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## The effect of antioxidant supplementation on human sperm cryopreservation

Katrina E. Taylor  
*Edith Cowan University*

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# **The Effect of Antioxidant Supplementation on Human Sperm Cryopreservation**

**Katrina E. Taylor**

**Supervised by:**

**Dr Peter Roberts and Dr Peter Burton**

This Thesis Submitted in Partial Fulfillment of the Requirements for  
the Award of

**Bachelor of Science (Human Biology) Honours**

In the Faculty of Computing, Health and Science,  
Edith Cowan University, Joondalup, Western Australia.

**Date: 4<sup>th</sup> November 2005**

## ABSTRACT

Semen cryopreservation has an important role in assisted reproductive technology however, the cooling, freezing and thawing processes often result in a significant loss of sperm motility, viability and nuclear integrity. The destructive effects of cryopreservation are significantly exacerbated in samples exhibiting low sperm number and poor morphological characteristics. Recent research into infertility has focused on the correlation between excessive oxidation and subfertility, in particular radical induced lipid peroxidation within the phospholipid bilayer of the spermatozoon plasma membrane and the promotion of cellular damage as a result of antioxidant insufficiency. The aim of this study was to evaluate the effect of vitamin E on the survival and integrity of sperm from oligozoospermic and teratozoospermic men following cryopreservation.

Ejaculated semen samples from 43 men undergoing assessment for infertility were identified as normal (n=23) or abnormal (n=20) according to WHO standards. Each semen sample was divided into 3 aliquots: The first fraction remained untreated and the second and third fractions were treated with cryo-media containing either 100 $\mu$ M or 200 $\mu$ M of the vitamin E analogue Trolox (6-hydroxy-2,5,7,8-teramethylchroman-2-carboxylic acid) prior to freezing. Post-thaw analysis included sperm survival rate, vitality staining, and assessment of DNA fragmentation using the TUNEL assay.

Motile sperm concentration and morphological normality was significantly higher in normozoospermic semen compared to the abnormal samples ( $P < 0.001$ : unpaired *t*-tests). Whole semen volume was significantly higher in abnormal samples ( $P < 0.05$ : unpaired *t*-tests). Post-thaw analysis found significant differences in post-thaw vitality between the normozoospermic and abnormal samples ( $P < 0.05$ : Unpaired *t*-tests). Post-thaw survival and DNA

fragmentation assay revealed no differences between normal and abnormal semen groups.

The addition of vitamin E at concentrations of 100 $\mu$ M and 200 $\mu$ M did not significantly improve survival rate, post-thaw vitality or the degree of DNA fragmentation in the normal or abnormal semen samples. However, the results obtained were highly variable and an improvement in post-thaw survival was seen in 12 of the 43 semen samples from both the normal and abnormal groups. The variable response to vitamin E treatment observed in this study, suggests that the antioxidant ability of vitamin E during cryopreservation may depend on the oxidative status of individual semen samples.

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## ABBREVIATIONS

<b>3'OH</b>	Three prime hydroxyl
<b>ANOVA</b>	One-way analysis of variance
<b>Bp</b>	Base pairs
<b>CaCl<sub>2</sub></b>	Calcium chloride
<b>Ca Lactate</b>	Calcium lactate
<b>cAMP</b>	Cyclic Adenosine monophosphate
<b>DAB</b>	DiAmino Benzidine
<b>dH<sub>2</sub>O</b>	Distilled water
<b>DNA</b>	Deoxyribonucleic acid
<b>E&amp;N</b>	Eosin and nigrosine
<b>g</b>	Grams
<b>HCl</b>	Hydrochloric acid
<b>HSA</b>	Human serum albumin
<b>KCl</b>	Potassium chloride
<b>L</b>	Litre
<b>LN<sub>2</sub></b>	Liquid nitrogen
<b>M</b>	Molar
<b>MgCl<sub>2</sub></b>	Magnesium chloride
<b>MgSO<sub>4</sub></b>	Magnesium sulphate
<b>mL</b>	Millilitre
<b>mM</b>	Millimole
<b>n</b>	Number
<b>NaCl</b>	Sodium chloride
<b>NaH<sub>2</sub>PO<sub>4</sub></b>	Sodium dihydrogen phosphate
<b>NaHCO<sub>3</sub></b>	Sodium bicarbonate
<b>NaOH</b>	Sodium hydroxide
<b>°C</b>	Degrees centigrade
<b>p</b>	Probability of chance occurrence
<b>PBS</b>	Phosphate buffer saline

<b>pH</b>	Hydrogen ion (H <sup>+</sup> ) concentration
<b>ROS</b>	Reactive oxygen species
<b>TdT</b>	Terminal deoxynucleotidyl transferase
<b>TUNEL</b>	Terminal deoxynucleotidyl transferase dUTP nick end labeling
<b>v:v</b>	Volume per volume
<b>w/v</b>	Weight per volume
<b>WHO</b>	World Health Organisation
<b>μg</b>	Micrograms
<b>μL</b>	Microlitre
<b>μM</b>	Micromole
<b>%</b>	Percent
<b>x</b>	Times

**CHAPTER 1:**

**INTRODUCTION**

## 1.1 BACKGROUND

Since the discovery of the cryoprotective capabilities of glycerol in the 1950's, human spermatozoa have been successfully cryopreserved and used during assisted reproductive technology. The cryopreservation of sperm has since become an integral part of the IVF program, although most of the improvements to the freezing and thawing processes over this time have been based on practical experience rather than scientific study (Brinsden & Rainsbury, 1992, p. 183; Royere et al., 1996). More recent developments in improving the outcomes of sperm cryopreservation include manipulating the biochemical and physical conditions of semen preparation techniques pre and post-thaw (Royere et al., 1996). Although many advances in semen cryobiology such as the introduction of alternate cryomedia and handling media, swim-up and density gradient separation and automated freezing have shown an overall improvement in sperm survival, a significant loss of motility and vitality of human sperm following cryopreservation can still be observed (Burton, Bellinge & Counsel, 2003; Gao et al., 1993).

During the cryopreservation of spermatozoa, the cooling, freezing and thawing processes have the potential to cause ultrastructural damage to various regions of the cell. The spermatozoa generally exhibit a significant loss of motility and viability following cryopreservation, and the fertilization capacity of the sample decreases *in vivo* due to the reduced number of motile sperm remaining in the sample (McLaughlin, 2001, p. 264). On average, 50% of live spermatozoa are injured during the cryopreservation process (Burton, Bellinge & Counsel, 2003; Gao et al., 1993).

A normal human ejaculate contains more morphologically abnormal sperm than any other mammalian species (Aitken & Sawyer, 2003) and few post-thaw samples are adequate for some forms of assisted reproduction (McLaughlin, 2001, p. 264). The individual suitability to cryogenic preservation cannot be entirely predicted (Giraud et al 2000) and the degree of functionally intact sperm remaining after freezing varies between individuals. Additionally,



minor inconsistencies in post-thaw survival rate can be detected between different ejaculates from the same person (Heuchel, Schwartz & Czyglik, 1983; Mc Laughlin, 1993). Furthermore, the percentage of cellular damage observed following cryopreservation of oligozoospermic samples is substantially higher than that of normal samples (Counsel, Bellinge & Burton, 2004; Hughes et al., 1999).

Over the past fifteen years, there has been an increasing requirement for sperm cryopreservation. Donor sperm is routinely quarantined for recipient protection, and those undergoing medical treatment such as vasectomy or chemotherapy can store sperm for future use. Individuals who are unable to produce samples at the essential time in their partners ovulatory cycle due to absence or pressure, are able to utilize sperm cryopreservation for later use in assisted reproduction (McLaughlin, 2002). In addition, the stress of producing a semen sample for assisted reproduction on the day of oocyte collection can be significantly reduced if cryogenically preserved samples can be used (Brinsden & Rainsbury, 1992, p. 183).

The World Health Organisation (1999) has acknowledged a decline in the average sperm counts in men from several developed countries, while the incidence of testicular cancers and urogenital abnormalities is thought to be increasing. The optimization of the cryopreservation process is particularly significant to oligozoospermic men and to individuals who require medical treatment that reduces or permanently destroys their reproductive capability, such as chemotherapy, radiation therapy, or specific surgical procedures (Chaudhary & Hallas, 2003; Lass et al, 1998; Tournaye et al., 2004).

## **1.2 SIGNIFICANCE OF THE STUDY**

Recent research into subfertility has focused on the involvement of an excessive production of endogenous reactive oxygen species during spermatogenesis and sperm maturation (Aitkin & Sawyer, 2003; Anzar et al., 2002; Gil-Guzman et al., 2001; Hendin et al., 1999; Parinaud et al., 1997).

The findings have shown a correlation between excessive oxidation and male subfertility and an investigation by Aitken & Krausz (2001) has indicated that the ability of any pregnancy to continue to full term is greatly reduced in samples that have been subject to oxidative stress. Spermatozoon oxidation has been implicated as a major cause of mitochondrial and plasma membrane damage in addition to chromosomal injury and DNA fragmentation. Lipid peroxidation in the spermatozoon plasma membrane is thought to be further promoted by cryopreservation, a process that appears to be compounded by the poor semen parameters of oligozoospermic samples (Aitken et al., 1998; Allamaneni et al., 2005; Gil-Guzman et al., 2001).

The adverse effects of oxidation can be reduced by antioxidants that are produced in the germinal epithelia, can be found within spermatozoa, and are present as a component of seminal plasma. In addition, the effect of endogenous antioxidants in poor semen samples is often diminished while the concentration of reactive oxygen species is abnormally high (Aitken et al., 1998).

An animal study involving boar sperm by Pena et al, (2004) has established that antioxidant supplementation (specifically vitamin E) of the cryo-media prior to cryopreservation, significantly improved spermatozoon post-thaw survival rate and motion parameters. In the boar, the ejaculate is produced in two portions. The first contains the majority of sperm, whilst the second portion is of reduced number and of poorer quality. Vitamin E supplementation in the pre-freeze media caused an increased sperm survival rate in the second portion but not in the first. In the human, vitamin E supplementation of cryomedia has produced variable results (Askari et al., 1994). This could equate to the distinct differences in the number and quality of sperm found in normozoospermic and oligozoospermic samples.

### **1.3 AIM OF THIS STUDY**

The overall aim of this study was to optimize cryopreservation conditions for human sperm by testing the hypothesis that antioxidant supplementation to the pre-freeze media will improve the post-thaw sperm characteristics in samples from oligozoospermic men.

### **1.4 EXPERIMENTAL DESIGN**

Excess semen samples were obtained from Concept Fertility Clinic (King Edward Memorial Hospital, Subiaco, Western Australia), following routine semen analysis. Normozoospermia and oligozoospermia was determined during routine assessment by Concept laboratory staff. Each sample was divided into three aliquots. The first and second fractions were exposed to cryomedia containing vitamin E at two different concentrations prior to cryopreservation in liquid nitrogen. The third fraction functioned as a control group. Post-thaw analysis of each fraction included assessment of motile sperm to determine survival rate, the eosin-nigrosine dye exclusion staining protocol to measure post-thaw vitality, and the degree of DNA fragmentation was determined using the TUNEL assay. Statistical assessment determined any variation in post-thaw survival parameters between treated and control groups, and established any differences between the normal and abnormal semen samples.

