART	Assisted reproductive technology
BCF	Beat-cross frequency (Hz)
CASA	Computer-assisted sperm analysis
CPA	Cryoprotective agent
CPM	Cryoprotective medium
DGC	Density gradient centrifugation
DSW	Direct swim-up
ICMART	International Committee for Monitoring Assisted Reproductive Technologies
ICSI	Intracytoplasmic sperm injection
IM	Immotile sperm, as per WHO 5th edition
IUI	Intra-uterine insemination
IVF	In-vitro fertilisation
LIN	Linearity
NP	Non-progressively motile sperm, as per WHO 5th edition
PR	Progressively motile sperm, as per WHO 5th edition
ROS	Reactive oxygen species
SFR	Seaforia^{TM} Sperm Separation System

STR	Straightness
VAP	Average path velocity (µm/s)
VCL	Curvilinear velocity (µm/s)
VSL	Straight line velocity (µm/s)
WOB	Wobble

List of Tables

List of Figures

Publications and presentations

The following publications and presentations are a direct result from the work

submitted in the current thesis:

Publications

Robinson, C., Roberts, P., Matson, P. (2018). Sperm motility assessment using computer assisted semen analysis (CASA): a comparison of standard microscope slides and coverslips and the 20 µm MicroCell™. *Journal of Reproductive Biotechnology and Fertility,* 7, 1-8.

Robinson, C., Roberts, P., Reynolds, K., Matson, P. (2018). The effect of glycerol and a glycerol-containing cryoprotective medium upon the motility of human sperm prior to freezing, and subsequent difficulties in assessing sperm motility following dilution. *Journal of Reproductive Biotechnology and Fertility,* 7, 9-14.

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Male infertility and assessment of semen

1.1.1 Diagnosis and treatment of male infertility

A couple will be defined as being infertile after having regular unprotected coitus for 12 months with no clinical pregnancy achieved (Zegers-Hochschild et al., 2009). It is estimated that 8-12% of couples worldwide are infertile and 40-50% of cases are due to male infertility (Kumar & Sing, 2015). There can be various aetiological reasons for male infertility, all with varying treatment options and outcomes (Tournaye, 2012).

Azoospermia is the absence of sperm in the ejaculate and approximately 10-15% of infertile men have this condition (Marmar, 2011). Azoospermia manifesting from a physical blockage of the sperm passage in the epididymis, vas deferens or ejaculatory duct is known as obstructive azoospermia (Ammar, Sidhu, & Wilkins, 2012), representing 15-20% of all azoospermic cases (Engin, Kadioğlu, Orhan, Akdöl, & Rozanes, 2000). Obstructions can result from bacterial infections that have caused tissue scarring, as well as cyst formation, where surgical intervention may be required to remove the source of the blockage (Marmar, 2011). Non-obstructive azoospermia is more prevalent than obstructive azoospermia and results from the interference of spermatogenesis, either by testicular physiology disruption or the compromise of gonadotropin production (Chiba, Enatsu, & Fujisawa, 2016).

Cryptorchidism is the most common male urogenital tract congenital deformity and results from the failure of the testes to descend from the torso into the scrotal sac during foetal development (Ammar, et al., 2012). The proximal temperature of the

undescended testes is higher than that of the scrotal sac, leading to the disruption of spermatogenesis (Kurpisz, Havryluk, Nakonechnyj, Chopyak, & Kamieniczna, 2010).

Inflammation or an autoimmune response is thought to account for 5-10% of male infertility, resulting in orchitis or epididymo-orchitis (Hedger, 2011). Testicular atrophy can occur following events of pathogenic infections, due to the lack of regenerative capabilities of the epididymal and testicular epithelium (Dohle et al., 2005). Epididymitis can result from infections by C*hlamydia trachomatis, Neisseria gonorrhoeae, Escherichia coli* and less commonly from *Salmonella spp, Mycobacterium tuberculosis* and *Haemophilus influenza* (Hedger, 2011).

Testicular varicocoele is a dilation of the veins within the spermatic cord resulting in an increase in temperature with the rise in proximal blood flow to the testes (Pastuszak & Wang, 2015). 19-41% of infertile men will present with testicular varicocoele and it is the most common surgically-treatable male infertility factor (Pastuszak & Wang, 2015).

1.1.2 Routine semen analysis

The first step in investigating male fertility is a semen analysis (Leushuis et al., 2014). Although semen analysis can provide a snapshot of a man's current fertility status, it is not able to conclusively identify the aetiology for infertility, and alone is a poor predictor of successful pregnancy outcomes (Guzick et al., 2001; Leushuis, et al., 2014).

Parameters observed in a semen analysis include the total volume of semen, pH, colour and appearance, and the viscosity of the sample; all of which are conducted prior to microscopic observation. Following this macroscopic investigation, the core parameters being observed are sperm count, motility and morphology; as well as comments on any abnormal phenomena such as the presence of round-cells,

agglutination and clumping, the presence of somatic cells and cleaved heads or tails (WHO, 2010).

A prominent limiting factor of semen analyses is the variability of the semen samples an individual can produce, with factors such as febrile illness experienced or the consumption of some medications in the 120 days prior to the sample being produced, the season, the fraction of the ejaculate observed and the period of abstinence maintained prior to ejaculation being shown to influence measured parameters (Brezina, Yunus, & Zhao, 2012; Carlsen, Andersson, Petersen, & Skakkebæk, 2003; De Giorgi et al., 2015; Hebles, Dorado, Gallardo, González-Martínez, & Sánchez-Martín, 2015; Leushuis et al., 2010; Splingart et al., 2012). Hence, a minimum of two semen analyses are recommended when investigating male fertility (World Health Organization, 2010). As well as sample variability from the individual, a large potential difference in semen analysis measurements can occur due to operator bias, where two operators may measure the same sample differently based on their scoring criteria and methods of analysis (Giwercman, Spano, Laehdetie, & Bonde, 1999; Pacey, 2010). To keep operator bias to a minimum, adherence to a standardised system of analysis is recommended and has been implemented by the World Health Organisation (World Health Organization, 1980, 1987, 1992, 1999, 2010), as well as the introduction of internal and external laboratory quality assurance schemes to provide ongoing feedback and comparison of results for laboratories (Matson, 1995; Pacey, 2010; World Health Organization, 2010). The purpose of these laboratory guidelines and quality assurance schemes is to ensure laboratories are producing both accuracy and precision in the measurements made. Due to the variable nature of semen samples, and the subjectivity of measured parameters in a semen analysis, there is the

consistent need to keep methods standardised to reduce operator bias, and to adhere to the same scoring criteria to ensure precision and reproducibility of results.

1.1.3 Computer-assisted sperm analysis

Computer-assisted sperm analysis (CASA) aims to reduce operator bias when conducting semen analyses and has grown in popularity since its introduction in the mid 1990's (Johnson, 1997; Lu, Huang, & Lü, 2014; Mallidis et al., 2012; Morrell, 1997; Mortimer & Swan, 1999). They are automated systems that allow the real-time processing of images to be interpreted by a computer program, allowing the rapid measurement of sperm kinetic parameters as well as concentration and morphology (Lu, et al., 2014). These systems are beneficial as they are able to give consistent, reproducible measurements of a sample, and can measure kinematic values such as velocity, that would otherwise be difficult to achieve by manual methods.

Although much of the diagnostic work conducted in the medical industry has become automated, fertility laboratories have, on the whole, been resistant to using CASA systems for routine semen analyses, with CASA software having a larger presence in the research field (Amann & Katz, 2004; Tomlinson & Naeem, 2018). Much of the early criticisms of these systems arose from the software's inability to differentiate between sperm cells and non-sperm cells (Davis & Latz, 1993). This not only produced skewed concentration measurements, but also affected motility parameters. This occurs as non-sperm cells are misclassified as immotile sperm, giving them an incorrect higher percentage compared to progressive and non-progressive sperm.

For CASA software to provide accurate and reliable results, there first must be a number of factors controlled and accounted for. Studies have highlighted non-

software variables that can influence CASA measurements such as the chamber type used to house the sample, time between the sample being loaded and analysed, what the sample is diluted with, and at what dilution factor (Del Gallego et al., 2017; Garner et al., 2001; Liu, Foote, & Brockett, 1998; Yeste, Bonet, Rodríguez, & Rivera Del Álamo, 2018).

The reluctance to use CASA software in diagnostic fertility laboratories is still felt to this day, however if the variables that can influence CASA software's ability to perform semen analyses can be controlled, then they have the potential to be a powerful tool in diagnostic andrology. What is needed to overcome this inaccuracy is further research investigating CASA application in routine diagnostic andrology, and potential situations that can occur in day-to-day situations in a clinical andrology laboratory.

1.1.4 Supplementary tests

As well as the fundamental measurements of sperm concentration, motility and morphology observed in semen analyses, there are also supplementary tests that can be performed to further investigate potential causes of male infertility. One such parameter is sperm DNA fragmentation, which has been suggested to represent sperm function more accurately than traditional semen analysis parameters (Bounartzi et al., 2016). Several tests are available to assess sperm DNA integrity including the sperm chromatin structure assay, sperm chromatin dispersion, acridine orange staining technique and the TdT-mediated-dUTP nick end labelling procedures; all showing various relationships to sperm function and fertility (Chohan, Griffin, Lafromboise, De Jonge, & Carrell, 2006).

Subpopulations of sperm with DNA damage are present in normal, healthy ejaculates, however some infertile men are seen to have a higher proportion of those with DNA fragmentation (Lewis et al., 2013). The cause of this damage is thought to stem from various origins, such as oxidative stress, apoptotic DNA degradation and defective spermatogenesis (Shafik, Shafik, Sibai, & Shafik, 2006). Some sperm isolation techniques used for ART procedures have been shown to contribute to DNA damage, such as density gradient centrifugation (DGC) (Ghaleno et al., 2014; Volpes et al., 2016).

Immunological based infertility can arise from the presence of antisperm-antibodies and is thought to be present in 1.2-19% of fertile men, and 8-21% of infertile men (Bozhedomov et al., 2015). There are various tests available for the detection of antisperm antibodies, one being the direct immunobead test, a screening test designed to detect antibodies bound to the surface of a spermatozoon (Koriyama et al., 2013).

Similar to routine semen analyses, both of these supplementary tests are largely conducted manually in fertility laboratories, however CASA software modules have been developed to analyse these criteria (Sadeghi et al., 2016; Yu et al., 2018). Studies have indicated the ability of CASA to give similar results to manual counting methods when detecting the presence of immobilising antisperm antibodies and the reading of certain sperm DNA fragmentation tests, therefore eliminating the need for the time consuming manual counting method (Mortimer, Horst, & Mortimer, 2015; Yu, et al., 2018).

1.2 Definition of ART and procedures

After a couple has been diagnosed with a fertility problem, they may then seek to achieve pregnancy through assisted reproductive technologies (ART). ART refers to the procedures and treatments involving the in-vitro handling of human gametes and embryos to achieve pregnancy (Zegers-Hochschild, et al., 2009). In 2015 according to the Australian and New Zealand Assisted Reproductive Database (Fitzgerald, Harris, Paul, & Chambers, 2017) a total of 77,721 treatment cycles were reported from Australia and New Zealand fertility clinics, seeing a 5.6% increase from 2014. 22.8% of these initiated cycles resulted in a clinical pregnancy, with 18.1% resulting in a live delivery.

Intrauterine insemination (IUI) is the least invasive ART treatment which is often used when first treating unexplained infertility, or if the female vagina is hostile to spermatozoa (Quaas & Dokras, 2008). It involves the introduction of sperm directly into the cervix via a catheter, where the sperm will continue their hazardous journey to fertilise a potential oocyte (Tournaye, 2012). It can be further improved through ovarian stimulation with the use of gonadotropins and clomiphene citrate; however this can result in multiple gestations as it increases the chance of multiple oocytes being released into the fallopian tubes (Sutter et al., 2009). The International Committee for Monitoring Assisted Reproductive Technologies (ICMART) acquired data from fertility clinics in over 37 countries in 2010 reporting 193,523 husband-donated sperm IUI cycles were conducted, 12.1% of these resulted in a pregnancy with an 8.8% successful delivery rate (Dyer et al., 2016).

In-vitro fertilisation (IVF) involves the isolation and harvesting of oocytes through ovarian stimulation, the isolation and purification of spermatozoa, and the subsequent fertilisation of an oocyte in-vitro; where the most viable embryos are transferred into the uterus or can be frozen for transfer at a later time (Sunderam et al., 2015). IVF can be utilised when repeated cycles of IUI have failed, if the male presents with obstructive azoospermia or if semen parameters such as count and motility are deemed to be too low for IUI (Merchant, Gandhi, & Allahbadia, 2011). IVF cycles accounted for approximately 99% of ART procedures undertaken in the United States of America (Sunderam, et al., 2015).

Related to IVF is intracytoplasmic sperm injection (ICSI), a technique that requires greater micromanipulation of gametes for insemination. This involves the same preliminary steps of gamete isolation, however as opposed to IVF where multiple spermatozoa are introduced in-vitro to the oocyte which actively penetrate the zona pellucida, ICSI involves manually injecting a single spermatozoon into the oocyte (Malter, 2016). ICSI is suitable for samples with extremely low sperm motility, count and morphology as only a single spermatozoon is required for insemination (Boulet et al., 2015).

For the year 2010, ICMART reported 781,626 cycles of IVF and ICSI using fresh semen, with pregnancy and successful delivery rates of 27.0 and 20.1% respectively (Dyer, et al., 2016).

1.3 Semen composition and washing

1.3.1 Semen composition

Within the seminal plasma is a heterogeneous mixture of spermatozoa with variable mobility and morphology, cellular debris, leukocytes, epithelial cells and sperm germinal cells (Mann, 1954). The sexual accessory glands of the male contribute largely to semen composition with the seminal vesicle secretions contributing 65-75%, the prostate 15-30% and the bulbo-urethral glands secreting 1-5% (Mann & Lutwak-Mann, 1981; Owen & Katz, 2005).

