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The Effects of Different Cryoprotectants on Stallion Epididymal Spermatozoa

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ABSTRACT

Any event that makes semen collection or mating impossible, such as death, castration, or injury, may terminate a stallion's breeding career. Fortunately, stallion sperm which are capable of fertilization can be harvested from a region adjacent to the testicles called the epididymis, and frozen for future use. However, the fertility of frozen-thawed epididymal sperm has been found to be lower than that of ejaculated sperm.

This thesis investigated the effects of different cryoprotectants and dilution after thawing with SW3 media (SW3, JEL Media, NZ), which contains complex amino acids, sugars and no protein, on the fertility and sperm characteristics of frozen-thawed epididymal sperm.

Epididymal sperm were obtained from 5 colts at the time of castration and each collected sample was split into different treatment groups and frozen with different cryoprotectants (glycerol, formamides or a combination) in the same base extender. The sperm were frozen in liquid Nitrogen until the time of insemination or post-thaw analysis. The effects of different cryoprotectants and the addition of the post-thaw dilution medium on motility parameters, morphology, acrosome status, plasma membrane integrity and DNA integrity were evaluated after thawing.

The reproductive tract of mares were examined by ultrasound during oestrous and the mares were inseminated at 4 hours prior to ovulation with epididymal sperm frozen in either glycerol or a glycerol and formamide combination. Fertility was determined at either 8 days after ovulation when the uterus of each mare was flushed for an embryo or at 14 days after ovulation when the mare was scanned to determine if there was a pregnancy.

The post-thaw dilution medium (SW3) was found to have a significant beneficial effect on the motility of frozen-thawed epididymal sperm frozen with glycerol *in vitro*. Pregnancy data revealed that the glycerol and formamide combination cryoprotectant was superior to glycerol alone, with 50% versus 10% pregnancy rates recorded respectively. These results suggest that the higher pregnancy rates

are the effect of the cryoprotectant and not the complex amino acids of the base extender (which was the same for all samples) which contribute to the improved fertility of the frozen epididymal sperm. Furthermore, because good pregnancy rates without the addition of SW3 were obtained prior to insemination, this supports the conclusion that the cryoprotectant is a key factor altering the fertilizing ability of epididymal sperm.

Investigations into the fertility of epididymal sperm will enhance our understanding of factors which affect sperm fertility whilst in storage in the cauda epididymius. Furthermore, the opportunity to preserve the fertility of these gametes at the time of gelding or death of a stallion will have long-lasting beneficial effects on the size of the gene pool for selection. This is not only important for the domestic horse but for endangered equine species also. Ultimately, the fertility of epididymal sperm should be optimised to ensure that the maximum numbers of doses are available of this limited resource.

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LIST OF ABBREVIATIONS

ABP	Androgen Binding Protein
AI	Artificial Insemination
ANOVA	Analysis of Variance
AR	Acrosome Reaction
AV	Artificial Vagina
BC	Botu-Crio
CASA	Computer Assisted Semen Analysis
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene Diaminetetracetic Acid
EG	Ethylene Glycol
FITC	Fluorescein Isothiocyanate
FSH	Follicle-Stimulating Hormone
GLY	Glycerol
GnRH	Gonadotrophin Releasing Hormone
hCG	Human Chorionic Gonadotrophin
HOS	Hypo-osmotic Swelling
LH	Luteinizing Hormone
MF	Methyl-Formamide
PBS	Phosphate Buffered Saline
PM	Progressive Motility
PNA	<i>Arachis hypogaen</i> (Peanut) Agglutinin
PSA	<i>Pisum sativum</i> (Pea) Agglutinin
rpm	Revolutions Per Minute
S.D.	Standard Deviation
TM	Total Motility
TPM	Total Progressive Motility
UTJ	Utero-tubual Junction
x10 ⁶	Million per ml

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Sudden death, catastrophic injury, castration or any other event that makes semen collection or mating impossible may prematurely conclude a stallion's reproductive life. Horse owners may request a final semen collection in the former two instances if their horse was a champion, or in the latter instance they may request a sperm collection for storage following castration so that the genetic potential is safeguarded in case the animal later becomes a champion. One may ask why do breeders refrain from collecting semen by conventional means (mating a phantom mare and collecting the ejaculate in an artificial vagina) from their young colts prior to castration for possible later use? The simple answer is that training young colts to produce semen can lead to management issues later due to the acquisition of the "mating behaviour" which can lead to complications in relation to their schooling.

Fortunately the use of frozen semen can preserve the genetics of an individual male. Furthermore, it is possible to recover and freeze sperm from a region adjacent to the testicles called the epididymis at the time of gelding or death for future use (Barker & Gandier 1957; Johnson et al. 1980). Indeed, the first pregnancy from frozen – thawed stallion sperm was produced after insemination of seven mares with frozen epididymal sperm (Barker & Gandier 1957). However, the fertility of frozen thawed epididymal sperm has also been shown to be lower than that of ejaculated sperm (Morris et al. 2002c).

This study aimed to optimize the fertility of frozen epididymal stallion sperm by investigating the effects of different cryoprotectants and post-thaw dilution on the motility, morphology, acrosome status and membrane integrity of epididymal sperm. Subsequently, the DNA stability and fertility rates of epididymal sperm frozen in different cryoprotectants were investigated. The establishment of a practice which enhances the fertility and utilization of epididymal sperm will

mean it can be used more widely to preserve the genetics of prized horses as well as give insight to ways in which the genetics of endangered species may be protected.

1.2 STRUCTURE OF THE SPERMATOZOON

The spermatozoon was first acknowledged by Anton van Leeuwenhoek and Johan Hamm in 1677 with the assistance of a microscope (Davies Morel, 1999). Sperm were described as “animalacula”, meaning “innumerable minute bodies with the power of active forward motion” (Pesch & Bergmann 2006). Structurally sperm consist of three primary regions: the head, mid-piece and tail (Figure 1.1), with the entire spermatozoon being enveloped by a plasma membrane which is characterised by a regional specific glycoprotein and lipid constitution (Pesch & Bergmann 2006).

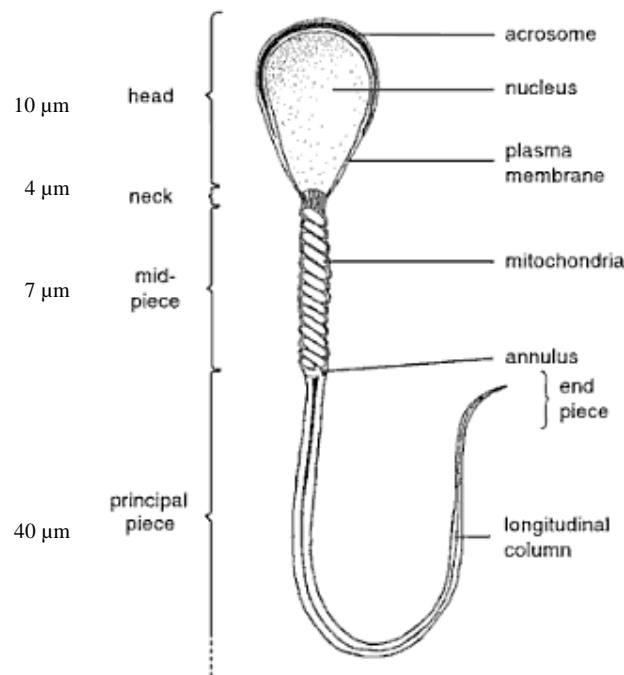


Figure 1. 1 Diagrammatic representation of a Stallion Spermatozoon including dimensions (Adapted from Davies Morel, 2008).

The head of the stallion spermatozoon is broad, relatively flat in shape, and contains highly condensed chromatin within its nucleus (Amann & Graham 1993; Pesch & Bergmann 2006). Only in the neck region, there is a small area of uncondensed chromatin where transcription, translation and protein-biosynthesis are still possible (Dadoune et al. 2004; Miller et al. 2005). The stallion spermatozoon has a double membrane, except in the anterior region, where there

is an additional membrane forming the acrosome cap (Davis Morel, 2008). This acrosome cap includes hydrolytic enzymes like acrosin, hyaluronidase and esterases necessary for the acrosome reaction (see Section 1.4: Capacitation and the Acrosome reaction) (Pesch & Bergmann 2006). Below the acrosome cap is an equatorial sector, this is the region which fuses with the plasma membrane during the acrosome reaction, preceding fertilisation (Amann & Graham 1993).

The mid-piece of the spermatozoon is in fact part of the tail (discussed below), and is attached to the head by a ball and socket arrangement, referred to as the implantation fossa (Amann & Graham 1993). In stallions, morphologically normal sperm commonly have an abaxial (off centre) location of the mid-piece (Dowsett et al. 1984; Graham 1996). The midpiece is characterised by a mitochondrial sheath (Pesch & Bergmann 2006) and this dense population of energy-producing mitochondria is needed for metabolism and to drive the tail during the sperm movement (Davies Morel 2008).

The longest part of a spermatozoon, the tail, is said to include the neck, mid-piece, the principal piece and the end piece (Barth & Oko 1989). It is made up of a series of microtubule fibrils that form the contractile elements, doublets, which use the energy provided from the mid-piece to flex the tail in a helical pattern and drive the spermatozoon (Eddy 1988; Barth & Oko 1989; Amann 1993).

1.3 SPERMATOGENESIS

1.3.1 The Physical Steps of Spermatozoatogenesis

Spermatogenesis is a process which involves the maturation of male spermatogonia to spermatozoa or 'sperm' and is thus the male version of gametogenesis. It occurs in the testes and epididymis in a stepwise fashion and involves three intergrated processes; spermatocytogenesis, meiosis and spermiogenesis (Johnson et al. 1997; Martini 2006) to produce mature elongated spermatids (Barth & Oko 1989). In the stallion this entire progression takes 57 days (Johnson et al. 1997; Davies Morel 2008).

The first step of spermatogenesis, spermatocytogenesis, is the mitotic division of spermatogonia to produce stem cells and primary spermatocytes and takes 19.4 days in the stallion (Barth & Oko 1989; Johnson et al. 1997; Davies Morel 2008).

Unlike the following step; meiosis, spermatocytogenesis does not result in the reduction in chromosome number, and hence diploid (64 chromosome) spermatogonia give rise to diploid primary spermatocytes. In meiosis however, a single diploid cell divides to produce two haploid cells, which are round spermatids (Barth & Oko 1989; Davies Morel 2008). Meiosis may be divided into the first and second meiotic division and together these also take 19.4 days. Subsequently, spermiogenesis, a slightly shorter process taking 16.6 days, involves the differentiation of the round spermatid to produce elongated sperm (Barth & Oko 1989; Johnson et al. 1997). This step can be later broken down into four steps, namely, the Golgi, cap, acrosome and maturation phases.

The Golgi phase refers to the stage when the golgi apparatus produces a spherical acrosome granule which migrates to the end of the nucleus (Barth & Oko 1989). Additionally the centriole migrates to the opposed side of the nucleus (Barth & Oko 1989). The cap phase is the period where the acrosomic head cap of the spermatids is growing. Here the acrosome shape changes to form a cap over the nucleus and also flagellum begin to form (Barth & Oko 1989; Johnson et al. 1997). During the acrosome phase the most dramatic morphological transformations to the nucleus and acrosome occur (Barth & Oko 1989). Here the manchette form; these are temporary organelles only found in spermatids, composed of linked microtubules which form a sheath around the lower half of the morphologically changing nucleus and extend to the developing flagellum (Johnson et al. 1997). The nucleus is further condensed as it is transported through the epididymis (Barth & Oko 1989). In the final phase of spermatid development, the maturation phase, spermatids complete their differentiation, and the final formation of the flagellum and neck piece occur as well as maturation and shaping of the nucleus (Barth & Oko 1989). The manchette migrate caudally to possibly provide a shaft to support the flagellar canal, before dissolving (Johnson et al. 1997). In addition the mitochondria migrate and group on the flagellum at the midpiece region. Finally a membrane forms to complete the spermatozoan (Barth & Oko 1989; Johnson et al. 1997).

1.3.2 The Hormonal Control of Spermatogenesis

Regular male reproductive function requires neural communication between the central nervous system, the hypothalamus and reproductive organs (Amann 1986).

Hormonal and neurochemical signals relay information between the hypothalamus, anterior pituitary gland, Leydig cells, Sertoli cells, and germinal epithelium. Therefore the functioning of each of these components of the male reproductive system, as well as the epididymis, accessory sex glands, and ejaculatory mechanism is controlled by signals from one or more distant sites. In theory, fertility and fecundity might be suppressed by altered function at any of these regions (Amann 1986).

The hormonal control of spermatogenesis is illustrated in Figure 1.2. Follicle stimulating hormone (FSH) and testosterone are essential for successful spermatogenesis (McLachlan et al. 1995). The anterior lobe of the pituitary gland releases luteinizing hormone (LH) and FSH (Martini 2006). The pituitary release of these hormones occurs is stimulated by gonadotropin-releasing hormone (GnRH) (Matsumoto et al. 1983), a peptide synthesised in the hypothalamus. GnRH is secreted in pulses and as the levels of GnRH varies so does the rates of secretion of FSH and LH, and testosterone, which is released in response to LH (Martini 2006). Both FSH and testosterone exert synergistic actions on germ cells, but it is testosterone which has specific effects on the later stages of epididymal maturation (McLachlan et al. 1995).

LH stimulates the secretion of testosterone and other androgens by the interstitial cells of the testes (Martini 2006). Testosterone is the most likely regulator of spermatogenesis in the stallion, as it had been shown to be vital for the continuation of spermatogenesis in the adult testes (Setchell 1978). FSH targets primarily the Sertoli cells of the seminiferous tubules. Under FSH stimulation and in the presence of testosterone from the interstitial cells, Sertoli cells 1) endorse spermatogenesis and spermatogenesis, and 2) secrete androgen-binding protein (ABP) (Martini 2006). Furthermore, the sperm output of the adult testes is determined by the number of Sertoli cells (McLachlan et al. 1995).

The rate of spermatogenesis is controlled by a negative feedback mechanism involving GnRH, FSH and inhibin. Under the stimulation of GnRH, FSH promotes spermatogenesis along the seminiferous tubules. As spermatogenesis accelerates, however, so does the secretion of inhibin from the Sertoli cells. Inhibin reduces FSH production in the anterior lobe of the pituitary gland and may

also suppress the secretion of GNRH at the hypothalamus (Martini 2006). The overall effect is that when FSH levels become high, inhibin production rises until FSH levels return to equilibrium. If FSH levels fall, inhibin production drops, so the rate of FSH production accelerates (Martini 2006).

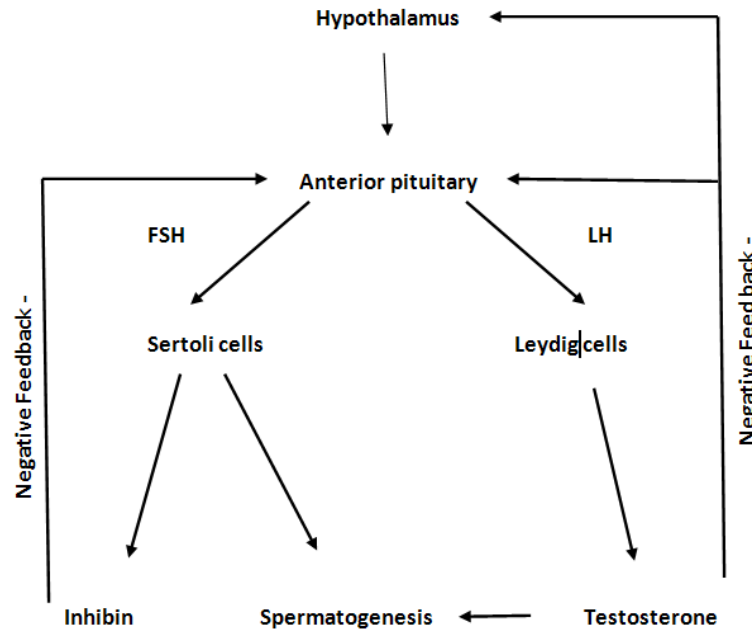


Figure 1. 2 The hormonal control of spermatogenesis (Davies Morel 2003; Weston 2005)

1.4 CAPACITATION AND THE ACROSOME REACTION

In mammals, sperm immediately post ejaculation, while motile, are incapable of fertilization until they proceed through capacitation and the acrosome reaction. The capacitation step takes place *in vivo* in the female genital tract and which is the precursor to the acrosome reaction in the egg zona pellucida (Hamamah & Gatti 1998). The changes produced during capacitation, together with the subsequently induced acrosome reaction (AR), are irreversible exocytotic events which are essential if sperm are to bind and penetrate the zona pellucida and thereafter fuse with the oocyte plasma membrane (Yanagimachi 1994).

The capacitation phenomenon was first described by Austin (1951) and Chang (1951), and it is a progression of biochemical and physiological events that a spermatozoon must undergo to render it capable of fertilization (Austin 1951; Chang 1951; Barth & Oko 1989; Amann & Graham 1993; Yanagimachi 1994;

Samper 2000; Davies Morel 2008). In the female reproductive tract ‘capacitation factors’ (Gadella et al. 2001; Colenbrander et al. 2002), result in the reorganisation of membrane proteins, metabolism, rearrangement of phospholipids (Rathi et al. 2001), a fall in cholesterol levels (Gadella et al. 2001) and hyperactivation of sperm motility (Yanagimachi 1994; Colenbrander et al. 2001) enabling sperm to move away from the oviductal epithelium, and to provide the thrust needed for them to penetrate the zona pelucida (Rathi et al. 2001). It is thought that seminal plasma components may have a ‘washing’ effect and help remove ‘de-capacitation’ factors that coat the sperm during storage in the epididymis; alternatively seminal plasma may contain factors that more directly promote sperm activation (Sostaric et al. 2008).

Once the semen is deposited into the female reproductive tract and capacitation has been induced, it progresses through the uterus by uterine contractions (Katila 2001b) towards the site of fertilization in the ampullary isthmus junction of the oviduct (Hunter & Nichol 1983). When a connection has been made with the egg, the spermatozoon undergoes the acrosome reaction releasing acrosomal hydrolytic enzymes, which hyperactivate motility and aid in penetration of the oocyte (Cummins & Yanagimachi 1986; Pesch & Bergmann 2006).

1.5 FUNCTION OF THE EPIDIDYMIS

In the reproductive system of all male mammals is a long tightly coiled tube called the epididymis through which sperm leaving the testes must transit (Sullivan et al. 2005; Sostaric et al. 2008). Embryologically, the epididymis is derived from the tissue that formed the mesonephros in some ancient animals, a primitive kidney (Sostaric et al. 2008). The stallion epididymis has been shown to be approximately 80 m long (Mannely 1959) and it is divided into three major regions: the caput, corpus and cauda (Reid & Cleland 1957), each section having a particular function. The caput is concerned with the re-absorption of fluid and solutes from the rete testes, the corpus with sperm maturation and the cauda in the storage of fertile sperm until ejaculation occurs (Amann & Graham 1993). During the storage period the cauda epididymides accumulate sperm to ensure that a sufficient number is available at the time of ejaculation. In bulls and stallions the number of stored sperm is approximately enough for 10 ejaculates (Sullivan et al. 2007).

Sperm are transported through the three regions by the combination of epididymal epithelial secretions, ciliary activity, and smooth muscle activity (Swierstra et al. 1974). Reports by Swistera *et al.* (1974) have stated that the time for sperm to pass through the caput to the cauda is approximately 4.1 days in the stallion. However, others have stated that the duration of epididymal transit varies between 5 and 14 days in the stallion (Franca et al. 2005). Throughout this passage the sperm undergo vital morpho-functional alterations (Papa et al. 2008). This is due to the sequential exposure of sperm to epididymal fluids (Orgebin-Christ 1967), a process controlled by androgens (Mann & Luwak-Mann 1981). As well as the acquisition of potential for motility, the midpiece is stabilized (Barth & Oko 1989; Hafez & Hafez 2000), modifications occur in the chromatin of the sperm nucleus (Johnson et al. 1980), changes in the plasma membrane proteins occur (Sullivan et al. 2005), there are increases in total surface negative charges in the number of disulfide bonds (Sullivan et al. 2005) occur, reorganisation of surface antigens occurs (Sullivan et al. 2005) and the acrosome is stabilised and its size is altered (Hafez & Hafez 2000). The major visible change is the migration of the cytoplasmic droplet from the proximal to distal midpiece. Sperm in the cauda epididymis still possess the droplet of residual cytoplasm in the distal midpiece that remains after the reshaping of the cell during spermiogenesis (Sostaric et al. 2008).

The *in vivo* fertility of cauda epididymal sperm tends to be less than that of ejaculated sperm in cattle and in sheep (Dacheux & Paquignon 1980). In the majority of species studied, these sperm are immotile and unable to either 'recognise' the zona pelucida or undergo acrosomal reaction, even when a calcium signal is provided artificially in the form of the ionophore, A23187 (Williams et al. 1991; Yanagimachi 1994; Sirivaidyapong et al. 2001). The absence of exposure to seminal plasma and the continued exposure to inhibitory factors in epididymal secretions have been thought to contribute to this low motility and poor fertility. Moreover, the addition of seminal plasma has been shown to improve the motility of epididymal sperm in both fresh samples (Braun et al. 1994) and samples following the freeze-thaw process (Stout et al., 1999). However other studies observed no significant effect after the addition of seminal

plasma to epididymal sperm on motility (Bruemmer et al. 2002) or fertility (Morris et al. 2002b).

1.6 COLLECTION OF EPIDIDYMAL SPERMATOZOA

The appropriate epididymal sperm collection techniques for use in terminal cases in horses has not been fully evaluated (Cary et al. 2004). However, several methods of sperm recovery from the cauda epididymis have been described including aspiration (Sharma et al. 1997), floatation (Hewitt et al. 2001), and retrograde flushing of the cauda epididymis (Morris et al. 2002b). Retrograde flushing is a technique where a syringe is attached to the vas deferens and sperm are carried by the extender and expelled at the junction between the cauda and corpus where a cut has been made (Garde et al. 1994). This method has been compared to the floatation technique by Martinez-Pastor *et al.* (2006) and was shown to be the superior technique as it obtained higher numbers of sperm, and was less contaminated with other cell types (Martinez-Pastor et al. 2006). Similarly Verberckmoes et al., (2004) concluded that flushed samples are better protected not only because the sperm is pushed by the extender, but also because the contact with blood and other fluids, which can be damaging, is reduced due to the few cuts performed. The sperm acquired from this technique can be used for artificial insemination of either fresh or frozen semen, and result in pregnancy (Barker & Gandier 1957; Morris et al. 2002c).

A modified retrograde flush technique, reported by Graneman, (2006), consists of the separation of the testes-epididymis complex, removal of surrounding tissues by blunt dissection and straitening the epididymal duct. After this, the duct is cut in three parts to facilitate flushing. Flushing is carried out by injection of extender into the upper portion of the lumen until sperm cells are recovered at the lower extremity (Graneman 2006). This technique allows the recovery of a higher number of sperm than compared with a single collection with an artificial vagina and was used in the present study.

1.7 CRYOPRESERVATION OF STALLION SPERMATOZOA

Since the beginning of the 20th century, the cryopreservation of semen has extensively been used for animal breeding and conservation of endangered species (Pesch & Bergmann 2006). In stallions, the first pregnancy obtained from the

insemination of frozen-thawed sperm was reported by Barker and Gandier (1957) and was in fact obtained from epididymal sperm. Yet, since this time most reports of the fertility of frozen semen are based on ejaculated samples (Heitland et al. 1995; Samper & Morris 1998). It was quickly recognised that sperm cannot be frozen raw and must therefore be frozen in semen extenders which contain nutrients, buffers, antibiotics and a cryoprotective agent. Nutrients, such as glucose serve to provide an energy source for the sperm (Graham et al. 1978; Mottershead 2000). Buffers are added to balance pH and reduce the production of acid (Graham et al. 1978; Mottershead 2000). Various antibiotics are added to control bacterial content and the cryoprotectant assists in stabilizing the cell during the freezing and thawing process (Hafez & Hafez 2000; Mottershead 2000). In addition, egg yolk is often used in extenders as its presence has been shown to protect against cold shock (Phillips & Lardy 1940; Bedford et al. 1995) and to reduce the concentration of glycerol required (Watson & Martin 1974). Semen extenders may also be based on milk solids as opposed to egg yolk, or a combination of those (Aurich 2008).