## **CHAPTER 2:**

## **LITERATURE REVIEW**

## 2.1 SPERMATOZOON ANATOMY

Mature sperm are highly specialized cells approximately 50µm long. The spermatozoon is organized into three major regions; the head, midpiece, and tail, with each region further arranged into various functional subdivisions. (Figure 1). Spermatozoa lack most of the intracellular structures that are seen in somatic cells, which facilitates streamlining for optimal motility. During normal sperm development, the excess cytoplasm and most organelles from the originating spermatid are compressed downward from the head and detached from the flagellum during spermiogenesis (Martini, 2001, p. 1025). Morphologically abnormal spermatozoa arise when this process is defective or incomplete (Bearer & Friend, 1990).

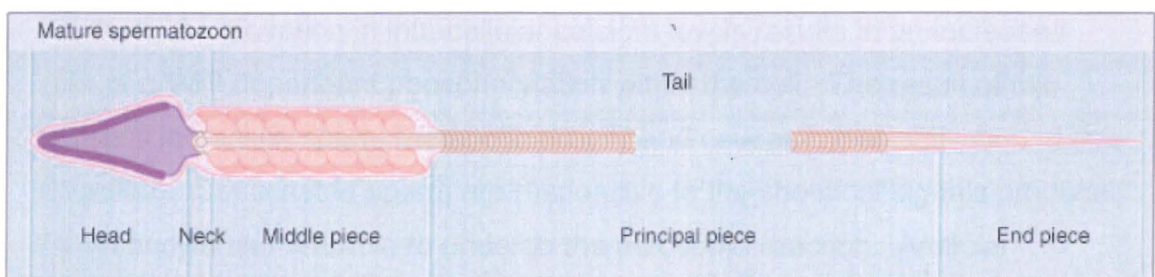


Figure 1: Regional Organisation of the Spermatozoon (Ref: Young & Heath, 2002)

### 2.1.1 The Sperm Head

The sperm head contains the acrosome and nucleus and is subdivided into the acrosomal cap, equatorial region, and post-acrosomal region. Each subdivision becomes functional during different stages of fertilization (Johnson & Everitt, 2000, p. 58-59). The nucleus is elongated and flattened, and contains highly condensed chromatin and a little nucleoplasm (Martini, 2001, p. 1025). During spermiogenesis, chromatin condensation begins beneath the acrosomal cap and gives rise to the various spermatozoon shapes observed between species. As the spermatozoon takes form during this process, the golgi apparatus migrates across one end of the nuclear membrane and fuses to become the acrosomal cap (Figure 2a) (Johnson & Everitt, 2000, p. 58-59). The acrosomal cap contains a high concentration of glycoprotein granules

(involved with capacitation), carbohydrates (energy), and the lytic enzymes required for the acrosome reaction (Bearden, Fuquay & Willard, 2004, p. 175).

#### **2.1.1.1 Capacitation**

Capacitation normally occurs once the sperm have come in to contact with the uterine and follicular fluids of the female reproductive tract and provides the spermatozoa with the ability to penetrate the zona pellucida during fertilization (Johnson & Everitt, 2000, p. 58-59). During sperm capacitation, the configuration of the glycoproteins and lipids on the spermatozoon surface are rearranged and the membrane potential is altered. This causes a decrease in the stability of the sperm plasma membrane, produces an increase in intracellular cAMP and allows an increase in membrane permeability to calcium. An elevation in intracellular calcium levels results in an increased rate of cAMP-dependent phosphorylation within the cell. The result of this process increases sperm metabolic activity and causes hyper-activation of the flagellum. Capacitated sperm are responsive to the chemical signals produced by an oocyte and are able to undergo the acrosome reaction. Artificial capacitation can be induced and reversed *in vitro* (Johnson & Everitt, 2000, p. 161-162; Kalthoff, 2001, p. 77).

#### **2.1.1.2 Acrosome reaction**

Capacitated sperm normally undergo the acrosome reaction when they encounter the zona pellucida of an oocyte. The sperm however, must first pass through the cumulus and corona radiata that surround the zona pellucida. The sperm plasma membrane contains a membrane protein (PH20) that is capable of digesting a passage through the cellular layers (Kalthoff, 2001, p. 83). Additionally, the chemical signals of progesterone are thought to cause the sperm plasma membrane to become leaky and allow the release of the acrosomal enzyme hyalouronidase that is also capable of digesting a path through the oocytes protective outer cellular layers and allow sperm/zona interaction. Some sperm can prematurely undergo the acrosome reaction in

response to progesterone, however only sperm with intact acrosomes are capable of zona binding and fertilization (Johnson & Everitt, 2000, p.163).

The acrosome reaction occurs when capacitated sperm bind via a sperm membrane binding site ( $\beta 1,4$ -galactosyl transferase) to a glycoprotein receptor (ZP3) on the zona pellucida. The association occurs at the tip and equatorial region of the acrosome and causes a further influx of calcium, increase in cAMP and a rise in pH within the spermatozoa. Once bound, the interaction of ZP3 with the sperm plasma membrane initiates acrosomal swelling and fusion of the outer acrosomal membrane with the surface membrane. The lytic enzymes of acrosome vesicles are exocytosed and digest a section of the intercellular matrix of the zona pellucida. The ZP2 binding sites of the inner acrosomal membrane are exposed during this process and maintain a bond between the spermatozoon and zona pellucida. This action ends as the remaining equatorial and postacrosomal regions of the sperm plasma membrane acquire the potential to fuse with the plasma membrane of the oocyte. Acrosome reacted sperm only remain viable for a short time (Johnson & Everitt, 2000, p.162-163).

### **2.1.2 The Midpiece**

Two centrioles are aligned at the base of the nucleus and mark the initial section of the spermatozoon midpiece. The proximal centriole is indicative of the spermatozoon neck, while a flagellum extends from the distal centriole (Johnson & Everitt, 2000, p. 58-59). Condensed mitochondrial rods form a spiral around axial filaments (the axoneme) and provide the energy required for motility (Figure 2b). The axoneme forms the framework of the flagellum (Martini, 2001, p. 1025). The entire midpiece is separated from the sperm head by a striated ring. In immature sperm cytoplasmic droplets are often retained at the midpiece and can demonstrate some of the same biochemical properties as the sperm head. In most species cytoplasmic droplets of immature sperm are completely removed during sperm maturation in the epididymis. A deficiency in this process will allow immature or abnormal

sperm into the ejaculate and impede normal sperm function (Bearer & Friend, 1990).

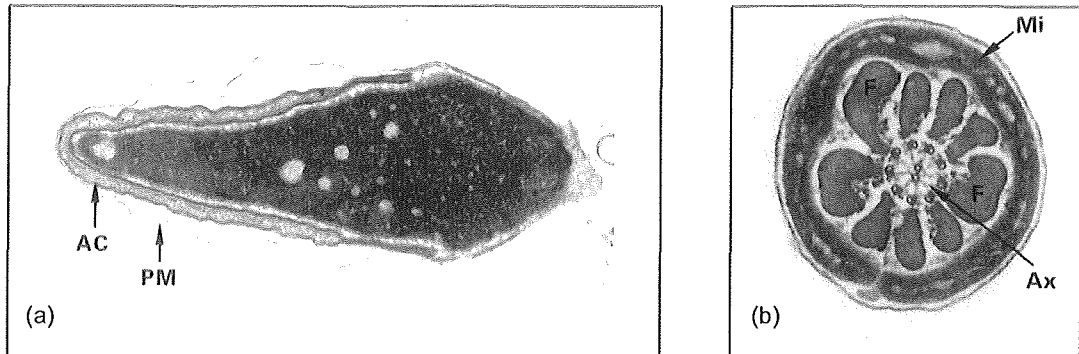


Figure 2: a) Spermatozoon Head (x14 000). The *acrosome* AC and *plasma membrane* PM, and b) Midpiece Section (x48 000). The *mitochondria* Mi, *outer dense fibres* F, and *axoneme* Ax. (Reference: Young & Heath, 2002)

### 2.1.3 The Tail

The tail is comprised of the principal piece, (axoneme surrounded by a fibrous sheath), and the end piece in which the sheath terminates (Johnson & Everitt, 2000, p. 58-59). Sperm become motile when the axonemal fibrils contract producing a lashing motion in the tail (Bearden, Fuquay & Willard, 2004, p.176). The movements in the tail become more rapid after capacitation and hyperactive after the acrosome reaction (Bearer & Friend, 1990).

### 2.1.4 The Plasma Membrane

Each region of the spermatozoon plasma membrane exhibits a unique structural, functional and biochemical property (Bearer & Friend, 1990; Jones, 1998). The resting potential and antigenic arrangement of the membrane is also region specific (Johnson & Everitt, 2000, p. 59). In comparison to somatic cells, there is a considerable fraction of polyunsaturated phospholipids in the membrane. These are primarily involved with motility (Jones, 1998).

Cytoskeletal interactions, boundary proteins or other structures are thought to maintain this unusual arrangement, which is continually modified from the initiation of spermiogenesis, through to oocyte fusion (Bearer & Friend, 1990; Johnson & Everitt, 2000, p. 59; Jones, 1998).



## 2.2 SEMEN

A single ejaculate normally contains spermatozoa and seminal plasma derived from the accessory glands of the male reproductive tract. The composition of seminal plasma varies between species though most contain a unique combination of energy substrates, buffering agents, reducing agents, prostaglandins, lymphocytes and potentially infectious agents (Bearden, Fuquay & Willard, 2004, p. 177-178; Johnson & Everitt, 2000, p. 155).

### 2.2.1 Semen Analysis

The initial macroscopic examination prior to semen preparation consists of measuring volume, viscosity, pH, appearance, and liquefaction properties of whole semen. Microscopic examination involves an estimation of sperm concentration, motility, morphology, agglutination properties, and the presence of other cellular elements. A simplified comparison of normal and abnormal semen values are outlined below in Table 1. In clinical cases, an individual can fall into one category or possess a combination of the various sperm irregularities (WHO, 1999; Brinsden & Rainsbury, 1992, p.178-183).

Table 1. Normal & abnormal states of fertility (WHO, 1999)

	Number of Spermatozoa / ml	Total Semen Volume	Total Number Sperm in Ejaculate	% Motility	% Normal Morphology
<b>Normozoospermia</b> (Normal)	≥ 20 million	≥2 ml	≥ 40 million	≥ 50% or >25% rapid	≥ 15%
<b>Oligozoospermia</b> (Low sperm count)	< 20 million	≥ 2 ml	< 40 million	≥ 50%	≥ 15%
<b>Asthenozoospermia</b> (Low motility)	≥ 20 million	≥ 2 ml	≥ 40 million	< 50% progressive motility or < 25% rapid progressive motility	≥ 15%
<b>Teratozoospermia</b> (Poor morphology)	≥ 20 million	≥ 2 ml	≥ 40 million	≥ 50%	< 15%
<b>Azoospermia</b> (Total absence of sperm)	0	—	0	—	—

### 2.2.2 Oligozoospermia

Oligozoospermia is one of the most common reasons for male infertility and can be characterized by a significantly reduced number of sperm in the ejaculate. In most clinical cases, the condition tends to be accompanied by a higher degree of morphologically abnormal sperm. The etiology of the

condition is largely unknown although it can result from childhood illnesses, drug use, chemotherapy, urogenital infection, and varicocele (Brinsden & Rainsbury, 1992, p. 65). Characteristically, the samples of oligozoospermic individuals demonstrate a reduced survival rate following cryopreservation (Counsel, Bellinge & Burton, 2004; Grizard *et al.*, 1999; Oehninger *et al.*, 2001). It is important therefore to continue to work towards optimizing sperm cryopreservation to ultimately improve pregnancy outcomes for severely oligozoospermic men requiring assisted reproduction.