The semen is in a liquid state as it is ejaculated but coagulation occurs immediately after, mostly by components from the seminal vesicles (Lilja & Laurell, 1984). Liquefaction begins in-vivo over a 5 minute period, and after 15-30 minutes liquefaction is usually complete after the action of prostate-specific antigen and plasminogen; this decoagulation process is important for the spermatozoa as it allows increased exposure to factors within the seminal fluid that enhance motility and fertilising capabilities (Puppo & Puppo, 2016).

1.3.2 Sperm washing

Seminal plasma has an interesting and at times conflicting role in the longevity and protection of spermatozoa. Cellular components such as leukocytes and dead spermatozoa have been shown to produce reactive oxygen species (ROS), causing oxidative stress to healthy sperm cells, which with increased exposure negatively impact on fertilisation capabilities (Agarwal, Prabakaran, & Said, 2005; Griveau, Dumont, Renard, Callegari, & Le Lannou, 1995; Kovalski, de Lamirande, & Gagnon, 1992). However, some oxidation of the sperm plasma membrane is required for key

fertilisation events such as capacitation and the acrosome reaction to occur; this scenario has coined the term the 'antioxidant paradox' (Henkel, 2011).

Under normal in-vivo conditions healthy motile sperm separate from non-fertile subpopulations through active migration, with cervical mucus blocking and preventing continued exposure to ROS-generating cells from potentially damaging healthy motile spermatozoa (García-Herreros & Leal, 2014). For the application of semen for ART purposes, this natural filtering and selection process of sperm passing through the female reproductive tract is absent, and so must be mimicked in order to achieve higher chances of fertilisation.

There have been several sperm washing techniques developed to isolate healthy sperm for use in ART procedures. A processing technique should be gentle, so as to not damage the delicate spermatozoa and aim to yield an increased proportion of physiologically and morphologically normal sperm than was in the original sample (Yumura, Iwasaki, Saito, Ogawa, & Hirokawa, 2009). Ideally, it should also be easy and quick to perform and cost-effective (Henkel & Schill, 2003).

Separation techniques are classified by the method of action of how separation is achieved. Examples include: active migration, such as the classic swim-up, pellet swim up and migration-sedimentation; DGC using density gradients; filtration techniques such as glass wool filtration, glass bead filtration and transmembrane migration; and electrophoresis (Ainsworth, Nixon, & Aitken, 2011; Henkel & Schill, 2003; Sakkas, 2013). Each separation technique has its own merits and disadvantages, which method is the most ideal may change depending on the quality of the ejaculate and the ART procedure to be undertaken (Henkel, 2012).

1.4 Sperm isolation techniques

1.4.1 Swim up

The swim up method of separation is one of the oldest and most widely used isolation techniques in fertility laboratories (Rappa et al., 2016). According to the World Health Organisation (2010), it is effective in quickly and easily isolating motile spermatozoa. In a common swim-up up procedure the semen sample is washed and pelleted, then a separation media overlays the pellet and is then incubated for 60 minutes at a 45˚ angle (Volpes, et al., 2016). Motile spermatozoa travel through the pellet into the overlying media, which is mostly void of immotile cells and debris, yielding a clean fraction of recovery with highly motile spermatozoa (Younglai, Holt, Brown, Jurisicova, & Casper, 2001).

Due to the densely packed cell pellet, motile sperm towards the bottom of the pellet can have difficulty in reaching the overlying media, and as it relies on initial sperm motility and the pellet-overlay media surface area, total yield of sperm is less than other separation techniques (Henkel & Schill, 2003).

Cell pelleting has been shown to increase ROS activity in neighbouring spermatozoa (Homa, Vessey, Perez-Miranda, Riyait, & Agarwal, 2015), however this can be avoided by using liquefied semen samples instead of a washed pelleted sample, known as a direct swim-up (DSW) (Al Hasani et al., 1995; Homa, et al., 2015). As the swim up procedure relies on the active migration of spermatozoa, its efficacy in males with low motility is limited; however with female infertility and normozoospermia it is a reliable method of sperm isolation for ART procedures (Henkel, 2012).

DNA fragmentation has long been associated with poor ART outcomes (Evenson & Wixon, 2006; Spanò et al., 2000; Tesarik, Greco, & Mendoza, 2004; Virro, Larson-Cook, & Evenson, 2004). One major advantage of the swim up method is its reduced level of DNA fragmentation on isolated spermatozoa. Volpes et al. (2016) have shown that the pellet swim-up separation technique yields spermatozoa with an improved DNA fragmentation index compared to other techniques, such as DGC, reinforcing previous studies suggesting the same (Zini, Finelli, Phang, & Jarvi, 2000).

The direct swim-up method is a popular method of sperm isolation in non-specialised fertility laboratories or clinics because of it's simplicity, and that it can be performed in the absence of specialised laboratory equipment such as a centrifuge. In order to increase its applicability and to allow greater access of fertility treatment, several commercial devices have been designed in order to increase the ease with which it can be performed. One such device is the Seaforia^{TM} Sperm Separation System (SFR) (Lotus Bio^{TM} (Nymphaea) Ltd., Israel).

1.4.2 Density gradient centrifugation

DGC is a routinely used separation technique in fertility laboratories around the world (World Health Organization, 2010). The centrifugation process separates spermatozoa based on their density, where mature, motile and morphologically normal spermatozoa form a pellet as they have a higher density compared to immature, morphologically abnormal spermatozoa. Seminal plasma components are also omitted from the cell pellet (Malvezzi, Sharma, Agarwal, Abuzenadah, & Abu-Elmagd, 2014).

There are two common gradient techniques that can be used in this process: continuous where there is a gradual density increase in media from the top to the

bottom; and discontinuous where there is a clearly defined boundary between two gradients of varying density (Bolton & Braude, 1984; Pousette, Akerlof, Rosenborg, & Fredricsson, 1986). In both cases, gradients are overlain with liquefied semen samples and centrifuged. The total yield of motile sperm cells has been seen to be higher in DGC than swim-up methods (Ricci et al., 2009).

A concern with the use of DGC for sperm isolation is the potential risk of DNA damage due to the presence of transition metals in the density media (Aitken et al., 2014). Previous studies have reported the presence of DNA damage in spermatozoa following DGC using Percoll® (Zini, et al., 2000) and ISolate® (Stevanato et al., 2008). Malzezzi et al. (2014) compared three density gradient media and reported comparable DNA damage in all cases. Aitken et al. (2014) sought to observe the underlying causes of this and discovered that colloidal silicon gradients of PureSperm® contained free radical generating metals such as Al, Cu and Fe that caused oxidative stress to sperm DNA. Aitken et al. (2014) observed that this phenomenon is not just restricted to PureSperm®, indicating a contaminative toxic effect of transient metals in density gradient preparations used in sperm preparation.

1.5 Preservation of male fertility

1.5.1 History of sperm cryopreservation

Attempts at storing gametes in a frozen state can be traced back to the early 20th century (Gosden, 2011). Polge, Smith and Parkes (1949) demonstrated that glycerol added to semen allowed the successful freezing, thawing and recovery of motile sperm. Polge and Rowson (1952) advanced this research by demonstrating glycerol's

protective role in freezing and thawing bull spermatozoa, which was then used to successfully inseminate oocytes. The following year, Bunge and Sherman (1953) applied the same principles to human semen and achieved pregnancies in women using frozen-thawed sperm.

Other than glycerol, there have been multiple cryoprotective agents which have been investigated in the use of freezing gametes. Ethylene glycol, dimethyl sulfoxide and 1, 2-propanediol are such examples; however on the whole glycerol has been the most common cryoprotective agent used in the freezing of human sperm (Leibo & Pool, 2011; Lovelock, 1954; Sherman & Lin, 1958; Smith, 1952).

As various cryoprotective agents were investigated, so too were freezing techniques. Mazur (1963) observed that cells had a higher likelihood of surviving the cooling process when temperature was lowered at a slower rate. It became apparent through several trial and error processes that the biggest danger to the cell during cryopreservation was the periods of cooling and thawing; where at the storage temperature of liquid nitrogen (-196°C) they are relatively stable (Gosden, 2011; Zhang et al., 2016).

1.5.2 Cryopreservation

Cryopreservation aims to store cells at temperatures below freezing for extended periods of time and the subsequent thawing of them, restoring original cell function and capability (Leibo & Pool, 2011). This process however presents many difficulties to cells that would otherwise not normally be exposed to such extreme temperatures. During faster than optimal cooling, intracellular ice can form leading to cell death (Liu, Cheng, & Silversides, 2013), alternatively if the cooling process is below a critical value,

the formation of extracellular ice concentrates the solute, causing cell dehydration and chilling injury to cells (Meryman, 2007).

To negate these effects, cryopreservation media (CPM) are used to protect cells from the freezing process. These usually include a cryoprotective agent (CPA) such as glycerol, a buffer, sugars and salts (Barbas & Mascarenhas, 2009). There are a plethora of commercially-available CPM available to freeze sperm, each being composed of the above elements in various concentrations. However these media are far more expensive than the CPA glycerol alone, and with laboratory expenditure being a constant issue, it is of interest to compare how these commercial CPM compare with the seminal CPA glycerol, which has been used previously in the cryopreservation of human sperm (Tyler, 1973).

Although the use of glycerol has been shown to allow survival during sub-zero temperatures, it is itself toxic to sperm (Critser, Huse-Benda, Aaker, Arneson, & Ball, 1988; McLaughlin, Ford, & Hull, 1992). CPAs both protect cells during cryopreservation, but also exert toxicity that can have a deleterious effect upon sperm motility. The survival of sperm following cryopreservation is therefore a product of these two properties of a CPA which can be measured at two time intervals, being the initial addition of cryoprotective agent, and the post-thaw recovery. To date, there has been a lack of literature comparing the effect of various CPM on sperm motility and degradation prior to cryopreservation and after thawing. Understanding this delicate relationship between toxicity and cryoprotection is needed to further the refinement of CPM.

1.5.3 Applications of cryopreserved sperm

There are many applications of fertility cryopreservation, ranging from ART procedures in humans, to the Livestock industry for breeding purposes (Bagchi, Woods, & Critser, 2008; Mara, Casu, Carta, & Dattena, 2013). Patients undergoing chemotherapy are encouraged to store semen prior to undergoing therapy, as it is damaging to the spermatozoa (Thomson et al., 2009). Spermatozoa in the early stages of development are particularly vulnerable to chemotherapeutic agents; however quiescent sperm precursor cells can also be damaged following multiple chemotherapy sessions (Gandini et al., 2006). Men working with potentially toxic agents that may disrupt spermatogenesis are also advised to store semen (Di Santo, Tarozzi, Nadalini, & Borini, 2012). Males in couples who are undergoing ART procedures are also recommended to cryopreserve samples, in the event of the man being either absent on the day of insemination, or being unable to produce a semen sample on the day of the procedure due to a variety of reasons (Di Santo, et al., 2012).

The ability to store donor semen is also of clinical importance to fertility laboratories, as this allows the global transportation of samples, allowing greater choice of donors to patients. The cryostorage of semen also grants the ability to screen for potential infectious diseases, such as HIV, Hepatitis B and C prior to samples being used for insemination (Centola, 2002). Also with the recent changes in various countries laws regarding same-sex couple's accessibility to fertility treatments, there is a need to improve cryopreservation methods on the limited resource that is donated semen (Moskovtsev et al., 2013; Prokai et al., 2015).

CHAPTER 2

AIMS AND HYPOTHESES

2. Aims and hypotheses

The study sought to compare the cryopreservation capabilities and toxicity of neat glycerol and a commercially available CPM on human sperm. Sperm isolation techniques of DGC, DSW, and a commercial device that utilises the swim-up method were compared for their effectiveness in isolating motile sperm from frozen-thawed semen samples. Measurements were conducted using CASA software and a pre-experiment technical validation phase was also conducted. Here, variables such as the chamber type to be used in conjunction with CASA software, time interval between loading a chamber and taking CASA measurements, diluents and dilutions factors to be used for the semen sample, and operator corrected scores versus original CASA measurements were investigated.

2.1 Aims

The specific aims of the study were to:

1. Select an appropriate chamber type to be used in the study and to validate experimental protocols by:
    a) Comparing the motility and kinematic values of the MicroCell chamber and a standard microscope slide and coverslip with 5µl and 10µl of semen applied.
    b) Evaluating the effect of time on kinematic values for the above three chamber configurations from when the chamber is first loaded, and when the CASA measurement is taken

c) Measuring the difference between the CASA original score for concentration in the above three chamber configurations, and operator-corrected concentration score.

2. Measure the toxic effect of glycerol and a commercial cryoprotective medium on sperm motility, prior to cryopreservation.
3. Measure the effect of seminal plasma and two common gamete handling media on the motility of neat semen samples, and samples containing a cryoprotective agent.
4. Measure the recovery of motile, frozen-thawed semen samples using glycerol or a commercial CPM
5. To compare the efficacy of the DSW, SFR and DGC in the isolation of post-thawed motile sperm.

2.2 Hypotheses

The study will investigate the following null hypotheses:

1. There will be no significant difference between the standard microscope slide and coverslip motility and concentration when compared to the MicroCell chamber.
2. There will be no significant difference between motility and concentration of sperm based on how long a sample has been loaded before being measured.
3. No significant difference will occur between the CASA's original score for concentration and operator corrected scores for concentration.

4. There will be no significant difference in motility and concentration of sperm, after being exposed to glycerol or the commercial CPM prior to freezing.
5. Seminal plasma and the two common gamete handling media will have no influence on the motility of neat semen and cryoprotectant containing semen.
6. Commercially available CPM will result in similar survival rates of sperm post-thaw compared to glycerol.
7. DGC will yield a similar concentration of spermatozoa when compared to DSW and SFR.
8. DSW and SFR will yield a similar number of motile spermatozoa compared to density gradient centrifugation.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and methods

3.1 Ethics

Ethics approval for the current study was granted by the Human Research Ethics Committee of Joondalup Health Campus and the Edith Cowan University Human Research Ethics Committee, **Appendices A** and **B** respectively. A copy of the consent form and project information participants were given is attached in **Appendix C**.