A significant challenge to successful sperm cryopreservation is that a complete arrest in the development and metabolic processes within the sperm is essential (Pesch & Bergmann 2006). So, the cells must be held in a state of “suspended animation” until needed for fertilisation (Pesch & Bergmann 2006). Acquisition of this state can result in reduced fertility of some stallion’s which limits our ability to cryopreserve semen from some stallions (Squires et al. 2004). The damage which can occur during the process can be attributed to a suite of factors such as the changes in temperature, ice crystal formation, oxidative damage, alterations in the sperm membrane, DNA damage, toxicity of cryoprotectants, osmotic stress associated with the addition and removal of molar amounts of cryoprotectants and alteration in solute concentrations during freezing (Watson 2000). Moreover, frozen-thawed stallion sperm has been reported to take longer to reach the oviduct (Bader 1982), and Dobrinski et al. (1995) reported that frozen-thawed sperm do not adhere to the epithelial lining of the oviduct as well as fresh ejaculated sperm do.

1.7.1 Cryopreservation of Epididymal Spermatozoa

Frozen equine epididymal sperm have been shown to be fertile; however recent efforts at achieving successful pregnancy rates have been challenging (Morris et al., 2002). Sperm recovered from the stallion epididymis has been shown to display progressive motility equal to, or better than, ejaculated sperm (Tiplady et al. 2002). It is speculated that the variation in morphology and function between epididymal sperm contribute to differences in membrane stability, cold shock susceptibility and resistance to osmotic stress. Therefore, methods used for cryopreservation of ejaculated sperm might not be appropriate for epididymal sperm (Hewitt et al. 2001). While individual stallion variation exists, overall per-single cycle pregnancy rates following the use of frozen ejaculated sperm range from 30% to 50.6% (Loomis et al., 1999; Samper, 1990). The pregnancy rates following the use of frozen epididymal sperm are anecdotally extremely low using standard insemination methods. However, per cycle pregnancy rates from 17% to 30% have been obtained by depositing the sperm hysteroscopically close to the uterotubal junction (Morris et al., 2002).

The limitations experienced in the freezing procedures for ejaculated sperm are more exaggerated and less developed for epididymal sperm. (Perez-Osorio et al. 2008) The limited success in both cases may be partially attributed to the most commonly used cryoprotectant, glycerol. For this reason research has been conducted into other forms of cryoprotectants including formamides and derivatives of formamides such as dimethylformamide and methylformamide or combinations of formamides with glycerol such as Botu Crio®.

1.7.2 Glycerol

The pivotal advancement in the field of cryopreservation was the introduction of glycerol as a cryoprotective agent in bull sperm (Polge et al. 1949). Glycerol replaces water within the sperm cell cytoplasm and this reduces intracellular ice crystal formation (Fayrer-Hosken et al. 2008). However, the use of glycerol has limitations in the cryopreservation of stallion sperm, and large variability in its fertility is reported between individual stallions (Guay et al. 1981). It has been tested at multiple concentrations in egg-yolk extenders, and lower concentrations of glycerol are more effective in the survival of sperm cells, (Cochran et al. 1984; Cristanelli et al. 1985), typically ranging between 2 and 5% (Alvarenga et al.

2005) in the stallion compared with bull sperm, which require a glycerol concentration of 7% (DeJarnette et al. 2000). Indeed, in horses, Ball and Vo (2001) showed deleterious effects of increasing glycerol concentration and the addition and removal of glycerol on motility and acrosome integrity of fresh equine semen. Moreover, Vindament et al. (2009) demonstrated significant deleterious effects on fertility of high glycerol (4.8%) on fertility of frozen semen. Furthermore, it has been revealed that molar concentrations of glycerol can affect the physical features of the cytoplasm, the permeability and stability of the membrane bilayer(s), and the noncovalent attachment of proteins to the sperm surface (Hammerstedt & Graham 1992).

1.7.3 Dimethylformamide (DMF)

In an effort to minimise osmotic damage as well as the toxic effects (Fahy 1986; Hammerstedt & Graham 1992) of glycerol on sperm and its contraceptive effects in the mare (Pace & Sullivan 1975; Demick et al. 1976; Bedford et al. 1995), alternative cryoprotectants, with lower molecular weights and greater membrane permeability than glycerol, have been assessed in effort to improve our ability to freeze equine sperm (Squires et al. 2004; Perez-Osorio et al. 2008). Since these compounds permeate the plasma membrane more effectively than glycerol, they should in theory cause less osmotic damage to stallion sperm than glycerol does. Therefore, these compounds may prove very effective in the cryopreservation of stallion sperm, and could be particularly useful for stallions that produce sperm with poor post-thaw qualities when glycerol is used as the cryoprotectant.

Recent studies have demonstrated beneficial effects of using amides such as dimethylformamide (DMF) and methylformamide (MF) as cryoprotectants. For example, Squires et al., (2004) found that methylformamide and dimethylformamide protected stallion sperm from cryodamage as effectively as glycerol did. Furthermore, recent fertility trials have also been performed that revealed a significant improvement on fertility of stallion semen frozen with dimethylformamide when compared with glycerol (Medeiros 2003; Moffet et al. 2003). Medeiros (2003) found after artificial insemination of 30 mares, those who were inseminated with 800×10^6 sperm frozen in DMF had greater pregnancy rates (40% pregnant) than those inseminated with the same number of sperm frozen in glycerol (0 pregnant). Furthermore Gomes et al. (2002) stated that DMF

improved most variables evaluated by computer-assisted sperm analysis (CASA) when compared with glycerol. In contrast, other experiments have shown no differences in fertility rates for mares inseminated with semen that was frozen with extenders containing DMF or glycerol, such as that by Vindament et al., (2002) in which mares were inseminated daily with resulting pregnancy rates for mares inseminated with semen frozen with extender containing 2% glycerol or 2% DMF recording at 46% and 50%, respectively (Vidament et al. 2002).

The benefits of DMF are therefore controversial as are the concentrations in which it is most effective. It has been demonstrated that 2% DMF provided better post-thaw motility than 1%, 3% and 5% when 'good freezer' stallions were used (Vidament et al. 2002). In contrast, Gromes et al. (2002), Medeiros et al., (2002), and Squires et al. (2004) showed that greater concentration of DMF (5%) resulted in enhanced post-thaw motility, particularly with so called 'bad freezer' stallions.

1.7.4 Botu Crio®

In recent times a new freezing extender Botu Crio® (Biotech Botucantu-Brazil) has been made commercially available for stallions (Samper & Garcia 2008). This extender combines glycerol and methylformamide as the cryoprotectant, and includes 20 different amino acids in the base diluent. The combination cryoprotectant and the base diluent can be purchased separately and therefore used in combination with other cryoprotectants or base extenders. *In vitro* evaluation of semen frozen in Botu Crio® suggests that motility parameters are increased in comparison with thawed semen processed in extenders containing only glycerol as the cryoprotectant. Samper and Garcia (2008) compared semen frozen in 3.5% glycerol and Botu Crio® extender containing 1% glycerol and 4% methylformamide and found no effect of cryoprotectant on fertility for good freezers but semen frozen in Botu Crio® was significantly better than semen frozen in glycerol for the poor freezing group. They concluded that Botu Crio® extender appears to enhance post-thaw quality and fertility of stallions regarded as poor freezers.

Other combination cryoprotectants have also been assessed, some proving advantageous. For example Vindament et al. (2002) reported that the combination of DMF and glycerol was more effective than either alone. They found that

motility decreased with 5% DMF (0.66 mol/l) when combined with 3 or 5% glycerol and sperm motility was high after freezing in 1% or 3% DMF combined with 1% or 3% glycerol.

1.7.5 Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) has been found to be the optimal cryoprotectant for rabbit (Wales & O'Shea 1968) and elephant (Jones 1973) sperm. Chenier et al. (1998) explored the five cryoprotectants; glycerol, ethylene glycerol, diethylene glycol, propylene glycol and DMSO at four molarities (0.5, 1.0, 1.5 and 2.0 mol/l respectively) on multiple ejaculates from seven fertile breeding stallions. Overall DMSO gave the best post-thaw motility and viability values, and superior post thaw results for stallions whose semen froze poorly in glycerol. In the same study a fertility trial was conducted on 1 mol/l DMSO which gave pregnancy rates of 78% (Chenier et al. 1998). In contrast, Alvarenga et al. (2000) froze semen from 10 ejaculates each from 10 stallions frozen in 0.55 mol/l GLY, 0.9 mol/l ethylene glycol (EG), 1 mol/l DMSO or 0.6 mol/l DMF and found that after thawing the percentage of total and progressive motile sperm after thawing was less ($p < 0.5$) in the semen frozen in the presence of DMSO compared with other cryoprotectants. Consequently they concluded that DMSO was not effective in preserving stallion semen (Alvarenga et al. 2000).

1.7.6 Ethylene glycol

Ethylene Glycol (EG) is the cryoprotectant of choice for bovine embryos, but limited studies have been conducted in its use for freezing semen, in particular equine sperm. Alvarenga et al. (2000) compared different cryoprotectants and found that EG and DMF provide similar cryoprotection as glycerol, based on post-thaw motility and flow cytometry analysis. Kotjangia et al. (1963) reported that three of five mares (60%) conceived when inseminated with semen frozen using 6% EG as a cryoprotectant (Kotjagina et al. 1963). Rombe and Kotjagina (1968) obtained similar pregnancy rates for mares inseminated with semen frozen in extenders containing glycerol or EG (Rombe & Kotjagina 1968). Squires et al. (2004) found that EG at the concentration of 0.9 mol/l resulted in similar percentages of motile cells (43%) in comparison with glycerol (52%) at 0.55 mol/l. Nevertheless, larger trials using EG as the cryoprotectant should be conducted. (Squires et al. 2004).

1.8 ASSESSMENT OF THE FERTILIZING POTENTIAL OF SPERMATOZOA

Semen evaluation is an extremely important tool that is used to calculate insemination doses as to assess what defects are present in a semen sample and determine why these defects have occurred (Graham & Mocé 2005). Assessments of total progressive motility (TPM), morphology and acrosome status are the most common parameters used to predict the fertility of an ejaculate, however there are also other tests available to assess the viability and fertility of a semen sample such as the assessment of plasma membrane integrity, DNA fragmentation, mitochondrial function and fertilizing ability. Ultimately, the gold standard for fertility function and evaluation is to investigate the ability of the sperm to produce a pregnancy (Samper et al., 2007). Yet, because fertility is a binomial variable it has been reported that these tests must be repeated at least 100 times to be statistically significant and enable conception rates to be used as an assessment of fertility (Graham & Mocé 2005). Furthermore, mare pregnancy rates can be strongly influenced by the age and history of the inseminated mares as well as by breeding management (Colenbrander et al. 2003). For the above reasons, combining the results of sperm function tests improves the reliability of fertility estimation (Colenbrander et al. 2003). Refer to chapter 3 for an elaboration on external semen assessment and methods, chapter 4 for DNA assessment procedures and chapter 4 for artificial insemination techniques and experiments.

1.9 THE TIMING AND NUMBER OF SPERMATOZOA USED IN ARTIFICIAL INSEMINATION

Many factors influence the pregnancy rate in horses bred by artificial insemination, including the inherent fertility of the mare and stallion, the type of semen inseminated (i.e. fresh, cooled-transported or frozen-thawed) (Jasko et al. 1992c), the timing of insemination, the number of sperm in the insemination dose (Pickett & Voss 1975b) and the concentration of extended semen (Jasko et al. 1992c).

The timing of insemination must be considered with respect to the time of ovulation of the dominant follicle to optimise pregnancy rates. In the case of frozen-thawed semen, efforts are made to deposit the sperm closer to the time of ovulation as the longevity of this type of frozen-thawed sperm is reduced in

comparison to fresh or chilled sperm. The use of human chorionic gonadotrophin (hCG), which has an LH-like action in mares, to induce ovulation aids to optimise the time of insemination relative to ovulation (Sieme & Klug 1996). Sieme et al. (2003) compared the outcomes on pregnancy rates after inseminating cooled (at a dose of 500×10^6) and frozen-thawed (at a dose of 800×10^6) sperm once, twice or three times. They found that mares inseminated once with cooled semen during an oestrus had pregnancy rates comparable to those inseminated two (56.5%) or three (71.4%) times at 24 hour intervals. Likewise in the same study, a single frozen-thawed semen insemination between 12 hours before (41.3%) and 12 hours after (50%) ovulation produced similar pregnancy rates to those achieved when mares were inseminated either two (50%) or three (33.3%) times at 24 hour intervals (Sieme et al. 2003). Similarly, a study by Barbacini and colleagues found no difference in per-cycle pregnancy rate for mares that were inseminated 24 and 40 hours after hCG with 400×10^6 total sperm (46%) compared with those inseminated once post-ovulation with 800×10^6 total sperm (47%) (Barbacini et al. 2005). For epididymal sperm single insemination of 150×10^6 fresh epididymal sperm resulted in per-cycle pregnancy rates of 45% (Morris et al. 2002c). In the same study a single insemination of 200×10^6 frozen-thawed epididymal sperm resulted in 18% pregnancy rates for hysteroscopic insemination and 8% pregnancy rates for conventional insemination. In the same study, however, when the frozen-thawed epididymal sperm was washed after thawing, and the dose was reduced to 5×10^6 sperm it was possible to achieve pregnancy rates of 29%.

It is almost generally accepted that $250-500 \times 10^6$ progressively motile sperm is a satisfactory and effective insemination dose, to compensate for variability in the stallion's fertility (Brinsko 2006). To optimise pregnancy rate Pickett et al. (2000) recommends that 800×10^6 total frozen sperm with at least 30% post-thaw progressive motility should be used. However recently, when using conventional insemination techniques, breeding mares with less than 500×10^6 sperm frozen from fertile stallions can result in acceptable pregnancy rates (Pickett et al. 2000). Nevertheless, when using frozen-thawed sperm it must be considered that this sperm may have suffered sub-lethal cryodamage which may adversely affect fertility. Furthermore, reducing the insemination dose can increase the efficient use of semen but the optimal dose required to produce satisfactory fertility rates will

vary among stallions (Brinsko 2006). Indeed this was illustrated by Volkmann and VanZyl (1987) when they achieved 44% per-cycle pregnancy rates with $137\text{-}210 \times 10^6$ sperm that were progressively motile post-thaw, but the pregnancy rate increased to 73% when the mares were inseminated with $>220 \times 10^6$ progressively motile sperm post-thaw (Volkmann & vanZyl 1987).

1.10 RATIONALE AND AIM OF THIS STUDY

The lower fertility of frozen-thawed epididymal sperm compared to ejaculated sperm is interesting and may also shed some light on factors affecting the overall fertility of stallions. Recently, the beneficial effects of using formamides as cryoprotectants in combination with a variety of amino acids and complex energy sources for the freezing of epididymal sperm has been shown to be a valuable tool for both epididymal sperm and sub-fertile stallions.

The primary aim of this research project is to focus on determining the source of these beneficial effects, to determine if they are due to different cryoprotectant type or the base semen extender. This will be tested by comparing 2.5% glycerol, 2.5% DMF, 5% DMF and a glycerol and formamide combination cryoprotectant (Botu Crio[®] cryoprotectant) frozen in the same base extender (Botu Crio[®] extender).

It has also been proposed that epididymal sperm may be less fertile due to its lower motility. Based on this observation, stimulation of motility in epididymal sperm would theoretically enhance the ability of sperm to reach the ampullary isthmic junction, the site of fertilisation. This theory will also be tested in this project *in vitro* by exposing frozen-thawed epididymal sperm to the post-thaw medium SW3 (Jel Media, Auckland NZ), a protein free Tyrodes which contains complex amino acids and sugars.

As Papa *et al.* (2008) state, it is of great importance that experiments involving epididymal sperm are performed with techniques that allow higher pregnancy rates using low doses of sperm, in order to maximise the use of semen that is in limited supply. For this reason deep horn insemination as a means of artificial insemination (AI) will be investigated as this method, compared to the conventional methods of AI requires 500-fold less sperm and therefore would

maximise the number of doses of semen available from a limited supply of epididymal sperm. Mares will be inseminated once with 600×10^6 sperm based on research illustrating little variation in per-cycle pregnancy rates when mares are inseminated more than once (Sieme et al. 2003; Barbacini et al. 2005). Furthermore this dose will optimise the use of this finite supply of epididymal sperm.

CHAPTER 2

MATERIALS AND METHODS

2.1 ETHICS

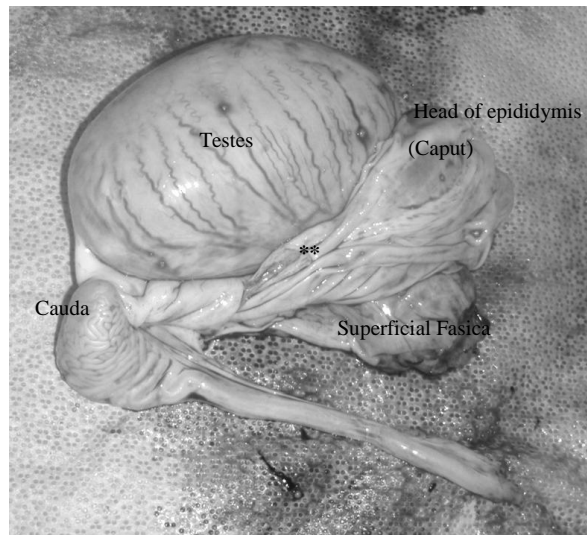
Ethical consent was obtained from the Animal Ethics committee from the School of Science and Engineering at the University of Waikato before this study was conducted.

2.2 RECOVERY OF EPIDIDYMAL SPERMATOZOA

Testicles were obtained from five 2 year old colts at the time of castration (Figure 2.1). The cauda epididymis and the vas deferens were dissected away from the proximal corpus and caput of the epididymis (Figure 2.2). The epididymal tubules were carefully separated by blunt dissection to facilitate elongation (Figure 2.3 and 2.4). A 21G needle (Appendix 2) was inserted into the vas deferens, and 20ml of room temperature Kenney's solution (Appendix 1) was infused through the epididymis using a syringe (Figure 2.5) (Appendix 2) and the semen was collected in a 50ml Falcon tube (Appendix 2).



Figure 2. 1 Castrated testicle with tunica dartos surrounding testicle proper. The testicle is contained in the scrotal sac. The scrotum consists of a thin layer of skin and the underlying superficial fascia. Beneath this is the cremaster muscle.



*Figure 2. 2 Testicle removed with cauda epididymis pulled to the side. underneath that labelled ** lies the corpus of the epididymis.*



Figure 2. 3 Blunt dissection removing the tunica albuginea around the cauda epididymis

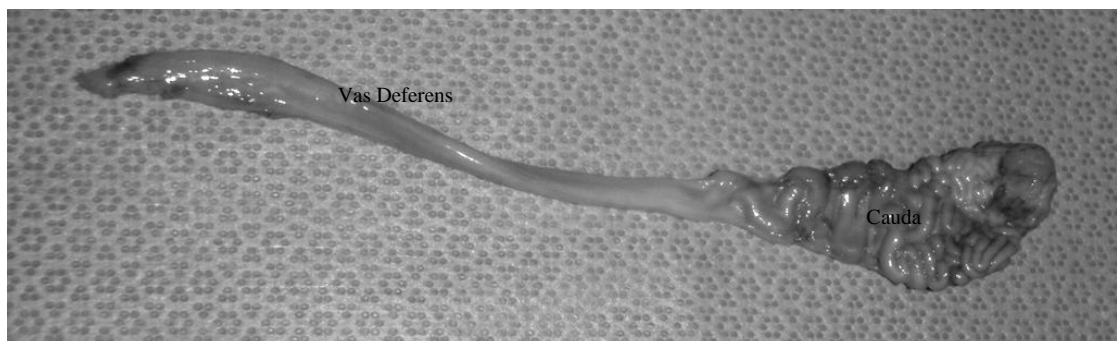


Figure 2. 4 Cauda epididymis with tunica albuginea removed.

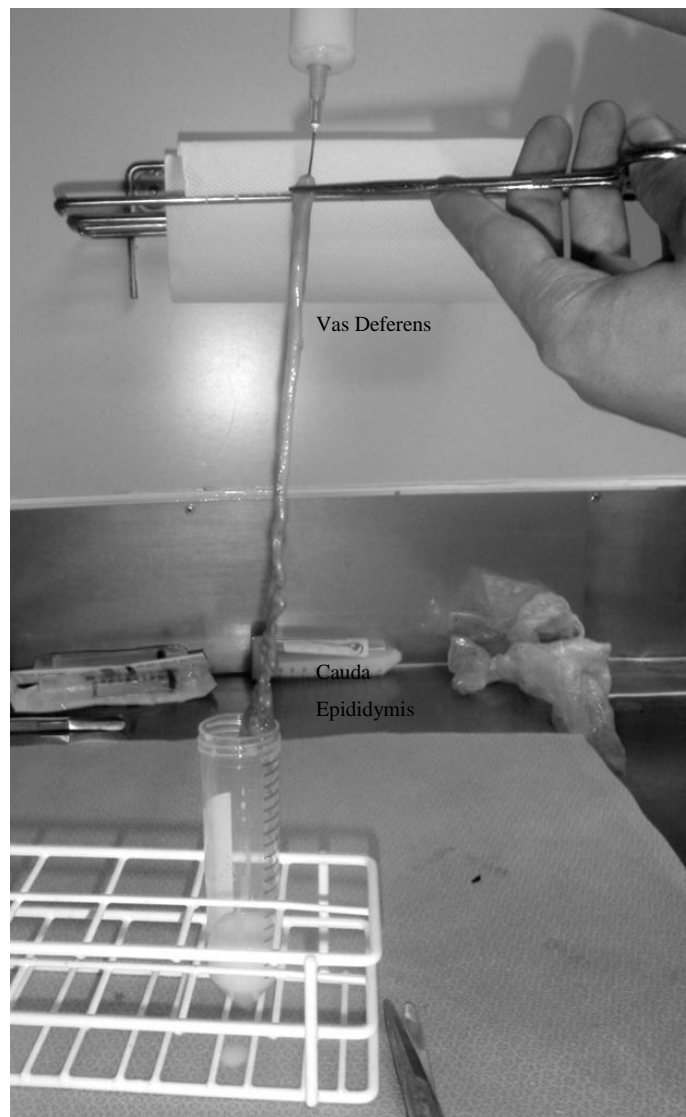


Figure 2. 5 Flushing of the cauda epididymis with 20ml of Kenney's extender.

2.3 CRYOPRESERVATION OF EPIDIDYMAL SPERMATOZOA

2.3.1 First Extension and Centrifugation of Sperm

Sperm was extended 1:1 (v/v) with room temperature Kenney's solution in a 50 ml centrifuge tube (Appendix 2) and then a subjective assessment of sperm motility (Section 3.2.3.1: motility assessment) was performed at 400 x magnification on an Olympus microscope (Appendix 2). At the bottom of the diluted sample 3ml of Optiprep axi-shield (Appendix 1) was drawn into a 10 ml syringe and this was slowly expelled into the bottom of the semen sample to cushion the solution when centrifuged. The sample was then centrifuged at 2400 rpm (10 cm radius) for 15 minutes. The supernatant was carefully removed using a 3ml disposable pipette (Appendix 2) leaving approximately 7 ml of working

pellet. A blunt end, 18 G, 1.5 inch needle (Appendix 2), was used to remove the cushion. The sperm suspension was mixed gently.