## **2.3 CRYOPRESERVATION OF SPERM**

### **2.3.1 Semen Preparation**

Spermatozoa can only survive in unprocessed semen for a limited time and once the temperature cools to 5°C many sperm will die. Early studies have shown that extenders are an important factor in prolonging the life of spermatozoa, and the addition of permeating and non-permeating cryoprotectants aims at reducing the damage caused by the freeze/thaw process (Bearden, Farquay & Willard, 2004, p. 199).

#### **2.3.1.1 Extenders**

The role of an extender is protective so it must possess several specific properties. The solution must be isotonic to semen, have buffering capacity to control fluctuations in pH, have the ability to reduce cold shock injury during cooling, provide nutrients for sperm metabolism, and protect against microbial contamination. The buffering capacity is commonly achieved using sodium citrate, tris, or milk. Cold shock injury is reduced through the protective mechanisms of lecithin and lipoproteins found in egg yolk or milk, and energy is supplied by egg yolk, milk, or simple sugars. Antibiotics are added to prevent microbial contamination, and the addition of cryoprotectants aims to minimize the damage that occurs through ice crystal formation, rising osmolarity and mechanical injury (Bearden, Farquay & Willard, 2004, p. 199-200).

### **2.3.1.2 Permeating cryoprotectants**

Permeating cryoprotectants in the freezing media decreases the damage caused by the freezing process. Permeating cryoprotectants lower the freezing point of solutions through water binding which reduces the number of ice crystals that can form as the temperature decreases. This minimizes the cellular damage associated with ice crystal formation (Bearden, Fuquay & Willard, 2004, p.207). The most common permeating cryoprotectants are glycerol, dimethyl sulfoxide (DMSO), and propanediol (Bras et al., 1996, p. 239). Glycerol is currently the primary cryoprotectant used for human sperm cryopreservation although ethylene glycol has received recent attention due to its relatively fast permeability speed. Glycerol however is efficient within specific concentration limits, although it can be toxic at higher concentrations (McLaughlin, 2001). The addition of 1.5M glycerol to semen causes water to move out of the cell towards the higher concentration gradient, which decreases the cells volume by dehydration and reduces the risk of ice crystal formation. As the glycerol solution begins to permeate the cell, water will follow by osmosis, and the cellular volume will increase until the intercellular and intracellular environments have been equalized (Bearden, Fuquay & Willard, 2004, p. 207; Gao et al, 1993).

### **2.3.1.3 Non-permeating cryoprotectants**

Non-permeating cryoprotectants act to reduce the damage that occurs during thawing as post-hypertonic injury can occur as the cells return to isotonic conditions. In the absence of non-permeating cryoprotectants, the cellular integrity of sperm would be compromised as the cells swell and lyse in response to the osmotic movement of water towards the concentration gradient within the cells (Gao et al, 1993). The two most commonly used non-permeating cryoprotectants for sperm rehydration are sucrose and dextran. These substances do not permeate the cell and so allow the concentration of glycerol to decrease whilst the osmotic pressure remains relatively stable (Bras et al., 1996, p. 239-240). The integrity of the acrosomal and plasma membranes are protected to a larger degree if rehydration is performed as a

gradual process that carefully lowers the osmotic concentration of the medium and subsequent cytoplasmic component of the spermatozoon back to normal osmolarity (Gao et al, 1993).

### **2.3.2 The Freeze/Thaw Process**

Semen samples are preserved in liquid nitrogen at a temperature of  $-196^{\circ}\text{C}$ . Following the addition of the cryo-media, the samples are either suspended in uncirculated liquid nitrogen vapor to reduce the temperature, or placed in a programmable freezer that circulates liquid nitrogen vapor at a controlled rate until the temperature reaches between  $-30$  to  $-80^{\circ}\text{C}$ . The samples are then plunged directly into liquid nitrogen (McLaughlin, 2001).

#### **2.3.2.1 Packaging**

Prior to freezing, the hyperosmotic sperm suspension is packaged into freezable containers. There are three main types, cryovials, straws and syringes, each of which has advantages and disadvantages. Cryovials and syringes are difficult to seal effectively and tend to be bulky in storage. Straws are commonly used, come in two sizes 0.25mL and 0.5mL, and are convenient for sample identification (through colour coding) and help maximize storage capacity (McLaughlin, 2001).

#### **2.3.2.2 Cooling speed**

The dehydration properties of the cell determine the optimum cooling speed, and the dehydration speed of a cell is dependant on its individual surface area/volume ratio. This suggests that cooling at a slower rate should result in less intracellular water at lower temperatures, less ice crystal formation within the cell and less ultrastructural damage to an average volume cell. The cytoplasmic volume of spermatozoa however is low; therefore cooling at a fast rate can prevent excessive water loss from the cell and avoid permanent dehydration damage (Bras et al., 1996, p. 233-235). In addition, the spermatozoon osmotic tolerance is higher when cellular exposure to the hyperosmotic medium is short as opposed to slow cooling where the

concentration of an isotonic solution peaks due to ice formation at around 4-0°C (Gao et al, 1993).

Holt and North (1984) examined the effect of slow cooling on intramembranous particle arrangement over the acrosome, post-acrosomal region, and flagellum portion of the spermatozoon plasma membrane. This study found that the plasma membrane rearranged as the temperature decreased to 5°C and only partially reversed to its original configuration upon warming. The partial reversion of the intramembranous particles of the plasma membrane was particularly significant on the spermatozoon tail and was found to be associated with the loss of motility seen after freezing (Holt & North, 1984).

Other studies have shown the optimum cooling speed for spermatozoon survival is very rapid in comparison to the freezing rates of other cell types. Although the timing can vary, human semen samples are generally cooled rapidly at around 10°C per minute to around -30°C or -80°C at which point the samples are plunged into liquid nitrogen for ultra-rapid freezing to -196°C (Bras et al., 1996, p. 233-235). Rapid freezing reduces gas bubble formation that can appear simultaneously with the development of ice crystals. During thawing, the gas bubbles can combine and rapidly become vacuoles large enough to distend and burst the cell (Bras et al., 1996, p. 238; Hughes et al., 1999).

#### **2.3.2.3 Thawing**

The rate at which the samples are cooled determines the rate at which they are thawed. Samples cooled at 10°C per minute should be thawed at 400°C per minute. If the samples are contained in straws, this conveniently equates to removing the straws from the liquid nitrogen and placing them on the bench to warm at room temperature of approximately 22°C (McLaughlin, 2001).

### **2.3.3 Post-Thaw Sperm Analysis**

Cryo-induced cellular damage can be assessed by measuring a set of parameters provided by the World Health Organisation (1999, p. 6-7). The post-thaw recovery rate can be assessed by measuring the percentage of motility, ultrastructural damage (plasma membrane and acrosome integrity), intracellular damage (chromatin and chromosome stability), energy status, and biochemical consistency (Royere et al, 1996). Post-thaw functional tests include capacitation induction, spermatozoon binding capacity, and induction of the acrosome reaction. There is however, no true predictive test to determine the fertilization capacity of the sample following cryopreservation except *in vitro* fertilization and pregnancy outcome (Bras et al., 1996, p. 62-65).

## **2.4 CRYO-INDUCED CELLULAR DAMAGE**

The cryopreservation process introduces mechanical, osmotic, thermal, and oxidative stresses to the spermatozoa and has the ability to cause structural anomalies to the plasma membrane, acrosome, and nuclear components that ultimately lead to functional deficiencies or cell death (Burton, Bellinge & Counsel, 2003).

### **2.4.1 Ice Crystal Formation**

Ice crystal formation is one of the primary reasons for cell death during the cryopreservation process. The presence of any solid material within the cell (i.e. dust particle) will induce the formation of intracellular ice crystals. Spontaneous ice crystal formation will occur at temperatures below -10°C, increasing in the rate of formation as the temperature cools to produce a crystal lattice capable of perforating the plasma membrane and other structures. All of the water present will have crystallised at around -130°C, although some substances may vitrify, producing a glass like structure rather than a crystalline one (Bras et al., 1996, p. 231).

### **2.4.2 Vitrification**

Vitrification is due to the high osmolarity of the remaining substances. For most cells, the activation energy for water permeability doubles with every 10°C drop in temperature. As more water molecules form ice crystals with the decreasing temperature, the osmolarity of cells suspended in an isotonic solution increases due the reduction of water remaining in liquid form. An extracellular increase in osmotic pressure will cause more water loss from the cells, the water moving into the intracellular spaces will in turn form extracellular ice crystals, and the osmolarity of the medium will remain high. The solution vitrifies when the osmolarity of the remaining fluid is more than 30 times normal value. The outcome of this process is cellular dehydration and damage due to the high osmolarity of the medium (Bras et al., 1996, p. 232-235).

### **2.4.3 Osmolarity Fluctuation**

The use of cryoprotectants and other cryo-agents during the freezing and thawing process is integral to minimizing the potential changes in sperm volume and damage in response to osmotic fluctuations. However, there is an association between the degree of spermatozoon damage and an increase in concentration of the medium. A significant percentage of spermatozoa experience hyperosmotic stress when exposed to hypertonic solutions (Gao et al, 1993). Free water decreases due to the formation of ice crystals and the medium surrounding the cells becomes hypertonic. As the temperature drops to between 4°C and 0°C, the medium reaches an osmotic concentration that can potentially destroy 50% of the spermatozoa (Noiles, Bailey and Storey, 1995).

High osmotic solutions can have a deleterious effect on the spermatozoal plasma membranes. Cold-induced dehydration can cause the intracellular volume to drop below the minimal limit, which permanently disables the cell (Bras et al., 1996, p. 233-235). As observed in other cells types, irreversible damage of spermatozoa follows a water loss of 65%. Membrane

fusion, phospholipid loss; protein denaturation, and unnatural cytoskeletal interactions have all been implicated as a cause of spermatozoon death relating to extreme volume loss, however the explanation is yet to be determined (Gao et al, 1993). Similarly, a reduction in spermatozoon motility by up to 60% is noted if the intracellular volume exceeds 1.38 times its normal value (Gilmore et al, 1997).

#### **2.4.4 Cryo-Capacitation and Acrosomal Damage**

Partial cryo-capacitation can occur in some spermatozoa during the cooling and warming processes. Spermatozoa artificially induced into capacitation lose their fertilization potential quickly and therefore compound the reduced fertility seen in these samples following cryopreservation. By reducing the interval between sample thawing and use in IVF procedures, the fertility rate may increase (Pena et al., 2004).

Acrosomal damage is evident where the acrosomal contents are swollen or lost (Royere et al, 1996). Semen samples outside the normal WHO parameters can be susceptible to cold-induced acrosomal damage without any obvious alteration to sperm motility (Pukazhenthil et al, 1999). In the bull, 50-55% of the spermatozoa will have intact acrosomes following cryopreservation (Bearden, Fuquay & Willard, 2004, p. 194), nevertheless a relationship between acrosome integrity and vitality or motility is yet to be established (Royere et al, 1996). The gradual addition of glycerol in pre-freeze media to the semen sample provides greater acrosomal integrity following cryopreservation (Fiser & Fairfull, 1989).