3.2 Subjects and participants

Men undergoing routine semen analysis at Fertility North as part of their fertility investigation were recruited to participate in the project. Permission was sought to use the remainder of their semen sample after the Fertility North analysis, ensuring participants that their fertility treatment was not compromised in any way and involvement was voluntary. It was emphasised that the sample would only be used in the listed experiments and not to be used for insemination, and that their participation was confidential. Only men who had no record of previous infectious diseases including HIV, Hepatitis B and C or Syphilis were invited to participate in the study. A recommended two day period of abstinence, as required for Fertility North's semen analysis was implemented, and production of the sample was either at Fertility North or completed off-site in a sterile container, no more than one hour prior to submission to Fertility North. All collections followed Fertility North's standard procedure for collection of semen samples, patients also filled out a Fertility North collection form (**Appendices D** and **E** respectively).

Fertility North's electronic diary Artemis (v1.0.590) was used in order to investigate participants clinical history and to ensure no infectious samples were used in the study. Data was stored throughout the study in a password protected Excel spreadsheet that only the principal author had access to. All men who participated in the study had their names removed from this Excel spreadsheet and were assigned a project number to protect their identity.

A total of 78 men were recruited for the study. Pooled semen samples were used to ensure an adequate sample size in experimental stages.

3.3 Laboratory techniques and semen analyses

3.3.1 Manual semen analysis

Manual motility measurements throughout the project were conducted according to the WHO 5th (2010) guidelines for semen analysis. Sperm motility was classified as either being progressive (PR), non-progressive (NP) or immotile (IM). All samples were allowed to liquefy for >20 minutes, whereafter 10µl was applied to standard 76.2 x 25.4 mm slides and 22x22mm coverslips (Livingstone, Roseberry, NSW, Australia) and analysed using phase contrast microscopy with a 25x objective lens. All measurements were conducted at room temperature. Technical variability was reduced by having the same operator score each of the manual semen analyses. The laboratory was enrolled in an external quality assurance scheme for semen analysis (see **Appendix F**). The operator attended a semen analysis workshop (**Appendix G**) and was employed at an accredited fertility laboratory with competency demonstrated in performing diagnostic semen analyses.

3.3.2 Computer-assisted sperm analysis parameters and settings

The CASA system used in this study was the Sperm Class Analyzer (Microptic S.L., Barcelona, Spain) coupled through an acA780-75gc GigE camera (Basler AG, Ahrensburg, Germany) to a Nikon microscope (ECLIPSE E200MV R, Nikon, Tokyo, Japan) using a x10 phase contrast objective, as shown in **Figures 1** and **2**. The software used in conjunction with the Sperm Class Analyzer was the SCA® Research edition (v.6.2.0.1) module for motility and concentration of human semen samples. This module allows the measurement of sperm concentration, sperm kinematic values and identifies sperm motility status according to the current World Health Organization criteria (2010). The interface of the SCA® Motility and Concentration program is depicted in **Figure 3**. The chamber used in conjunction with the CASA software was the MicroCell 20µm 4- chamber slide (Vitrolife Sweden AB, Göteborg, Sweden), as shown in **Figure 4.** All measurements were conducted at room temperature.

Prior to the slides being measured, the microscope was configured to account for the measured chamber's depth. Minimum sperm head area to be classified as a sperm head was 1µm^2 and the maximum area was 100µm^2. The chamber was allowed to sit after being loaded in order to allow the sample to settle and minimise drift. Drift was accounted for and was initially adjusted at 5µm/s to differentiate drifting immotile cells from actively motile cells. Operator corrections were applied if an immotile cell's velocity was greater than 5µm/s. Images were captured at 25 frames per second, and five fields of view were captured in order to analyse motility. Sperm motility was classified as being PR, NP or IM. In the instance where the CASA system misclassified non-sperm cells as sperm cells or a sperm cell's motility status, operator corrections were applied (excluding the technical validation phase where both original CASA and

operator-corrected scores were recorded). Examples of the CASA system misclassifying non-sperm cells as sperm cells is depicted in **Figures 5** and **6**

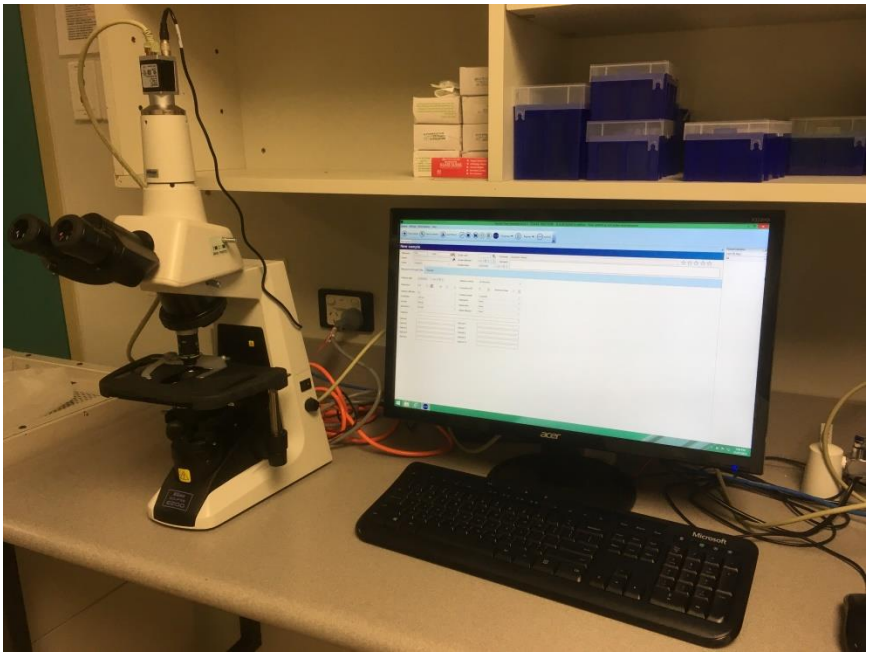

Figure 1. *The Sperm Class Analyzer and the SCA motility interface*

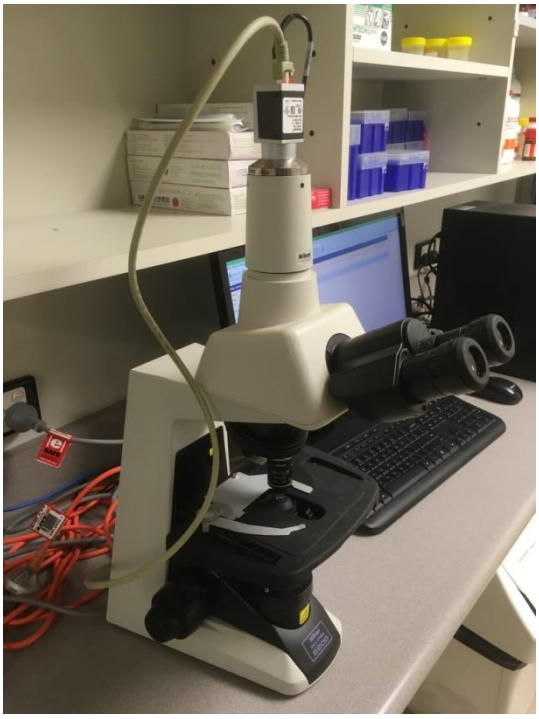

Figure 2. *The Sperm Class Analyzer coupled through an acA780-75gc GigE camera*

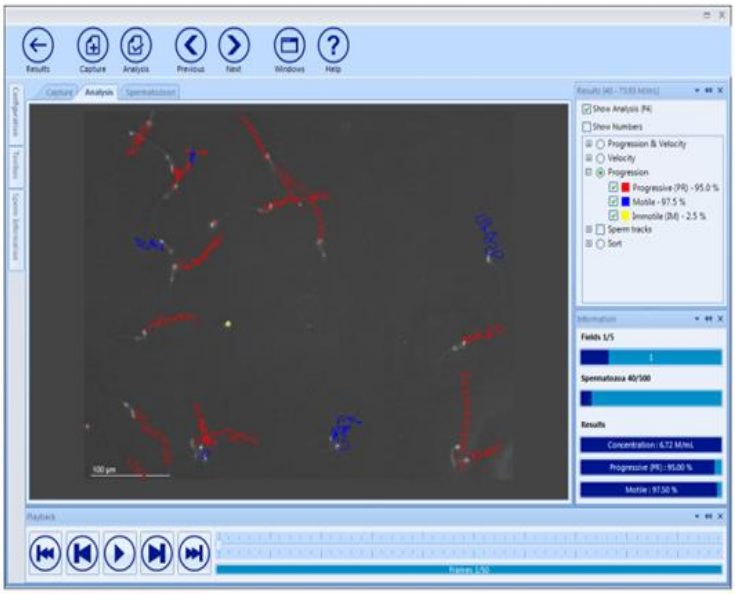

Figure 3. *SCA® Motility and Concentration module measuring a semen sample's sperm motility. Red tracks identify progressively motile sperm trajectories, blue depicts non-progressive sperm and yellow identifies immotile sperm cells.*

Figure 4. *MicroCell 20µm chamber. Slides are available as either 2 chambers, or 4 chambers per-slide.*

Sourced from: (https://www.vitrolife.com/globalassets/support-documents/product-manuals/microcell-user-manual)

Figures 5 *and* **6.** *The CASA system misclassifying non-sperm cells as sperm cells.* **Figure 5** *shows the original CASA field and* **Figure 6** *shows the operator corrected field.*

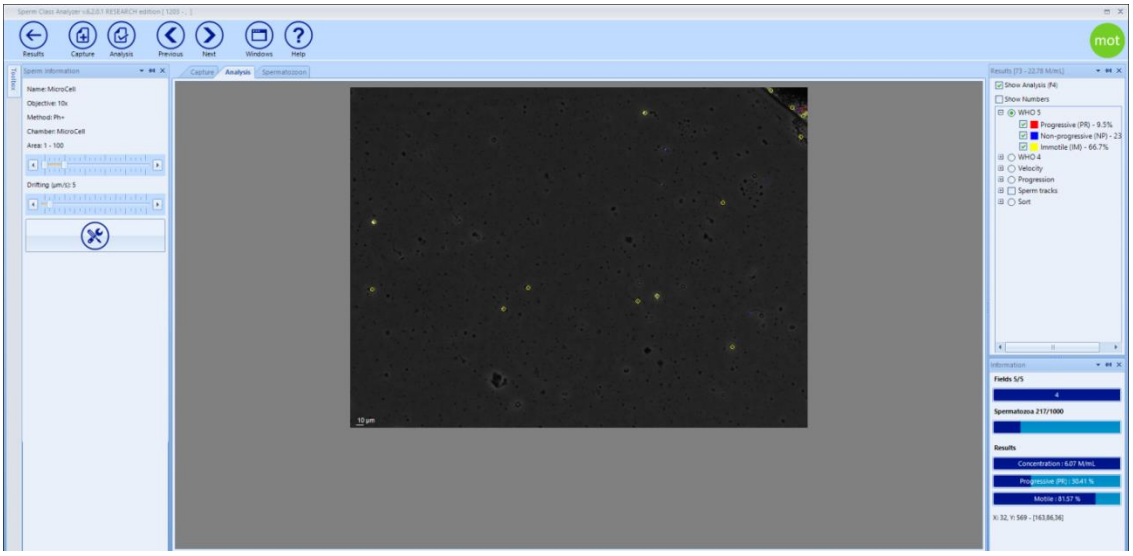

Figure 5. *Original CASA score for sperm concentration*

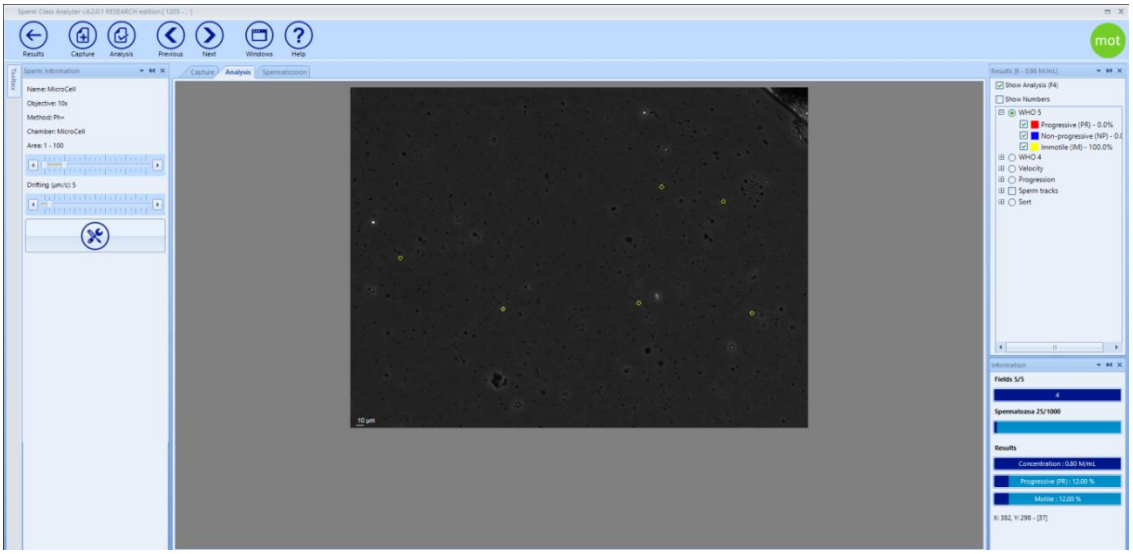

Figure 6. *Operator-corrected score for sperm concentration*

3.3.3 Addition of cryoprotectants and diluents

The addition of all CPAs and diluents was performed at room temperature and in a drop-wise fashion to reduce osmotic shock to sperm cells. As osmotic shock has been shown to reduce sperm motility (Abraham-Peskir, Chantler, Uggerhøj, & Fedder, 2002), this was avoided by the slow addition of all diluents and all resulting suspensions were thoroughly mixed to ensure equal distribution of diluent and sample. Seminal plasma was obtained by centrifuging semen at 1400g for 10 minutes and observed microscopically to ensure no sperm were present in the seminal plasma supernatant.