2.3.2 Calculation of Sperm Concentration

In a separate 5 ml glass test tube (Appendix 2) a 1 in 200 dilution was made by the addition of 3980 μ l of purified water to 20 μ l of the centrifuged epididymal sperm sample (to kill the sperm by osmotic rupture) and this was mixed gently. Using a 20 μ l pipette (Appendix 2) a haemocytometer chamber (Appendix 2) was filled with 10 μ l of this suspension. After 3 minutes sperm were counted in 5 large squares of the haemocytometer to determine sperm concentration. Sperm located on two of the borders were included in the count and sperm on the other two borders were excluded.

2.3.3. Second Extension and Addition of Cryoprotectant

To dilute the sample to the desired 600×10^6 sperm/ml for freezing the appropriate amount of the different cryoprotectants were added to the appropriate amount of Botu Crio[®] freezing extender to get the following final concentrations: glycerol 2.5%, DMF 2.5%, DMF 5%, and glycerol/formamide combination 5% (abbreviated to 'BC' in as it is supplied by Botu Crio[®], not to be confused with the Botu Crio[®] freezing extender) This extender/cryoprotectant mixture was then added slowly to the sperm suspension. (see Appendix 3, equations 1-4 for detailed equations).

2.3.4 Equilibration using Glycerol

For glycerol, the diluted sample, contained in a 50 ml centrifuge tube, was allowed to equilibrate to 5 °C in the fridge over 60 minutes, placed in a 500 ml beaker (Appendix 2) containing 200 ml of room temperature (20 °C) water. The semen was then loaded into 0.5 ml straws (Appendix 2) in a cabinet cooled to 5 °C and these straws were sealed with PVC powder (Appendix 2), placed onto cooled freezing racks and frozen at 3cm above the liquid Nitrogen (Appendix 2) in the vapour for 10 minutes. Straws were then plunged directly into liquid Nitrogen (-196 °C) and placed into goblets for long term storage in liquid Nitrogen tanks.

2.3.5 Equilibration using DMF and the Glycerol and Formamide Combination Cryoprotectant (Botu Crio[®])

For DMF (2.5% and 5%) and the glycerol and formamide combination cryoprotectant, the diluted sample was loaded into 0.5ml straws (Appendix 2) at

room temperature, and these were sealed with PVC powder (Appendix 2). The straws were then placed onto room temperature freezing racks and were then allowed to equilibrate to 5°C in the fridge over 20 minutes. As for straws cryopreserved with glycerol, these were then frozen 3cm above liquid Nitrogen vapour for 10 minutes. Straws were then plunged directly into liquid Nitrogen (-196 °C) and placed into goblets for long term storage in liquid Nitrogen tanks.

2.4 THAWING OF SEMEN

The straws were thawed for 30s in a 37°C water bath. They were then dried to prevent any water mixing with the sperm. The ends of the straws were then cut allowing the contents to run into a warmed 15ml Falcon tube (Appendix 2).

2.5 SEMEN ANALYSIS

Individual straws were thawed and different methods of analysis were conducted to assess the effects the cryoprotectant has on the semen integrity including: motility (Chapter 3), morphology (Chapter 3), acrosome status (Chapter 3), membrane integrity (Chapter 3), DNA integrity (Chapter 4) and fertility (Chapter 5) Refer to the referenced Sections for details on the methods used in these experiments.

CHAPTER 3:

THE EFFECTS OF DIFFERENT CRYOPROTECTANTS AND THE ADDITION OF THE POST-THAW MEDIUM SW3 ON EPIDIDYMAL SPERMATOZOA

3.1 INTRODUCTION

The ability to predict the fertility of a stallion on his semen would enhance any breeding programme. This has however posed difficulties due to the multi-factorial nature of fertility, predictability and accuracy of available tests and the limitations of the type of semen available such as ejaculated sperm, epididymal sperm, fresh, chilled or frozen semen (Graham & Mocé 2005). Furthermore limitations exist with our ability to manage the highly variable mares inseminated (Graham & Mocé 2005). For a sperm to be capable of fertilising the female gamete it must possess a suit of attributes as outlined by Graham and Mocé (2005) including: progressive motility, active mitochondria to deliver the energy required for motility, an intact acrosomal membrane which is capable of undergoing capacitation changes thereby permitting the acrosome reaction to occur at the correct moment, antigen receptor molecules in the plasma membrane to permit the binding of the sperm to the zona pellucida and oocyte membrane (oolemma), a plasma membrane that permits fusion with the oolemma and a nucleus that is capable of proper de-condensation, nuclear reorganization, and genetic performance to maintain zygotic and embryonic development. Because each sperm cell requires so many attributes to fertilize an oocyte, an assay measuring only a single attribute will fail to detect sperm defective in a different attribute, and therefore overestimate the number of fertile sperm in the semen sample. Furthermore, due to the inter- and intra-stallion variability observed (Love et al. 2000), there is no guarantee of acceptable fertility rates even if all of these parameters seem to be normal (Colenbrander et al. 2003). Because of this there is no single means of analysis that correlates perfectly with stallion fertility or

infertility, and hence it has become accepted that a combination of thorough evaluation methods be performed on each semen sample (Katila 2001b; Meyers 2001; Colenbrander et al. 2003; Graham & Mocé 2005; Kirk et al. 2005; Samper et al. 2007). A good combination of these techniques is thought to provide a practical alternative to fertility data (Kenney et al. 1983). However, it must be acknowledged that it is also unreasonable to conduct all semen parameter analyses due to financial and time restraints on most semen experiments (Graham & Mocé 2005).

3.1.1 Motility

Sperm motility is often measured in semen analyses, high motility being a prerequisite for judging semen to be normal. To initiate fertilization, mammalian sperm rely on the propulsive forces generated by their flagella to get to the site of fertilization in the oviduct and to penetrate the zona pellucid of the egg (Mortimer 1997). The flagellum attains its energy provided in the form of ATP. from the mitochondria (Pesch & Bergmann 2006).The direct source of energy for movement must be acquired from the sperm diluent as the spermatozoon itself houses no significant energy reserves. Effectual movement is active and progressive, enabling the cell to move in a linear direction. Despite the apparent importance of motility, in some studies it has been observed that there is little correlation between motility and fertility (Kirk et al. 2005), nevertheless, because it is an economical and efficient way to assess semen, motility assessment is conducted widely in semen laboratories (Graham 1996; Katila 2001b; Rota et al. 2004) and is particularly useful to monitor whether problems during processing have occurred (Crabo 2001).

Visual motility assessments are conducted by placing a drop of diluted sperm suspension on a pre-warmed glass slide and subsequently placing a cover slip over the sample. Using a light microscope each sample is viewed in the centre of the cover slip (as motility declines more rapidly at the edges than in the centre as a result of drying and exposure to air) (Samper et al. 2007), and the percentage of motile and progressively motile sperm is assessed in each sample. Non-diluted semen is not usually used for motility assessments due to agglutination or fusion of the sperm cells to the glass (Samper et al. 2007). Analysis of sperm longevity

is conducted by assessing sperm motility at 6, 12, 24 and 48 hours by preparing new slides at these points in time from stored samples (Samper et al. 2007).

Some investigators prefer computer assisted motility assessments as they are thought to be less subjective (Graham 1996), however there is little evidence to suggest that computer assisted analysis of motility has a better correlation with fertility (Colenbrander et al. 2003). In addition computer assisted motility equipment is costly and not present in many semen laboratories. Numerous investigators have endeavoured to find different post-thaw criteria that could best predict fertility of frozen semen, but very few studies exist with a sufficient number of stallions or mares (Samper et al. 1991; Kirk et al. 2005)

3.1.2 Morphology

The morphological assessment of sperm has a long history and it is generally accepted that certain morphological structural deviations correlate with male sub-fertility and infertility (Pesch & Bergmann 2006). Numerous factors can result in abnormal sperm morphology, for instance; genetic defects, disease, nutrition, or temperature (Barth & Oko 1989). Any morphological defect which affects the progressive motility of a spermatozoon can reduce its ability to reach the oocyte and fertilize. However, only some, but not all defects are associated with sub-fertility and infertility (Barth & Oko 1989), and unlike in other species such as bulls, boars and rams, morphology does not appear to correlate perfectly with fertility in stallions (Long et al. 1990). Despite this, it is still considered an important means of semen assessment to perform routinely as it may diagnose the origin of particular fertility problems in a stallion (Dowsett et al. 1984). Stallions with good fertility will often have >60% normal sperm and <5% abnormalities occurring in the acrosome and midpiece (Samper et al. 2007). The main morphological defects observed in this study are outlined in Section 3.2.3.2.

The morphology of sperm can be assessed after they have been stained with dyes such as eosin-nigrosin (Hancock 1951) or eosin-aniline blue (Shaffer & Almquist 1948). Eosin-nigrosin dyes are commonly used as they are effective, simple and in addition to allowing sperm to be readily visualized, these are so-called a “live or dead” stain, allowing the membrane integrity to be assessed at the same time as morphology (Long et al. 1990; Samper et al. 2007). Smears are made on warmed

slides with little drying time to lessen the exposure time as live and dead sperm react differently; live sperm will exclude the dye whilst dead cells allow passive diffusion of the dye across their non-functional cell membranes and appear purple (Long et al. 1990; Samper et al. 2007).

3.1.3 Acrosome Status

A large proportion of sperm in stallions with reduced fertility have been linked to a high percentage of sperm not being capable of undergoing the acrosome reaction when stimulated to do so *in vitro*. With the aid of specialised stains such as chlortetracycline stains (Varner et al. 1987) and lectin-binding stains (Cross et al. 1986; Cheng et al. 1998) The population of acrosome-reacted sperm can be recorded. Sperm which have undergone a premature acrosome reaction are incapable of fertilization, whilst capacitated sperm are believed to have reduced existence in the female reproductive tract (Watson 1995b).

Two common non toxic lectins include: FITC-PSA (Fluorescein isothiocyanate conjugated *Pisum sativum* agglutinin) (Overstreet et al. 1995; Sabeur et al. 1998; Meyers 2001) and FITC-PNA (Fluorescein isothiocyanate conjugated *Arachis hypogea* agglutinin) (Cross & Meizel 1989; Katila 2001a). FITC-PSA, which binds to glycoconjugates in the acrosome, has been used to explore acrosomal status in stallion sperm. However, only two patterns of acrosome status are shown with this stain, either intact or lost (Farlin et al. 1992). FITC-PNA on the other hand is more descriptive, this stain binds to β -galactose moieties associated with the outer acrosomal membrane, and is therefore able to stain acrosome-reacting sperm also (Cheng et al. 1996). Additionally, FITC-PNA also stains brighter (Graham 1996). With this stain the acrosome status is able to be classified into one of five patterns: intact acrosome, patchy acrosome, 75% reacted acrosome, reacted acrosome and equatorial segment. Examples of these four staining patterns are shown in Section 3.2.3.3.

To conduct acrosome analysis a smear of the sample is made and stained with one of the above mentioned dyes. When dry fluorescent enhancers or anti-fade solutions are often added (Meyers 2001). Reacted acrosomes will show no fluorescence, intact acrosomes will fluoresce brightly over the sperm head to the equatorial region and partly reacted acrosomes appear patchy or only fluoresce at

the equatorial segment when assessed under a fluorescent microscope (Cross & Meizel 1989; Cheng et al. 1996).

3.1.4 Plasma membrane integrity – HOS test

Plasma membrane integrity is imperative for sperm metabolism and for successful fertilization (Jeyendran et al. 1984). To measure the integrity of the sperm membrane the hypoosmotic swelling test (HOS) can be conducted to assess the percentage of swollen sperm which correspond to the percentage of intact and biochemically active plasma membranes (Jeyendran et al. 1984; Colenbrander et al. 2003). When sperm are exposed to a hypoosmotic solution those with intact, functional membranes swell to establish an osmotic equilibrium (Neild et al. 1999). In boars, a modified HOS correlated fairly well with pregnancy rates ($r=0.43$) (Perez-Llano et al. 2001) whereas in stallion sperm, although its use has been validated (Nie & Wenzel 2001) there are no reports which correlate the HOS test with fertility.

The HOS test is conducted by placing a sample of sperm in a hypo-osmotic solution. Here, fluid is transported across the plasma membrane as the sperm attempts to reach an osmotic equilibrium. If the membranes are functional and intact the cells will swell which puts tension on the tail fibres resulting in them coiling as observed under the microscope (Jeyendran et al., 1984; Katila, 2001b; Samper et al., 2007). If the membranes are damaged the tails appear straight (Figure 3.1).

A study conducted by Nie and Wenzel (2001) found that 100 μ l of stallion semen added to 1.0 ml of pre-warmed 100 mosmol/l sucrose solution produced the optimal number of plasma membrane swellings. Their study also revealed that the temperature and time in which this test was conducted did not significantly alter the results (Nie & Wenzel 2001).

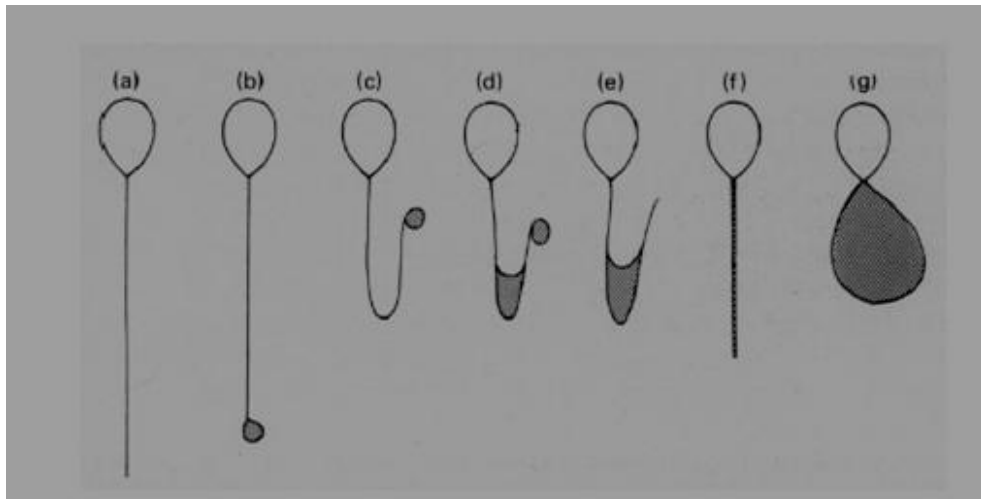


Figure 3. 1 An illustration of the diverse degrees of swelling observed when performing the HOS analysis (a) Is an uncoiled tail which represents a damaged sperm membrane, (b-g) are various degrees of tail coiling indicating that the membrane is intact (Image obtained from Jeyendran, et. al., 1984).

3.1.5 Aims

The aims of the series of experiments in this chapter were to evaluate the post-thaw characteristics of epididymal sperm frozen with different cryoprotectants and to assess the effects of the addition of the protein-free Tyrodes medium, SW3, after thawing.

Firstly, epididymal samples frozen in Botu Crio[®] base diluent with one of 2.5% glycerol, 2.5% DMF, 5% DMF or 5% glycerol/formamide cryoprotectant were compared to commercially frozen ejaculated samples frozen with 4% glycerol, DMF or a combination. The sperm characteristics that were assessed included: motility, morphology, plasma membrane integrity and acrosome status. Secondly the effects of time on the motility of ejaculated sperm and epididymal sperm frozen with the different cryoprotectants were investigated over a 30 minutes. Finally the effects of SW3 medium on ejaculated and epididymal sperm frozen with the different cryoprotectants were investigated.

The experimental design and the different sperm parameters assessed in this study are outlined in Figure 3.2.

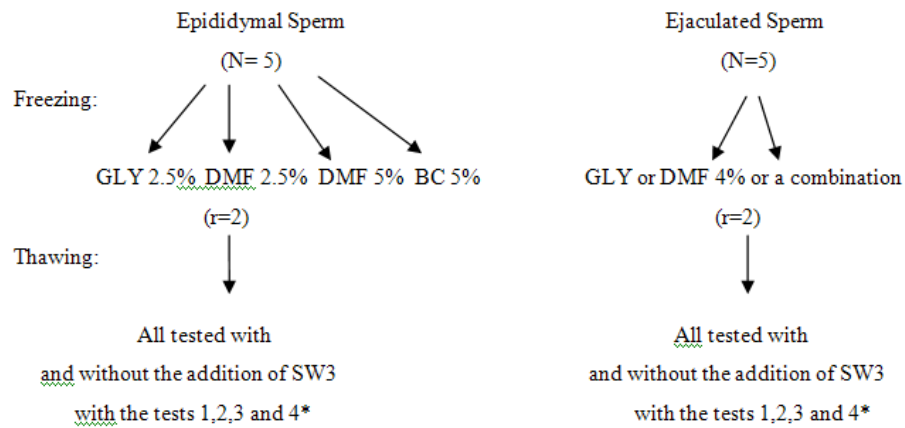


Figure 3. 2 Flow diagram representing the experimental design used in this study. *N* = number of stallions, *r* = replicates, 1 = motility assessment, 2= morphology assessment, 3= acrosome status analysis and 4 = plasma membrane integrity analysis. GLY = glycerol, DMF = dimethylformamide, BC = glycerol/formamide combination

3.2 METHODS

3.2.1 THAWING OF EPIDIDYMAL SPERM

The 0.5ml straws were thawed for 30s in a 37°C water bath. These were then dried with a paper towel to prevent any water mixing with the sperm and one end was then cut to allow the contents to run into a warmed 15ml Falcon tube (Appendix 2).

3.2.2 Post-thaw Treatments

After thawing, each sample was divided into two. One half remained undiluted whilst the other half was diluted 1:2 with SW3 medium (100 µl : 200 µl). Both aliquots were assessed as described below.

3.2.3 Assessment of Spermatozoa

The following sperm parameters were evaluated after thawing to provide information on sperm viability, longevity and the effects of SW3 medium. Each sample was tested twice for each stallion and each treatment group, with means being calculated to ensure non-biased results. An assistant with no previous knowledge of the effects of cryoprotectant thawed the straws into a 1 ml pre-warmed test tube and labelled them with a number code to ensure the assessment was blind. Five ejaculated semen samples frozen using glycerol, DMF or a

combination from different stallions were used as controls, and means from these were also calculated.

3.2.3.1 Motility

Sperm motility was determined by visual assessment at 400x magnification under a microscope (Olympus BHB) (Appendix 2) on a 37 °C heated stage. One 10 µl drop of sperm suspension was placed at each end of a warmed glass slide and covered with a 22 mm x 22 mm warmed cover-slip (Appendix 2). The percentage of sperm which were motile in any way (including those moving in circles), defined as 'total motility' (Equation 5 - Appendix 3), and the percentage of sperm that were motile and moving in a forwards direction, defined as 'progressive motility' (Equation 6 - Appendix 3) were estimated for each of the two drops per sample and the average was determined. This was done at both 0 minutes and 30 minutes.

The overall motility, expressed as total progressive motility (TPM) (Equation 7- Appendix 3) was then equated. This is an estimate of the percentage of motile sperm that were progressively motile in the field of view.

3.2.3.2 Morphology

A 10 µl drop of sperm suspension was placed onto a labelled glass slide, along with a 10 µl drop of eosin-nigrosin stain (Appendix 1), which were mixed and together smeared thinly over a glass slide and air dried quickly by placing it on a warming plate. The sample was assessed under a microscope (Olympus BHB), and in the region found to have a high proportion of sperm a drop of oil was placed followed by the cover slip and another drop of oil. One hundred sperm were visually assessed under the microscope for morphology using phase contrast 1000 x magnification under oil immersion. Sperm were classified into the following categories:

- Normal morphology (Figure 3.3)
- Head defect (Figure 3.4)
- Mid-piece defect and principal piece defect (Figure 3.5)
- Proximal droplet (Figure 3.6)
- Distal droplet (Figure 3.6)

- Curved tail (Figure 3.7)
- Bent tail (Figure 3.7)
- Coiled tail (Figure 3.7)

If more than one morphological defect was observed the most obvious defect was recorded.

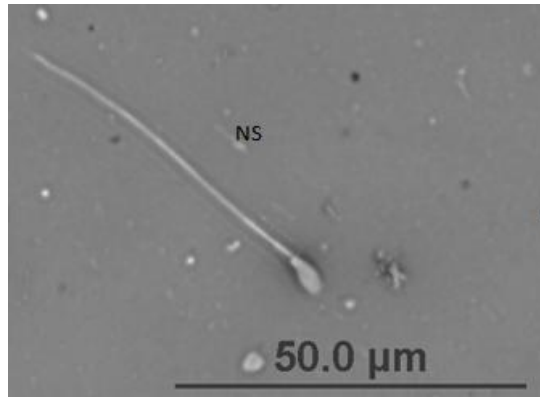


Figure 3. 3 Morphologically normal stallion sperm.

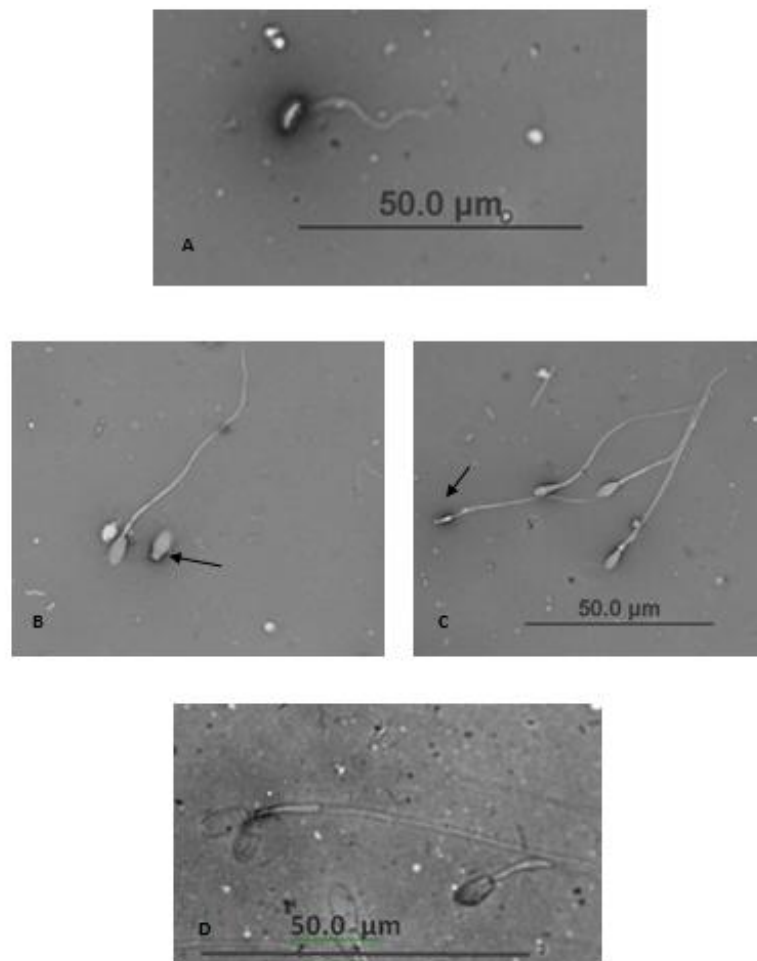


Figure 3. 4 Head defects observed in stallion sperm. A= Misshapen head; B = Loose head; C = Misshapen head; D= Double head.