#### **2.4.5 Plasma Membrane Injury**

Roughly 40% of the total fatty acids in the spermatozoon plasma membrane are polyunsaturated fatty acids. This arrangement is unique to spermatozoa and is essential to facilitate sperm motility (Kaneko et al., 2002). The integrity of the plasma membrane is also crucial for the early events of fertilization (Bearer & Friend, 1990).



#### **2.4.5.1 Membrane fluidity**

There is a relationship between pre-freeze membrane fluidity and post-thaw spermatozoon survival (Giraud et al, 2000). Investigations by Noiles, Bailey and Storey (1995) on mouse spermatozoa have revealed that once the temperature reaches 4°C the fluidity of the plasma membrane decreases and it becomes an insubstantially brittle structure. With the decrease in temperature, the probability of stress fracture increases as the movement of water through the membrane is hindered. Distinct differences in membrane fluidity can be seen in sperm samples from different ejaculates. This is thought to be one of the reasons for the variation in survival rate following cryopreservation (Giraud et al, 2000).

Comparative studies of sperm from several species have identified that all lipid composites and interactions of the plasma membrane become unstable at lower temperatures, however the mammalian glycolipid fraction becomes particularly inconsistent when compared to other species (Parks & Lynch, 1992). The addition of cryoprotectants (particularly glycerol) to the media allows the spermatozoon plasma membrane to remain fluid and cryo-damage is minimised (Noiles et al, 1995).

#### **2.4.5.2 Phospholipid translocation**

An investigation into the position of phospholipids in intact and damaged ram sperm cells by Muller et al (1999) has revealed that the post-thaw organisation of the plasma membrane and post-thaw survival rate varies between subpopulations of spermatozoa within the same sample. Phosphatidylserine is thought to be one of four phospholipids central to spermatozoon capacitation and the acrosome reaction that act by destabilizing the plasma membrane. Early destabilization of the membrane and translocation of phosphatidylserine in a significant percentage of the cryopreserved sample can lower fertilization capability as the processes of capacitation and the acrosome reaction must be intimately timed (Muller et al, 1999).

Phosphatidylserine is a phospholipid positioned randomly on the cytoplasmic side of the plasma membrane of most cells and is involved with calcium dependent interactions between protein kinases and the membrane (Duru et al., 2001). It can be phosphorylated to become glycerol, phosphoric acid, serine and fatty acids and is capable of ATP-dependent movement between the internal and external layers of the membrane (Muller et al, 1999). When phosphatidylserine is translocated to the external layer of the membrane, the enzyme aminophospholipid translocase rapidly returns the molecule to the inner layer. It is an ATP-dependent process (Duru et al, 2001). In normal conditions, aminophospholipid translocase is highly efficient in maintaining membrane composition, and ATP levels must be almost exhausted to slow its activity. However, the activity of this enzyme in post-thaw intact sperm remains irreversibly slow and unresponsive to increased ATP concentrations. The reason for this is unknown (Muller et al, 1999).

When spermatozoa are cryogenically preserved, the cell membrane is distended and phosphatidylserine is artificially translocated to the outer layer (Anzar et al, 2002; Duru et al, 2001). Because the activity of aminophospholipid translocase simultaneously slows with the decrease in temperature and fails to re-establish full function upon warming, phosphatidylserine expression on the outer surface of the plasma membrane is prolonged (Muller et al, 1999). In the testes of a normozoospermic male, the extended presence of phosphatidylserine on the membrane surface of immature sperm induces their phagocytosis by sertoli cells. This phagocytic course of action may be deficient or absent in oligozoospermic samples containing elevated numbers of abnormal or dead sperm (Muller et al, 1999), and prolonged expression of this molecule as a result of cryopreservation not only promotes sperm phagocytosis, but is also indicative of the early apoptotic pathway (Anzar et al, 2002).

## **2.4.6 DNA Fragmentation**

Injury to sperm DNA follows incubation at low temperatures in the absence of cryoprotectants. Additionally, the extent of DNA damage and sperm viability is inversely correlated over time (Linfor & Meyers, 2002). Cryopreservation further generates a considerable level of DNA fragmentation, the severity of which intensifies with an increased number of morphologically abnormal and damaged spermatozoa commonly seen in oligozoospermic samples (Anzar et al, 2002; Host et al, 1999). On a clinical level, DNA injury and inaccuracies essentially foreshadow chromosomal aberrations, spontaneous abortion, and birth defects, and recent investigations have now associated spermatozoal DNA damage with childhood cancers (Aitken & Sawyer, 2003).

### **2.4.6.1 Apoptosis**

An 'apoptosis-like phenomenon' has been found to occur in some bovine sperm following cryopreservation induction. Apoptosis is normally a function of cell population control during spermatogenesis and is characterized by an intracellular increase in caspase levels. The caspase enzymes are directly involved in the apoptotic pathway and their presence results in DNA denaturation (Martin et al., 2004). During the initial stages of apoptosis, endonucleases reduce the DNA into small fragments consisting of around 180 base pairs (Anzar et al., 2002). The freezing and thawing processes of cryopreservation can artificially activate the apoptotic machinery in immature sperm through the disruption of the mitochondrial transmembrane potential, or through the prolonged expression of phosphatidylserine markers on the membrane surface. Remarkably, mature sperm have been shown to be unaffected by this process (Anzar et al, 2002; Muller et al, 1999).

Experiments performed by Paasch et al (2004), and Anzar et al (2002), using the annexin V surface ligand (a fluorescently conjugated protein that binds to the phospholipids on cell membranes, in particular phosphatidylserine), have found that a subpopulation of mature sperm do not over-express phosphatidylserine on the surface of their plasma membrane. More

importantly, the results suggested that the mature sperm fraction were not irreversibly disturbed by cryopreservation and do not enter into the apoptotic pathway. The studies proposed that isolates of this fraction of sperm may freeze with greater survival rate and may be more appropriate for post-thaw assisted reproductive techniques, as opposed to the decreased lifespan (both in vivo and in vitro) of the remainder of the sample (Paasch et al., 2004; Pena et al., 2004).

Though the former trials appear clinically valuable for normozoospermic samples, there is a significantly greater level of DNA fragmentation in oligozoospermic samples prior to, and following cryopreservation due to the increased percentage of immature sperm in the sample (Host et al, 1999). More recent research selectively isolating the annexin V negative fraction of mature sperm, has found that although the overall percentage of isolated sperm has a higher survival rate during cryopreservation, the total number of spermatozoa present in the isolated fraction is minimal. This may not be clinically supportive for oligozoospermic samples that contain a high proportion of immature and abnormal spermatozoa (Paasch et al., 2005).

#### **2.4.6.2 Necrosis**

Cryopreservation disintegrates most necrotic cells as their plasma membranes are weak, however the process does not remove apoptotic cells from the media because their membranes remain intact. Upon thawing, the apoptotic sperm cells undergo secondary necrosis, which causes an increase in plasma membrane permeability, distension of the cytoskeleton and an increase in peroxidation of the membrane phospholipids (Anzar et al, 2002). However, research suggests that freeze activated apoptosis may be less significant in regard to decreasing the viability of the sample than other destructive mechanisms of cryopreservation, although the reactive oxygen species produced through an increase in membrane lipid peroxidation can be a major contributor of oxidative stress (Moustafa et al., 2004).

### **2.4.7 Reactive Oxygen Species**

Reactive oxygen species (ROS) have a central role in normal sperm function and at low levels are involved with capacitation, the acrosome reaction, stimulating hyper-activation, and binding to the zona pellucida of the oocyte (Ford, 2004; Kaneko et al., 2002). Continual ROS generation demonstrates that spermatozoa are capable of producing endogenous reactive oxygen species through the reduction/oxidation system in their plasma membrane (Aitken & Sawyer, 2003; Ford, 2004; Muller et al., 1999), a process that is largely unavoidable in aerobic systems (Pena et al, 2004).

Normally, as the cells are maturing in the epididymis of the male reproductive tract, the potential level of motility and functional capacity increases as the sperm nucleus and tail are oxidised (Kaneko et al., 2002). The concentration of reactive oxygen species is controlled by antioxidants produced in the epithelia of the male reproductive tract and secreted in seminal plasma (Hendin et al, 1999). The effect of antioxidants within this system will be addressed in a later section.

Reactive oxygen species appear elevated in sperm samples as a result of cellular necrosis (Anzar et al, 2002), morphological error (Parinaud et al, 1997), and following cryopreservation (Baumber et al, 2003). Any decrease in endogenous antioxidant ability can allow an increase in ROS concentrations (Aitken & Sawyer, 2003). Oxidative stress during cryopreservation forces cells into a highly activated state, the consequence of which can stimulate glycolytic enzymes, disrupt the plasma membrane, reduce sperm motility, cause acrosomal damage, damage the mitochondrial genome and induce DNA fragmentation (Aitken & Krausz, 2001; Counsel, Bellinge & Burton, 2004; Gill-Guzman et al., 2001).

Spermatozoa and lymphocytes are thought to be responsible for producing significantly harmful levels of oxidants in selectively poor semen samples (Hendin et al., 1999). Of the three major reactive oxygen species involved in

spermatozoon damage (superoxide anion, hydrogen peroxide and the hydroxyl free radical), the peroxide anion is thought to be responsible for most nuclear and membrane injury that occurs during the cryopreservation process (Baumber et al., 2003; Pena et al, 2004). Notably, the peroxidation of lipid proteins (primarily the unsaturated fatty acids) in the plasma membranes of spermatozoa, has been revealed as a major source of the peroxide anion in semen samples (Kaneko et al, 2002). The loss of membrane fluidity as a consequence of oxidative insult to the unsaturated fatty acids, results in a loss of motility and binding capacity (Aitken & Sawyer, 2003). In addition, the egg-yolk extenders commonly used in cryo-media contain transition metals (as does seminal plasma) that can facilitate lipid peroxidation by enhancing hydrogen peroxide activity in cryopreserved samples (Aitken & Krausz, 2001; Bilodeau et al., 2000).

As mentioned earlier, lipid protein peroxidation appears to be predominant in the plasma membranes of necrotic cells (Anzar et al, 2002), and in cells that display morphologically abnormal cytoplasmic retention (Parinaud et al., 1997). Immature sperm with abnormal head morphology and sperm retaining excess cytoplasm are generally released prematurely from the germinal matrix as a result of inefficient spermatogenesis and are commonly seen in the samples of subfertile men. The enzymes contained in the excess cytoplasm on the immature sperm facilitate ROS production, which further exacerbates free radical generation and cellular injury. The process is cyclic and compounding and results in the poor semen quality commonly associated with low sperm count, poor morphology and reduced motility (Aitken & Sawyer, 2003; Gil-Guzman et al., 2001). Furthermore, oligozoospermia is generally characterized by deficient antioxidant levels in the semen (Ford, 2004; Gil-Guzman et al., 2001; Hendin et al, 1999).

Lymphocytes are able to produce significant levels of oxidative substances, however their removal from subfertile samples does not prevent the loss of spermatozoon functional ability (Anzar & Krausz, 2001). Correspondingly,

oligozoospermic samples are not often dominated by an elevated lymphocyte fraction, endorsing the sperm cell as a major contributor of reactive oxygen species within poor samples (Hendin et al., 1999).