3.3.4 Cryopreservation and thawing

Following the addition of CPA, samples were loaded into 0.5ml CBS High Security Sperm Straws (Cryo Bio System SAS, Paris, France) and sealed using the SYMMS III Sealer (Cryo Bio System SAS, Paris, France). Straws were then loaded into a Cryologic CryoChamber (CryoLogic Pty Ltd, Blackburn VIC, Australia) which sat inside a liquid nitrogen filled CryoBath (CryoLogic Pty Ltd, Blackburn VIC, Australia) and coupled to the CL-2000 freeze control system (CryoLogic Pty Ltd, Blackburn VIC, Australia). Initial temperature began at a baseline of 20 ˚C with a subsequent cooling rate of -10 ˚C/min. Once the samples reached -40 ˚C, straws were plunged into liquid nitrogen and stored in a liquid nitrogen Dewar until thawed. **Figures 7** and **8** shows the cryopreservation equipment used in the present study. The safe handling of liquid nitrogen is important to reduce risk to the operator; this was identified in the current study with the operator attending a liquid nitrogen safe handling course (**Appendix H**).

Samples were thawed in a water bath for three minutes at 37 ˚C before being assessed.

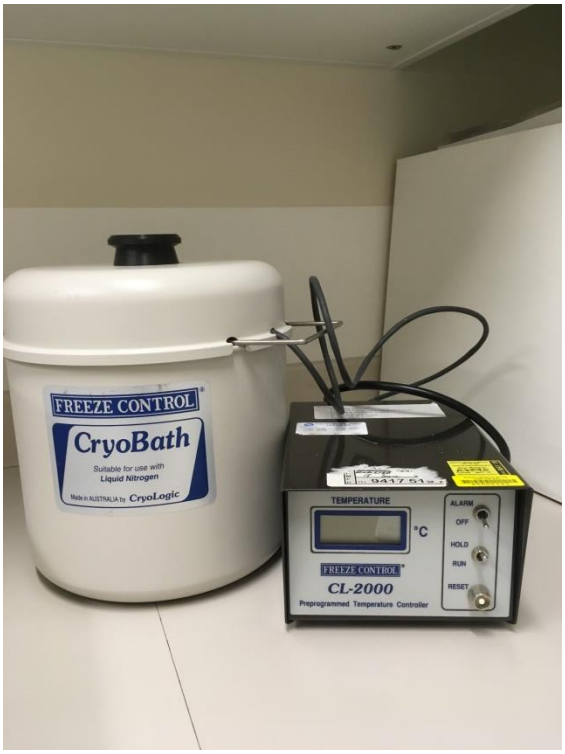

Figure 7. The CryoBath and CL-2000 freeze control system.

Figure 8. The CryoChamber within the CryoBath coupled to the CL-2000 freeze control system.

3.4 Experimental design

This study was comprised of four related experiments. The experimental designs of each are as follows.

3.4.1 Technical validation

This phase sought to validate several technical variables when using CASA systems that would be controlled throughout the remainder of the study. 20 samples were included in this phase.

Three chamber/slide configurations were evaluated for their effect on sperm motility parameters, namely the MicroCell 20µm chamber (Vitrolife Sweden AB, Göteborg, Sweden) loaded with 3µl semen, and standard 76.2 x 25.4 mm slides and 22x22mm coverslips (Livingstone, Roseberry, NSW, Australia) loaded with semen volumes of 5µl (CV.5µl) and 10µl (CV.10µl). Initially, samples were observed at time intervals immediately, 2.5 minutes, 5 minutes and 20 minutes after loading to observe the effect of time on each chamber in terms of (i) the number of sperm viewed by the CASA system before and after operator correction, and (ii) the sperm motility. Each chamber configuration was stored at room temperature on a benchtop between readings. Once an optimum time was selected for the assessment of the chamber/slides after loading, the effect of the chamber/slide configuration upon sperm kinetics was determined.

Five random fields of a sample were captured by the CASA and fields were individually reviewed before corrections were applied by the operator where the CASA software had misclassified a sperm cell or its motility status. The corrected results were then separately recorded to allow for comparisons between the original CASA report and an

operator corrected score. Sperm motility status was assigned as PR, NP or IM. In addition, kinetic parameters assessed were curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF), as shown in **Figure 9.**

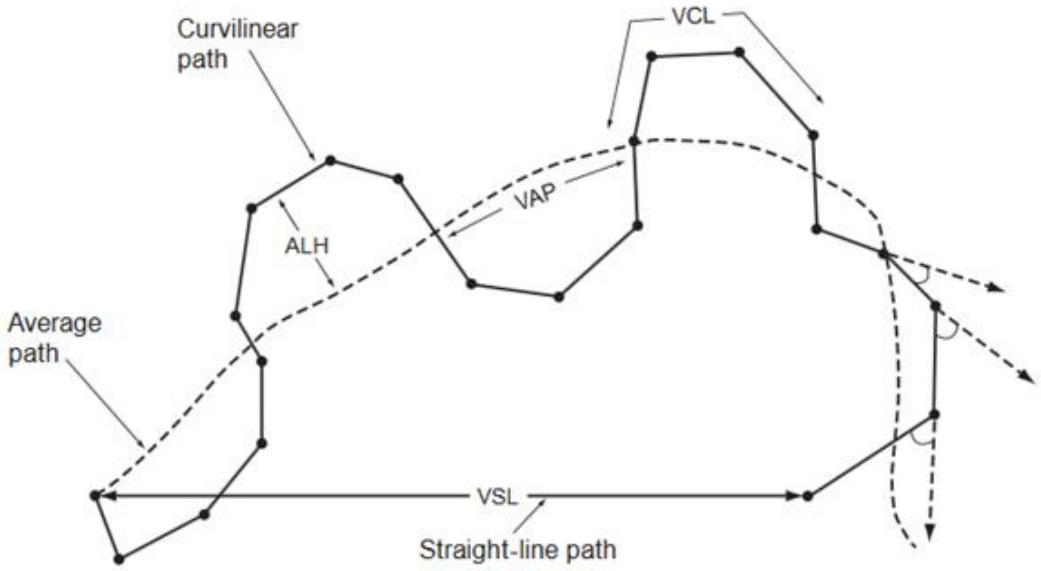

Figure 9. *Kinematic values CASA software are able to measure, as per the WHO 5th edition (2010):* ***VCL****, curvilinear velocity (µm/s). Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope;* ***VSL****, straight-line (rectilinear) velocity (µm/s). Time-averaged velocity of a sperm head along the straight line between its first detected position and its last;* ***VAP****, average path velocity (µm/s). Time-averaged velocity of a sperm head along its average path;* ***ALH****, amplitude of lateral head displacement (µm). Magnitude of lateral displacement of a sperm head about its average path;* ***LIN****, linearity. The linearity of a curvilinear path, VSL/VCL;* ***WOB****, wobble. A measure of oscillation of the actual path about the average*

path, VAP/VCL; ***STR****, straightness. Linearity of the average path, VSL/VAP;* ***BCF****, beat-cross frequency (Hz). The average rate at which the curvilinear path crosses the average path.*

3.4.2 The effect of cryoprotectants and diluents on sperm motility prior to cryopreservation

This phase sought to investigate the toxicity that CPAs exert on sperm motility prior to cryopreservation. Further investigations were made to elucidate potential artefacts that could occur when CPA containing semen samples were diluted with common gamete handling media and seminal plasma. 14 samples were included in this phase. Motility assessments were conducted manually for this phase according to the WHO 5th classification (World Health Organization, 2010).

After the initial motility assessment, aliquots of each sample were divided into the following treatment groups: (i) neat semen with nothing added, (ii) an equal volume of CPM (Quinn's Advantage™ Sperm Freezing Medium; Origio Australasia Pty Ltd, Thornleigh NSW, Australia) mixed with the semen, (iii) glycerol (Sigma-Aldrich Pty Ltd, Sydney NSW, Australia) at 5% v/v mixed with the semen, and (iv) 10% v/v glycerol mixed with the semen. Each aliquot was then observed after one minute and the motility recorded as above. Following this, seven men's samples (of the 14 total) containing the above CPAs were diluted (1:5) with G-MOPS^{TM} PLUS medium (Vitrolife Pty Ltd, Sydney NSW, Australia), which contains human serum albumin, and a motility measurement was taken within one minute. The other seven men's samples were diluted (1:5) with Quinn's Advantage™ Medium with HEPES (Origio Australasia Pty Ltd, Thornleigh NSW, Australia) supplemented with 5% human serum albumin (Origio

Australasia Pty Ltd, Thornleigh NSW, Australia), or seminal plasma (SP) (1:5 dilution), and a final motility measurement recorded.

3.4.3 The effect of neat glycerol and a commercial cryoprotectant on the recovery of motile frozen-thawed sperm

This phase sought to continue the investigation of the cryoprotectants used in the previous experiment, this time observing their cryoprotective capabilities in recovering motile sperm from frozen-thawed samples. Twenty samples were included in this phase.

Initial pre-freeze analyses of samples were conducted using the CASA system with the 20µm MicroCell chamber. After the initial motility assessment, aliquots of each sample were divided into the following treatment groups: (i) neat semen with nothing added, (ii) an equal volume of CPM Quinn's Advantage™ Sperm Freezing Medium mixed with the semen, (iii) glycerol at 5% v/v mixed with the semen, and (iv) 10% v/v glycerol mixed with the semen. Samples were then frozen according to the study protocol and subsequently thawed. Following thawing, samples were then loaded into the MicroCell chamber and a final analysis was conducted using the CASA system.

3.4.4 Comparing density gradient centrifugation, direct swim-up method, and a commercial device utilising the swim up method in the isolation of motile sperm

This phase aimed to investigate the efficacy of DGC, the DSW, and the commercial Seaforia^{TM} Sperm Separation System (SFR) (Lotus Bio^{TM} (Nymphaea) Ltd., Israel) that utilises the swim-up method. Semen samples were frozen 1:1 v/v with Quinn's Advantage™ Sperm Freezing Medium and stored in liquid nitrogen. Post-thawed

samples were pooled in this phase to allow sufficient volume to be processed through each isolation method. Following thawing, initial analyses of pooled samples were conducted using CASA software in conjunction with the 20µl MicroCell chamber. 20 pooled post-thaw samples were included in this phase.

PureSperm (Nidacon AB, Mölndal, Sweden) supplemented with Quinn's Advantage™ Medium with HEPES and 5% human serum albumin to give a 1ml 60% density gradient was overlayed with 1ml of frozen-thawed semen. Centrifugation was at 350g for 15 minutes, the resulting pellet was extracted and placed into a fresh centrifuge tube which was then made up to 2ml with G-MOPS^{TM} and spun at 500g for 5 minutes. After this wash stage the supernatant was removed and reduced to 0.3ml with the cell pellet being resuspended, followed by a motility and concentration measurement.

For the DSW, 1ml of sample was applied to a centrifuge tube and was carefully overlaid with 1ml of G-MOPS^{TM}, ensuring that a clear interface was present between the medium and semen. The tube was then angled to 45° and incubated at 37°C for 30 minutes. 0.3ml of the top-most supernatant containing motile sperm was then removed, ensuring the interface was not disturbed, and a motility and concentration measurement was made.

The operation of the SFR was in compliance with its user manual (**Appendix I**). Two 0.5ml chambers were used, where 0.5ml of sample was applied to the semen well, and 0.8ml of G-MOPS^{TM} (Vitrolife Pty Ltd, Sydney NSW, Australia) was overlayed via the handling media insertion. Both chambers were then placed onto the Seaforia^{TM} incubation unit and incubated at 37°C for 30 minutes. After incubation 0.3ml of semen

containing supernatant was extracted, followed by a motility and concentration measurement.

3.5 Statistical analysis

Statistical comparisons using the SPSS statistics package (IBM v.23) were made in the current study. Data sets were first explored and considered to be either normally or abnormally distributed based on the Shapiro-Wilk test for normality score (α=0.05). If these normally distributed data sets contained no outliers and met Mauchly's tests for sphericity, repeated measures ANOVA testing with Bonferroni post-hoc analysis was applied to investigate where differences occurred between groups. If data sets failed to meet the assumptions required for repeated measures ANOVA, non-parametric Friedman's test was employed to identify possible significant differences between data groups. A Sign pair-wise comparison test was then used to identify where the differences occurred between measurements. For all tests, differences were considered significant at $p<0.05$.

CHAPTER 4

RESULTS

4. Results

4.1. Technical validation

4.1.1 Sperm number in operator-corrected and uncorrected fields

The number of sperm counted in 5 fields of view at 0 minutes, 2.5 minutes, 5 minutes and 20 minutes after being loaded are shown in **Table 4.1,** with both the uncorrected and operator-corrected results. At each time interval for each chamber type, there was a statistically significant ($p<0.05$) reduction observed between the CASA's original measurement (uncorrected) and an operators applied corrections (corrected).

The number of sperm counted on the corrected CV.10µl was significantly higher than the corrected CV.5µl at 0 minutes ($p<0.05$), 2.5 minutes ($p<0.01$) and 5 minutes ($p<0.01$). There were also significant differences observed between the uncorrected CV.10µl and uncorrected CV.5µl at 0 minutes ($p<0.01$), 2.5 minutes ($p<0.01$) and 5 minutes ($p<0.05$). **Figure 10** shows the consistently reduced count for operator-corrected concentration measurements in the MicroCell chamber.