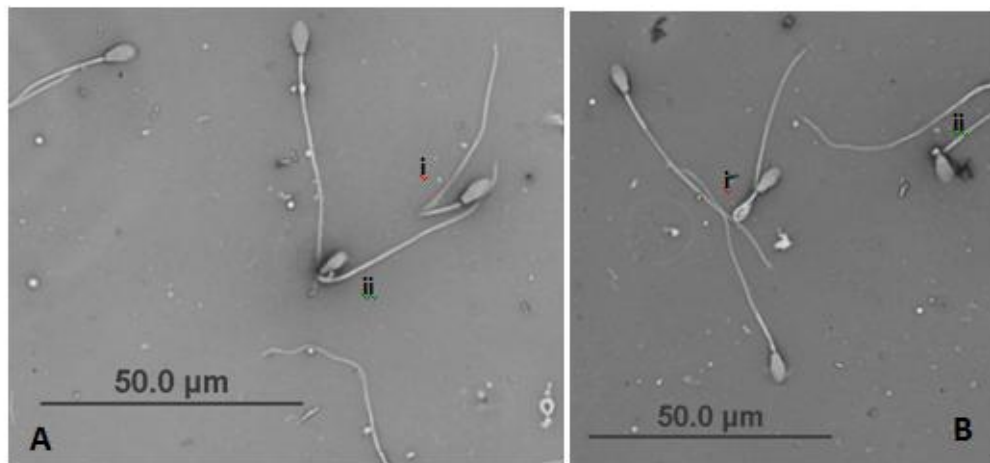


Figure 3. 5 Midpiece and principal piece defects of stallion sperm. A (i) = defect of the distal end of the midpiece (ii) = Defect of the proximal end of the midpiece; B (i) Bent midpiece, folded onto itself, otherwise classified as a Distal midpiece reflex – a bending of flagella around a distal droplet (see distal droplets) (ii) Proximal piece defect

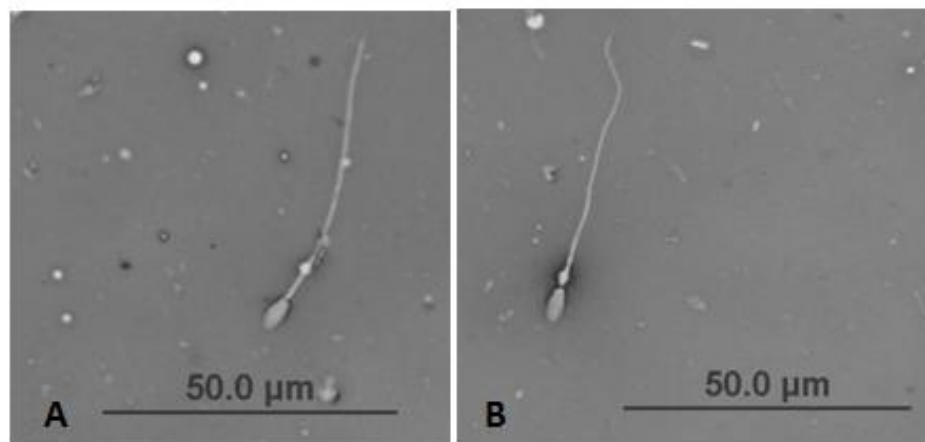


Figure 3. 6 Cytoplasmic droplets of sperm. A= Distal droplet; B= Proximal droplet.

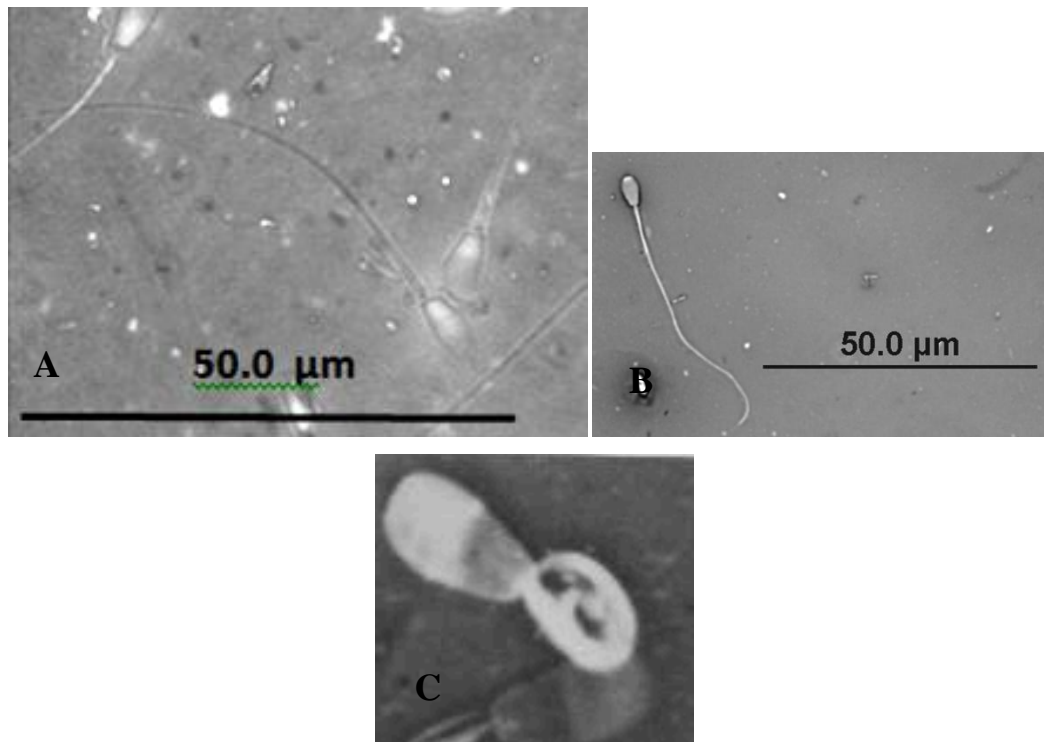


Figure 3. 7 Tail defects of stallion sperm. A = Curved tail; B= Bent tail; C= Coiled tail

3.2.3.3 Acrosome Status

A 20 µl drop of sperm was placed onto a labelled glass slide. The sperm sample was smeared thinly over the slide and air dried and then stored in the refrigerator at 5°C until ready for assessment.

On the glass slide was placed 20 µl of FITC-PNA (Appendix 1) and allowed to distribute over the sperm. These slides were then incubated in a humidified chamber for 30 minutes at 39 °C. These were subsequently rinsed with Phosphate Buffered Saline solution (PBS) (Appendix 1) and allowed to air dry in the humidified chamber. Two drops of UCD mounting medium was then placed on the slide and a cover-slip were placed over top and lightly pressed with a paper towel to allow even spreading of the mounting medium. The acrosome status of the sperm was then assessed under 400 x magnification using a Leica DMRE fluorescence microscope (Appendix 2). The acrosome status of each sperm cell was recorded as intact, patchy, 75% reacted, reacted or equatorial segment (ES) (Figure 3.8).

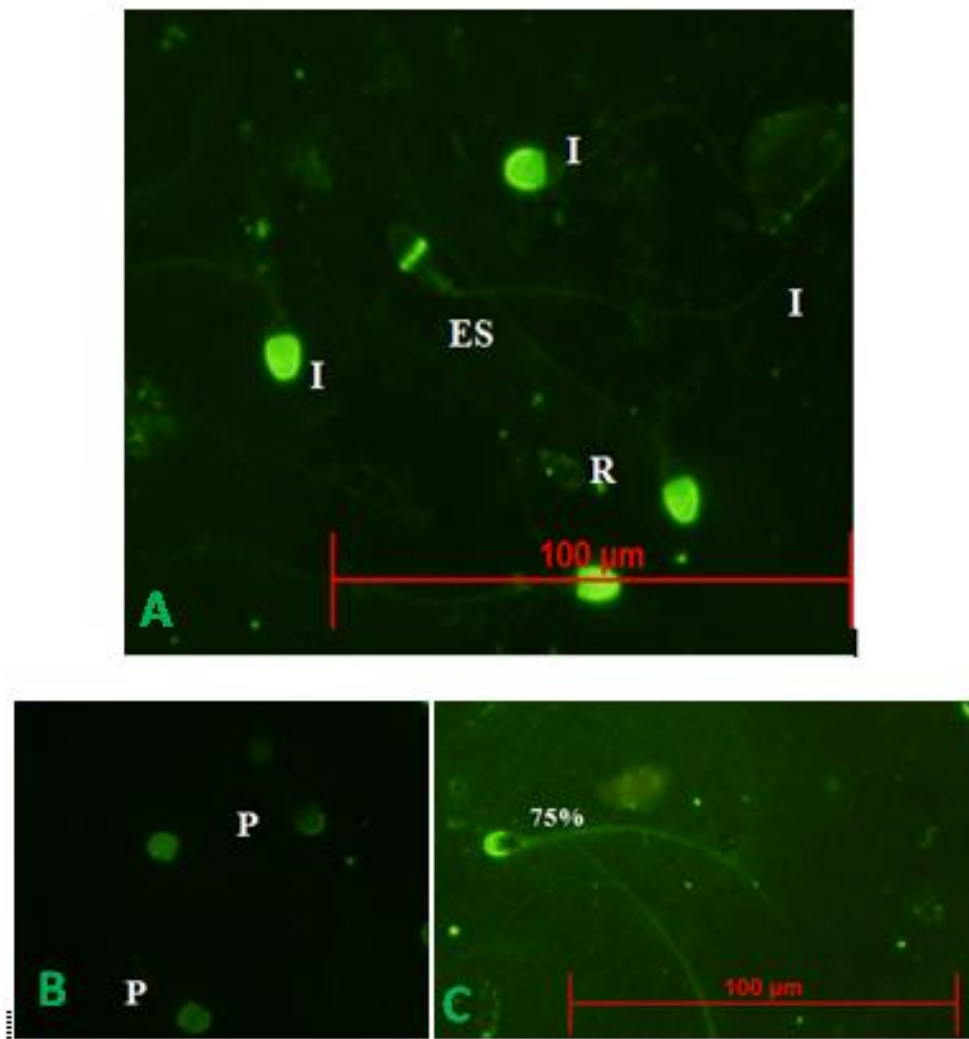


Figure 3. 8 Stallion sperm stained with FITC-PNA and viewed under fluorescence at 400x magnification. A = intact acrosomes (I), Reacted acrsome (R), Equitorial Segment (ES); B= Patchy acrosomes (P); C = 75% Reacted (75%).

3.2.3.4 Plasma Membrane Integrity – Hypo-Osmotic Swelling Test

Aliquots of 100 µl of the sperm suspension were added to 1.0 ml of pre-warmed 100 mosmol/l hypo-osmotic swelling solution (Appendix 1) in a 1.5 ml microcentrifuge tube (Appendix 2). Each sample was incubated for 30 minutes at 37 °C. After incubation, a 10 µl drop of the sperm suspension was placed into a haemocytometer slide (Appendix 2). The sample was examined microscopically at 10x, 20x, 30x and 40x magnifications. A total of 100 sperm were counted, recording the number with straight and coiled tails.

3.2.4 Statistical Analysis

Data from motility, morphology, acrosome status analysis and plasma membrane integrity analysis was investigated using the statistics programme STATISTICA (StarSoft Inc., 2008). The independent t-test, one-way ANOVA and two-way ANOVA with repeated measures were used to analyse:

1. Epididymal sperm compared to ejaculated sperm
2. The effect of time on the motility of epididymal sperm and ejaculated sperm
3. The effects of cryoprotectant on epididymal spermatozoa
4. The effects of the post-thaw medium SW3 on epididymal and ejaculated sperm.

If significant effects were detected by one-way ANOVA or two-way ANOVA then the Newman-Kleus multiple range test was conducted to determine what differences existed between groups. When undertaking analysis of variance tests it was checked that all samples tested met the criteria of ANOVA in that the data is normally distributed and of equal variance. The difference was considered significant if the p-value was < 0.05

3.3 RESULTS

3.3.1 Motility

3.3.1.1 Epididymal Sperm compared to Ejaculated Sperm

Analysis of total and progressive motility at time 0 and 30 minutes after thawing illustrated that the total motility of ejaculated sperm was significantly higher than epididymal sperm immediately after thawing ($p= 0.0216$) and 30 minutes after thawing ($p= 0.038$). Post-hoc comparisons showed that ejaculated sperm differed significantly from all epididymal sperm cryoprotectant treatment groups immediately after thawing (Figure 3.9). At 30 minutes ejaculated sperm was significantly different to all epididymal sperm treatment groups except 2.5% DMF. There were no significant differences in progressive motility amongst treatment groups.

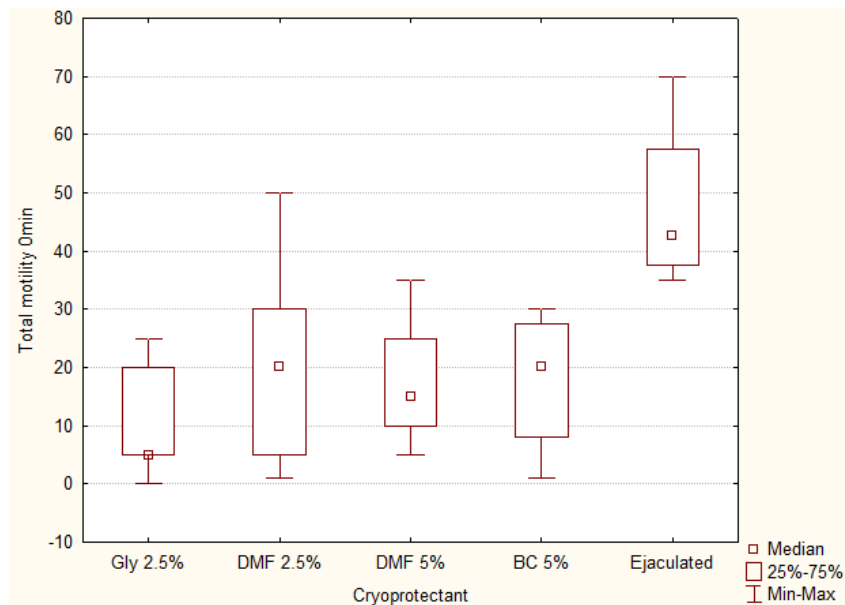


Figure 3. 9 Total motility immediately after thawing. Gly = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

3.3.1.2 The Effects of Cryoprotectant on Epididymal Sperm

There were no significant effects of the type of cryoprotectant on the total motility or progressive motility at 0 or 30 minutes after thawing for samples treated ($p= 0.550$) and not-treated ($p= 0.650$) with SW3 medium.

3.3.1.3 The Effect of Time on the motility of Epididymal Sperm and Ejaculated sperm on motility

The change in total motility and progressive motility over the 30 minute period immediately after thawing was assessed for each treatment groups and there were no significant differences in the change between 0 and 30 minutes. Nor were there any significant differences in total motility between cryoprotectants at time 0 and 30 minutes. The mean percentage TPM estimates from epididymal samples at 0 and 30 minutes are shown in Figures 3.10 - 3.11 (Also see Appendix 4, Figure 1)

3.3.1.4 The Effects of the post-thaw medium SW3 on Epididymal and Ejaculated Sperm

Comparison of the TPM of all samples with and without the treatment of SW3 medium demonstrated a significant overall variation between treated and untreated samples ($p < 0.0001$). With an increase in motility occurring for all groups. Further analysis revealed that sperm frozen with glycerol 2.5% glycerol had a significant response to SW3 medium ($p = 0.016$) when compared to other treatment groups. (DMF 2.5% $p = 0.1059$; DMF 5% $p = 0.1547$; glycerol/formamide combination 5% $p = 0.0907$). These differences are depicted in Figures 3.10 and 3.11.

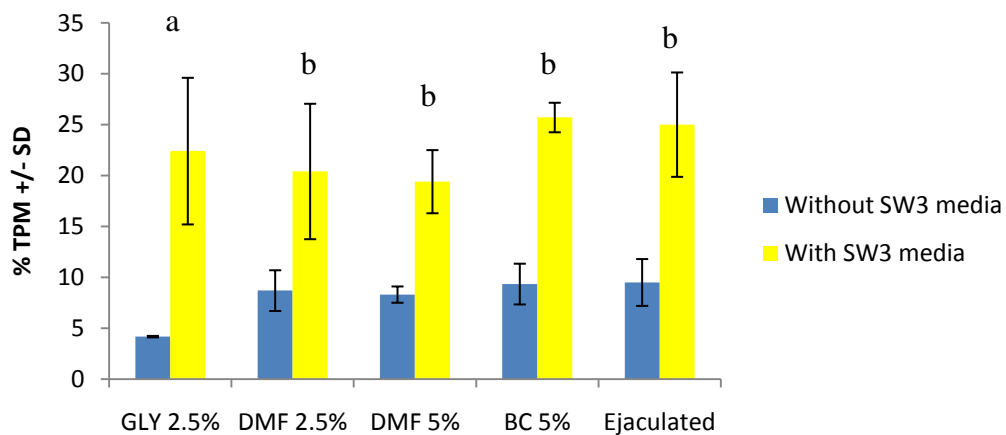


Figure 3. 10 Mean percentage TPM immediately after thawing, with and without SW3 medium added immediately after thawing. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination. Superscripts ^{a,b} denote significant and non-significant effects of SW3 medium respectively.

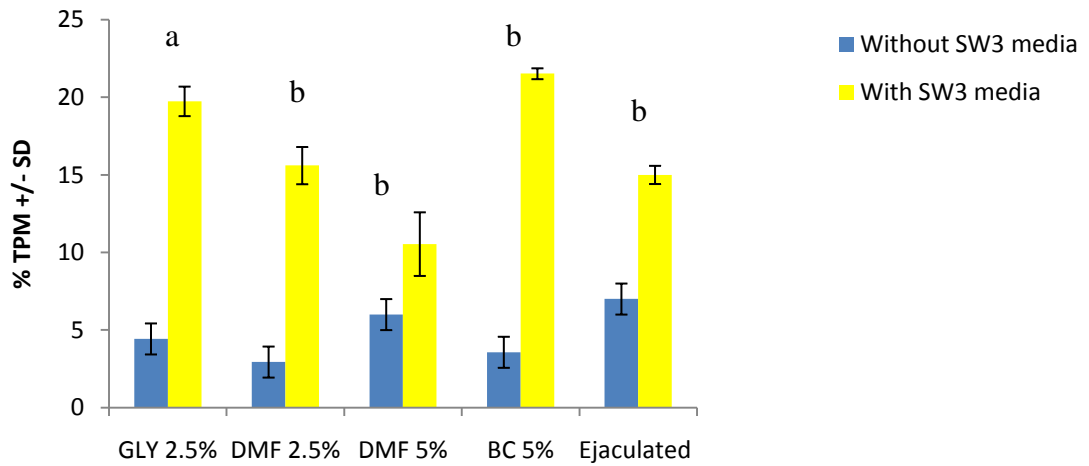


Figure 3. 11 Mean percentage TPM with and without SW3 medium at 30 minutes. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination. Superscripts ^{a,b} denote significant and non-significant effects of SW3 medium respectively.

3.3.2 Morphology

3.3.2.1 Epididymal Sperm compared to Ejaculated Sperm

Ejaculated sperm had a mean normal morphology of 44.5% and epididymal samples had a mean normal morphology of 33%. The percentage of morphologically normal sperm was statistically similar between epididymal sperm and ejaculated sperm (Figure 3.12). The predominant defect was bent midpieces.

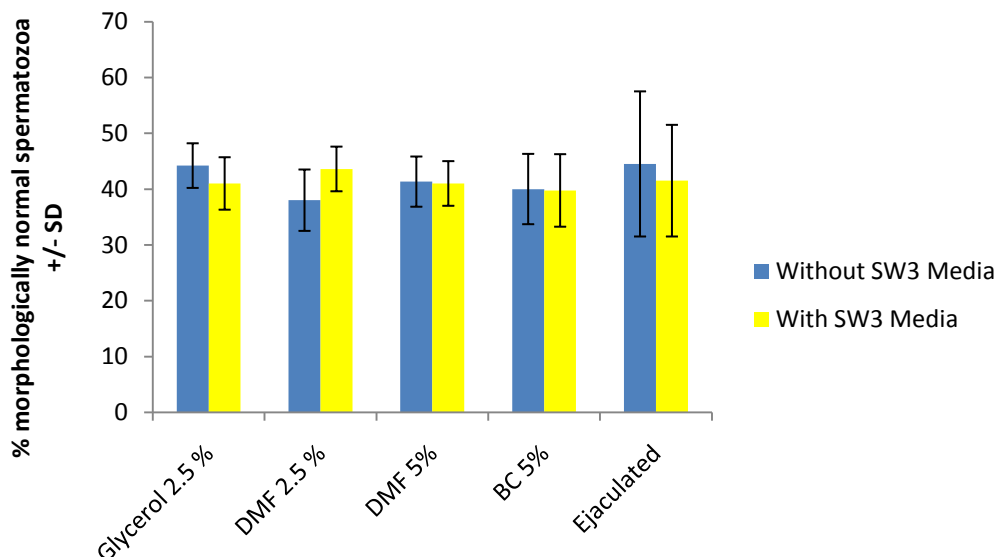


Figure 3. 12 Percentage of morphologically normal sperm thawed after freezing cryoprotectants. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

Statistical analysis comparing the morphology of epididymal sperm and ejaculated sperm found no significant differences between the two semen types for any of the morphological defects except for the occurrence of distal droplets (Figure 3.13). Post hoc comparisons demonstrated that the significant differences were between ejaculated sperm and sperm frozen in the glycerol/formamide cryoprotectant (BC) ($p = 0.0128$) and ejaculated sperm and DMF 5% ($p = 0.0056$).

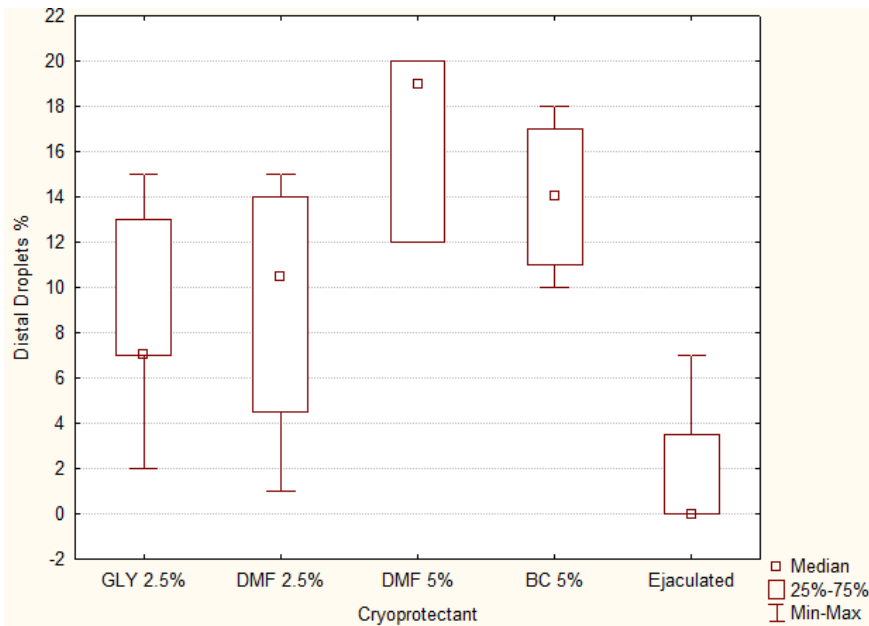


Figure 3. 13 Box and Whisker plot depicting the effects of cryoprotectant type on the occurrence of distal droplets (DD) in SW3 untreated sperm. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

3.3.2.2 The Effect of Cryoprotectant on Epididymal sperm

Statistical analysis comparing the effects of the cryoprotectants on the occurrence of the different morphological defects between epididymal treatment groups found a significant effect of cryoprotectant type on the occurrence of distal droplets ($p=0.0019$). Post-hoc comparison illustrated that the significant differences were between DMF 5% and all other treatment groups (glycerol 2.5% vs DMF 5% [$p= 0.0008$], DMF 5% vs DMF 2.5% [$p= 0.0047$] and DMF 5% vs BC 5% [$p=0.013$], with DMF 5% having the highest rate of occurrence of distal droplets (Figure 3.13).