A fundamental concern is that oxidative damage to DNA is not equally reflected on the spermatozoon plasma membrane, and many spermatozoa with significantly damaged DNA retain their fertilization ability (Aitken & Krausz, 2001). Childhood cancers are thought to be predisposed through oncogene activation in the Y chromosome that is particularly susceptible to oxidative damage because of the lack of ability for mutation correction during homologous recombination. This implies that oxidative DNA injury in samples that are cryopreserved for artificial reproductive techniques such as intracytoplasmic sperm injection (ICSI), carry a substantial risk of producing an embryo with an undefined genetic insufficiency that must be repaired by the oocyte prior to the first cleavage. Failure to do so would result in homogenous mutagenesis that will continue through the germ line. DNA injury in the male germ line through oxidative damage has now been associated with childhood cancers, and various infertility pathologies (Aitken & Krausz, 2001).

#### **2.4.8 Antioxidants**

The production of functional sperm requires protection against oxidative stress (Kaneko et al, 2002). Several antioxidants are found in the male reproductive tract and are present in seminal plasma to varying degrees between individuals. Ascorbate (vitamin C), tyrosine, urate, tocopherol (vitamin E) and hypotaurine are found predominantly in the seminal plasma, and catalase, superoxide dismutase, xanthine oxidase and glutathione peroxidase can be located in the cytoplasm within the sperm cells (Aitken & Krausz, 2001; Hendin et al., 1999). The antioxidants vitamin C, tyrosine, urate and vitamin E, in addition to albumin (a component of seminal plasma) are particularly efficient in reducing the free radicals found in semen. These substances act to prevent lipid peroxidation and are commonly found in inefficient concentrations in infertile or sub-fertile men (Aitken & Krausz, 2001).

#### **2.4.8.1 Glutathione peroxidase**

Glutathione peroxidase is a phospholipid located on the mitochondrial plasma membrane and its under-expression has been noted to cause a loss of sperm motility (Aitken & Krausz, 2001). Rodent studies have shown that glutathione is normally produced in the epithelia of the male reproductive tract and functions as a powerful antioxidant during the maturation and storage of sperm (Kaneko et al, 2002). Glutathione peroxidase provides cellular resistance against lipid peroxidation by preventing the oxidation of sulphhydryl groups and unsaturated fatty acids in the plasma and mitochondrial membranes (Kaneko et al., 2002). The semen samples found to have the highest sperm survival rate following cryopreservation have a significant level of glutathione peroxidase activity prior to the freezing process (Pena et al, 2004).

Glutathione also acts as an amino acid donor during spermatogenesis (Kaneko et al., 2002). The chromosomal histones that normally package DNA into the nucleus are replaced by protamines (donated by glutathione), which condense the chromatin dramatically to minimize the size of the nuclear components within the spermatozoon head (Kalthoff, 2001, p.58). This process reduces the concentration and activity of free antioxidant within the cell. If the levels of glutathione are too low during spermatogenesis, the number of mature and morphologically normal spermatozoa generated will decrease, and the sperm will be vulnerable to apoptosis induced by ROS or another event such as cryopreservation (Kaneko et al., 2002). As the level of antioxidants found in the cytoplasm of spermatozoa is too low to protect the cells from further oxidative damage, the cells must rely on antioxidants found in the seminal plasma once they have matured or left the male reproductive tract (Pena et al., 2004).

#### **2.4.8.2 Tocopherol**

Tocopherol (Vitamin E) is an effective antioxidant in semen samples and can protect the integrity of the spermatozoa after they have left the testes. It is thought to act by preventing the level of oxidative damage occurring to the



spermatozoon plasma membrane whilst it remains fluid. A study by Pena et al., (2004), has assessed the degree of protective efficiency of a water soluble vitamin E analogue in separate fractions of a single boar ejaculate. The initial fraction (the sperm rich portion) contains a much higher concentration of spermatozoa than the second portion of the sample (the post-spermatic portion). The same group identified three major observations in an earlier investigation. 1) The sperm rich portion of the sample had a higher ability to survive the cryopreservation process, 2) the motility and progression parameters of the post-spermatic portion were much lower than the sperm-rich portion, and 3) a higher degree of translocated phosphatidylserine on the outer membrane was detected in the post-spermatic portion (Pena et al., 2003b). The sperm count, motility, progression parameters and plasma membrane disruption in the post-spermatic portion of the boar ejaculate is therefore comparable to that seen in human oligozoospermic samples.

The more recent study by Pena et al. (2004), established that antioxidant supplementation to the initial, sperm-rich portion of the boar ejaculate did not have a significant effect on the improvement of spermatozoon integrity, motion restrictions or cell death. However, the addition of vitamin E to the post-spermatic portion significantly improved the integrity of the plasma membrane and reduced cellular damage by approximately 20%. The improved survival rate seen in the post-spermatic portion of the boar semen following the addition of antioxidants, support the notion that improving the cryopreservation survival rate in human oligozoospermic samples may involve decreasing the level of oxidation through the addition of antioxidants to the pre-freeze media.

## **2.5 SUMMARY**

The use of frozen sperm for human artificial reproduction has existed for over 50 years, and today assisted reproductive technology uses cryopreserved samples for convenience or for donor insemination. The quality of semen samples can be organized into several categories according to standards set by the World Health Organisation. Oligozoospermia, characterized by a

reduction in the number of motile sperm, is generally accompanied by a higher level of morphologically abnormal sperm, and demonstrates a higher degree of oxidative damage and necrotic sperm than normal samples. Cryopreservation introduces a compounding environmental disadvantage to profoundly poor semen samples and has been shown to further increase the concentration of reactive oxygen species, the level of which is negatively correlated to sperm motility, DNA integrity, oocyte binding capacity and overall fertilization potential. Recent animal research has focused on the potential protective action of antioxidant addition to buffer and cryo-media solutions prior to the freezing process with promising results, however the absolute value and protective capacity of exogenous antioxidant addition to cryo-media for human sperm cryopreservation is yet to be established.

# **CHAPTER 3:**

## **MATERIALS AND METHODS**

### **3.1 ETHICAL ENDORSEMENT**

Written approval for this study was granted by the King Edward Memorial Hospital Internal Ethics Committee (EC05-43.1) and by the Edith Cowan University Faculty of Computing, Health and Science Ethics Sub-Committee for the Conduct of Human Research.

### **3.2 SEMEN SAMPLES**

This study utilized surplus semen remaining after routine semen analysis at Concept Fertility Clinic, Subiaco, Western Australia. In total 43 semen samples were collected and used in this study. The samples were collected by masturbation into sterile containers. Patient consent to use surplus semen was obtained prior to use (see Appendix 1 for Consent Form).

### **3.3 PRELIMINARY ANALYSIS**

Normozoospermia (n=23) and oligozoospermia (n=10) were classified during semen analysis by Concept staff in accordance with guidelines determined by the World Health Organisation (WHO, 1999). Semen samples displaying a high percentage (>85%) of morphologically abnormal sperm relative to WHO values (teratozoospermia, n=10) were included in this study. The parameters measured during semen analysis included the examination of whole semen volume, consistency, and pH. A Makler Chamber (Sefi-Medical Instruments, Israel) was used to measure both sperm concentration (total and motile), and percentage of rapid linear, slow/non-linear, and non-progressive motility under light microscopy (Olympus Australia Pty Ltd, Welshpool, Western Australia) at 100x magnification. Sperm morphology was conducted in accordance with WHO criteria to determine the percentage of head, midpiece and tail defects within each sample.

### **3.4 SAMPLE PREPARATION**

Following liquefaction and preliminary assessment, 0.6mL of each semen sample was aliquoted into three 12mL Falcon round bottom tubes (Falcon,

Becton Dickinson Labware, USA). The first semen fraction was a control, and received an equal volume (1:1) of standard semen cryomedia (Appendix 2). The second fraction received an equal volume of cryomedia containing 100 $\mu$ M of a water soluble vitamin E analogue (6-hydroxy-2,5,7,8-tetramethylchromon-2 carboxylic acid), Trolox (Sigma-Aldrich Sweden, Tyreso, Sweden), and the third fraction received an equal volume of cryomedia containing 200  $\mu$ M of vitamin E. See Appendix 3 for control and vitamin E working solutions. Cryomedia was added drop wise to the semen samples to reduce osmotic fluctuations. The semen preparations were drawn by syringe into labeled 0.5 mL semen freezing straws (Genetics Australia, Australia), and plugged (Poudre de Bouchage, IMV Technologies, France). Two straws were prepared from each fraction. The samples were cooled in a Planar Kryo 10 control rate-freezer (Planar, UK) at -10°C/minute to a temperature of -80°C prior to rapid plunging into liquid nitrogen (-196°C). The samples were then stored in LN<sub>2</sub> storage dewars (Air Liquide GT40, Western Australia) where they remained until analysis.

### **3.5 POST-THAW ANALYSIS**

To assess post-thaw sperm parameters, straws were removed from liquid nitrogen and allowed to thaw at room temperature (~25°C). Three experimental end-points were assessed:

- a) Sperm survival rate determined the extent of sperm motility following the cryopreservation process.
- b) Post-thaw vitality assessed the ultrastructural integrity of the spermatozoon plasma membrane between the fractions.
- c) DNA fragmentation was examined to identify any variation in DNA integrity.

### **3.5.1 Sperm Survival Rate**

Sperm survival rate is the percentage of motile sperm remaining in a sample following a cycle of freezing and thawing. Survival rate was determined for the 100 $\mu$ M and 200 $\mu$ M vitamin E treated fractions and for the control groups in the 43 semen samples included in this study. Post-thaw motile and immotile sperm were counted using a Makler Chamber under light microscopy at 100x magnification.

To provide a more accurate assessment of sperm survival, sperm counts were performed on each straw immediately after thawing to provide two post-thaw motility counts from which an average of post-thaw motility was determined. In total, 258 post-thaw sperm counts were performed. Survival rate for each fraction was then calculated using the formula:

$$\text{Post-thaw motility} / \text{Pre-freeze motility} \times 100.$$

### **3.5.2 Post-Thaw Vitality**

The eosin-nigrosine dye exclusion staining assay was used to determine post-thaw vitality. Eosin permeates the membranes of non-viable sperm, which appear as red bodies in the sample. Live sperm are impermeable to eosin and do not stain. Nigrosine provides a darkened background to help visualize the cells. For stain solutions see Appendix 4.

#### **3.5.2.1 Cell staining**

Two drops of 1% eosin stain (BDH Laboratory Supplies, UK) were added to one drop of the thawed semen preparation from the various sperm fractions. Three drops of 10% nigrosine (BDH, UK) were added to each solution after 30 seconds. A drop of each fraction was smeared over glass microscope slides. The slides were allowed to air-dry.

### 3.5.2.2 Identification of viable and non-viable sperm

The prepared slides were assessed by oil immersion light microscopy at 1000x magnification (Figure 3). In accord with WHO guidelines (1999), vitality was quantified by counting a minimum of 200 sperm on each slide and the degree of live sperm was expressed as a percentage. In total, at least 25,800 sperm were counted on 129 slides to determine the post-thaw vitality of the 100 $\mu$ M, 200 $\mu$ M vitamin E treated and untreated control groups.