Table 4.1. *The number of sperm (mean ± sem) counted by the analyser in 5 fields of view immediately (0 mins), 2.5 mins, 5 mins and 20 mins after loading when the recognition of sperm was uncorrected or corrected. The three configurations used were a 20µm Microcell chamber, and slides/coverslips with either 5µl (CV.5µl) or 10µl (CV.10µl) semen applied.*

	0 mins		2.5 mins		5 mins		20 mins	
Slide	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
MicroCell	291 ± 53	228 ± 48^{b*}	302 ± 59	244 ± 52	308 ± 55	237 ± 45^{g*}	305 ± 65	241 ± 54
CV.5µl	176 ± 36^{a**}	135 ± 29^{b*c*}	177 ± 21^{d*}	131 ± 19^{e**}	206 ± 30^{f*}	147 ± 26^{g*h**}	188 ± 28	135 ± 22
CV.10µl	374 ± 59^{a**1**}	281 ± 49^{c*2**}	344 ± 50^{d*}	261 ± 44^{e**}	330 ± 45^{f*}	257 ± 40^{h**}	269 ± 50^{1**}	207 ± 44^{2**}

All values of corrected vs uncorrected are significantly different to each other and hence do not have superscripts. Other values with the same superscript are significantly different from each other. (p<0.05), ** (p<0.01), *** (p<0.001).*

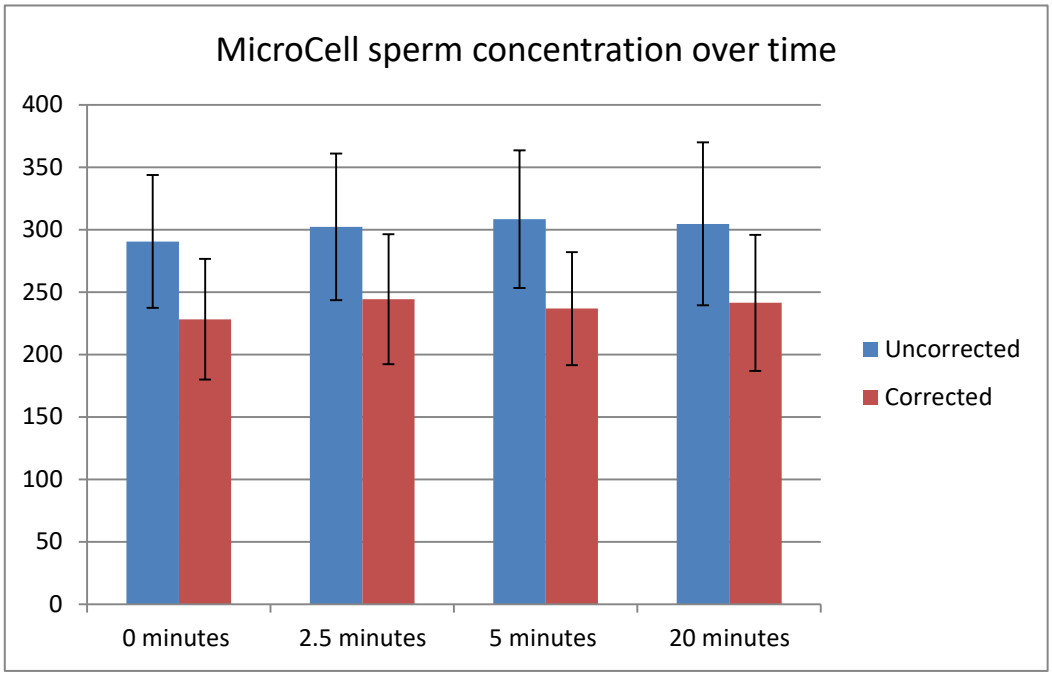

Figure 10. *The sperm concentration measured in the MicroCell chamber at time intervals of 0 minutes, 2.5 minutes, 5 minutes and 20 minutes. Operator corrections are shown where the CASA software misclassified non-sperm cells as sperm cells.*

4.1.2 Influence of time on sperm motility between chamber types

After semen was loaded on the MicroCell chamber, the CV.5µl and CV.10µl slides, operator-corrected measurements of sperm motility were made at time intervals of 0 minutes, 2.5 minutes, 5 minutes and 20 minutes after loading, these results are shown in **Table 4.2**. At 0 minutes, there were no statistical differences observed between slide types for IM, NP or PR sperm. Microcell chambers, CV.5µl and CV.10µl slides all gave stable readings for all categories of motility up to 5 mins. A number of changes were then seen after 20 minutes with both the Microcell and CV.5µl showing a significant reduction in motility compared to time 0 minute ($p<0.001$ for both). CV.5µl and

CV.10µl differed significantly to each other at 20 minutes for IM and NP (both $p<0.01$), the MicroCell and CV.10µl differed significantly from each other at the 20 minute time interval for both PR and IM cell proportions (both $p<0.05$).

4.1.3 Sperm motility and kinetic parameters

Based upon the results above showing stability of motility readings over the first 5 minutes, motility measurements were made at a standard 2 minutes after loading for each chamber type with operator corrections applied; these results are shown in **Table 4.3**. Neither total motility nor progressive motility were significantly different between chamber types, consistent with the previous round of tests above. However, the MicroCell values were significantly different to the CV.5µl for VAP ($p<0.05$) and the CV.10µl for VCL ($p<0.001$), VAP ($p<0.001$) and VSL ($p<0.01$). No significant differences were observed between the Microcell chamber and the slides with either CV.5µl or CV.10µl in the STR, LIN, WOB and ALH.

Table 4.2. *The proportion (mean ± sem) of sperm that were immotile (IM.), non-progressively motile (NP) or progressively motile (PR.) immediately, 2.5 mins, 5 mins and 20 mins after loading. The three configurations used were a 20µm Microcell chamber, and slides/coverslips with either 5µl (CV.5µl) or 10µl (CV.10µl) semen applied.*

Slide	0 mins			2.5 mins			5 mins			20 mins		
	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)
MicroCell	56 ± 8	12 ± 2	32 ± 9[1]	56 ± 7	14 ± 2	30 ± 9[2]	54 ± 7[3]	16 ± 2[a]	30 ± 9	60 ± 8[3b]	15 ± 2	25 ± 9[12e]
CV.5µl	52 ± 8	13 ± 4	36 ± 8[4]	54 ± 8[56]	10 ± 2	35 ± 9[7]	58 ± 9[58]	10 ± 1[a]	32 ± 9[9]	63 ± 8[68c]	11 ± 1[d]	26 ± 8[479]
CV.10µl	54 ± 9	11 ± 1	35 ± 8	52 ± 9	10 ± 2	38 ± 9	51 ± 8	12 ± 2	37 ± 8	52 ± 8[bc]	17 ± 2[d]	31 ± 9[e]

Values with the same superscript are significantly different from each other, letters indicate differences between the same chamber at different time intervals, letters dictate differences between different chambers at the same time. (p<0.05), ** (p<0.01), *** (p<0.001). 1***, 2**, 3*, 4***, 5*, 6**, 7**, 8*, 9**, a**, b*, c**, d**, e*.*

Table 4.3. *The kinematic results (mean ± sem) for 10 semen samples obtained with 20µm Microcell chambers and microscope slides with coverslips (CV.5µl: 5µl semen; CV.10µl: 10µl semen) at two minutes.*

Motility and kinetic parameters	Chamber/slide		
	Microcell	CV. 5µl	CV. 10µl
Total motility (%)	49.8 ± 6.2	50.1 ± 7.7	57 ± 7.5
PR motility (%)	30.6 ± 7.3	34.1 ± 9.3	39.9 ± 9.1
VCL (µm/s)	36.7 ± 4.9^{a***}	41.4 ± 5.9	42.4 ± 5.5^{a***}
VAP (µm/s)	20.7 ± 2.6^{b*c***}	24.2 ± 3^{b*}	24.4 ± 2.8^{c***}
VSL (µm/s)	13.6 ± 2.1^{d**}	15.3 ± 1.8	16.6 ± 2.1^{d**}
STR (%)	58.9 ± 2.5	58.2 ± 1.7	62.5 ± 1.7
LIN (%)	33.9 ± 2.4	35.6 ± 1.9	38 ± 2
WOB (%)	55.6 ± 2	57.5 ± 1.6	58.6 ± 2
ALH (µm)	1.9 ± 0.3	2.0 ± 0.3	2.2 ± 0.2

Values with the same superscript are significantly different from each other. (p<0.05), ** (p<0.01), *** (p<0.001).*

4.2. The effect of cryoprotectant and diluent used on sperm motility prior to cryopreservation

4.2.1 Effect of cryoprotectants on sperm motility

The overall PR motility of sperm for all samples, with or without the cryoprotectants, is shown in **Table 4.4** and **Figure 11**. Following the addition of each cryoprotective agent

there was a significant decrease in PR observed across all types of CPA added ($p<0.001$). There was no statistical difference seen between the CPM and 5% glycerol, but the PR of 10% glycerol was significantly lower than both the CPM ($p<0.001$) and 5% glycerol ($p<0.001$). The majority of the reduction in PR motility of samples seen was a shift directly to IM cells, although the samples containing 10% glycerol had a significantly higher proportion of NP cells than the neat semen ($p<0.05$) or that containing CPM ($p<0.05$).

Table 4.4. *Sperm motility (mean ± sem) following the addition of no cryoprotectant (nil), cryoprotective medium (CPM), and glycerol at 5% and 10% v/v. (n=14).*

Cryoprotectant	Sperm motility (%)		
	PR	NP	IM
Nil	$64.2 \pm 4.0^{a***b***c***}$	$0.6 \pm 0.2^{f*}$	$34.9 \pm 3.9^{h***i***j***}$
CPM	$45.4 \pm 6.1^{a***d***}$	$1.1 \pm 0.4^{g*}$	$53 \pm 5.8^{i***k***}$
5% glycerol	$39.9 \pm 4.9^{b***e***}$	3.4 ± 1.2	$56.6 \pm 4.7^{j***l***}$
10% glycerol	$23.2 \pm 3.9^{c***d***e***}$	$3.2 \pm 0.9^{f*g*}$	$73.6 \pm 3.8^{j***k***l***}$

Values with the same superscript are significantly different from each other. ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).*

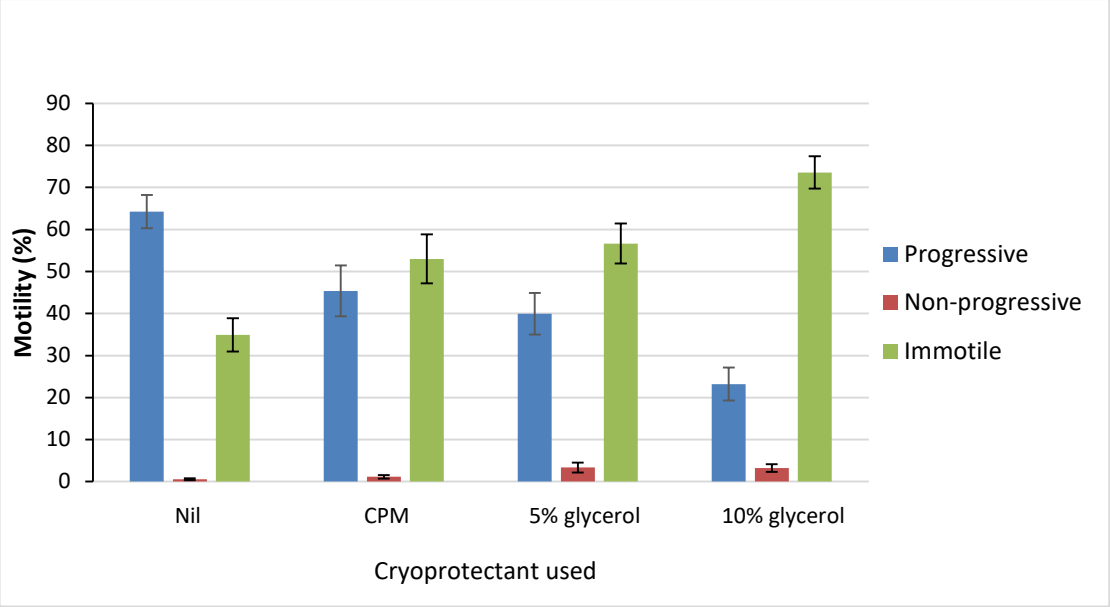

Figure 11. *Sperm motility (mean ± sem) following the addition of no cryoprotectant (nil), cryoprotective medium (CPM), and glycerol at 5% and 10% v/v.*

4.2.2 Dilution with handling media and seminal plasma

Seven semen samples containing an equal v/v CPM, 5% glycerol or 10% glycerol were subsequently diluted with G-MOPS^{TM} PLUS (1:5 dilution) and loaded onto a glass slide, with a motility measurement being made within 2 minutes of the dilution with G-MOPS^{TM} PLUS. These results are shown in **Table 4.5**. The addition of G-MOPS^{TM} PLUS to the neat semen sample did not impact on the PR motility, but the dilution of samples containing each of the cryoprotectants resulted in a significant decrease in PR motility, relative to the cryoprotectant alone.

Table 4.5. *Sperm progressive motility (mean ± sem) following the addition of cryoprotectants and subsequent dilution using G-MOPS™ PLUS medium. The cryoprotectants were none (nil), Quinn's Advantage Sperm Freezing Medium (CPM), and glycerol at 5% and 10% v/v.*

Cryoprotectant	Diluent	
	Nil	G-MOPS™ PLUS
Nil	62.6 ± 6.4	58.3 ± 7.5
CPM	46.1 ± 9.3[1***]	25.9 ± 7.2[1***]
5% glycerol	34.0 ± 7.6[2*]	16.7 ± 5.9[2*]
10% glycerol	17.0 ± 3.7[3*]	3.4 ± 1.2[3*]

Groups are significantly different when they have the same superscript letter. (p<0.05), ** (p<0.01), *** (p<0.001).*

The remaining seven men's semen samples were diluted with Quinn's Advantage™ Medium with HEPES, or seminal plasma; results are shown in **Table 4.6** and **Figure 12**. The neat semen sample did not show a significant reduction in PR motility when diluted with Quinn's Advantage™ Medium with HEPES or SP. The addition of SP to samples with CPM or 10% glycerol did not show a statistically significant decrease in the PR motility of sperm, although addition to the 5% glycerol group did see a slight but not statistically significant decrease in PR motility. Quinn's Advantage™ Medium with HEPES had a negative impact on PR within all samples containing cryoprotectant, significantly reducing the proportion of PR spermatozoa.