3.3.2.3 The Effects of the post-thaw medium SW3 on Epididymal and Ejaculated Sperm

The addition of SW3 medium had no significant effect on the percentage of morphologically normal sperm ($p = 0.869$). Similarly, no significant interactions existed between cryoprotectant type and SW3 medium on the percentage of morphologically normal sperm.

3.3.3 Acrosome status

3.3.3.1 Epididymal Sperm compared to Ejaculated Sperm

The percentage of intact acrosomes demonstrated no significant differences between epididymal sperm and ejaculated sperm (Figure 3.14). No statistically significant differences were found between the other acrosome status categories between epididymal sperm and ejaculated sperm.

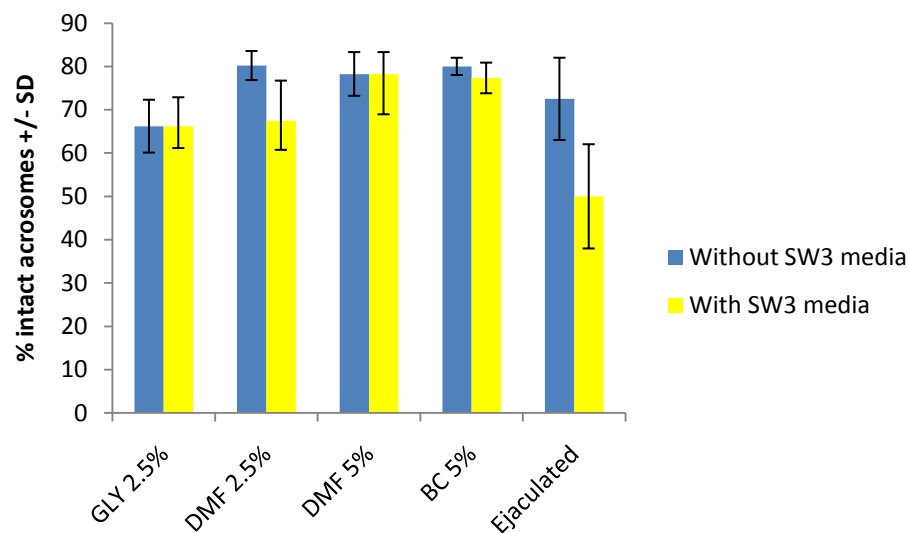


Figure 3. 14 The percentage of thawed sperm with intact acrosomes after freezing with various cryoprotectants. Error bars show 95% confidence intervals. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

3.3.3.2 The Effects of Cryoprotectant on Epididymal Sperm

Analysis demonstrated that there were no significant differences found in acrosome status categories between cryoprotectant treatment groups (Figures 3.15 – 3.19). For this reason no further post-hoc comparisons were necessary.

3.3.3.3 The Effects of the post-thaw medium SW3 on Epididymal and Ejaculated Sperm

As Figure 3.14 depicts, there appears to be only a slight treatment effect of the addition of SW3 medium on the percentage of intact acrosomes on both epididymal and ejaculated sperm and this effect was found to be insignificant. DMF 5% had the highest percentage of intact acrosomes when treated with SW3 medium (78.5%), but the formamide and glycerol combination differed only very slightly (77.3%) (see Tables 2-5 Appendix 4).

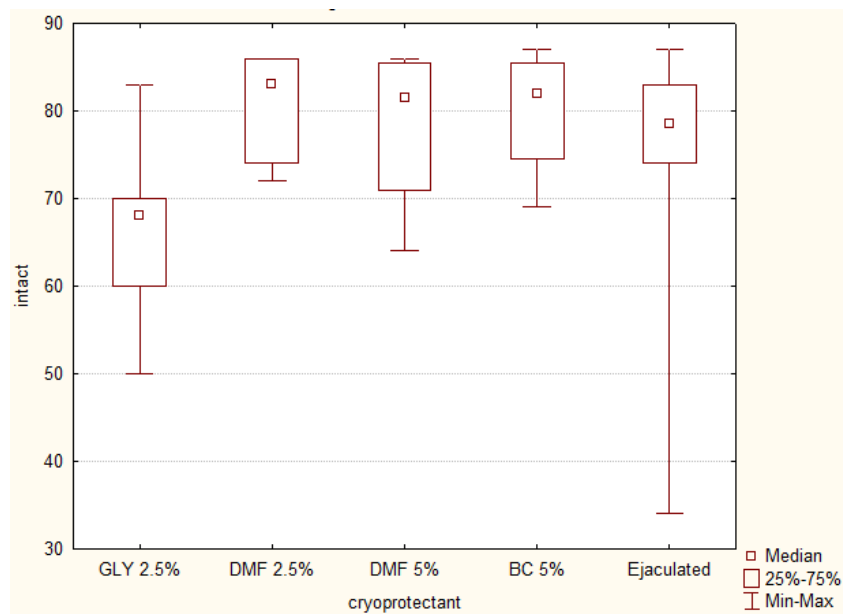


Figure 3. 15 Box and Whisker graph depicting the effect of cryoprotectant type on the percentage of sperm with intact acrosomes, without SW3 medium $p = 0.406$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

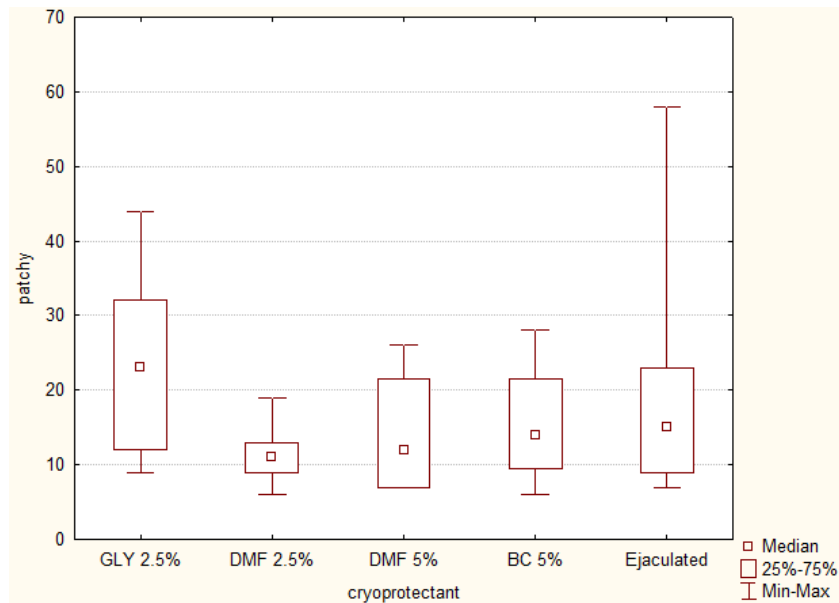


Figure 3.16 Box and Whisker graph depicting the effect of cryoprotectant type on the percentage of sperm with patchy acrosomes, without SW3 medium added $p = 0.556$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

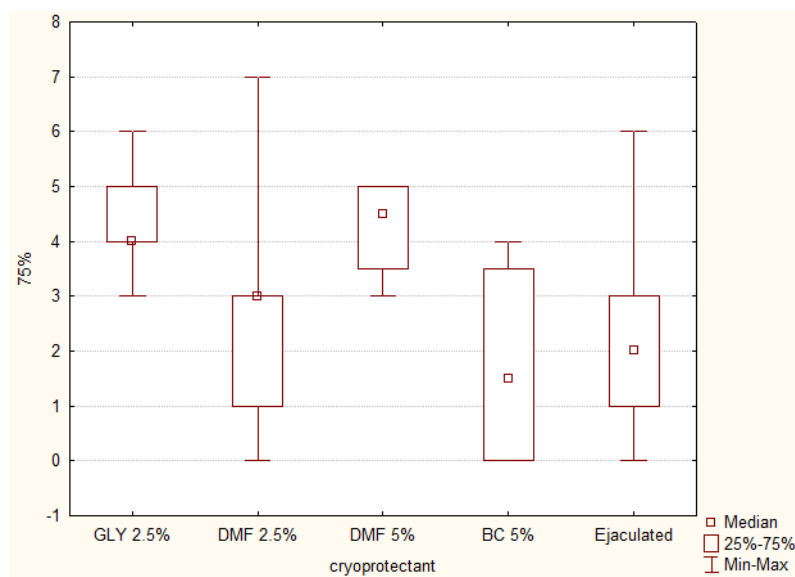


Figure 3.17 Box and Whisker graph depicting the effect of cryoprotectant type on the percentage of sperm with 75% reacted (75%) acrosomes, without SW3 medium added $p = 0.207$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

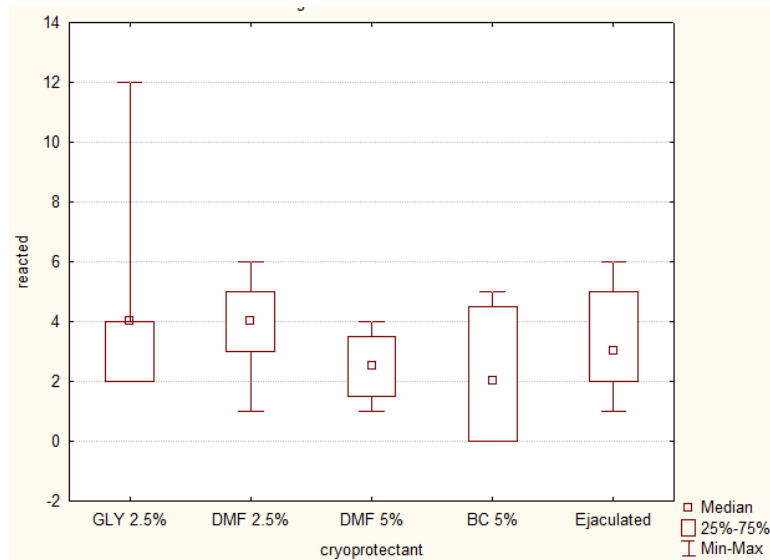


Figure 3.18 Box and Whisker graph depicting the effect of cryoprotectant type on the percentage of reacted acrosomes, without SW3 medium added $p = 0.597$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

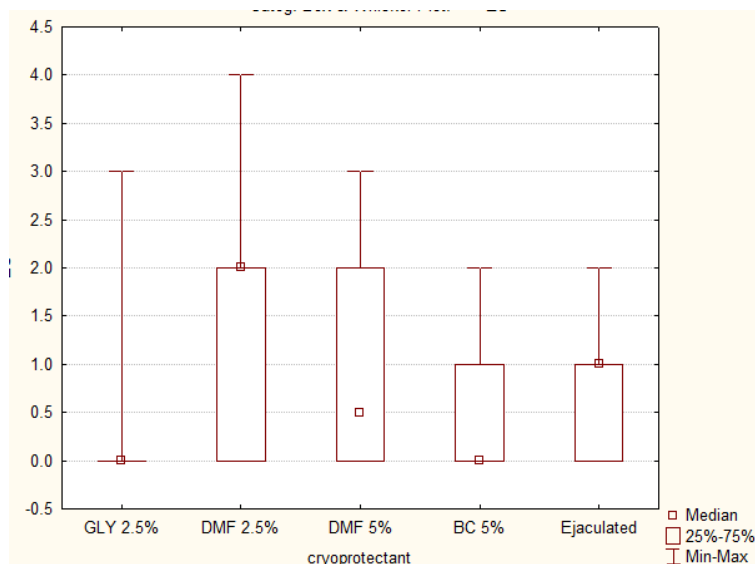


Figure 3.19 Box and Whisker graph depicting the effect of cryoprotectant type on the percentage of equatorial segments, without SW3 medium added $p = 0.690$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

3.3.4 Plasma membrane integrity

3.3.4.1 Epididymal Sperm compared to Ejaculated Sperm

Statistical analysis demonstrated a significant difference in the percentage of intact plasma membranes (% coiled) between ejaculated sperm and all epididymal sperm treatment groups ($p = 0.008$). Epididymal sperm had greater intact plasma membranes (Figure 3.20).

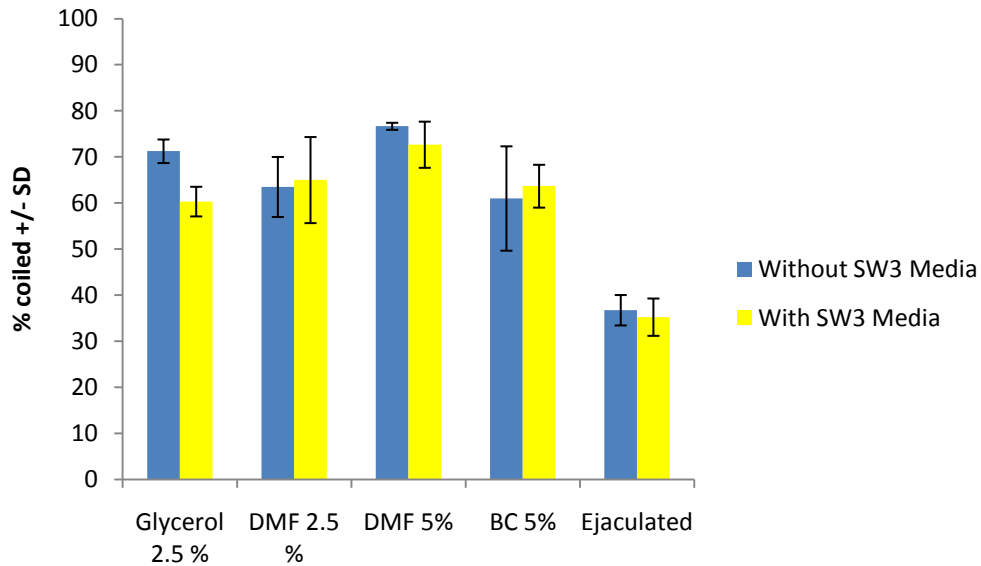


Figure 3. 20 The percentage of thawed sperm with coiled tails after thawing with various cryoprotectants, with and without the addition of SW3 medium after thawing. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

3.3.4.2 The Effects of Cryoprotectant on Epididymal Sperm and Ejaculated sperm

The variation between the epididymal samples was not significant (Figure 3.19) (Also see Table 5, Appendix 4)

3.3.4.3 The Effects of the post-thaw medium SW3 on Epididymal and Ejaculated Sperm

Statistical analysis outlined that there was a significant difference within groups treated with SW3 medium ($p = 0.004$), however as above this was due to the ejaculated samples having significantly less coiled tails than epididymal sperm. Glycerol 2.5% had the biggest reduction in the percentage of coiled tails after the addition of SW3 medium (-10.92%) (Table 5, Appendix 4). DMF 5% gave the highest percentage of coiled tails in both treated and untreated sample groups (Table 5, Appendix 4)

3.4 DISCUSSION

3.4.1 Summary of Results

The aim of this study was to investigate the effects of different cryoprotectants and the addition of the post-thaw medium SW3 on epididymal sperm from castrated colts in comparison with ejaculated sperm from stallions of proven fertility. Epididymal sperm and ejaculated sperm differed in that ejaculated sperm displayed greater total motility than epididymal sperm immediately after thawing and 30 minutes after thawing. Additionally epididymal sperm had a significantly greater percentage of intact acrosomal membranes than ejaculated sperm. Cryoprotectant type, influenced the proportion of distal droplets as there was a significant difference between samples frozen in DMF 5% compared with those frozen in glycerol 2.5%, DMF 2.5% and the glycerol/formamide combination 5% (BC), with DMF 5% having a much higher proportion of distal droplets. The predominant finding was that SW3 medium was observed to have beneficial effects on sperm motility if the sperm was frozen with glycerol as the cryoprotectant ($p= 0.016$). Morphology analysis found that the addition of SW3 medium had no effect on percentage of morphologically normal sperm or particular defects. These results suggest there is a beneficial effect of SW3 medium on motility that warrants further study. For future prospective studies a large number of stallions and mares should be included and factors analysed by way of a logistic regression to account for the variables encountered in this study.

3.4.2 Motility

Ejaculated sperm had greater total motility than epididymal sperm whilst the percentage of progressively motile sperm were similar. In a similar study which compared frozen ejaculated sperm to frozen epididymal sperm of stallions it was found that total and progressive motility were similar between groups post-thaw (Weiss et al. 2008), which differs to the total motility results of this study but agrees with the progressive motility results. Similarly sperm recovered from the stallion epididymis has been shown to display progressive motility equal to, or better than, ejaculated sperm by Tiplady et al. (2002). In contrast Heise et al (2011) found a statistically significant difference in motility between frozen-thawed ejaculated and frozen-thawed epididymal spermatozoa, with ejaculated sperm displaying greater motility. The difference's found in this study and others,

is likely to be attributed to the presence and absence of motility factors (Braun et al. 1994) in seminal plasma in ejaculated and epididymal sperm respectively.

Cryoprotectant type had no significant effect on the change in motility parameters over 30 minutes, or these parameters independently at time zero and 30 minutes. The results of this study are in agreement with other experiments with horses, where DMF was found to be as efficient in preserving equine semen for freezing as glycerol was (Keith 1998; Alvarenga et al. 2000). In contrast Medeiros et al. (2002) found that the post-thaw motility of sperm frozen with glycerol was significantly lower than that of those frozen in glycerol combined with DMF and with DMF alone. These results agree with those of a rabbit study (Hanada & Nagase 1980) that has shown that DMF preserves the sperm post-thaw motility better after thawing than glycerol does. On the other hand, Squires et al. (2004) compared glycerol (0.55M) to formamides such as dimethylformamide in stallion ejaculated sperm and found that use of glycerol resulted in higher percentages of motile sperm (61%) than DMF did (38%). However, when DMF was increased to 0.6 mol/l (5%) or 0.9 mol it resulted in percentages of motile cells similar to that with glycerol (54%). Others such as Vindament et al. (2002) have reported that combinations of cryoprotectants are more beneficial than DMF or glycerol alone. These authors compared post-thaw motility and fertility of stallion ejaculated sperm frozen in glycerol and/or DMF at different concentrations. Motility was high after freezing with 5% DMF (0.66 mol) when combined with 3 or 5% glycerol. In agreement with the work of Vidament et al. (2002), *in vitro* evaluation of semen frozen in Botu Crio[®] by Samper and Garcia (2008) found that motility parameters are increased in comparison with thawed semen processed in extenders containing only glycerol as the cryoprotectant.

The occurrence of lower motility parameters at low concentrations of DMF compared with glycerol found in some of the above studies is thought to be due to the fact that formamides contain a smaller quantity of lone electron pairs than glycerol, and as a result higher concentrations of these compounds should be needed to equal the cryoprotective potential of glycerol (Nash 1996). However, given that these compounds permeate the plasma membrane more efficiently than glycerol they should cause less osmotic damage to stallion sperm than glycerol

and thus despite the non-significant difference in motility found in the present study, and the contradictory results of other authors, the benefits of formamides may just be undetected by this motility analysis.

A comparison of results obtained from samples with and without SW3 medium illustrated that SW3 gave better motility across all cryoprotectant treatment groups, with the treatment group 2.5% glycerol experiencing significantly higher motility. In theory this enhanced motility may result from the increased level of sugar which is included in SW3 medium. Because epididymal sperm does not receive the benefits of sperm motility factors in seminal plasma (Bavister et al. 1978), and is subjected to the inhibitory effects of quiescence inducing factors, ionic constituents and osmotic pressure which depress motility (Mann & Luwak-Mann 1981), simple dilution of epididymal inhibitory factors may be beneficial. Why, however, it did not have a significant effect on the other treatment groups warrants further investigation.

Future studies could examine the factors affecting sperm longevity at 6, 12, 24 and 48 hours to enhance our understanding of the different effects of the cryoprotectants used in this study. For high-quality frozen-thawed sperm samples, visual motility analysis by microscopy may be sufficient to evaluate the sperm quality. However, both motion and flow cytometric analysis may be necessary to adequately evaluate the sperm quality from stallions perceived as “poor freezers”, or epididymal samples in this case. In theory, computerised analysis of sperm motility should offer more reliable, unbiased and repeatable means of assessing motility than examination by eye (Colenbrander et al. 2003) and offer the ability to evaluate sperm viability and DNA integrity of the sample for multifactorial analysis. In practice results of computer-assisted sperm motility analysis (CASA) of sperm has no superior correlation with fertility of fresh (Jasko et al. 1992a) and frozen (Samper et al. 1991) semen.

3.4.3 Morphology

Comparing epididymal sperm to ejaculated sperm and freezing epididymal sperm in different cryoprotectants had no significant effects on the percentage of morphologically normal sperm. However, ejaculated sperm and epididymal sperm differed significantly in the proportion of distal droplets with ejaculated sperm

having significantly less distal droplets than epididymal sperm frozen in 5% glycerol/formamide combination and 5% DMF. Such defects signify a failure of maturation whilst in transit through the epididymis, as normally the residual cytoplasm is released down the foot of the tail during spermatogenesis. Despite the early discovery of cytoplasmic droplets by Retzius in 1909 there are few published studies on these defects and their effects on fertility on any species (Althouse 1998; Retzius as early as 1909). Waberski et al. (1994) showed that extended porcine semen with high percentages of spermatozoa with proximal and distal cytoplasmic droplets had a negative correlation to pregnancy rate and litter size (Waberski et al. 1994). Furthermore retained droplets may encumber binding to the uterine epithelium in the pig (Peyrunkina et al. 2001). Nevertheless, in this study distal droplets were low in ejaculated sperm, whilst high in epididymal sperm and it is known that distal droplets are common in epididymal sperm due to their immaturity in comparison to ejaculated sperm and therefore these results are not unusual.

Further analysis within epididymal sperm treatment groups revealed that freezing epididymal sperm with 5% DMF resulted in a statistically significant higher proportion of distal droplets, especially when compared to 2.5% glycerol. Distal droplets are related to immaturity, however, the cryoprotectant would not be affecting maturation as this process occurs before cryopreservation, and therefore if it is causing this defect it must be acting some other way or the occurrence of the defect is unrelated to cryopreservation. DMF 5% was handled in the same manner as other samples so it is peculiar that it has a higher occurrence of this defect.

In boars the normal occurrence of both proximal and distal cytoplasmic droplets is between 10-15%, whilst published data on the occurrence of these for stallions is limited (Heise et al. 2011). In this study the percentages of these defects ranged from 19.6 % (glycerol 2.5%) to 25.55% (DMF 5%) for samples not treated with SW3 medium, and 12% (glycerol 2.5%) to 23% (DMF 5%) for those treated with SW3 medium. Good fertility is associated with semen samples with less than 5% abnormalities occurring in the acrosome and midpiece, and thus the incidence of these droplet defects alone is disturbingly high. In ejaculated semen samples

intended for insemination purposes, semen of this quality with high proportions of morphological defects would likely be excluded.

The addition of SW3 medium to thawed frozen epididymal sperm appeared to have no significant effects on morphology. As defects in morphology can be an indicator of male sub-fertility or infertility (Pesch & Bergmann 2006) this lack of effect caused by SW3 is a positive result. SW3 medium it is not having a detrimental effect on morphology and therefore it is less likely to negatively influence the functional integrity of the sperm, and therefore its fertilising capabilities.

Perhaps in future the analysis of the live to dead ratio revealed when staining the sperm would be of interest - especially as epididymal sperm motility can be low and viability high. Furthermore computerised technology is currently being used to measure the shape of the sperm heads (sperm head morphometry). In this respect, Gravance et al. (1996) reported that sub-fertile stallions had a significantly lower percentage of sperm with heads that conformed to the morphometric norm, and suggested that the analysis of sperm head morphometry may be a useful addition to routine semen examination for the prediction of fertility (Gravance et al. 1996). Further investigations into the defects found in this study may be of benefit to determine if they do in fact affect fertility. An insemination trial using DMF 5% may aid these conclusions.