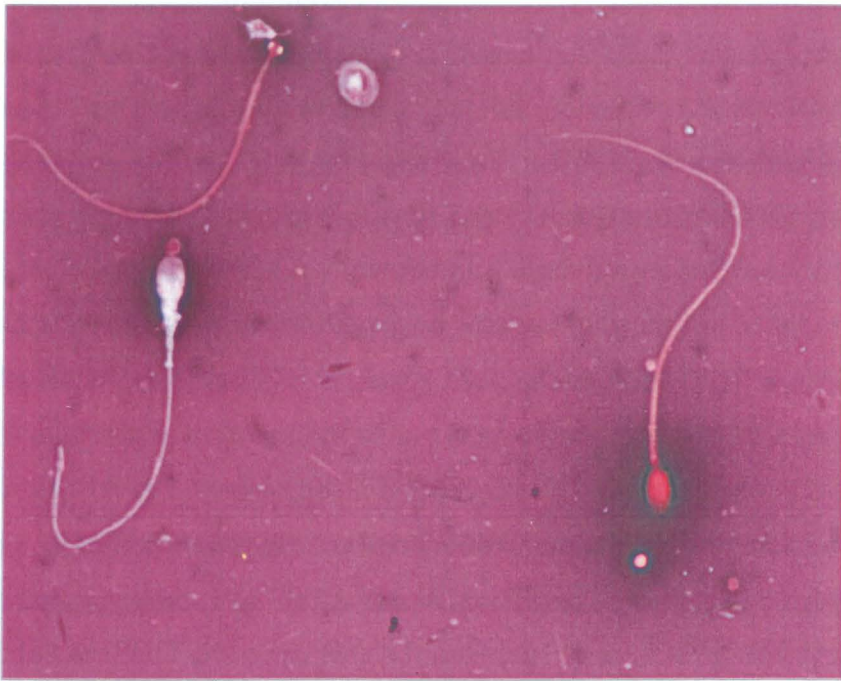


Figure 3. Eosin/Nigrosine Dye Exclusion Staining.1000x

The white spermatozoon is viable. The non-viable sperm membrane is damaged causing it to stain red.

### 3.5.3 DNA Fragmentation

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was utilized to selectively stain the nuclei of apoptotic sperm cells *in situ*. In principle, this assay binds labeled nucleotides to the broken 3'OH termini in single and double stranded DNA. Only apoptotic cells contain enough broken DNA fragments to reveal a darkened nucleus.

### 3.5.3.1 Cell Fixation

Following thawing at room temperature, 10 $\mu$ L of the sperm suspension from each of the three fractions was smeared over silanized glass microscope slides (Superfrost Plus, Menzel-Glaser, Braunschweig), and allowed to air-dry. The cells were fixed in a 3:1 ratio methanol/acetic acid solution at room temperature for 5 minutes, then washed in phosphate buffered saline (PBS, Amber Scientific, Australia, see Appendix 5) for 5 minutes.

### 3.5.3.2 Immunocytochemistry

Sperm with DNA strand breaks were revealed by TUNEL assay using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101 (Chemicon International, Australia). Reagents for the TUNEL assay were made to working strength according to the ApopTag® Plus Peroxidase Kit instructions and are shown in Appendix 5. Fixed sperm were permeabilized for 5 minutes in PBS-0.1% Triton X-100 solution then washed for a further 5 minutes in PBS. The cells were neutralised by applying 75 $\mu$ L of the ApopTag® Equilibration Buffer to each slide, and incubated in a humidified chamber for a minimum of 10 seconds at room temperature. Excess Equilibration Buffer was removed and 55 $\mu$ L of working strength Terminal deoxynucleotidyl Transferase (TdT) solution was applied. The TdT solution contained digoxigenin conjugated nucleotides and TdT enzyme, which functioned as a catalyst and resource for nucleotide triphosphate addition and elongation at the 3'-OH, fragmented ends of the DNA in damaged sperm. TdT solution was evenly distributed with plastic coverslips and the slides were incubated at 37°C for 1 hour. Slides were then incubated in the ApopTag® Stop/Wash Buffer for 10 minutes and washed for 5 minutes in 2 changes of PBS. To identify DNA elongation from the fragmented sites, the slides were treated for 30 minutes at room temperature with 65 $\mu$ L peroxidase conjugated anti-digoxigenin antibody, which acts by binding to the elongated regions of DNA. Slides were washed twice in PBS and stained for 4 minutes with 75 $\mu$ L of a peroxidase substrate containing 1.5 $\mu$ L DiAmino Benzidine (DAB). DAB is a stain with an affinity for peroxidase



and allowed the sperm with damaged nuclei to be viewed. This reaction was stopped by washing in dH<sub>2</sub>O.

#### **3.5.3.3 Positive and Negative controls**

Positive and negative control slides were produced during each TUNEL procedure to aid in the accurate identification of sperm with damaged and undamaged DNA. To create a positive control, 75µL of a 0.2µg/mL DNase I solution (Appendix 5; Reaction Buffer for DNase I and DNase I solution) was used to create multiple fragment sites in sperm nuclear DNA through enzymatic digestion after cell permeabilization in PBS-0.1% Triton X-100. The DNase exposed slides were isolated prior to treatment to avoid the contamination of other slides and incubated at 37°C for 10 minutes in a humidified chamber. The slides were then washed in PBS 2-3 times for 5 minutes before resuming the sperm TUNEL protocol. The induced DNA fragmentation allowed extensive antibody/antigen binding resulting in an increased degree of nuclear staining.

Negative control slides were not exposed to the TdT substrate and were treated with the ApopTag® Reaction Buffer only. In the absence of TdT, nucleotide elongation at the fragmented sites cannot be initiated, the cells lack the sites for antibody binding and will therefore not stain with exposure to the peroxidase/DAB substrate.

#### **3.5.3.4 Visualization of DNA damaged sperm**

To aid in exposing the nuclei of undamaged sperm, the cells were counterstained with methyl green (0.5%) for 10 minutes. Excess methyl green was removed with dH<sub>2</sub>O and the slides were air-dried. Sperm were viewed under 1000x brightfield microscopy using an oil immersion objective lens (Figure 4). Sperm that had entered the apoptotic pathway had brown stained nuclei. Unaffected cells were pale green. 200 spermatozoa were counted on each slide and a percentage of apoptotic sperm was determined for each of the three fractions.

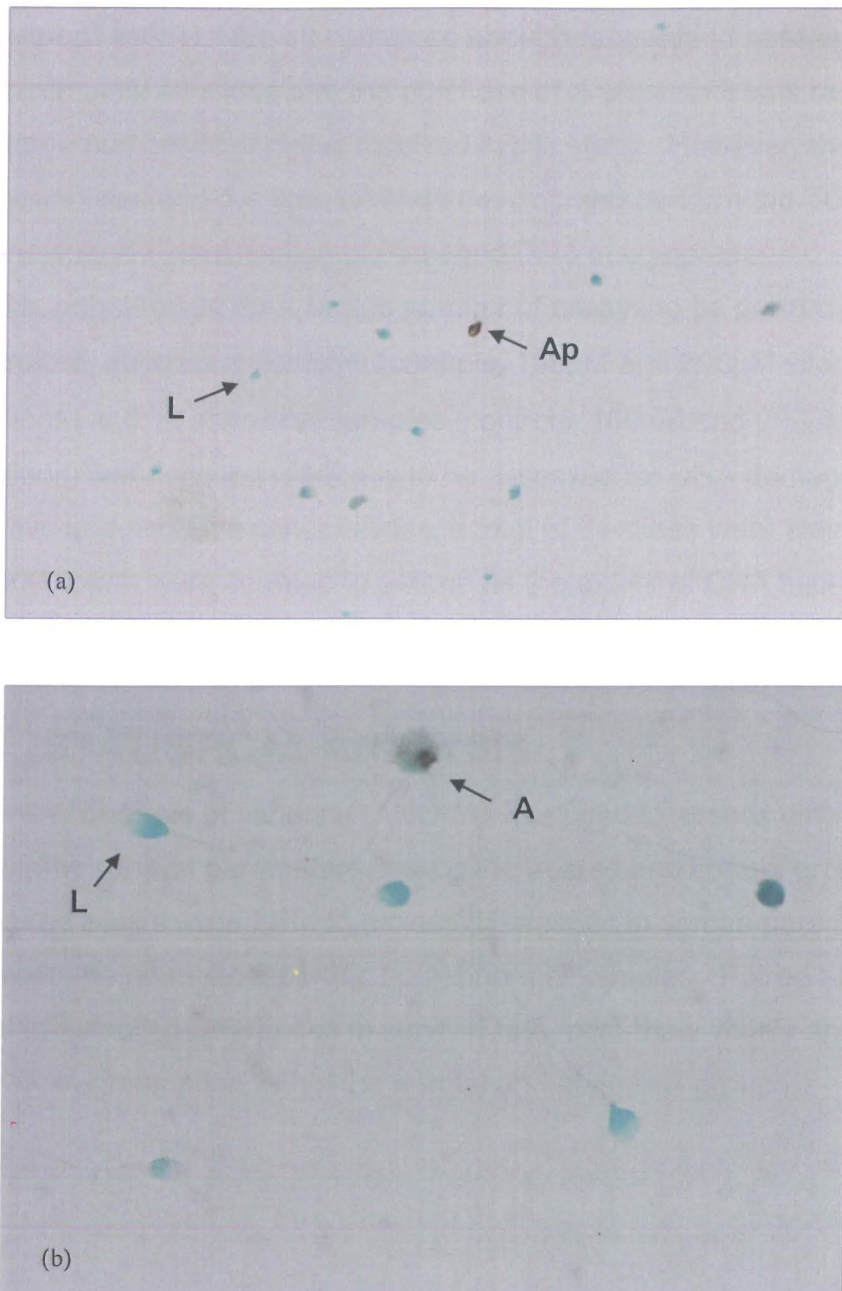


Figure 4: Pictomicrographs of the TUNEL assay a) Sperm with apoptotic nuclei viewed at 400x magnification. The darkened nucleus (Ap) characteristically seen following immunoassay in severely apoptotic sperm. b) Typical field of view for counting DNA damaged sperm. 1000x brightfield microscopy. A clearly defined region of DNA fragmentation can be seen on the sperm labeled (A) along with signs of plasma membrane injury. The blue/green stained sperm (L) show no signs of DNA damage.

#### **3.5.3.5 Restrictions**

The ApopTag® TUNEL kit contained enough reagents to assess approximately 40 slides and the purchase of a second kit was required due to the large number of samples involved in this study. However, the quantity of reagents used and the time taken to develop and perform the TUNEL assay for the specific identification of damaged DNA in cryopreserved sperm in cryo-media, only allowed for a limited number of assays to be performed.

Therefore, six normal samples (controls, 100µM and 200µM vitamin E fractions) and 12 abnormal samples (controls, 100µM and 200µM vitamin E fractions) were chosen randomly to be assessed for DNA damage. Excluding positive and negative control slides, a total of 54 slides were stained and 10,800 sperm were counted to determine the extent of DNA injury within and between the groups.

### **3.6 STATISTICAL ANALYSIS**

One-way analysis of variance (ANOVA) was used to assess differences in post-thaw survival parameters among the treated and control groups.

Unpaired t-tests were used to assess differences in semen parameters between the normozoospermic and abnormal samples. Paired t-tests were used to establish differences in survival rate, post-thaw vitality and the degree of DNA fragmentation within the normal and abnormal groups.