Table 4.6. *Sperm progressive motility (mean ± sem) following the addition of cryoprotectants and subsequent dilution using either Quinn's Advantage™ Medium with HEPES (HEPES) or seminal plasma. The cryoprotectants were none (nil), Quinn's Advantage Sperm Freezing Medium (CPM), and glycerol at 5% and 10% v/v.*

Cryoprotectant	Diluent		
	Nil	HEPES	Seminal plasma
Nil	65.9 ± 5.1	61.9 ± 7.1^{1*}	68.4 ± 5.6^{1*}
CPM	44.6 ± 8.5^{2***}	31.3 ± 8.6^{2***3***}	46.9 ± 8.8^{3***}
5% glycerol	45.9 ± 6.0^{4***}	20.1 ± 4.8^{4***5*}	37.4 ± 7.0^{5*}
10% glycerol	29.4 ± 6.3^{6**}	10.9 ± 3.3^{6**7*}	25.0 ± 6.8^{7*}

Groups are significantly different when they have the same superscript letter (between different cryoprotectants for same diluent) or number (between different diluents for the same cryoprotectant). (p<0.05), ** (p<0.01), *** (p<0.001).*

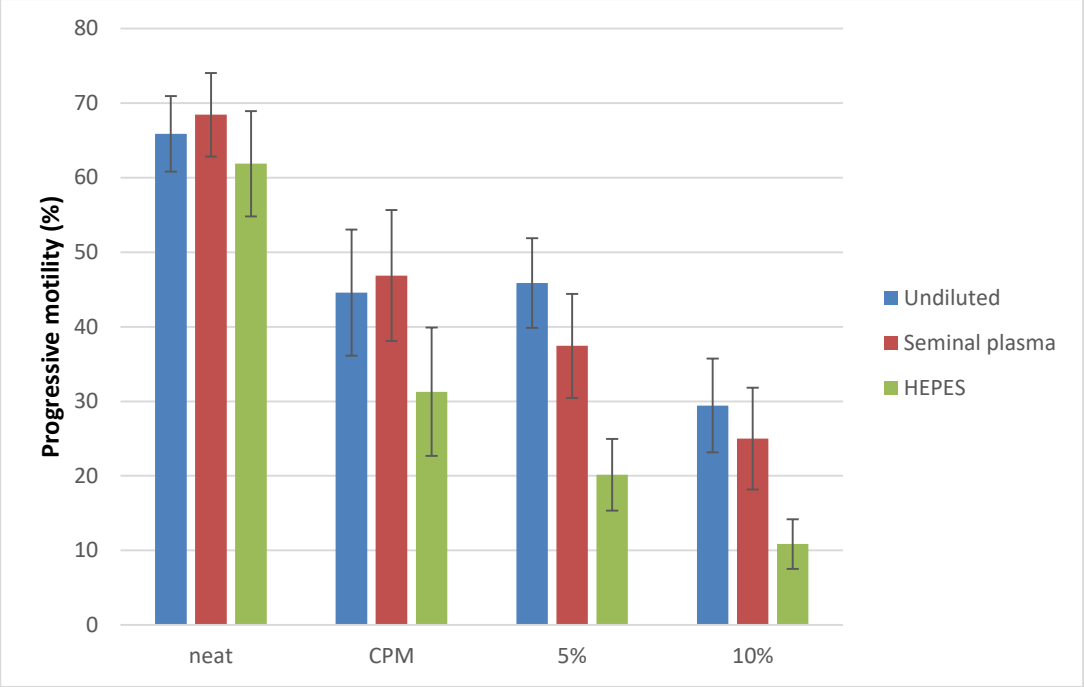

Figure 12. *Sperm progressive motility (mean ± sem) following the addition of cryoprotectants and subsequent dilution using either Quinn's Advantage™ Medium with HEPES (HEPES) or seminal plasma. The cryoprotectants were none (nil), Quinn's Advantage Sperm Freezing Medium (CPM), and glycerol at 5% and 10% v/v.*

4.3. The effect of neat glycerol and commercial cryoprotectant on the recovery of motile frozen-thawed sperm

There was a significant reduction seen in all kinematic parameters for each cryoprotective agent group observed between the pre-freeze control and the resulting post-thaw ($p<0.05$). The 10% glycerol group suffered the greatest decline in PR motility compared to the control pre-freeze ($p<0.001$), followed by 5% glycerol ($p<0.001$) and the CPM ($p<0.001$). The CPM progressive motility was significantly different to both glycerol groups, however between the 5% glycerol and 10% glycerol post-thaw progressive motility there was no significant difference. 10% glycerol scored

significantly lower on several kinematic values such as VCL, VAP, VSL, and WOB compared to both the CPM and 5% glycerol. These results are displayed in **Table 4.7.**

Table 4.7. *Pre-freeze control and post-thaw sperm kinematic measurements (mean ± sem) of samples when exposed to CPM (1:1 dilution) and 5% glycerol and 10% glycerol (v/v).*

Motility and kinetic parameters	Cryoprotective agent added			
	Pre-Freeze (neat)	**Post-thaw CPM**	**Post-thaw glycerol 5%**	**Post-thaw glycerol 10%**
PR motility (%)	26.3 ± 3.4^{abc}	10.8 ± 1.5^{ade}	4.2 ± 0.9^{bd}	3.1 ± 0.7^{ce}
VCL (µm/s)	46.9 ± 1.5	38.3 ± 3.0^{f}	39 ± 2.4	38.9 ± 2.2^{f}
VAP (µm/s)	29.2 ± 0.8	21.5 ± 1.9^{g}	20.9 ± 1.4^{h}	18.4 ± 1.0^{gh}
VSL (µm/s)	21.1 ± 0.7	14.2 ± 1.3^{i}	13.5 ± 0.9^{j}	11.8 ± 0.7^{ij}
STR (%)	71.4 ± 1.5	60.9 ± 4.7	66.6 ± 3.1	65.5 ± 2.0
LIN (%)	46.7 ± 1.1	32.9 ± 2.5^{k}	34.3 ± 2.2	31.5 ± 1.7^{k}
WOB (%)	63.2 ± 0.9	49.3 ± 3.5^{l}	51 ± 2.4^{m}	47 ± 1.4^{lm}
ALH (µm)	2.3 ± 0.1	2.0 ± 0.2	2.1 ± 0.1	2.2 ± 0.1
BCF (Hz)	6.9 ± 0.2	5.1 ± 0.4	5.4 ± 0.3	5.3 ± 0.5

Values with the same superscript are significantly different from each other. ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).a***, b***, c***, d***, e***, f*, g***, h*, i*, j*, k**, l**, m**.*

4.4. The isolation of motile sperm comparing density gradient centrifugation, direct swim-up method, and a commercial device utilising the swim up method

The yield of sperm concentration for each isolation method was significantly lower in comparison to the initial count of pooled post-thawed samples. DGC had the highest yield of 10.2 x 10^6 sperm/ml, followed by DSW of 1.8 x 10^6 sperm/ml, followed closely by SFR with 1.5 x 10^6 sperm/ml. Each isolation technique had a significantly higher population of PR sperm after processing in comparison to the initial pooled post-thaw ($p<0.005$). The SFR and DSW had comparable PR motile sperm at 42.8 and 39.1% respectively, where DGC was significantly lower than both of these at 20.5% ($p<0.001$ for both). In line with this, DGC had the highest proportion of immotile sperm at 68.3%, followed by DSW with 52.5%, and SFR with 48.2% IM. Non-progressively motile sperm were comparable between pre and post-isolation. VCL was significantly higher in each isolation group compared to the post-thaw (all $p<0.001$), however LIN, WOB and ALH were significantly higher in the post-thaw compared to each isolation group (all $p<0.001$). There were a range of non-statistically significant differences observed between other kinematic parameters between groups. These results are shown in **Table 4.8.**

Table 4.8. *Kinematic parameters (mean ±sem) of pooled, frozen-thawed semen samples that were processed through a continuous density gradient centrifugation, a direct-swim up, or the Seaforia™ commercial device.*

Kinematic parameters	**Pooled post-thaw**	**Density gradient centrifugation**	**Direct-swim up**	**Seaforia**
Count (x 10^6/ml)	$24.5 \pm 2.0^{a***b***c***}$	$10.2 \pm 1.4^{a***d***e***}$	$1.8 \pm 0.3^{b***d***}$	$1.5 \pm 0.3^{c***e***}$
Progressive (%)	$12.1 \pm 0.8^{a***b***c***}$	$20.5 \pm 1.5^{a***d***e***}$	$39.1 \pm 2.6^{b***d***}$	$42.8 \pm 3.3^{c***e***}$
Non-progressive (%)	$9.8 \pm 0.5^{a*}$	$11.2 \pm 0.6^{a*b*c*}$	$8.4 \pm 1.3^{b*}$	$9.0 \pm 1.5^{c*}$
Immotile (%)	$78.2 \pm 1.0^{a***b***c***}$	$68.3 \pm 1.8^{a***d**e**}$	$52.5 \pm 3.3^{b***d**}$	$48.2 \pm 3.7^{c***e**}$
VCL (µm/s)	$53.1 \pm 0.9^{a***b***c***}$	$61.3 \pm 1.6^{a***}$	$63.7 \pm 1.4^{b***}$	$62.9 \pm 2.2^{c***}$
VAP (µm/s)	$31.7 \pm 0.4^{a*}$	32.5 ± 0.5	$33.4 \pm 0.5^{a*}$	33.7 ± 0.8
VSL (µm/s)	21.5 ± 0.4	21.2 ± 0.7	22.2 ± 0.8	22.1 ± 0.7
STR (%)	68.4 ± 0.6	65.1 ± 1.9	66.3 ± 2.1	66.4 ± 1.8
LIN (%)	$44.6 \pm 0.8^{a*b***c***}$	$37.8 \pm 1.7^{a*}$	$37.4 \pm 1.6^{b***}$	$37.8 \pm 1.8^{c***}$
WOB (%)	$63.0 \pm 0.6^{a***b***c***}$	$56.0 \pm 1.0^{a***}$	$54.9 \pm 0.9^{b***}$	$56.8 \pm 1.0^{c***}$
ALH (µm)	$2.6 \pm 0.0^{a***b***c***}$	$3.0 \pm 0.1^{a***}$	$3.2 \pm 0.1^{b***}$	$3.2 \pm 0.1^{c***}$
BCF (Hz)	$6.6 \pm 0.1^{a*b**c*}$	$7.3 \pm 0.2^{a*}$	$7.6 \pm 0.2^{b**}$	$7.3 \pm 0.2^{c*}$

Values with the same superscript between post-isolation groups are significantly different from each other. (p<0.05), ** (p<0.01), *** (p<0.001).*

CHAPTER 5

DISCUSSION

5. Discussion

5.1 Variables influencing CASA measurements

CASA systems provide the benefit of being able to measure a number of kinematic parameters such as VSL and ALH that would otherwise be difficult to obtain through manual measurements (Mortimer, 2000). Whilst the results obtained with these automated systems are said to be more objective and precise than manual methods (Vyt et al., 2004), the values obtained are influenced by a variety of technical factors, such as the chamber used to house the sample (Gloria et al., 2013; Ibanescu et al., 2016) and the configuration of the CASA system (Boryshpolets, Kowalski, Dietrich, Dzyuba, & Ciereszko, 2013).

There are many options available when selecting the chamber to be used in conjunction with CASA systems. A cheap and convenient option often recommended by the manufacturers of CASA systems in measuring motility is the use of a simple microscope slide with coverslip, although the volume of semen added and hence the depth of the sample does need to be standardised, as the coverslip is effectively floating on the sample (Del Gallego, et al., 2017). The WHO 5th edition (2010) recommends disposable chambers of 20µm depth when measuring human sperm motility by CASA, thereby providing a monolayer of sperm cells that allows rotational flagella action (Kraemer, Fillion, Martin-Pont, & Auger, 1998; Le Lannou, Griveau, Le Pichon, & Quero, 1992). A professional consensus paper (ESHRE, 1998) recommended that chambers or slides used for human sperm be 10-20µm deep when assessing motility and kinematics. When using purpose-made chambers of fixed depth that use

capillary action to load the sample, there are different depths that can be purchased, which have been shown to affect measurements (Spiropoulos, 2001).

The technical validation phase of this thesis compared three chamber configurations, being (i) the MicroCell fixed 20µm depth chamber, where samples are loaded by capillary action, (ii) a standard microscope slide and a 22mm x 22mm coverslip with 10µl semen (CV.10µl), giving a depth of 20.7µm, and (iii) a standard microscope slide and a 22mm x 22mm coverslip with 5µl semen (CV.5µl), resulting in a depth of 10.3µm.

5.1.1 CASA concentration measurement versus operator-corrected scores

Sperm concentration measurements were compared between chamber types to determine if differences arose due to the non-uniform chamber depth of the coverslip slides and the fixed-depth of the MicroCell. At each time point for each chamber, there was a statistically significant difference between the operator's corrected count and the original CASA count, with most of the errors the CASA system made being the misclassification of non-sperm cells as sperm cells, including abrasions on the slide and cellular debris. The CASA system in the present study was configured to recognise sperm heads as 1µm^2 to 100µm^2, which may have resulted in an increase in non-sperm cells being recognised as sperm cells. This was the default setting of the SCA® Research edition (v.6.2.0.1) module for motility and concentration of human semen samples. However many of the errors where non-sperm cells were recognised as sperm cells by the CASA software were due to abrasions on the chamber and cellular debris, the majority of which were smaller than the average sperm cell. This demonstrated that cells larger than spermatozoa were not contributing to the increase in non-sperm cell

recognition, and so the large head area to be detected was not significantly impacting measurements.