3.4.4 Acrosome status

A comparison of the acrosome status of epididymal verses ejaculated sperm and sperm frozen in different medium tested with and without the addition of the post thaw medium SW3 illustrated that altering any of these treatment variables had no effect on acrosome status. Nevertheless sperm with 5% glycerol/formamide combination had the most intact acrosomes and those with DMF 2.5% were the most affected by SW3 medium, with the highest reduction in intact acrosomes. It is understood that the percentages of cells in a sperm sample with either intact plasma membranes or acrosomal membranes are more highly correlated with fertility than motility (Christensen et al. 2000). Cryoprotectants alone are recognized to have a detrimental effect on the ability of sperm to undergo the acrosome reaction, and frozen-thawed sperm may undergo premature break-down

of the acrosome prior to interaction with the oocytes, thus explaining the reduced fertility potential of cryopreserved semen (Centola et al. 2005). Although the results were non-significant, it may be so that the combination of glycerol and formamide at 5% preserves epididymal sperm best, illustrated by the least percentage of damaged acrosomes, and these should therefore have greater fertility when used for insemination, which is in fact confirmed in Chapter 5. In agreement with this, studies have compared glycerol to formamides such as DMA and found glycerol to be more detrimental to sperm acrosomes than DMA (Mocé et al. 2010).

At ejaculation, sperm mix with seminal plasma which contains the small membranous vesicles known as prostasomes, that may enable sperm to experience physiological activation (Sostaric et al. 2008). Therefore, epididymal sperm have not been exposed to these prostasomes, and so their potential to undergo the acrosome reaction *in vivo* may be compromised. In this study the percentage of intact acrosomes of epididymal sperm was between 66.2% (with glycerol 2.5%) and 80.2 (with DMF 2.5%) for untreated samples and 66.2% and 78.25% (with DMF 2.5%) for those treated with SW3 medium, hence over half of the sperm in the sample had not yet undergone a premature acrosome reaction. Indeed, these high proportions of intact acrosomes seen in epididymal sperm after thawing may not be a positive prediction of fertility.

Future investigations to verify acrosome status and the factors influencing the potential for the acrosome reaction in epididymal sperm could be performed by chemically inducing the acrosome reaction with calcium ionophore A23187 which induces acrosomal damage and can be used as a control to compare samples.

3.4.5 Plasma Membrane Integrity

Comparisons of epididymal sperm and ejaculated sperm revealed that epididymal sperm for all treatment groups had a mean of greater than 61% coiled tails, and thus intact plasma membranes, whereas the means of the ejaculated samples were less than 37%. This suggests that perhaps the later combination with seminal plasma and the process of ejaculation has harmful effects on the plasma membrane integrity of spermatozoa. Indeed, the process of sperm capacitation

begins upon release of the epididymal sperm from the quiescent and inhibitory environment while stored in the epididymis. On the other hand the differences may be due to the morphological and functional differences which exist between epididymal sperm and ejaculated sperm which influence membrane stability and cold shock susceptibility and the resistance to osmotic stress (Hewitt et al. 2001). Perhaps the plasma membrane of epididymal sperm is more resilient to cryopreservation than ejaculated sperm are.

Analysis of the functional integrity of sperm after freezing and thawing revealed no significant effects of cryoprotectant type or the addition of SW3 medium on the plasma membrane integrity of epididymal sperm. The plasma membrane and acrosome membrane are the most sensitive parts of the sperm (Friend & Rudolf 1974) and, accepting the premise that functional integrity of the plasma membrane is critical to normal sperm metabolism and function, it is logical to believe that the more sperm identified with normal characteristics in this region the better the quality of the semen sample.

Various studies have compared the membrane integrity of sperm using the HOS of semen samples frozen in glycerol and ethylene glycol (EG) (Mantorani et al. 2002; Rota et al. 2006; Futino et al. 2010). Comparison between glycerol and ethylene glycol for the cryopreservation of equine sperm established that ethylene glycol is a suitable replacement for glycerol as a cryoprotectant for stallion sperm if used at the same or lower concentrations (Mantorani et al. 2002). Unlike in the present study Futino et al. (2010) compared glycerol, MF and DMF in the cryopreservation of canine ejaculated semen and found that under the conditions of the HOS test glycerol was superior to MF and DMF ($p < 0.05$). Few other studies have compared semen frozen in different cryoprotectants using the HOS test and as it is a simple and effective test to perform, it could readily be integrated into routine semen analysis.

Fluorometry, an indirect method of assessing the percentage of membrane-intact cells, is another means in which cell membranes may be assessed and may be more sensitive in detecting differences between treatment groups, and thus future investigations into this method might be of benefit (Graham & Mocé 2005).

3.4.6 Future Advances

Other forms of analysis which may have been beneficial in the present study include the zona pellucida binding assay, oocyte penetration assay and flow cytometric analysis of sperm. The zona pellucida binding assay involves an *in vitro* experiment in which the number of sperm that bind to the zona pellucida after incubation for a set period of time is analysed. It is often hard to obtain equine zona pellucida and for this reason those from other species with molecular similarities are frequently used. The zona-free bovine oocyte binding assay has also been developed for stallion sperm penetration (Landim-Alvarenga et al. 2004). Since the zona pellucida is removed from the oocytes this assay can evaluate the capacitation/acrosome status of the sperm, as only sperm that have already undergone these changes can penetrate the oocyte (Yanagimachi 1972; Hanada & Chang 1976; Yanagimachi et al. 1976). This assay, however, does not evaluate the ability of sperm to bind and penetrate the zona pellucida, a formidable obstacle to sperm reaching the oocyte.

Analysing sperm by flow cytometry lessens variability associated with analysis (Kirk et al. 2005). However, it is unlikely that most stallion semen collection, processing and cryopreservation facilities will possess a flow cytometer to conduct the spermatozoal analyses. Though, the frozen straws could be shipped to facilities that possess a flow cytometer for these analyses to be conducted. These analyses would not likely be conducted for every ejaculate processed, but could be conducted at regular intervals, such as at the beginning, middle and end of the breeding season.

As for most studies the inclusion of more stallions and replicates of experiments would benefit analysis.

CHAPTER 4

ANALYSIS OF DNA AND METABOLIC OF FROZEN-THAWED STALLION EPIDIDYMAL SPERMATOZOA

4.1 INTRODUCTION

Chromatin is composed of DNA, RNA, and nuclear proteins (Wykes et al. 1997). It has a multifaceted arrangement of DNA and macromolecules with different degrees of compaction (Fuentes-Mascorro et al. 2000b). With the succession of spermatogenesis and spermiogenesis, the sperm chromatin starts to lose histones, which are exchanged with intermediate proteins and the sperm-specific protamines (Dadoue 1995; Kierszenbaum 2001). Simultaneously, DNA undergoes the structural changes crucial for a high degree of compaction, resulting in shrinkage of the overall nuclear volume (Ward & Coffey 1991; Fuentes-Mascorro et al. 2000b; Schlegel & Paduch 2005).

Evaluation of cryopreserved semen after thawing is primarily restricted to motility, membrane and acrosome parameters (Holt 2000). The damage to the DNA and the metabolic status of sperm after freezing and thawing has received less consideration (Aziz et al. 2005; Slowinska et al. 2008). Over the past decade, mounting evidence that chromatin organization and metabolic condition play an imperative role in fertilization and early embryo development has resulted in steadily increasing interest in these areas and their influences on fertility (Agarwal & Said 2003; Carrell et al. 2003; Aziz et al. 2005).

Because sperm chromatin structure is multipart, it is likely that more than one test is required to assess sperm chromatin integrity (Schlegel & Paduch 2005). Numerous techniques to assess sperm DNA integrity have recently been developed, some of the most robust including the sperm chromatin structure assay (SCSA), the terminal deoxynucleotidyl transferase (TUNEL) assay, the Comet

assay, and the chromatin integrity test. Each of these tests however measures different aspects of DNA damage (Lewis & Agbaje 2008). Nevertheless, it is agreed that DNA damage may be a good biomarker to understand fertility problems (Morris et al. 2002a).

It has been suggested that an additional cause for the low fertility observed in cryopreserved semen after artificial insemination is mitochondrial damage caused by the freeze/thaw process (Ruiz-Pesini et al. 1998). Studies with isolated mitochondria confirmed that freezing and thawing significantly impairs their bioenergetic functions (Dickinson et al. 1970; Mori et al. 1986). In consequence this results in compromised sperm energy metabolism which is critical for efficient movement along the uterine tube and penetration of the zona pellucida (Chandler et al. 2000).

4.1.1 Spermatozoa Chromatin Structure Assay (SCSA)

The sperm chromatin structure assay (SCSA) was introduced by Evenson in 1980 as a means of deducing the susceptibility of sperm to DNA denaturation following exposure to an acidic environment. Love (2005) investigated how the results of this assay relate to fertility (Love 2005). Sperm are labelled with the fluorescent dye, acridine orange (AO), which shifts fluorescence from green to red when associated with single-stranded DNA or RNA (Schlegel & Paduch 2005). This is analysed by a flow cytometer to evaluate the ratio of sperm with single stranded DNA (abnormal), fluorescing red, to those with double stranded DNA (normal), fluorescing green, present in a semen sample (Morrell et al. 2008). A computer adds up the percentage of green- versus red-labelled sperm and software allows for creation of a graphic plot of the percent of damaged cells giving an index known as the DNA fragmentation Index (DFI) (Morrell et al. 2008). The results of the assay can be illustrated on a scatter-plot, with the amount of red fluorescence (abnormal) on the X-axis and green fluorescence (normal) of the Y-axis (Love 2005). Experiments have shown that if the percentage of cells with abnormal ratios surpasses 30-40% then fertility is unlikely in humans (Evenson et al. 1999; Spano et al. 2000). Testing with SCSA is advantageous in that it evaluates a high number of sperm in a relatively short period of time and it can be a fast method for screening multiple samples (Schlegel & Paduch 2005). However this process requires expensive equipment and a relatively high concentration of sperm

(Schlegel & Paduch 2005). Furthermore it has been stated to be less specific than the alkaline comet or TUNEL methods (discussed below) in determining DNA fragmentation in that it identifies changes in protamine content and disulphide cross-linkage in addition to DNA strand breaks (Lewis & Agbaje 2008).

4.1.2 Terminal Deoxynucleotidyl Transferase-mediated dUDP Nick-End Labelling (TUNEL) assay

The terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) assay detects double and single stand DNA breaks by enzymatically labelling the free 3' OH end of the DNA with a fluorescent substrate (Morris et al. 2002a). TUNEL-positive cells are identified either by a microscope or by fluorescence-activated cell sorting (Morris et al. 2002a). The strength of the fluorescence corresponds with the number of incorporated dUTP, and thus the number of nicks in the DNA (Schlegel & Paduch 2005). TUNEL is advantageous in that it requires a small number of sperm, is relatively quick and easy to perform and it can, if required, be analysed by light microscopy using anti-fluorescein antibodies, and can thus be low cost and incorporated into a typical andrology laboratory (Schlegel & Paduch 2005). However, because individual cells are analysed directly by visual means, only limited numbers of sperm can be evaluated (Schlegel & Paduch 2005).

4.1.3 The Alkaline Comet Assay

The alkaline comet assay (or, the Single Cell Gel Electrophoresis assay) assesses actual DNA strand breaks (Lewis & Agbaje 2008). It has been extensively used in somatic cells to measure genome damage, especially single- and double-stranded breaks. This technique entails making microgels with cells suspended in agarose solution on specialized microscopic slides, lysing cells with large amounts of salt and detergents, digestion and removal of proteins from nuclear DNA, electrophoresis, staining of electrophoresed DNA in microgels with an intense fluorescent dye, and analysis of images under epifluorescence (Singh et al. 2003). Using some protocols, the presence or absence of a comet tail is scored, some semen samples producing hardly any 'cometed' sperm (Aravindan et al. 1997). In other protocols, all sperm produce a comet, so allowing the images to be analysed for percentage tail DNA, tail length and/or tail moment (Morris et al. 2002a).

Because sperm differ from somatic cells in that they have a unique tightly compacted chromatin structure the comet assay had to be modified for the use in sperm (Lewis & Agbaje 2008), originally being applied to sperm by Singh (Singh et al. 1989). The formation of disulphide bonds between protamines and DNA is the main reason high levels of DNA compaction exist in sperm. As sperm move through the epididymis, the protamines are cross-linked by disulphide bonds, condensing the chromatin to one-sixth the volume taken up in somatic cell nuclei (Fuentes-Mascorro et al. 2000a). In addition, following ejaculation more bonds are formed, further enhancing chromatin solidity (Molina et al. 1995). This dense compaction gives protection against exogenous assault to the sperm DNA but it also stops DNA strands migrating during electrophoresis (Lewis & Agbaje 2008). To enable the use of the comet assay in semen Lewis and Agbaje (2008) and associates have investigated different means of conducting this test (Hughes et al. 1997; Donnelly et al. 1999). Originally, the use of the conventional alkaline comet protocols designed for somatic cells failed to lyse the sperm cells. However in later studies the addition of the protease enzyme Proteinase K (PK) to the lysis solution to remove protamines allowed sperm DNA to de-condense and migrate. Although successful, PK was only effective at high concentrations (100mg/ml) and after long periods of incubation (overnight) (Lewis & Agbaje 2008).

Sawyer et al. (2003) have illustrated that millimolar concentrations of hydrogen peroxide brings about single stranded breaks and/or alkali-labile sites in human sperm (Sawyer et al. 2003). With increasing hydrogen peroxide concentrations the DNA becomes more and more fragmented. With this knowledge the addition of hydrogen peroxide to a subset of sperm samples when conducting experiments may be used as a control in the comet assay protocol and other protocols for comparative purposes.

The alkaline comet assay is advantageous in that it has been proven to be technically straightforward, inexpensive, rapid and reproducible (Morris et al. 2002a; Singh et al. 2003). One of its unique and powerful features is its ability to determine DNA damage within an individual cell as opposed to just one general measure of damaged cells versus undamaged cells as in the TUNEL assay. Furthermore the results from the comet assay are correlated with DNA damage

measured by the TUNEL and SCSA methods (Aravindan et al. 1997; Donnelly et al. 2000). The only hindrance to this method is that many problems have arisen, as previously mentioned, from the difficulties encountered in releasing the DNA from the sperm head due to the unique DNA compaction (Ward & Coffey 1991). Furthermore it requires a dedicated electrophoresis unit with an epifluorescent microscope, generally not present in most semen processing laboratories (Schlegel & Paduch 2005).

4.1.4 Chromatin Integrity test

Chromatin structure may also be assessed by the chromatin integrity test, described by Tejada et al., 1984, which involves the use of an acridine orange (AO) fluorescent probe, a DNA metachromatic stain that reveals DNA fragmentation by colour coding (Celeghini et al. 2008). It has been recommended as a screening test to estimate DNA damage in human sperm and to predict fertilization rates (Tejada et al. 1984; Hoshi et al. 1996). AO fluoresces green when it intercalates as a monomer into native, double stranded DNA, and red when it binds to denatured, single stranded DNA.

It involves treatment of sperm with acid to separate thiols from DNA, increasing DNA vulnerability to denaturation. The advantages of this test are that it is low cost, repeatable and takes little time. However it does require the use of a fluorescent microscope, and AO staining is still under debate due to the subjective interpretation of results, the problems of rapidly fading fluorescence and heterogeneous slide staining (Claassens et al. 1992; Duran et al. 1998; Evenson et al. 1999).

4.1.5 MTT Assay

Besides other factors, sperm motility depends on mitochondrial energy metabolism. Due to their unique chemical and biological properties tetrazolium salts have become some of the most commonly used tools in cell biology for measuring the metabolic activity of cells spanning from mammalian to microbial in origin (Berridge et al. 2005). This widespread usage is due to the positively charged quaternary tetrazole ring core with four Nitrogen atoms. After mild reduction tetrazolium structures transform from weakly coloured salts into brightly coloured formazan products by disruption of the tetrazole ring. MTT

(3[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow, water soluble tetrazolium salt (Aziz et al. 2005). The process of using MTT to measure metabolic activity in sperm cells was first described by Mosmann in 1983 (Mosmann 1983; Hansen et al. 1989). In this assay, MTT is changed 'into water-insoluble purple formazan on the reduction cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria' (Slater et al. 1963; Aziz et al. 2005). The amount of formazan formed can be collated spectrophotometrically and this number can be used as an estimate of the number of mitochondria and hence the number of living cells in the sample (Denizot & Lang 1986; Aziz 2006).

The MTT assay is straightforward, inexpensive, rapid and reliable method for estimating the percentage of viable sperm in mammalian species (Mosmann 1983; Naser-Esfahani et al. 2002; Gaczarzewicz et al. 2003)

4.1.6 Aims

The aim of the series of experiments in this chapter was to evaluate the post-thaw DNA and metabolic integrity of epididymal sperm frozen with different cryoprotectants to assess if different methods of cryopreservation affect these factors more, or less, than others which may ultimately affect sperms fertilising ability and longevity.

Epididymal samples frozen in 2.5% glycerol, 2.5% DMF, 5% DMF and 5% glycerol:formamide combination (Botu Crio[®]) were compared to ejaculated samples and samples treated with hydrogen peroxide (H₂O₂) (which induces damage to the cell and ultimately kills it) with the following tests:

1. The alkaline comet assay
2. The chromatin integrity test
3. The MTT assay

The alkaline comet assay enabled comparisons of actual DNA strand breaks in each sample, the chromatin integrity test allowed assessment of chromatin structure and the MTT assay allowed for the assessment of the metabolic activity required for motility.

4.2 METHODS

4.2.1 Initial methods applied to each straw

Three straws per stallion (to generate three repeats), per cryoprotectant treatment group were allocated to DNA analyses. Once the necessary preparations for each experiment had been completed, such as making medium, each 0.5ml straw was thawed at 37°C in a water bath for 30 seconds. The outside of the straw was then dried and drained into a 1.7ml centrifuge tube (Appendix 2), kept in a warm block of 37 °C. Three 125 µl aliquots from the sample were pipetted into three centrifuge tubes labelled '1', '2' and '3' in reference to the DNA analyses tests: the alkaline comet assay, the chromatin integrity test and the MTT assay. These tubes too were kept on the warm block during this process. The stallion number and cryoprotectant type were also labelled on the tubes. The three experiments were conducted simultaneously but their methods will be described separately for simplicity.

4.2.2 The Alkaline Comet Assay

4.2.2.1 Controls

For a negative control to generate samples positive for comet tails, 5 samples of epididymal sperm cells were treated with 70.4 mmol/l H₂O₂ (Appendix 2) in a Coplin jar (Appendix 2) for 10 minutes at room temperature after lithium diiodosalicylate treatment (refer below). For a positive control frozen-thawed ejaculated samples from 5 stallions with proven fertility were assessed.

4.2.2.2 Assay Protocol

The medium and agarose required were prepared and placed at the necessary temperatures for the experiment (Appendix 1). Slides were then prepared by labelling, soaking in ethanol (70%) (Appendix 2), flaming under the Bunsen burner (Appendix 2) to sterilise followed by cooling. Once cool they were immersed in 60°C 1% normal melting point agarose (Appendix 1) for 20 seconds and slowly removed. The excess agarose was gently wiped off the bottom with a paper towel and the slides were placed in a clean location to dry for at least 3 hours.

The group of aliquots of epididymal sperm labelled '1' (allocated to the comet assay) were washed by dilution with 250 μ l of PBS-SDS (Appendix 1), placed on a rotating wheel for fifteen minutes, spun at 1000 rcf for 5 minutes in the centrifuge (Appendix 2), followed by the removal of the supernatants. This process was then repeated three times to wash the sperm of excess proteins and residues.

The pellets were then re-suspended in 1ml of PBS-SDS-PK solution (Appendix 1) and incubated at 37 °C for an hour whilst rotating at 400 rpm. Next, 5 μ l from each sample were mixed with 75 μ l of 5% low melting point agarose within the 37 °C incubator to prevent solidification of the agarose. Continuing in the incubator a smear of each sample was made by pipetting 50 μ l of the cell/agarose solution onto the slide and immediately covering with a cover slip. The slides were then incubated at 4°C for 15 minutes. Whilst this was taking place the lysis solution was completed by measuring 40ml of the lysis base (Appendix 1) into Coplin jar and adding 1% triton X 100. The slides were then removed from incubation and the cover slips gently removed by sliding them off sideways. The Slides were then immersed in the lysis buffer and incubated at 4°C overnight. This was followed by incubation with the 2ME-DW solution (Appendix 1) at 4 °C followed by lithium diiodosalicylate (4 mmol/l) for 90 minutes at 20 °C to reduce the disulphide bonds. Whilst this was occurring the NAOH-EDTA solution (Appendix 1) was placed into the electrophoresis unit (Appendix 2), and when ready the slides were placed in it to equilibrate for 20 minutes at room temperature. The unit was then turned to 20 V and run for 20 minutes. Slides were removed gently and excess residue off the bottom of the slide was wiped off. This was followed by incubation in the 0.4 mol/l tris solution (Appendix 1) within a Coplin jar for 10 minutes. The slides were then transferred into a Coplin jar of ethanol for 5 minutes and then air dried. SYBR gold (Appendix 2) staining was then employed by placing 400 μ l of the stain diluted 1:10000 onto the slide in a square shape, followed by gentle spreading with the pipette tip. The stain was left for 5 minutes in the dark to adhere to the cells. A cover slip was placed onto the slide and it was viewed under a fluorescence microscope at 100x magnification using oil immersion. Because not all cells produced comets the presence or absence of comets on 200 sperm cells per sample was counted.

4.2.3 The Chromatin Integrity test

4.2.3.1 Controls

To generate samples positive for damaged chromatin, 5 control samples of epididymal sperm were treated with 250 μ l of H₂O₂ (70.4 μ mol/l) for ten minutes at 37 degrees. This sample was then centrifuged at 1000 rcf for 5 minutes and re-suspended in 250 μ l PBS. From this sperm suspension 20 μ l was smeared glass slide as described for untreated samples (see below). This H₂O₂ treated sample was the negative control and the positive control was frozen-thawed ejaculated samples from 5 stallions with proven fertility.

4.2.3.2 Assay Protocol

The second lot of aliquots of epididymal sperm labelled '2' were washed by diluting with 250 μ l of PBS and centrifuged at 1000 rcf for 5 minutes, followed by the removal of the supernatants. This process was then repeated three times. Smears were made of the slides and these were air dried and later fixed in Carnoys solution for 2 hours (Appendix 1). After fixation slides were air dried and stained with 400 μ l of freshly prepared acridine orange stain (Appendix 1) for 5 minutes in the dark. After staining, slides were washed with distilled water and a 20 μ l drop of mounting medium was placed on the slide followed by a cover slip. Slides were then immediately evaluated under a Zeiss fluorescence microscope (Appendix 2) with the excitation wavelength at 450-490 nm. A total of 200 sperm cells displaying different colours were counted on each slide by the same examiner and the duration of evaluation was not more than 40 seconds per field. Once this duration was up the field of view was changed to a different region of the slide. Sperm displaying green fluorescence were considered to contain normal DNA content, whereas sperm displaying a spectrum of yellow-orange to red fluorescence were considered to have damaged DNA.

4.2.4 The MTT Assay

4.2.4.1 Controls

Before incubation at 37 °C 5 control samples of epididymal sperm were centrifuged down at 1000 rcf for 5 minutes and suspended in 30% H₂O₂ for ten minutes followed by centrifuging and re-suspension in PBS. Frozen-thawed

ejaculated samples from 5 stallions with proven fertility were used as positive controls and treated the same as the epididymal samples (below).