## **CHAPTER 4:**

## **RESULTS**

4.1 SEMEN PARAMETERS

The semen parameters observed in this study are presented in Table 2. In accord with WHO (1999) semen values, 23 of the 43 samples were classified normal and 20 were abnormal. Individual samples from the abnormal group were classified oligozoospermic (n=6), teratozoospermic (n=9), oligoteratozoospermic (n=3) and oligoasthenoteratozoospermic (n=2). Differences between normal and abnormal groups were seen within motile sperm concentrations, in the degree of morphologically normal forms and in the total volume of ejaculate. Motile sperm concentration and morphological normality was significantly higher in the normozoospermic samples compared to abnormal samples (P < 0.001: unpaired *t*-tests). Ejaculate volume was significantly higher (P < 0.05: unpaired *t*-tests) in the abnormal samples compared to the whole semen volume of normozoospermic samples.

Table 2. Semen Variables

	Age (Years)	Ejaculate Volume (mL)	Total Sperm Concentration (million/Ml)	Motile Sperm Concentration (million/mL)	Motility (% forward progression)	Morphology (% normal)
Normal	36.7 ± 1.4	3.6 ± 0.3	71.8 ± 5.6	53.5 ± 5.1	91.4 ± 1.3	27.4 ± 2.0
Range	25 - 48	1.8 - 8.4	27 - 136.5	16 - 107.0	77 - 100	17 - 47
Abnormal	36.6 ± 1.3	5.0 ± 0.3 <sup>a</sup>	33.7 ± 5.1	20.6 ± 3.5 <sup>b</sup>	90.5 ± 1.7	12.9 ± 1.4 <sup>b</sup>
Range	29 - 51	2.3 - 7.7	0.1 - 92.0	0.1 - 71	83 - 100	5 - 27

Note: Values are the mean ± SEM and the range.

a P < 0.05 compared to normal semen samples.

b P < 0.001 compared to normal samples.

4.2 POST-THAW CHARACTERISTICS OF NORMAL AND ABNORMAL SEMEN SAMPLES

The post-thaw survival rate (Table 3) was similar between normozoospermic and abnormal untreated control samples, and differences in survival rate between normal and abnormal groups from the treated fractions were not statistically significant. However, significant differences in post-thaw vitality between normozoospermic and abnormal samples (Table 4) were observed.

For the untreated control group, post-thaw vitality was higher in the normozoospermic samples than in the abnormal samples ( $P < 0.05$ ; unpaired  $t$ -tests). Similarly, post-thaw vitality in treated semen fractions was significantly higher in the normozoospermic samples that received 100 $\mu$ M or 200 $\mu$ M vitamin E ( $P < 0.05$ ;  $P < 0.001$ ; respectively, unpaired  $t$ -tests).

DNA fractionation revealed by TUNEL assay (Table 5) found that the extent of DNA injury following cryopreservation was significantly higher in the abnormal semen samples treated with 200 $\mu$ M vitamin E than in the normal samples treated with the same vitamin E concentration ( $P = <0.05$ ; unpaired  $t$ -tests). Although not statistically different, controls ( $P = 0.061$ ) and 100 $\mu$ M vitamin E fractions ( $P = 0.074$ ) did show evidence of a slightly higher level of DNA damage in the abnormal samples after cryopreservation.

### 4.3 EFFECT OF VITAMIN E SUPPLEMENTATION OF CRYOMEDIA

The percentage of post-thaw survival (Table 3) in sperm from the normozoospermic group was not different between the control, 100 $\mu$ M vitamin E and 200 $\mu$ M vitamin E sperm fractions ( $P = 0.174$ ; one-way ANOVA). Similarly, no significant differences were observed between treated and untreated groups with abnormal semen parameters ( $P = 0.575$ ; one way ANOVA).

**Table 3. Survival Rate in Normal and Abnormal Semen Samples**

	<i>Control (% survival)</i>	<i>100<math>\mu</math>M Vitamin E (% survival)</i>	<i>200 <math>\mu</math>M Vitamin E (% survival)</i>
<b>Normal</b>	24.9 $\pm$ 2.1	28.6 $\pm$ 2.6	31.8 $\pm$ 3.0
<b>Abnormal</b>	22.4 $\pm$ 2.6	21.2 $\pm$ 3.1	25.9 $\pm$ 3.8

*Note:* Values are the mean  $\pm$  SEM.

Post-thaw vitality in normozoospermic and abnormal samples (Table 4) did not significantly improve with the addition of 100 $\mu$ M and 200 $\mu$ M vitamin E to the

cryomedia (P = 0.639; one-way ANOVA and P = 0.768; one-way ANOVA respectively).

**Table 4. Post-thaw Vitality of Normal and Abnormal Semen Samples**

	<i>Control (% vitality)</i>	<i>100µM Vitamin E (% vitality)</i>	<i>200 µM Vitamin E (% vitality)</i>
Normal	27.5 ± 2.0	28.1 ± 2.0	30.0 ± 2.0
Abnormal	19.3 ± 1.7 <sup>a</sup>	20.3 ± 2.1 <sup>a</sup>	19.4 ± 1.9 <sup>b</sup>

*Note:* Values are the mean ± SEM.  
*a* P < 0.05 compared to normal semen samples.  
*b* P < 0.001 compared to normal samples.

No differences in the degree of DNA fragmentation (Table 5) were detected in the control groups, 100µM and 200µM vitamin E fractions in either the normal (P = 0.93; one way ANOVA) or abnormal samples (P = 0.91; one way ANOVA).

**Table 5. DNA Fragmentation in Normal and Abnormal Semen Samples**

	<i>Control (% DNA injury)</i>	<i>100µM Vitamin E (% DNA injury)</i>	<i>200 µM Vitamin E (% DNA injury)</i>
Normal	24.9 ± 3.8	22.8 ± 4.7	24.2 ± 3.1
Abnormal	38.8 ± 4.5	38.2 ± 5.2	41.1 ± 5.3 <sup>a</sup>

*Note:* Values are the mean ± SEM.  
*a* P < 0.05 compared to normal semen samples.

## **CHAPTER 5:**

## **DISCUSSION**



## 5.1 DISCUSSION

Semen cryopreservation has become an essential component of infertility programs, however exposure to sub-zero temperatures and re-warming can result in the loss of structural integrity and functional capabilities in around 50% of the sperm (Gao et al., 1993; McLaughlin, 2001, p. 264; Woods et al., 2004). Spermatozoon injury as a result of cryopreservation reduces post-thaw motility and viability by disrupting the integrity of the acrosomal and plasma membranes, inducing biochemical alterations and by decreasing DNA stability (Pena et al., 2004; Royere et al., 1996). The degree of spermatozoon injury in cryopreserved samples is particularly amplified in oligozoospermic samples and samples with an unusually high proportion of morphologically abnormal or immature sperm (Counsel, Bellinge & Burton, 2004; Grizard et al., 1999; Oehninger et al., 2000).

Abnormal semen samples are also more likely to be subject to a higher level oxidation (Hendin et al., 1999). Normally, low levels of endogenous reactive oxygen species are functional during capacitation, hyper-activation, the acrosome reaction, and zona binding (Ford, 2004; Kaneko et al., 2002). However, cryopreservation can induce an increased rate of lipid peroxidation in the sperm plasma membrane causing an overall increase in the concentration of oxygen radicals in the sample (Baumber et al., 2003). Exposure to high ROS concentrations can result in the disruption of mitochondrial and plasma membranes, cause chromosomal and DNA fragmentation and bring about a reduction in sperm motility (Aitken & Krausz, 2001; Anzar et al., 2002; Baumber et al., 2003; Gill-Guzman et al., 2001; Parinaud et al., 1997). The adverse effects of oxidation can be reduced by antioxidants, however antioxidant defenses can often be deficient in abnormal semen samples, and the cells can be exposed to a potentially harmful level of oxidation (Aitken & Krausz, 2001; Hendin et al., 1999). The aim of the present study was to determine if the addition of an antioxidant to the semen cryo-media could improve the post-thaw integrity of cryopreserved human sperm from oligozoospermic men.

The results obtained in this investigation found that vitamin E supplementation of semen cryomedia in concentrations of 100 $\mu$ M and 200 $\mu$ M did not improve post-thaw survival, vitality or the degree of DNA fragmentation in samples with abnormal or normal semen parameters.

Twenty of the 43 semen samples included in this study were classified abnormal and as expected revealed a significantly lower number of motile sperm and a lower number of normal morphologies than those in the normal group. Data analysis also found that whole semen volume was significantly higher in samples containing poorer quality sperm compared to the normal semen samples. In the present study, post-thaw vitality in abnormal semen samples was found to be significantly lower than the post-thaw vitality seen in the normozoospermic samples. This finding confirms previous studies, which have found that the damaging effect of cryopreservation is significantly higher in samples with abnormal semen parameters (Aitken et al., 1998; Allamaneni et al., 2005; Counsel, Bellinge & Burton, 2004).

Spermatozoa from subfertile men have been shown to be less resistant to the damage induced by cryopreservation and correlations between low motility counts, poor morphology and a reduced post-thaw vitality have been established in earlier studies (Counsel, Bellinge & Burton, 2002; McLaughlin, 2002; Oehninger et al., 2000; Perez-Sanchez et al., 1994).

In the present study, small variations in post-thaw DNA fragmentation were observed between the normozoospermic and abnormal semen groups, although the differences found were not large enough to be statistically significant. Observations in relation to the extent of DNA fragmentation in semen samples have varied depending on the various clinical manifestations of subfertility measured. DNA strand breaks have been found to occur at a significantly higher level following cryopreservation in samples from men with oligozoospermia (Host et al, 1999), whereas no significant differences in the degree of DNA injury have been found between samples collected from

asthenozoospermic and normozoospermic men (Hughes et al., 1996). In human semen samples, morphologically abnormal sperm are known to possess higher levels of DNA fragmentation (Anzar et al, 2002; Sakkas et al, 1999). DNA injury in the current assay may have been masked by merging oligozoospermic, asthenozoospermic and teratozoospermic semen samples into one study group.

No significant differences were observed in response to the vitamin E treatments of semen samples from both normal and abnormal groups in the present study. Dalvit, Cetica & Beconi (1998) have described similar findings with 1g/mL vitamin E addition to bovine sperm prior to cryopreservation and found no significant differences in post-thaw motility or motion parameters. Conversely, treating boar semen with 100 $\mu$ M and 200 $\mu$ M vitamin E in cryomedia has been shown to significantly improved post-thaw sperm motility (Pena et al, 2004). In the human, vitamin E supplementation has produced improvements in post-thaw sperm integrity using 10mM vitamin E in the cryo-media (Askari et al, 1994). A concern with using higher vitamin E concentrations has been shown by Dalvit, Cetica & Beconi, (1998) who found that treating sperm with vitamin E can interfere with *in vitro* fertilization by inhibiting changes in the sperm plasma membrane that are normally induced by ROS. Assessment of post-thaw functional ability such as the acrosome reaction following cryopreservation with vitamin E supplementation at varying concentrations may help validate the clinical relevance of this antioxidant.

Interestingly in the present study, around a third of the semen samples improved post-thaw parameters considerably with vitamin E supplementation whilst the remainder were negatively or unaffected by vitamin E treatment. The improved response to vitamin E supplementation was found in samples from both normal and abnormal groups and demonstrates a variation in the cryogenic properties of individual semen samples that cannot be predicted by assessing normal semen values. Askari et al, (1994) observed similar variations between human semen samples treated with cryo-media

supplemented with vitamin E. This group however, as mentioned earlier, observed significantly improved sperm survival rates using an increased concentration of vitamin E.