Given the differences in the geometry of each chamber arising from different sample depths, it was not surprising that in the current study significant differences in the operator-corrected count were observed between the MicroCell chamber and CV.10µl (both 20µm depth) and the CV5µl (10µm depth). As time progressed, the MicroCell and CV.5µl slide did not have any observed significant differences in corrected count at 0 minutes and 20 minutes. The CV.10µl however showed a significantly reduced corrected count at 20 minutes. A possible explanation for this significant reduction is the effect of evaporation from the edges of the coverslip negatively influencing the apparent count in some way, whereas the MicroCell chamber and CV.5µl slide have less exposure to the atmosphere, possibly providing some sort of protection.

Chamber types that are loaded by capillary-action, such as the MicroCell, have been shown to potentially have reduced levels of sperm concentration compared to droplet-loaded chambers when measured in conjunction with CASA software (Amann & Waberski, 2014). Due to the Segre-Silberberg effect (Segré & Silberberg, 1962a, 1962b) sperm cells will often congregate towards the periphery of chambers.

These significant differences in counts between operator-corrected scores and the original CASA scores highlight the need for post-analysis corrections to be applied by a human operator, as miscounted non-sperm cells can result in an increased proportion of immotile cells, giving skewed motility and kinematic parameters when using the CASA software.

5.1.2 The effect of time on sperm motility between loading into a chamber and being measured

The time between loading of each slide and CASA measurement was also investigated in this phase. The PR, NP and IM motility was stable for all the chambers during the first 5 minutes, in contrast to studies using goat sperm (Del Gallego, et al., 2017) where total motility of a capillary-loaded chamber was influenced after just 2 minutes, and bull sperm (Contri, Valorz, Faustini, Wegher, & Carluccio, 2010) where a capillary-loaded chamber's motility status suffered from time deterioration more severely than a droplet-loaded chamber. Taking into account these findings, an interval of 2 minutes from loading of the chamber and measuring was used to give sufficient time to allow the chamber/slides to settle and equilibrate.

5.1.3 Chamber effect on sperm motility

The data gained from this study shows that chamber configuration used with CASA software is a variable that can influence motility measurements. Although there was no significant difference observed for PR motility and total motility between chamber types, there were significant differences observed for sperm kinematic measurements such as VCL, VAP, and VSL, all of which occurred between the MicroCell and the drop-loaded coverslip slides. No significant differences were observed between the two drop-loaded coverslip slides for motility parameters, as seen in a similar study (Palacín, Vicente-Fiel, Santolaria, & Yániz, 2013).

The main contributing factor to these differences in results occurs due to the loading nature of the chambers. The MicroCell uses capillary action to load the sample, whereas the slide-coverslips are loaded by droplet displacement (Bompart et al., 2018). Capillary loading of samples is determined by a force known as the laminar

Poiseuille flow, which dictates that particles within a fluid travel perpendicular to the direction of flow, resulting in the heterogeneous distribution of particles within a suspension (Bompart, et al., 2018; Douglas-Hamilton, Smith, Kuster, Vermeiden, & Althouse, 2005; Vasseur & Cox, 1976). Segré and Silberberg first documented this phenomenon noting that particles will often concentrate towards the meniscus when this force is present (Segré & Silberberg, 1962a, 1962b).

The influence on sperm motility and distribution/concentration of sperm within capillary-loaded chambers and droplet-loaded chambers has been previously investigated in human sperm (Peng, Zou, & Li, 2015; Tomlinson, Turner, Powell, & Sakkas, 2001) and a variety of animal species (Christensen, Stryhn, & Hansen, 2005; Gloria, et al., 2013; Hoogewijs et al., 2012; Lenz, Kjelland, VonderHaar, Swannack, & Moreno, 2011; Palacín, et al., 2013). Capillary-loaded chambers were largely found to have reduced levels of motility and concentration when compared to droplet-loaded chambers, however care should be taken when comparing these studies as (i) sperm characteristics differ across species, (ii) differences between chamber types used that utilise the same loading method, and (iii) differences in CASA software used across the studies. Although differences in results have been well documented for these chamber types, they are unable to define which chamber type is inherently more accurate than another, but merely that differences in results can occur depending on the chamber configuration used (Bompart, et al., 2018; Kuster, 2005). As such, care should be taken when making comparisons between studies.

5.2 Diluting cryoprotectant containing semen samples in conjunction with CASA software

To effectively measure sperm kinematics using CASA software, there must be a defined maximum working sperm concentration of a measured sample in order to reduce collisions of spermatozoa (Contri, et al., 2010; Garrett, Liu, Clarke, Rushford, & Baker, 2003; World Health Organization, 2010). Dilution of samples containing sperm concentrations above the nominated upper limit is recommended to be done with sperm-free seminal plasma from the same man whom produced the sample, to avoid changing the environment spermatozoa are exposed to (World Health Organization, 2010). Various media have been found to be suitable in diluting neat semen samples (Farrell, Foote, McArdle, Trouern-Trend, & Tardif, 1996), and whilst the use of media of defined composition is simpler than preparing sperm-free seminal plasma, there is a paucity of work on the negative aspects of diluting the semen and potential artefacts that may be introduced; including the dilution of semen containing cryoprotectants.

5.2.1 Diluting neat semen samples

The initial addition of Quinn's Advantage™ Medium with HEPES, G-MOPS^{TM} PLUS and SP to neat semen samples did not alter the PR motility significantly, as seen in a similar study by Mostafapor and Farrokhi (2014) who compared SP and two common handling media on sperm motility. Farrell et al. (1996) also concluded that the initial addition of three handling media did not significantly alter the PR motility of human sperm. The addition of both commercial handling media caused a slight decrease to the PR motility of the neat sample, whereas the SP addition saw a slight increase to the PR

motility; however none of these were statistically significant compared to the original neat sample. The use of SP as a diluent comes with some inherent issues, as seminal plasma components can vary between samples and men, as will the consistency and viscosity of the seminal plasma (Puppo & Puppo, 2016; World Health Organization, 2010). The viscosity of the medium through which spermatozoa are suspended may have a direct result on kinematic patterns (Ishimoto, Gadêlha, Gaffney, Smith, & Kirkman-Brown, 2018; Wang, He, & Zhang, 2016), which is a potentially uncontrolled variable and so should be kept in mind when comparing studies. This was avoided in the present study as the same pooled SP was used for the dilution of all samples.

5.2.2 The effect of cryoprotective agents on sperm motility, prior to cryopreservation

The cryopreservation of spermatozoa has always resulted in detrimental effects on post-thaw survival and fertilisation capabilities (Nijs & Ombelet, 2001; Sharma, Kattoor, Ghulmiyyah, & Agarwal, 2015). However even before spermatozoa are exposed to such conditions, cytotoxicity is present due to the exposure of spermatozoa to cryoprotective agents that are obligatory for surviving the freezing process (Gao et al., 1995). Studies have previously illustrated the increased alteration to acrosomal morphology with the increased exposure of spermatozoa to glycerol (Buhr, Fiser, Bailey, & Curtis, 2013; Di Santo, et al., 2012; Si, Zheng, Li, Dinnyes, & Ji, 2004), occurring in tandem with alterations to sperm head membrane fluidity (Gao et al., 1995; Pettitt & Buhr, 2013).

The data gained from this study highlights glycerol's direct negative influence on the motility of spermatozoa. Even after a short exposure time, glycerol's affect can be seen

with a statistically significant decrease in samples PR motility across all concentrations of glycerol used in the present study. This also appeared to occur in a dose-dependent fashion, in that the higher concentration of glycerol added to the semen sample, the largest decrease in progressive motility was seen. The CPM had a statistically similar effect on PR motility to the 5% glycerol even though the CPM includes several constituents beneficial for sperm motility, such as glucose (Amaral, Paiva, Baptista, Sousa, & Ramalho-Santos, 2011). Whilst the exact mechanism for the loss of motility was not identified in the present study, alterations to spermatozoon membrane fluidity, disruption to the mitochondrial membrane, and osmolarity changes leading to cell death would be consistent with this increase in shift from PR to IM spermatozoa.

5.2.3 Dilution of cryoprotectant-containing semen samples with handling media

The addition of both of these handling media to cryoprotectant-containing samples had a statistically significant decrease on the PR motility, including the commercially engineered CPM. Quinn's Advantage™ Medium with HEPES and G-MOPS^{TM} PLUS had a similar detrimental effect on PR motility on cryoprotective-containing samples, suggesting that the decrease in PR results was through a similar interaction for both reagents. The 10% glycerol containing sample saw the largest decrease in PR motility when diluted with both of the handling media.

One possible factor explaining the decreased motility following dilution is a phenomenon known as dilution effect. This occurs when semen samples are diluted with artificial extenders to lowered concentrations, resulting in decreased motility patterns, compromise of acrosomal integrity and sperm viability (Pinyopummin et al.,

2018). This effect of dilution has been demonstrated in many animal models, including stallion (Hayden et al., 2015), boar (Lipensky, Lustykova, Frydrychova, Rozkot, & Vaclavkova, 2013), bull (Garner, et al., 2001), tomcat (Prochowska, Niżański, Ochota, & Partyka, 2014) and rabbit (Johinke, Graaf, & Bathgate, 2015). Human sperm has been demonstrated to be more resistant to these dilution effects than some animal models, such as rabbit sperm (Farrell, et al., 1996). The dilution effect is thought to be brought about by the decreased exposure of spermatozoa to beneficial components of seminal plasma, resulting in reduced motility and functional capacitation leading to premature cell death in-vitro (Maxwell & Johnson, 1999). In animal models, the apparent reversal of the dilution effect has been observed when diluted semen samples have been supplemented with seminal plasma restoring sperm motility (Bernardini et al., 2011; Hernández et al., 2013; Mata-Campuzano et al., 2015; Neuhauser, Dörfel, & Handler, 2015).

In the current study however, the dilution effect alone does not explain the dramatic decrease in PR motility of the neat glycerol containing samples. As the initial addition of both handling media in the 1:5 dilutions to neat semen did not see a decrease in PR motility, the further small addition of either 5% or 10% v/v neat glycerol would not cause such a vast decrease to motility if it were the influence of dilution alone. This would suggest that there is another mechanism involved, most likely the alteration to membrane fluidity caused by the addition of glycerol, then followed by osmolarity changes brought about by the dilution with non-seminal plasma diluents. Supporting this notion is that the 10% glycerol containing semen sample was seen to have a statistically significant reduction in PR motility when diluted with both handling media, when compared to the CPM containing samples. If the dilution of non-seminal plasma

diluents was the sole factor causing a reduction in PR motility, then the CPM would have had a larger decrease, as this was a 1:5 dilution, opposed to a 10% v/v addition of glycerol. Alterations to spermatozoa membrane that are caused by glycerol (Keel & Webster, 1990) may leave spermatozoa more vulnerable to dilution effects brought about by further diluting with non-seminal plasma diluents. More research is needed to further uncover if this is a result of glycerol leaving spermatozoa more susceptible to osmotic alterations when further diluted with non-seminal plasma diluents; or that there is an undiscovered interaction between glycerol and constituents of the handling media that has yet to be revealed.

5.3 The cryopreservation of human semen

With the further development of cryoprotective media in the long-term storage of spermatozoa, there has been a lack of literature comparing modern media to the seminal cryoprotective agent glycerol. Following Polge, Smith, and Parkes (1949) discovery of the use of glycerol in the cryopreservation of sperm, companies have furthered this phenomena and engineered CPM, containing CPAs and various extenders with the aim in increasing post-thaw sperm viability (Kalthur et al., 2012). As these CPM are further developed and refined, the costs associated with them also rise, and as fertility laboratories use them on a regular basis it is of interest to compare their abilities when cheaper and effective alternatives are available; such as neat glycerol.

The cryopreservation process imposes oxidative stress on sperm membranes, resulting in many functional implications such as a reduction in anti-oxidant enzyme activity,

sperm organelle damage and a reduction in sperm motility (Bucak, Sarıözkan, Tuncer, Ulutaş, & Akçadağ, 2009; Bucak, Tuncer, Sarıözkan, & Ulutaş, 2009; Partyka, Lukaszewicz, Nizanski, 2012), as well as a reduction in intracellular antioxidant capability following the thawing process (Atessahin, Bucak, Tuncer, & Kızıl, 2008; Tuncer et al., 2010). Mammalian spermatozoa in particular are subject to increased oxidative stress resulting in membrane-lipid peroxidation when compared to other species (Atessahin, et al., 2008; Büyükleblebici et al., 2014). With CASA software being able to identify kinematic values of sperm far more accurately than an operator, this gives the ability to detect how environmental changes that sperm are exposed to during cryopreservation and thawing can influence sperm kinematic values (Yeste et al., 2018).

5.3.1 The effectiveness of neat glycerol and a commercial CPM on the cryopreservation of human semen

The current study has illustrated the survival capabilities of human spermatozoa when cryopreserved with neat glycerol at 5 and 10% v/v and a commercial CPM, followed by subsequent thawing. As expected, the freezing process had negative impacts on sperm kinetic measurements, seeing a reduction in PM regardless of the cryoprotective agent used. The most effective in preserving the PM of spermatozoa was the CPM, with a reduction of ~60% in total sperm motility, as seen in similar studies suggesting an acceptable degree of drop-off of ~50% decrease in sperm motility post-thaw (Oberoi, Kumar, & Talwar, 2014; World Health Organization, 2010). There was a linear relationship observed between reduced survival capabilities of the spermatozoa post-thaw with the increase in neat glycerol introduced to the system prior to the freezing

process, with the 5% glycerol providing greater recovery of motility when compared to 10% glycerol.