4.2.4.2 Assay Protocol

The third group of aliquots of epididymal sperm labelled '3' were diluted to 100×10^6 cells/ml in PBS. A volume of 25 μ l of MTT (5 mg/ml) (Appendix 1) was added to the sperm samples and these were heated to 37 °C and rotated for one hour, noting the colour change. A volume of 150 μ l of lysis solution (Appendix 1) was added to the sample and this was again put on a rotating wheel at 37 °C for one hour. Samples were then centrifuged at 9000 rcf for 3 minutes and then diluted 1:4 in 4.5 ml spectrophotometer cuvettes (Appendix 2). The spectrophotometer was then blanked with 1 ml of lysis solution in a cuvette at a wavelength of 570 nm and the rate of reduction of MTT was measured at 570 nm as an end-point reading to compare the amount of MTT reduced over the time of 60 minutes.

3.2.4 Statistical Analysis

Data from the comet assay, chromatin integrity test and the MTT assay was investigated using the statistics programme STATISTICA (StarSoft Inc., 2008). The independent t-test, one-way ANOVA and two-way ANOVA with repeated measures were used to the data. If significant effects were detected by one-way ANOVA or two-way ANOVA then the Newman-Kleus multiple range test was conducted to determine what differences existed between groups. When undertaking analysis of variance tests it was checked that all samples tested met the criteria of ANOVA in that the data is normally distributed and of equal variance. The difference was considered significant if the p-value was < 0.05.

4.3 RESULTS

4.3.1 Alkaline Comet Assay

The alkaline comet assay measured the percentage of sperm giving comet tails in samples post-thaw. Figure 4.1 shows the image for a typical cometed sperm cell after sperm have been processed in the comet assay. A medium amount of tail fluorescence can be observed in this photograph. Comparison of the baseline data illustrated that mean cometed sperm (damaged DNA) was similar between epididymal sperm froze with different cryoprotectants and to the ejaculated control samples (Figure 4.2). The differences between the four treatment groups were non-significant ($p = 0.376$). Hydrogen peroxide cells were observed to have suffered DNA damage in ejaculated semen controls and the difference between these samples and the four treatment groups was significant (Table 4.1). Hence hydrogen peroxide was seen to have measurable damage.

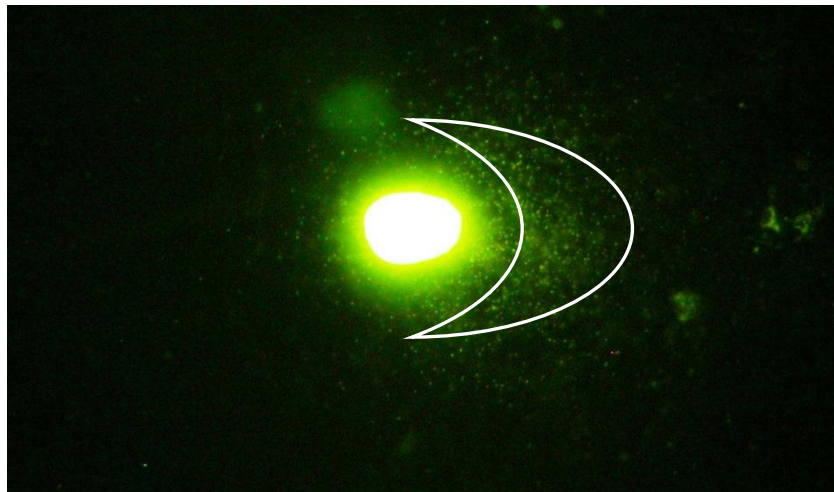


Figure 4. 1 Image demonstrating a stallion epididymal sperm positive for the occurrence of a comet tail. Outlined region represents the mass of ‘dots’ considered to be the comet tail.

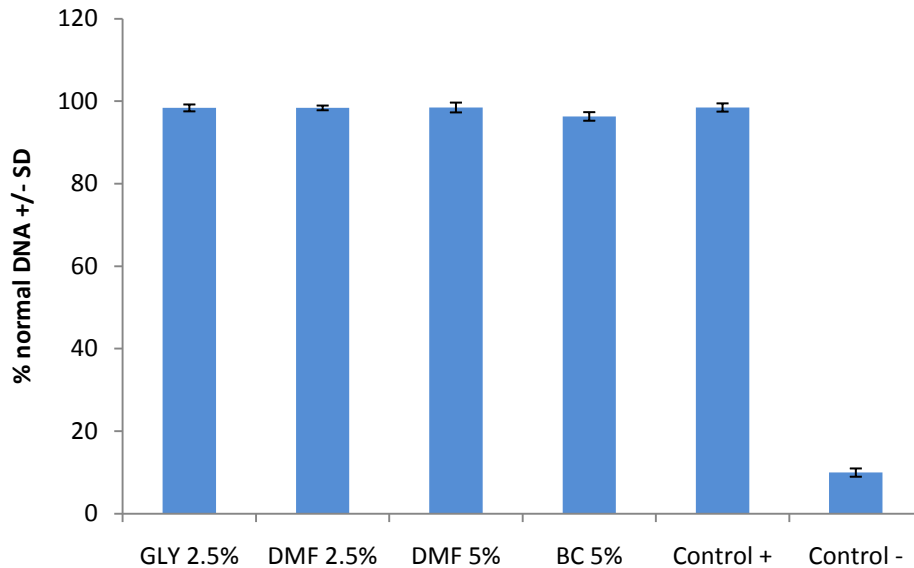


Figure 4. 2 The Percentage (%) of cells with normal DNA under the conditions of the comet assay in thawed epididymal sperm that had been frozen with various cryoprotectants. The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

Table 4. 1 Post-Hoc Newman-Keuls test for the occurrence of statistical differences between treatment groups for the comet assay. The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. Differences marked in bold are significant at $p < 0.05$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol:formamide combination.

	GLY 2.5%	DMF 2.5%	DMF 5%	BC 5%	Control +	Control -
GLY 2.5%		1.0000	0.9953	0.0717	0.9997	0.0001
DMF 2.5%	1.0000		0.9275	0.1637	0.9953	0.0001
DMF 5%	0.9953	0.9275		0.2226	1.0000	0.0001
BC 5%	0.0717	0.1637	0.2226		0.3031	0.0001
Control +	0.9997	0.9953	1.0000	0.3031		0.0001
Control -	0.0001	0.0001	0.0001	0.0001	0.0001	

4.3.2 The Chromatin Integrity Test

The chromatin integrity test measured the percentage of normal sperm (green) in each of the cryoprotectant treatment groups. The differences between groups is shown in Figure 4.3, which shows that the treatment groups and the positive control differ only slightly. However, the negative control treated with hydrogen peroxide had 0% normal sperm (all red). The difference between treatment groups was non-statistically significant, although the negative controls were statistically

unique (Table 4.2). Figure 4.4 shows that the IQR is smallest when samples are treated with 5% glycerol/formamide combination, and largest for those treated with 2.5% glycerol. The dispersion in the dataset was also considerably less for samples treated with the 5% glycerol/formamide combination than for other treatment groups.

Table 4. 2 Post-Hoc Newman-Keuls test for the occurrence of statistical differences between treatment groups for the chromatin integrity test. The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. Differences marked in bold are significant at $p < 0.05$ GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination

	GLY 2.5%	DMF 2.5%	DMF 5%	BC 5%	Control +	Control -
GLY 2.5%		0.5029	0.3921	0.3133	0.2957	0.0001
DMF 2.5%	0.5029		0.6259	0.4252	0.5562	0.0001
DMF 5%	0.3921	0.6259		0.9060	0.6971	0.0001
BC 5%	0.3133	0.4252	0.9060		0.8652	0.0001
Control +	0.2957	0.5562	0.5562	0.8652		0.0001
Control -	0.0001	0.0001	0.0001	0.0001	0.0001	

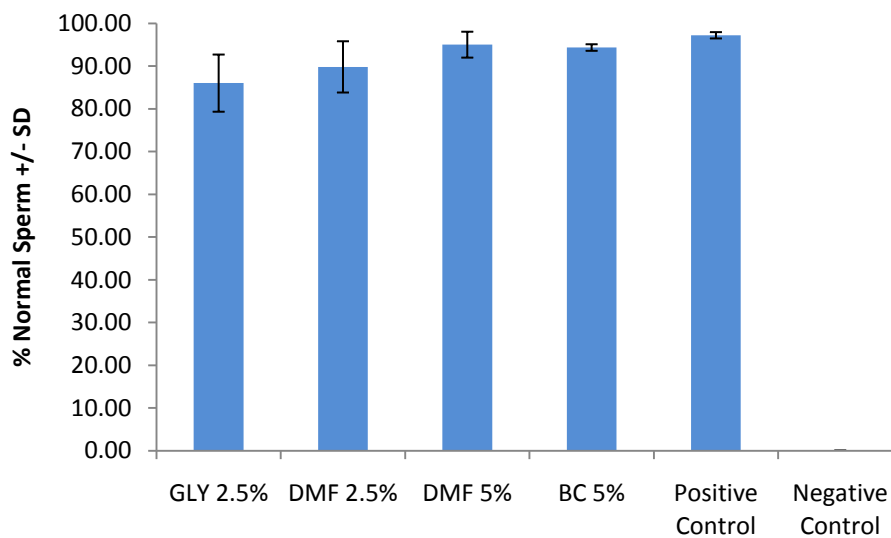


Figure 4. 3 Percent of normal sperm under the conditions of the chromatin integrity test in thawed epididymal sperm that had been frozen with various cryoprotectants. The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

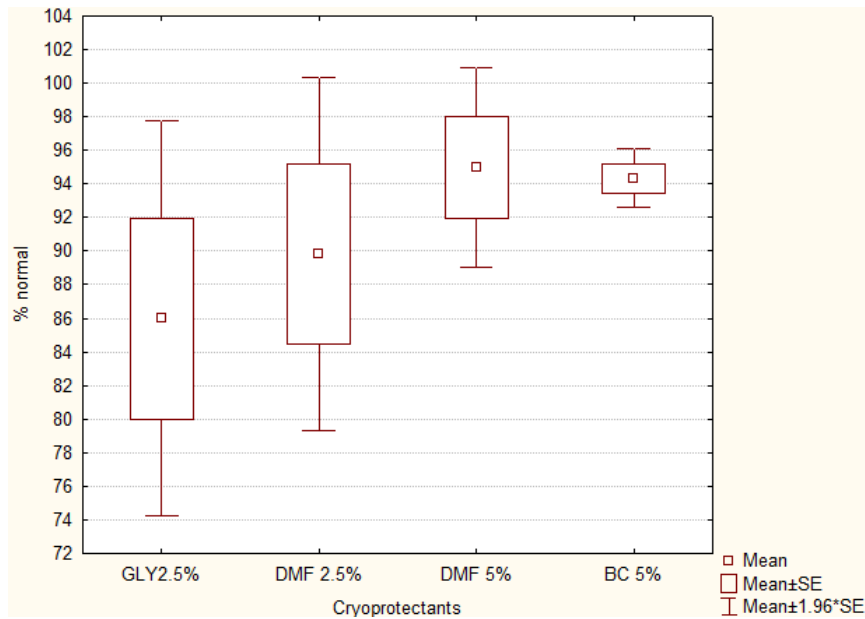


Figure 4. 4 Box and Whisker graph of the percentage of normal sperm under the conditions of the chromatin integrity test. *GLY* = Glycerol, *DMF* = dimethylformamide, *BC* = combination cryoprotectant (*Botu Crio*[®])

4.3.3 The MTT Assay

The MTT assay measured the percentage of viable sperm depending on the accumulation of formazin. The spectrophotometer absorbance reading means are displayed in Figure 5.5, used as a measure of viability. The different cryoprotectant treatment groups had a non-statistically significant difference between them (Table 4.3), however the positive (ejaculated semen) and negative control (epididymal sperm treated with hydrogen peroxide) were significantly different from each of the treatment groups. Glycerol 2.5% and 5% glycerol/formamide combination were very similar and had the lower MTT absorbance rates, whilst DMF 2.5% and DMF 5% were higher and also very similar.

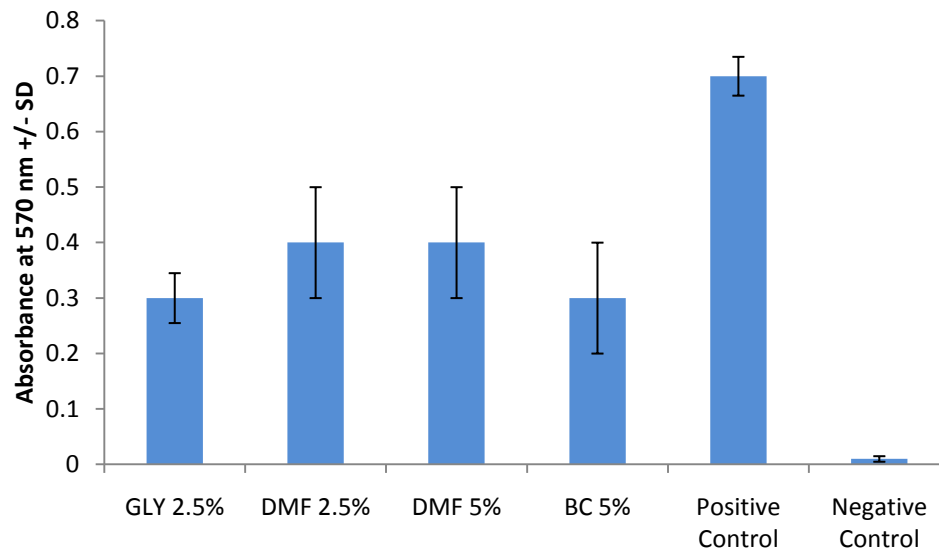


Figure 4. 5 Absorbance at 570 nm in 60 minutes of epididymal sperm that had been frozen with various cryoprotectants. The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination.

Table 4. 3 Post-Hoc Newman-Keuls test for the occurrence of statistical differences between treatment groups for the MTT assay . The positive controls were ejaculated frozen-thawed samples from stallions with proven fertility and the negative controls were epididymal samples treated with hydrogen peroxide. Differences marked in bold are significant at $p < 0.05$. GLY = Glycerol, DMF = dimethylformamide and BC = glycerol/formamide combination

	GLY 2.5%	DMF 2.5%	DMF 5%	BC 5%	Control +	Control -
GLY 2.5%		0.7523	0.6478	0.8474	0.0185	0.0201
DMF 2.5%	0.7523		0.6558	0.6031	0.0312	0.0199
DMF 5%	0.6478	0.6558		0.5967	0.0315	0.0117
BC 5%	0.8474	0.6031	0.5967		0.0184	0.0337
Control +	0.0185	0.0312	0.0315	0.0184		0.0002
Control -	0.0201	0.0199	0.0117	0.0337	0.0002	

4.4 DISCUSSION

None of the DNA tests showed significant differences between treatment groups, although all of these groups were significantly different from the negative controls (samples treated with H₂O₂). Ejaculated samples also appeared to be significantly different to treatment groups in the MTT assay.

4.4.1 Alkaline Comet Assay

In agreement with the results found here studies of spontaneous and induced alterations, in mouse sperm DNA, undertaken by Sega et al. (1986) found relatively low levels of damage in these cells. In a study by Solwinska et al. (2008) cryopreservation of bull ejaculated sperm caused a significant, but low (3.8%) reduction in the percentage of DNA in the comet head and an increase (5.3%) in tail length. Hence in this case the cryopreservation of bull semen brought about a low (4-5%) level of fragmentation in DNA when compared with non-frozen samples. In the present study cryopreservation of epididymal sperm appeared to cause little DNA fragmentation to the cells, however, perhaps DNA damage was present and undetected. In a study of frozen human and mouse sperm an abundance of DNA breaks when studied with the alkaline comet assay was found (Sega et al. 1986; Sega & Generoso 1988; Singh et al. 1988) and thus it would be predictable that more DNA breaks should have been detected in stallion sperm, especially from an epididymal sample. In future a more precise scoring system may be employed, for example: Fenestra Comet version 2.2. (Kinetic Imaging Ltd.), Komet 5.5 Software (Kinetic Imaging, Bromborough Wirral, United Kingdom) or measuring the percentage of head DNA (McKelvey-Martin et al. 1997). Perhaps the utilisation of a comet assay reagent kit for single cell gel electrophoresis (Trecigen Inc., Gaithersburg, MD, USA) (Anonymous. 2001) specialised for sperm would be advantageous to ensure consistent results.

Alterations of the protocol is another area for future research as it is well known that the DNA compaction of sperm prevents DNA strands migrating during electrophoresis in conventional alkaline comet assays. Possible changes include the option to incubate the slides overnight in 100 µg/ml proteinase K as practised by other authors (McKelvey-Martin et al. 1997). The lysis solution may have benefited from containing other chemicals such as sN-lauroyl sarcosinate 0.01%, tetrasodium ethylenediaminetetraacetic acid 50 mmol/l, reduced glutathione 2

mg/ml (Singh et al. 2003) as used in human sperm. Another option is to put lysis solution on ice and leave for at least 30 minutes overnight for maximum sensitivity (Trevigen® 2010).

4.4.2 The Chromatin Integrity Test

Because of the variability of protocols used in previous studies, the acridine orange staining technique has not been widely accepted as a screening test to predict DNA damage in human sperm, and thus studies for comparison are limited. However, protocols have become more refined in time (Chohan et al. 2004). When comparing protocols, Chohan et al. (2004) found that using Carnoys as the fixation medium was the best. They also found that the use of the SCSA to evaluate DNA damage was not beneficial and might be the result of less time utilised for denaturation. The same study additionally found that some individuals are more susceptible to DNA damage after freezing and thawing procedures than others and therefore a comparison to fresh ejaculated and fresh epididymal sperm samples to the frozen samples would have been beneficial in this study.

Findings from Evenson et al., (1999) revealed that natural pregnancy in humans is not possible when sperm have DNA damage $\geq 30\%$. Fortunately in this study DNA damage was shown to be below 30% in the chromatin integrity test for both epididymal and ejaculated positive control samples.

Reproducibility between the three slides which were prepared separately indicates that each of the techniques involved in the assay are consistent and that the preparation of a single slide is sufficient for the analysis of one sperm population. In addition to the reproducibility of the assay for control samples, assay results are also reproducible after treatment with hydrogen peroxide showing consistency of this damaging procedure.

4.4.3 The MTT assay

The MTT assay proved to be the most sensitive assay, distinguishing a difference between ejaculated and epididymal samples as well as those treated with hydrogen peroxide. Aziz et al., (2005) found that the amount of MTT reduction increased steadily with semen cell incubation time, and that increasing the volume of the sperm cells in a semen sample resulted in a proportional and significant

($P < 0.01$) decline in the rate of MTT reduction. It has also been deduced that cell number must also be precise as this alters MTT readings (Hansen et al. 1989). In this study times, volumes and cell numbers were managed carefully and therefore the possibility of altered results due to these issues are unlikely.

The results from Aziz et al., (2005) indicated a high correlation between the rate of MTT reduction and sperm viability. Furthermore the rate of MTT reduction decreased significantly with a rising proportion of killed sperm. They also concluded that the rate of MTT reduction rate may be used as an indicator of equine sperm fertility. The MTT reduction test is dependable for evaluating sperm samples. In addition can be used successfully, particularly in routine analysis where practical aspects such as time, costs and practicability are essential.

4.4.4 Future Advances

There is large scope for other DNA analysis techniques and comparisons of correlation with other methods of genomic analysis. For example it may be of research benefit to investigate the results produced from the SCSA and the TUNEL assay.

Morris et al. 2002a found that the higher the motility of sperm in semen, the higher the DNA damage load carried by sperm populations which is contradicts other studies (Barroso et al. 2000; Irvine et al. 2000; Zini et al. 2001) which report that sperm samples with low motility carried higher loads of DNA damage. An investigation into the correlation between motility and DNA damage of epididymal sperm would not only be interesting but if a strong correlation is discovered then the need to conduct time-consuming, expensive tests would be eliminated. Aitken et al. (2003) have reviewed the relationships between sperm DNA damage and fertilization. This group has shown that at low levels of oxidative stress, DNA damage is induced yet the fertilizing potential of sperm is actually enhanced (Aitken et al. 2003). These results are clinically significant since they support studies by Ahmadi and Ng (1999) that sperm showing damage to DNA can maintain their fertilizing potential which consequently means that damaged DNA becomes part of the next generation's genome.

CHAPTER 5:

ARTIFICIAL INSEMINATION WITH EPIDIDYMAL SPERMATOZOA

5.1 INTRODUCTION

The technique of artificially inseminating horses began when a group of Arabs stole stallion semen from a rival tribe, transporting it in a sponge to their domicile (Davies Morel 1999; Crabo 2001). The advantages of artificial insemination (AI) were quickly recognised and the process refined with frozen-thawed semen technology later being developed by Christopher Polge in the 1940s (Crabo 2001). AI was originally of research interest in dogs and horses until in the later 19th Century a more commercial use for the practice was developed in Russia and China (Foote 1982). It is now extensively used in sport horses, ponies, special breeds and standardbreds, which has increased the demand for research in this field (Weston 2005).

Artificial insemination is the only way in which epididymal sperm can be used for breeding in mares. But because little has been published on the fertility of frozen-thawed epididymal sperm, and that which has been published has mainly given poor results, the value of epididymal sperm for AI is still under debate. For example, in the first experiment using frozen-thawed epididymal sperm from stallions, only one of the seven mares inseminated conceived (Barker & Gandier 1957). Furthermore, since the semen quantities acquired from deceased, catastrophically injured or castrated stallions is limited, methodologies which require only small numbers of sperm are desirable. Reducing sperm numbers in the inseminate, the potential number of mares bred per ejaculate is increased and the incidence of post-breeding endometritis in mares with delayed uterine clearance is reduced (Sanchez et al. 2008). Conventional artificial insemination typically requires high numbers of sperm ($250-500 \times 10^6$), and is thus not practical in such cases.

5.1.1 Artificial Insemination with Cryopreserved Stallion Spermatozoa

Unfortunately using frozen equine semen for AI has been less successful than in other species, with reported pregnancy rates from some stallions being from 8 to 61% of those obtained with fresh semen from the same stallion (Amann 1984). Furthermore, because the fertile lifespan of oocytes (6-10h) and sperm (fresh 4-8 hours, frozen 6-12 hours) in the female genital tract is limited, to accomplish high fertility the timing of insemination relative to ovulation is vital (Wongtawan et al. 2006), particularly for frozen sperm (Papa et al. 2008).

In order to obtain satisfactory per-cycle pregnancy rates in mares, the minimum recommended number of sperm contained within a conventional insemination dose is usually $>300 \times 10^6$ progressively motile sperm for fresh semen (Pickett & Voss 1975a; Householder et al. 1981; Vidament et al. 1997; Gahne et al. 1998) and $> 200 \times 10^6$ for frozen semen (Pickett et al. 2000). The requirements for such high numbers of sperm limits the number of mares which can be inseminated per ejaculate. Some studies have had success in improving the fertility rates of stallions with low fertility using techniques of insemination which only require low numbers of sperm (Alvarenga & Leão 2002; Morris 2004). Furthermore the deposition of a low volume of concentrated sperm close to the uterotubal junction (UTJ), has proven more convenient than using larger volumes as it reduces the occurrence of backflow which can result in huge sperm losses (Wongtawan et al. 2006). To enable exploitation of low numbers of sperm available after freezing or sex pre-selection, and to aim at using small volumes of semen, both deep uterine (Buchanan et al. 2000) and hysteroscopic insemination techniques (Morris et al. 2000a) have been developed for horses.

5.1.2 Hysteroscopic Insemination

Recent studies have investigated the possibility of using a videoendoscope to deposit sperm on and around the uterotubal papilla (Manning et al. 1998; Vazquez et al. 1998; Lindsey 2000; Morris et al. 2000b). The crypts on the UTJ are an important site for preovulatory sperm storage.

Hysteroscopic insemination is conducted by guiding the videoendoscope through the cervix and pushing it forward through the uterine lumen of the estrous mare by the operators gloved arm in the vagina (Bracher & Allen 1992; Morris et al.