The level of ROS that results in pathological infertility have not yet been defined (Allamaneni et al, 2005). However, the point at which excessive oxidation causes clinical subfertility, and the levels of ROS that may significantly affect sperm during cryopreservation may vary, depending on the antioxidant abilities of semen samples on an individual level. Data collected from this investigation, has established that although vitamin E addition to cryomedia has no significant value in improving the post-thaw parameters of sperm in abnormal semen samples, the observation that individual samples may be responsive to vitamin E treatment necessitates further investigation.

## **5.2 FUTURE INVESTIGATIONS**

McLaughlin (1993) has observed that spermatozoa from different ejaculates of the same donor respond consistently to cryopreservation. Similarly, it would be interesting to determine the amount of variation between different ejaculates from the same donor in semen samples that respond well to vitamin E supplementation. Furthermore, vitamin E is often deficient in the seminal plasma of smokers who tend to excrete vitamin E at a higher rate than non-smokers (Hendin et al., 1999; Jeanes et al., 2004). Establishing an association between smoking and individual responses of semen samples to vitamin E may assist in improving methods of cryopreservation for some individuals undergoing treatment for infertility.

Investigations have suggested that exposure to low levels of oxidative stress may be an essential element in maintaining sperm DNA integrity (by inducing DNA compaction) and can increase oocyte fusion ability. Sperm not exposed to ROS can show DNA fractionation similar to that seen in sperm exposed to high ROS concentrations (Aitken et al, 1998). The present study found that some semen samples were negatively affected by vitamin E supplementation,

thus equilibrium between oxidant levels and antioxidant protection may be necessary. Previous studies have indicated that excessive ROS production induced by cryopreservation is a major cause of cryo-injury (Gill-Guzman et al., 2001; Moustafa et al, 2004; Twig et al, 1998), but have not established basal levels of antioxidant resistance collectively with the degree of oxidation as a result of these levels, before and after cryopreservation. Further studies are required to establish the source of this imbalance and thereby determine if excessive oxidation or deficient antioxidant mechanisms are the defining factor in sperm vulnerability during cryopreservation.

### **5.3 LIMITATIONS**

This investigation did not verify a) fertilization capacity, or b) establish an improved pregnancy rate. The parameters measured here assessed only the effects of vitamin E addition on spermatozoa throughout the cryopreservation process prior to fertilization. Therefore, the ultimate test of antioxidant action in cryomedia in the clinical setting was not determined.

### **5.4 BIAS**

The experimental procedures of this study were performed solely by the author. It was not a blind study, and although efforts were made to reduce subjectivity (such as coding, random thawing and random slide selection for counting), the results obtained may be subject to bias.

### **5.5 CONCLUSIONS**

In conclusion, the present study has shown that vitamin E supplementation of semen cryomedia does not result in improved post-thaw sperm parameters in poor quality semen samples. The unexpected positive effect of vitamin E on post-thaw sperm survival in some of the semen samples from both normal and abnormal semen groups, suggests that the activity of this antioxidant may be dependent on the level of oxidation occurring within the semen samples on an individual level that cannot be predicted by assessing normal semen values. It

is therefore important to further examine the exact nature of cryo-damage in relation to oxidative injury, to determine if excessive ROS generation or endogenous antioxidant deficiency is the cause of sperm damage during cryopreservation.

## **CHAPTER 6:**

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# **CHAPTER 7:**

## **APPENDICES**

7.1 APPENDIX 1

CONSENT FORM

STUDIES ON CRYOPRESEVATION OF HUMAN SPERMATOZOA

Our research group at Concept Fertility Centre would like to use some of your sperm for a research project. Normally, only a portion of your sperm is used for a semen analysis and the remainder is discarded. We would like to use this surplus sperm to study why many sperm die when they are frozen and to look at ways of improving the survival of frozen sperm. One way is to add an antioxidant to the freezing media. We are doing an experiment to see whether adding vitamin E to sperm freezing media improves survival after freezing. This research will provide us with valuable information that will increase our knowledge of the factors that affect sperm function and may therefore benefit couples with male factor infertility. No DNA will be extracted or stored.

I.....have  
(Given Names) (Surname)

read the information above explaining the study entitled "Studies on cryopreservation of human spermatozoa"

I have read and understood the information given to me. Any questions I have asked have been answered to my satisfaction. I agree to allow my sperm to be used for this research.

I agree that research data gathered from the results of this study may be published, provided that names are not used.

If you have any complaints regarding the conduct of this study please refer them to Dr Geoff Masters, Director of Clinical Services, who can be contacted on 9340 8222.

Dated..... day of.....20...

Signature .....

I,.....have explained the above to the signatory who has  
(Investigators full name)

stated that he understood the same.

Signature .....

## 7.2 APPENDIX 2

### SEMEN CRYO-MEDIA

#### SEMEN FREEZING MEDIA

The following disposable items are used:

- 50ml graduated pipettes
- Acrodisc Filters (0.2 $\mu$ m)

The following items are used and resterilized:

- 9x10ml, 1x600ml glass beakers
- Spatulas
- 500ml measuring bottle

The following equipment is used:

- Analytical balance
- Laminar flow hood
- Milli-Q water system

The following reagents are used:

NaCl	1.6g
KCl	0.08g
Ca Lactate	0.152g
MgSO <sub>4</sub>	0.024g
NaH <sub>2</sub> PO <sub>4</sub>	0.01g
NaHCO <sub>3</sub>	0.52g
Hepes	0.954g
Glucose	1.718g
Fructose	1.718g
Glycine	2.00g
Streptomycin	0.01g



Penicillin	0.01g
Glycerol	30ml
HSA	

- Turn on Milli-Q water to recirculate
- Milli-Q water must be at 18.2Ω before use. Discard water if the quality varies from 18.2Ω.
- Run 1 litre of Milli-Q to waste and then fill a 500ml measuring bottle.
- Dissolve the dry ingredients in 170ml Milli-Q water.
- Add HSA
- Add 30ml Glycerol
- Mix well and filter through a 0.2um filter using a 20ml syringe into 30ml aliquots into 60ml Nunk Flasks.
- pH media to 7.2 – 7.4 with NAOH solution.
- Aliquot approximately 30mls of media into 60ml flasks. Freeze and store @ -20°C.
- All the weights and the results of the pH are recorded on Semen media preparation sheet (4.10.31.1) in quality control file.

V4 Feb 2004

Reference: Concept Fertility Clinic. 4.2.1.7 PREPARATION OF BULK ART MEDIA

## **7.3 APPENDIX 3**

### **VITAMIN E AND CONTROL CRYO-MEDIA SOLUTIONS**

Vitamin E analogue: Trolox, FW – 250, Chemicon

1. 8mL semen cryopreservation media was added to 200 $\mu$ L Trolox to make a 0.5M vitamin E stock solution.
2. 200 $\mu$ L of 0.5M Vitamin E solution was added to 9.8mL semen handling media (HM) to produce a 10mM vitamin E/HM solution.
3. To make a 200 $\mu$ M vitamin E working solution, 0.5mL of the 10mM vitamin E/HM solution was combined with 24.5mL semen cryo-media.
4. To make a 100 $\mu$ M Vitamin E working solution, 9mL of 200 $\mu$ M vitamin E solution was added to 9mL semen cryo-media.
5. 24.5mL semen cryomedia and 0.5mL semen handling media made up the control cryo-media.

## **7.4 APPENDIX 4**

### **SOLUTIONS FOR EOSIN & NIGROSINE STAINING**

Eosin (34197)

Nigrosine (340584E)

1. Dissolve 0.5g of 1% Eosin in 10mL dH<sub>2</sub>O.
2. Dissolve 1g of 10% Nigrosine in 10mL dH<sub>2</sub>O.

## **7.5 APPENDIX 5**

### **REAGENTS AND SOLUTIONS FOR TUNEL ASSAY**

#### **PBS**

PBS 10x Concentrate (Amber Scientific, Perth, WA)

- 0.9% sodium chloride
- 0.02% potassium chloride and sodium phosphate
- Potassium phosphate buffer salts

50mL PBS 10x concentrate was added to 450mL dH<sub>2</sub>O. pH automatically adjusted to pH 7 on reconstruction.

#### **0.1% (w:v) Triton X-100 in PBS**

Triton X-100 - 10x concentrate (Sigma-Aldrich, St Louis, USA)

PBS

Add 1mL of Triton X-100 concentrate to 99mL PBS.

#### **Reaction Buffer for DNase I solution.**

10mM Tris HCL solution

2.5mM MgCl<sub>2</sub> solution

1. To make stock solutions dissolve:

3.03g of 500mM Tris (MW 121.1g) in 50mL dH<sub>2</sub>O.

0.48g of 100mM MgCl<sub>2</sub> (MW 95.2g) in 50mL dH<sub>2</sub>O.

0.28g of 50mM CaCl<sub>2</sub> (MW 111g) in 50mL dH<sub>2</sub>O.

2. The following volume of stock solutions was added to 75mL dH<sub>2</sub>O.

Tris: 2mL

MgCl<sub>2</sub>: 2.5mL

CaCl<sub>2</sub>: 1mL

3. pH was adjusted to 7.6 with HCl and made up to 100mL with dH<sub>2</sub>O.

### **DNase I solution**

DNase: Sigma (D4527) 10 000 Kunitz units (2020 units/mg)

1. To make a stock of 1mg/mL DNase I solution, 4.95mL of reaction buffer (above) was added to the bottle of enzyme. The bottle of DNase contained 4.95mg of enzyme)

2. 1mL of this stock was added to 9mL of reaction buffer to make a 0.1mg/ml solution. This was divided into 50μL and stored at -20°C. The aliquots could not be refrozen after thawing due to degradation of product.

3. When required for positive control, one 50μL aliquot was diluted 1:50 with reaction buffer to give a final concentration of 2μg/mL.

4. For long term storage at -20°C, 1mL of the 1mg/mL stock solution was mixed with 1mL glycerol (0.5mg/mL final concentration), and 1mL of the 0.1mg/mL stock solution was added to 1mL glycerol (50μg/mL final concentration).

### **Working strength Stop/Wash buffer**

1mL Stop/Wash buffer (90419, ApopTag ® Plus Peroxidase Kit) was mixed into 34mL dH<sub>2</sub>O in a coplin jar.

### **Working strength TdT enzyme**

Reagents included in ApopTag® Plus Peroxidase Kit

Concentrated TdT enzyme (90418)

Reaction Buffer (90417)

33 $\mu$ L TdT concentrate was added to 77 $\mu$ L Reaction buffer in a microcentrifuge tube. This was enough for 2 slides. Larger quantities were made in a 70% Reaction buffer to 30% TdT concentrate in 1.5mL eppendorf tubes.

### **Working strength peroxidase substrate**

Both reagents included in ApopTag® Plus Peroxidase Kit

50x DAB (90423)

1x DAB dilution buffer (90424)

For every two slides 3 $\mu$ L DAB substrate was added to 147 $\mu$ L DAB dilution buffer.

### **0.5% (w:v) Methyl green**

Methyl-Green (M8884) Sigma-Aldrich

1. Make up 0.1M sodium acetate, pH 4. Dissolve 1.36g  $C_2H_3O_2Na \cdot 3H_2O$  in 80mL of  $dH_2O$ , add acetic acid to obtain pH 4.0 and adjust volume to 100mL with  $dH_2O$ .
2. Dissolve 0.5g of methyl green in 100mL of the 0.1M sodium acetate solution.
3. Filter through 0.2 micron filter.