There have been few recent publications comparing glycerol's ability to effectively cryopreserve human spermatozoa at differing concentrations compared to modern commercial media; however there have been several studies investigating this in animal models. Bovine studies have investigated glycerol's effect at varying concentrations on sperm cryosurvivability with Büyükleblebici et al. (2014) noting that a 5% glycerol concentration yielded greater progressive motility post-thaw when compared to 7% glycerol. Villaverde et al. (2013) explored this same notion concluding that domestic cat sperm exposed to 5% glycerol offered greater post-thaw yield of motile spermatozoa to 3% and 7%, however there was no difference in other kinetic parameters. Buhr, Fiser, Bailey, and Curtis (2013) examined swine sperm survivability, which suffers greater toxicity from glycerol than other mammalian species, finding that 4% glycerol was the more successful compared to 0%, 2% and 8% dilutions. From this data it is apparent that a glycerol concentration of ~5% v/v yields the most successful post-thaw kinetic results in mammalian species.

Sperm kinematic parameters for the most part did not vary significantly between post-thaw groups, with the CPM usually providing slightly increased values in these ranges; however STR and BCF scores were higher in both glycerol groups than the CPM. VCL, VSL and VAP were each higher in the 5% glycerol group when compared to the 10% glycerol group, this is in line with a similar study investigating ursine semen diluted with glycerol, where lower concentrations of glycerol were seen to have increased measurements for VCL and ALH (de Paz et al., 2011).

Another factor that must be taken into account when making comparisons between studies is the rate of freezing (Gao & Critser, 2000; S. Kumar, Millar, & Watson, 2003) as faster than optimal freezing protocols can result in lethal cold shock (Watson, 2000). The formation of intracellular ice and osmotic stress are two main factors for reduced levels of sperm recovery post-thaw, with glycerol being directly related to both of these (de Paz, et al., 2011; Motamedi-Mojdehi, Roostaei-Ali Mehr, & Rajabi-Toustani, 2014; Sieme, Oldenhof, & Wolkers, 2015). However human sperm are relatively immune to rapid cooling between 1-25˚C/min, and as such the initial rate of cooling is more crucial in comparisons between animal models than human (Clarke, Liu, & Baker, 2004; Mortimer, 2004).

Our data suggests that for the conventional parameter of post-thaw progressive motility, the CPM offered greater protection compared to both the 5 and 10% glycerol (11%, 4% and 3% PR motility respectively). The kinetic parameters however did not vary significantly between groups with the CPM usually providing slightly increased values in these ranges; with the exception of STR being higher in both glycerol groups.

5.4 Sperm preparation for ART

The isolation of motile sperm for use in ART is a prerequisite for increasing fertilisation rates (Enciso et al., 2011; Fauque et al., 2014). Frozen-thawed semen samples are known to have decreased levels of motility and viability compared to fresh samples (Petyim, Neungton, Thanaboonyawat, Laokirkkiat, & Choavaratana, 2014), so the investigation of how to best prepare these samples for use in ART is of clinical importance. There are two methods of separation that are the most commonly employed due to their effectiveness in yielding motile sperm and their relative ease in

performing; these being DGC and the swim-up method (Jayaraman, Upadhya, Narayan, & Adiga, 2012; Rappa, et al., 2016; World Health Organization, 2010). The swim-up method in the present study was a DSW that involves no centrifugation, opposed to the conventional swim-up method where there is centrifugation and pelleting of the sample (Overstreet, Yanagimachi, Katz, Hayashi, & Hanson, 1980; World Health Organization, 2010). Each method of isolation has its own benefits and drawbacks in terms of sperm kinematics and concentration in the processed sample, with ultimately the insemination technique to be used dictating which isolation method is the most ideal (Henkel, 2012; World Health Organization, 2010).

One key feature of the present study was that semen samples were cryopreserved and thawed prior to sperm isolation, as the order in which sperm are cryopreserved and isolated is a topic of interest in the current literature and has conflicting conclusions as to which provides the greatest recovery of functional sperm (Esteves, Sharma, Thomas, & Agarwal, 2000; Palomar Rios, Gascón, Martínez, Balasch, & Molina Botella, 2018; Petyim, et al., 2014).

5.4.1 Comparison of density gradient centrifugation, direct swim-up and the Seaforia system

The results of the present study highlighted the capabilities of each of the isolation techniques and also provided kinematic values for the post-isolated sperm. The DSW and SFR had similar results in concentration and kinematics with no significant differences observed between them. This would be expected as the mechanism for sperm separation remained constant between the two, while differences in the methodology occurred in how the overlay media was applied and extracted. The SFR

has been specifically designed for non-specialised fertility laboratories and ease of use, with it being easier to perform than the DSW method. In the DSW method there are two notable stages that pose risk to the efficacy of the yield, these being the steps of overlaying the media on top of the liquefied sample, and also when extracting the overlay media containing the motile sperm post-incubation (Henkel & Schill, 2003; Mortimer, 2000). These stages pose risk as the operator may create an uneven interface between media and semen when overlaying, and by taking up the original seminal plasma as well as the overlay media when extracting post-incubation (Henkel & Schill, 2003). The SFR circumvents both of these flaws by the design of its chamber that houses the semen and overlay media, whereby the media is inserted through a secondary chamber that overflows into the main chamber, whereby subsequent migration of motile sperm occurs into the overlay chamber which is then extracted easily through a set-depth pipette, ensuring the interface is not disturbed. From its relative of use the SFR was the recommended extraction method compared to the DSW. However the SFR kit itself is much more expensive compared to the standard consumables that are required to perform the DSW.

Several differences in isolated sperm parameters occurred between the DGC and both swim-up methods. The most apparent difference lay in the parameters of sperm concentration and PR motility, with the swim-up methods having significantly higher PR motility, whereas the DGC had significantly higher sperm concentrations post-isolation. This echoes previous studies finding DGC to having superior yields of sperm concentration when compared to swim-up methods (Fácio, Previato, & Machado-Paula, 2016; Moohan & Lindsay, 1995; Ren, Sun, Ku, Chen, & Wu, 2004). Work from

Ghaleno et al. (2014) however is in contrast to this, with their group showing DGC to have increased populations of PR motile sperm when compared to DSW.

There are several difficulties when making comparisons between studies as to which sperm preparation method is superior to another. Some of these include differences in the methodologies used in isolation techniques, such as variances to centrifugal time and force, different handling media and density gradients used, the original quality of the semen sample, incubation times, CASA software used, chamber types used in measuring samples and DNA fragmentation tests employed, to name a few (Aitken, et al., 2014; Guimarães et al., 2014; Yeste, et al., 2018).

5.4.2 Clinical implications of isolation methodology

Research has been conducted on finding optimal concentrations and motility for sperm to be used for various insemination techniques, much of the time with conflicting results (Dickey, Pyrzak, Lu, Taylor, & Rye, 1999; Horvath, Bohrer, Shelden, & Kemmann, 1989; Merviel et al., 2010; Sakhel, Abozaid, Schwark, Ashraf, & Abuzeid, 2005; Van Voorhis et al., 2001). Recently there has been a focus on the functionality of sperm in isolated yields, rather than the rudimentary parameters of semen analyses, in predicting fertilisation outcomes (Barratt, Tomlinson, & Cooke, 1993; Borini et al., 2006; Bungum et al., 2004; Hirano et al., 2001; Liu, Clarke, & Baker, 1991; Seli, Gardner, Schoolcraft, Moffatt, & Sakkas, 2004; World Health Organization, 2010). Kinematic values for predicting pregnancy have been investigated with several parameters being identified as potential prognostic tools. Hirano et al. (2001) observed that VCL and the distance travelled by rapid sperm movement may be positively correlated with fertilisation capabilities, with Ren et al. (2004) concluding that VSL was

seen to be an indicator for fertilisation rates in IUI pregnancies. Even for manual insemination of oocytes using ICSI, research has highlighted the positive correlation with sperm VSL and successful fertilisation outcomes (Van den Bergh, Emiliani, Biramane, Vannin, & Englert, 1998). In the present study, both swim-up methods were found to have slightly higher levels of VAP, VSL, VCL and STR when compared to DGC and so the SFR would be the recommended isolation method when higher numbers of sperm concentration for insemination are not required.

Another indicator of sperm functionality and prognostic value of fertilisation is the level of DNA fragmentation within sperm subpopulations (Velez de la Calle et al., 2008; Zini, Boman, Belzile, & Ciampi, 2008). Although the current study did not assess DNA damage within post-isolated samples, research from other groups has shown that processing sperm through DGC results in increased levels of DNA fragmentation when compared to swim-up methods (Oguz et al., 2018; Volpes, et al., 2016). However there is no consensus in the literature as to which method yields sperm with lower DNA fragmentation, with contradictory findings from researchers stating that DGC provides a lower yield of sperm with DNA fragmentation (Amiri, Ghorbani, & Heshmati, 2012; Xue et al., 2014), or that there is no difference between the two (Jayaraman, et al., 2012; Zhao, Yang, Shi, Luo, & Sun, 2016). A possible factor influencing the comparison of these studies is that not all swim-up methods utilise a centrifugation step, however even this has been shown to not effect DNA fragmentation in isolated sperm using the direct swim-up or a pellet swim-up (Younglai et al., 2001). It is apparent that there is still no consensus in the literature as to which isolation method is superior to another; the present study has provided more data to the body of work in trying to elucidate

what is an often controversial area of research in selecting the best isolation technique for insemination.

CHAPTER 6

SUMMARY

6 Summary

6.1 Clinical relevance and implications

The benefit of this study was that the same pooled semen samples were used between control and treatment groups for each relative experimental stage, as opposed to using different samples for each treatment group. The technical investigation phase highlighted that many variables can influence CASA software in measuring semen samples, and as such an emphasis should be placed on these when conducting and comparing studies. Although CASA software can give reproducible results, these must still be viewed and corrected where necessary by an operator, with fully autonomous semen analyses from CASA software not achievable in the present study. The MicroCell was found to give consistent readings, was easy to use and disposable and was the preferred chamber type from this study.

In investigating cryoprotectant influence on sperm parameters prior to cryopreservation, this study has illustrated the toxic yet necessary influences of cryoprotectant agents, and that sperm survivability during cryopreservation is ultimately a result of the toxic and protective capabilities during this process. Due to this, in order to more effectively develop and refine these media, the initial stage of toxicity to sperm cells imposed by such media is vital. Further relationships between cryoprotectant containing samples, cell-free seminal plasma and the two commercial gamete handling media also alluded to the precarious stage of further diluting semen with non-seminal plasma components. As CASA software requires specific concentration limits of sperm samples to be accurately measured, this is a potential source of error.

The final phase of this study investigating isolation techniques for frozen-thawed sperm has provided data for addition to the current literature as to which isolation method is best. Both swim-up methods were found to have increased progressive motility and sperm kinematics such as VSL, VCL and VAP, which have been associated with positive fertility outcomes. Density gradient centrifugation allowed for a greater yield of sperm concentration, and ultimately the type of ART to be pursued to achieve fertilisation will determine which isolation method is the most effective.

6.2 Limitations

As semen samples were obtained from men undergoing fertility investigation, there may have been an increased bias in the sample population for decreased overall sperm parameters, however as the clinical relevance of the study is for ART, this bias is potentially not significant. To strengthen the data gained from the study, increased sample sizes for each phase of the study would have been beneficial. The setting of sperm head area 1-100um^2 determined by CASA may also have influenced the CASA readings.

6.3 Future research

Although the data in the present thesis contained many kinematic values for sperm motility, there were several sperm functionality tests that were not included. One sperm function test that would benefit the study is sperm vitality testing, done so through hypo-osmotic swelling testing. This method of testing exposes sperm to hypo-osmotic conditions whereby normally functioning sperm will swell, particularly the tail

area, as water molecules pass through the plasma membrane in an effort to reach osmotic equilibrium (Jeyendran, Ven, Perez-Pelaez, Crabo, & Zaneveld, 1984). This testing method could be applied to sections 3.4.2 and 3.4.3 of the current thesis, where the influence of cryoprotective agents and diluents on sperm motility was measured. The exact cause for the increase of immotile sperm populations when exposed to glycerol and common gamete handling diluents was not elucidated, and this testing method could give possible insights into this and if the sperm membrane was affected at these points. A morphology test could also be employed in these same experiments to add further evidence for sperm morphological disruptions.

Sperm DNA fragmentation has largely been incorporated in fertility laboratory testing for male factor infertility and there are a number of test types available to measure the level of DNA fragmentation in a semen sample (Evenson, Darzynkiewicz, & Melamed, 1980). The Sperm Chomatin Dispersion, or HALO test, measures the absence of sperm that are damaged by DNA fragmentation and benefits in being easy to perform and without the use of a flow cytometer, with only light microscopy required to analyse. With the focus on producing more functional sperm populations in processed samples to be used in ART, the inclusion of DNA fragmentation testing would add weight to the findings from section 3.4.4.

There are a plethora of sperm isolation devices available on the market, each relying on different methods of sperm separation. Many of these devices rely on a variation of the swim-up method, such as the Seaforia^{TM} device in the present thesis, however recently a new device has been introduced that utilises microfluidics to separate sperm. The FERTILE (Zymot) device (DxNow Inc., Gaithersburg, MD, USA) has been

shown to give yields of highly motile sperm, with concentration comparable to DGC and sperm with nearly no DNA fragmentation (Quinn et al., 2018). This device has only just been recently introduced to the market and requires further testing to compare its efficacy to other separation techniques. As it does not require a centrifuge and involves only two pipetting stages, it promises to be a convinient and easy method to separate sperm in non-specialised fertility laboratories.

CHAPTER 7

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7. References

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CHAPTER 8

APPENDICES

The appendices are not available in this version of the thesis,

at the author's request.