2000a). The endoscope is guided along the uterine horn ipsilateral to the ovary which contains the preovulatory follicle. When the tip of the endoscope is located within 3-5 cm of the papilla of the UTJ the small volume of inseminate is deposited onto the papilla.

With this technique Manning et al. (1998) reported a 22% pregnancy rate when 1×10^6 sperm were inseminated into the oviduct via the UTJ. Vazquez et al. (1998) reported a 33% pregnancy rate when 3.8×10^6 sperm were placed on the UTJ (Vazquez et al. 1998). Morris et al. (2000) also utilized the hysteroscopic insemination technique to deposit various numbers of sperm on the UTJ. They reported pregnancy rates of 29, 64, 75 and 60% when 0.5, 1, 5 and 10×10^6 sperm, respectively, were placed on the UTJ. Lindsey et al. (2000) demonstrated better fertility using a hysteroscopic insemination method (50% pregnant) when compared with no pregnancy obtained after deep uterine insemination with 5×10^6 sperm (discussed below) (Lindsey 2000).

Hysteroscopic insemination of mares with 150×10^6 fresh epididymal sperm produced pregnancy rates of 45% (Morris et al. 2000b) compared with pregnancy rates of 29% after insemination with 5×10^6 frozen-thawed epididymal sperm after hysteroscopic insemination (Morris et al. 2002c). Although these rates are less than those achieved with ejaculated sperm, they are higher than those reported (8%) after conventional insemination with frozen thawed epididymal sperm (8%) (Morris et al. 2000b).

Morris et al. 2002 observed a beneficial effect of washing frozen-thawed epididymal sperm in a modified Tyrode's balanced salt solution (sperm TALP) prior to hysteroscopic insemination. No pregnancies were obtained in the 20 mares inseminated with epididymal sperm not exposed to sperm TALP compared with a 29% conception in the 20 mares inseminated with epididymal sperm washed in sperm TALP after thawing. Indeed, this effect was seen in both the control group and the sperm which had been processed through the Percoll gradient.

5.1.3 Deep Intra-Uterine Horn Insemination

The deep intra-uterine horn insemination technique is another method used to inseminate low dose, highly concentrated, sperm close to the sperm reservoir (Buchanan et al. 2000; Woods et al. 2000; Brinsko et al. 2003; Wongtawan et al. 2006). Two methods of deep uterine insemination have been reported: in a study by Buchanan et al (2000), a flexible catheter was inserted into the uterine horn ipsilateral to the corpus luteum and the position of the catheter was verified by ultrasound. Insemination of 25×10^6 and 5×10^6 sperm produced pregnancy rates of 53 and 35%, respectively. Rigby et al. (2001) reported a pregnancy rate of 50% with deep uterine insemination.

Discussion continues over which technique produces higher pregnancy rates (Lyle & Ferrer 2005). Nevertheless, Brinsko et al. (2003) compared deep uterine insemination with hysteroscopic insemination. In this study mares were inseminated with 5×10^6 sperm and no statistically significant differences were observed between the pregnancy rates of the 21 mares inseminated hysteroscopically (62%) and the 20 by using the deep uterine method (50%). In an endeavour to improve the quality of the sperm used for deep uterine insemination, Nie and Johnson (2000) inseminated mares with 1×10^6 sperm that had been filtered through a glass wool/Sephadex column. However, their pregnancy results were disappointingly low (7-19%) and this may be because less than 1×10^6 sperm actually made contact with the UTJ (Nie & Johnson 2000). In contrast Lindsey et al. (2005) found that hysteroscopic insemination of 20×10^6 sexed sperm produced better pregnancy rates than deep uterine insemination. It may be that when sperm have been compromised by sex-sorting that hysteroscopic insemination is beneficial over deep uterine insemination.

Hysteroscopic AI requires specialized equipment and trained personnel, whereas transrectally guided AI is an inexpensive and fast technique. Sanchez et al. (2008) compared hysteroscopic insemination (HI) of mares to rectally guided insemination (RGI) for commercially guided insemination and established that HI resulted in a 45% pregnancy rate and RGI resulted in a 43% pregnancy rate, with no statistical differences between the two treatment groups. Although HI required a noteworthy reduction in the number of straws required they found that there was

no advantage of this technique when using commercially frozen semen from stallions of proven fertility and doses containing more than 50×10^6 sperm in a 0.5ml volume.

5.1.3 Management of the Mares for Insemination

The timing of insemination must be closely synchronised with the timing of ovulation to optimise pregnancy rates. The growth of the follicles is assessed by transrectal ultrasonographic examination of the ovaries (Palmer & Driancourt 1980). Ovulation induction hormone is typically administered when a follicle is >35 mm to ensure ovulation occurs within 48 hours (Duchamp et al. 1987), to ensure that insemination occurs at the ideal time.

Sperm motility the uterine contractions facilitate the movement of sperm throughout the reproductive tract (Katila 2001a), towards the caudal isthmus of the oviduct where fertilization occurs. Whilst awaiting the arrival of the oocyte after ovulation, the sperm are attached to the oviductal epithelium by virtue of their plasma membrane (Thomas et al. 1994; Weston 2005). This attachment ensures sperm are at the site of fertilization and capacitated, ready for when the egg is released (Thomas et al. 1994; Dobrinski et al. 1997). If fresh semen is being used for insemination, it is best deposited into the mare up to 48 hours before ovulation (Woods et al. 1990; Katila et al. 1997), as sperm are able to survive for long periods of time in the isthmus of the mares uterus (Hunter 1990). Insemination with cooled semen should occur 12 to 24 hours prior to ovulation or up to 12 hours after ovulation to ensure acceptable pregnancy rates (Sieme et al. 2003).

Ideally an insemination dose should be at the concentration of $25 - 50 \times 10^6$ sperm/ml (Varner et al. 1987) in a volume of approximately 50ml to ensure that there are 500×10^6 progressively motile sperm available (Day 1942; Pickett & Voss 1975b; Pickett et al. 2000). As cryopreservation diminishes the viability and longevity of the sperm (Watson 1995a; Bedford et al. 2000), it is suggested that at least 300×10^6 progressively motile sperm are inseminated when using frozen semen (Pickett et al. 2000).

For conventional insemination methods timing of insemination needs to be more precise with respect to ovulation and should occur approximately 6 hours before ovulation, or up to 6 hours after ovulation to optimize fertility rates (Pickett et al., 2000).

5.1.4 Recovery and Cryopreservation of Equine Embryos

Owners may elect to preserve valuable genetics from a particular mare and stallion combination, whilst other owners sell and purchase cryopreserved embryos and have them transferred at a certain time so that they can choose when the resulting foal is born (Hudson et al. 2008). The techniques of embryo collection and transfer were developed in the late 1970s and today this technique is permitted in most breeds (Squires et al. 2003). The main candidates for embryo transfer include older mares with poor reproductive histories that are not capable of producing a foal by conventional natural mating or artificial insemination, as well as show mares and those that are competing in racing, polo or other performance events (Squires et al. 2003). The techniques of cryopreserving equine embryos have advanced in the last decade to the point at which most practitioners can collect and vitrify embryos (Hudson et al. 2008). In recent studies, it has been established that equine embryos can be vitrified, warmed and then transferred, attaining pregnancy rates similar to fresh or cooled embryos (Eldridge-Panuska et al. 2005; Hudson et al. 2005). To be successfully vitrified, an equine embryo must be developmentally at the late morula or early blastocyst stage. Flushing embryos eight days after ovulation in the donor mare has proven to be ideal (Eldridge-Panuska et al. 2005; Hudson et al. 2005).

5.1.4 Aim

This chapter aimed to undertake a fertility trial using deep intra-uterine horn insemination to compare the pregnancy rates of epididymal sperm frozen in glycerol 2.5% to epididymal sperm frozen in a glycerol and formamide combination 5% (Botu Crio[®] [BC]).

Sperm were frozen at a concentration of 600×10^6 /ml to ensure that a total of 300×10^6 epididymal sperm were available for insemination when one 0.5 ml straw was used. This dose of 300×10^6 was chosen as it is suitable for deep-uterine insemination and optimises the number of doses available of the limited

epididymal sperm. The single insemination procedure rather than a double insemination procedure discussed previously was used to prevent unnecessary sperm loss and inseminations.

An embryo transfer was conducted to demonstrate that embryos produced from epididymal sperm can be successfully transferred, and develop normally, thus increasing the use of this finite sperm type. Embryos were also frozen for possible later research.

As conception rate alone is far the best indicator of fertility this study allowed conclusions on which cryoprotectant out of the two was best for freezing epididymal sperm.

5.2 METHODS

5.2.1 Selection and Treatment of Mares

Twenty fertile, oestrous mares (standardbred and thoroughbred) aged 3-12 years were selected for insemination. All mares were clinically examined prior to enrolment in the study to ensure they were fertile and healthy. Mares were inseminated using the deep intra-uterine horn method. This procedure was scheduled 30 or 36 hours after administration of either hCG or deslorelin respectively to induce ovulation once a preovulatory follicle > 35 mm was detected by ultrasound and the mare had good uterine oedema and a relaxed cervix.

5.2.2 Preparation of Mares for Insemination

Within four hours of ovulation the mares were restrained in stocks, the rectum was evacuated of feces by removal by hand and the side of the preovulatory follicle was confirmed via transrectal ultrasonography. The tail of the mare was then bandaged and tied aside and the perineum aseptically prepared using warm water containing Microsheild 4 (Appendix 2) and cotton wool, moving from the centre outwards until completely clean. The area was then dried with a white clean paper towel to ensure the area was completely clean.

5.2.3 Thawing of the Sperm Sample

The desired sample of frozen epididymal sperm was thawed for 1 minute prior to insemination in a 37°C water bath.

5.2.4 Deep intra-uterine Horn Insemination

Insemination was conducted with thawed sperm that had been frozen in glycerol 2.5% and thawed semen that had been frozen in a combination of glycerol and formamide at 5%. Only two treatment groups were evaluated due to the limited availability of mares. A flexible insemination pipette (Appendix 2) was inserted through the cervix into the uterine body by a veterinarian experienced in this technique. The thawed straw was then cut open, the outside dried with a paper towel and the straw inserted into the catheter for insemination.

5.2.5 Mare Management and Assessment

After insemination mares were managed routinely as for insemination with frozen semen. At day 8 or 15 mares were either flushed for embryos (see Section 5.2.6) to confirm pregnancy or examined for pregnancy at 15 days after ovulation (see Section 5.2.9). At both day 8 and 15 the appearance of the reproductive tract and ovaries was recorded. If the embryo was flushed and retrieved at day 8 and it was of suitable size for freezing the embryos were frozen for possible future experiments. In one instance the embryo was transferred non-surgically into a recipient mare to confirm the ability of epididymal sperm to produce developmentally normal embryos. Any mares which experienced haemorrhagic anovulations were excluded from the trial and replaced with another fertile mare. If a mare was not pregnant she was returned to the fertility trial. This was allowed to occur twice, after which the mare was then excluded from the study due to the possibility of her being infertile.

5.2.6 Embryo Recovery

In 3 mares on day 8 post-ovulation a non-surgical transcervical embryo flush procedure, using a flexible 33 FG Foley type catheter (Appendix 2) was performed. The catheter was inserted through the mare's cervix by a trained professional and the cuff was inflated with 50ml of air. The catheter was withdrawn against the internal os of the cervix and 2 litres of Vigro™ complete flush medium was introduced into the mare's uterus. After gentle manipulation of the uterus the fluid was allowed to drain through a MINIFLUSH™ filter (Appendix 2). The embryo was located using a stereomicroscope at 12x magnification and washed in ViGRO™ holding medium (Appendix 1).

5.2.7 Embryo Transfer

Using a nonsurgical method one embryo produced from the insemination of Stallion 5 sperm frozen in the glycerol/formamide combination was transferred from a donor mare into a synchronised recipient. Here the embryo was loaded into a flexible insemination rod (Appendix 2), The rod was covered in a protective sheath and placed through the cervix into the body of the uterus by a qualified veterinarian. This mare was managed routinely as for all pregnant mares, with ultrasound scans occurring up until day 68.

5.2.8 Embryo Freezing by Vitrification

Embryo vitrification was performed using a technique adapted from Carnevale. (2006). The embryo was washed in the ViGRO™ holding medium and placed in the first vitrification solution (S1). It was held in S1 for 5 minutes at room temperature and then transferred into S2 for 5 minutes. The embryo was then transferred into S3 for 45 seconds (Carnevale 2006). The embryo was loaded into a 0.25ml straw (as illustrated in Figure 5.1) The straw was then sealed with heated forceps, put into a goblet and held vertically in the liquid Nitrogen vapour for 1 minute prior to plunging into the liquid Nitrogen. A total of 2 embryos were vitrified in this study.

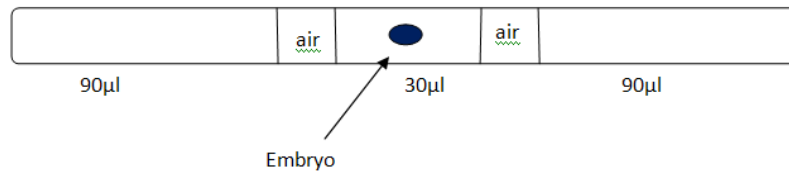


Figure 5. 1 Loading of the Embryo into a straw for vitrification – 90 µl of dilution solution (Carnevale 2006) is loaded into the straw followed by an air bubble followed by 30 µl of S3 containing the embryo followed by another bubble of air and 90 µl of dilution solution.

5.2.9 Treatment of mares after day 15

If a mare was confirmed pregnant at day 15 (i.e. they were not flushed at day 8) the mare was treated with a prostaglandin analogue (Prosolvin, Intervet – Appendix 2) to cause leuteolysis and induce oestrus thus resulting in abortion. This allowed them to be inseminated on a second oestrous cycle.

5.2.10 Statistical Analysis

Pregnancy data was plotted on simple histograms and the chi-square test for independent variables was used to determine any statistically significant differences between treatment groups.

5.3 RESULTS

5.3.1 Pregnancy data

One mare out of 10 (10%) conceived after insemination with epididymal sperm frozen with glycerol 2.5% as the cryoprotectant (Table 5.1 and Figure 5.1). The epididymal sperm frozen with glycerol/formamide combination at 5% produced 5 pregnancies (50%) after insemination of mares. Chi square analysis of these results reveals a significant difference between the two treatment groups ($p = 0.025$) and freezing epididymal sperm with the combined glycerol/formamide cryoprotectant gave higher pregnancy rates than when 2.5% glycerol was used as the cryoprotectant.

Table 4. 4 Pregnancy rates of mares inseminated with epididymal sperm frozen with 2.5% glycerol and 5% Botu Crio[®].

Treatment	Mares Inseminated	Mares Pregnant at 8 or 15 days	Pregnancy rate (%)	Pregnancies Aborted	Embryos Transferred	Embryos Frozen
Glycerol 2.5%	10	1	10% ^a	1	0	0
Glycerol/formamide 5%	10	5	50% ^a	2	1	2

^a Statistically significant difference ($P < 0.05$)

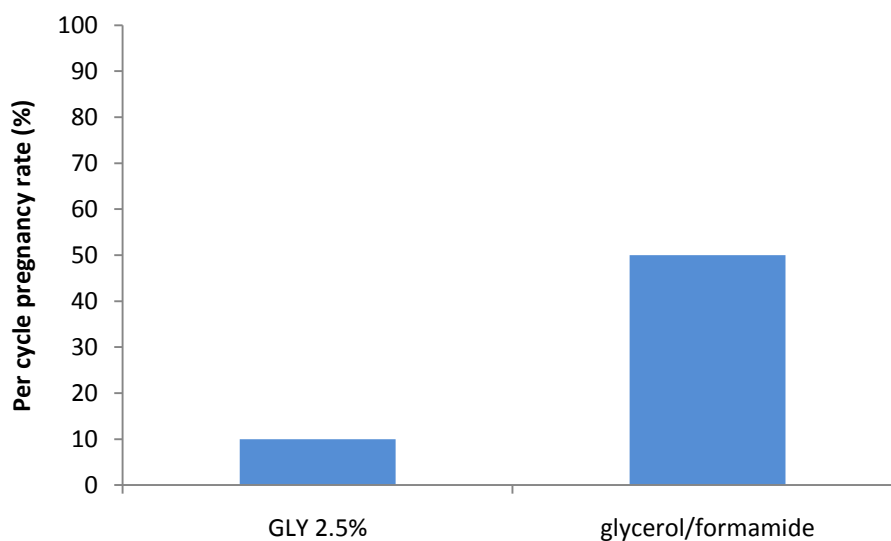


Figure 5. 2 Per-cycle Pregnancy rate of mares inseminated with epididymal sperm frozen with 2.5% glycerol (GLY 2.5%) and a glycerol/ formamide combination 5%(Botu Crio[®]).

5.3.2 Embryo transfer data

A successful pregnancy was produced from the one embryo transfer conducted using sperm epididymal frozen in glycerol/formamide 5%. Images showing pregnancy development are included to illustrate the changes in the embryo over time (Cortesy of Sandra Wilsher, The Equine Fertility Unit, New Market, Suffolk, UK) (Figures 5.3 – 5.6). The pregnancy developed normally and was documented as follows: At 13 days gestation the conceptus is found lying centrally in the uterine body (Figure 5.3). Following day 15 the vesicle became fixed at the base of the left horn. At 21 days the embryo proper (which becomes the foal) could be seen as a white bulge and the vesicle began to lose its spherical shape. The dorsal wall of the uterus has also begun to thicken (Figure 5.4). At 27 days of pregnancy the heartbeat was able to be detected as a flickering movement in the middle of the embryo. The allantoic cavity had begun enlarging beneath the embryo, lifting the embryo dorsally. The yolk sac had begun regressing. By day 38 (Figure 5.5) the embryo had moved to the dorsal part of the vesicle. By this stage the volume of the allantois greatly exceeded that of the yolk sac and the umbilical cord could be seen. Figure 5.6 shows a scan at day 45, at this stage the embryo had begun to show the form of a foetal horse.

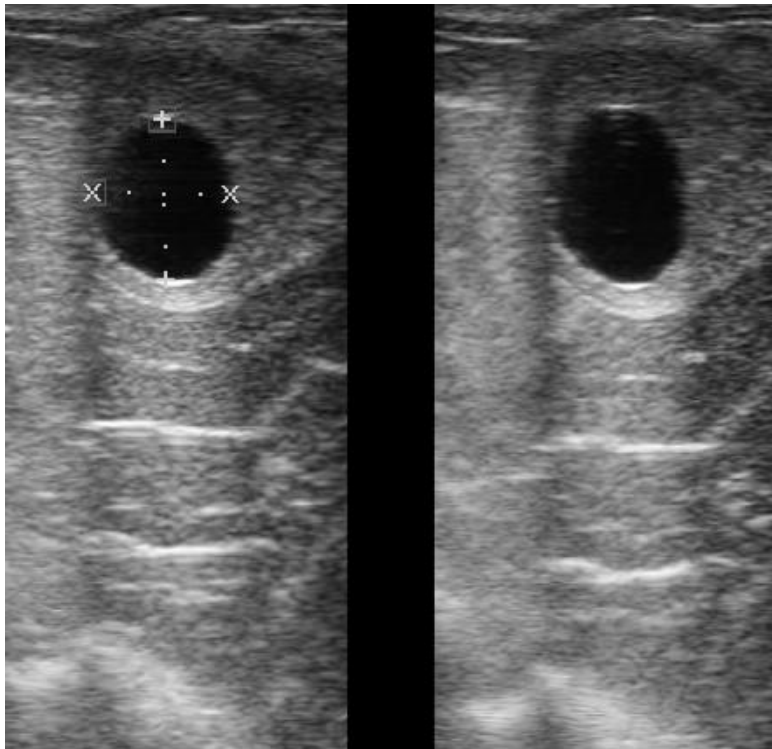


Figure 5. 3 Ultrasound pregnancy scan image of day 13 embryo.

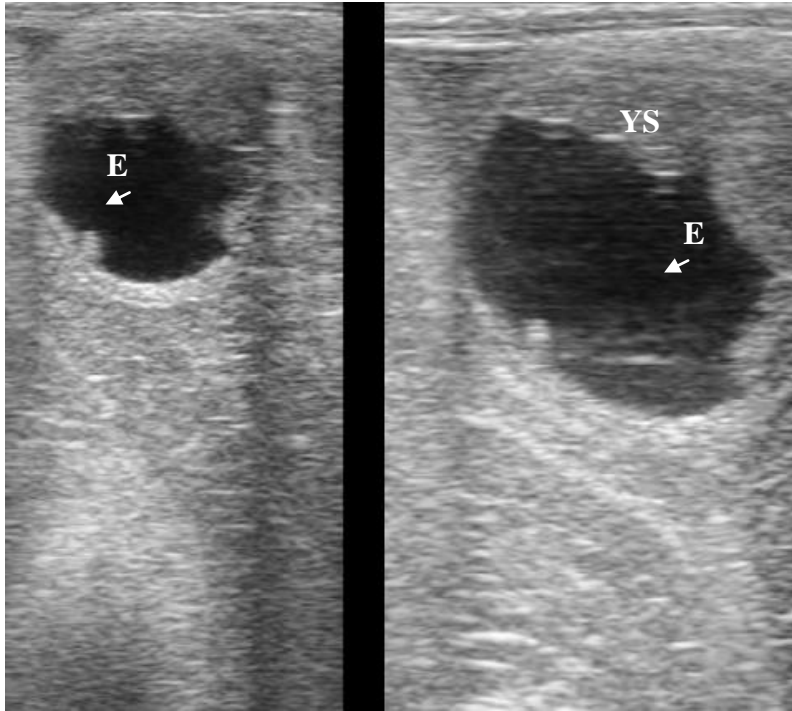


Figure 5. 4 Ultrasound pregnancy scan image of day 21 embryo: The outline appears irregular. The embryo proper (E) can be seen at the base of the yolk sac (YS) as a white bulge.

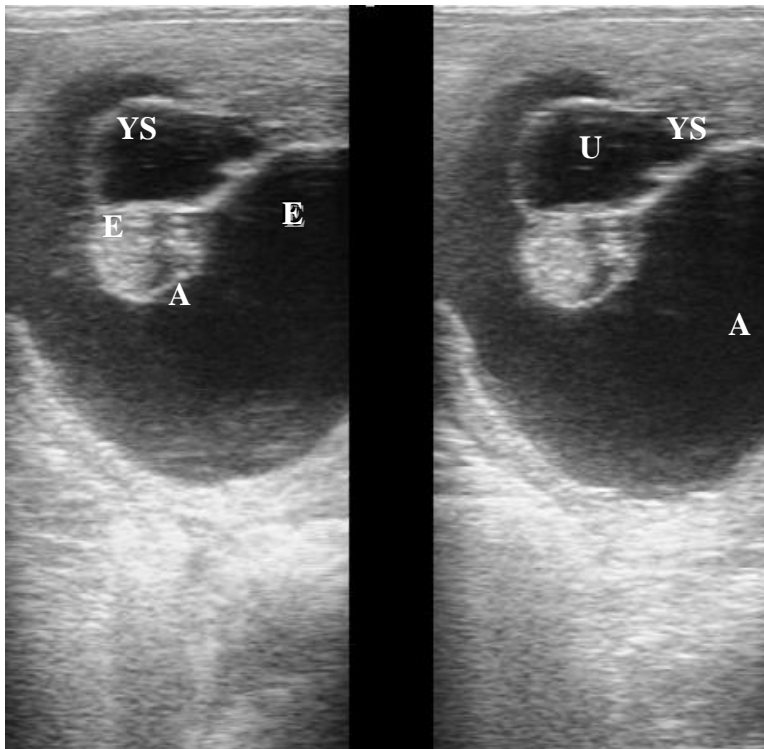


Figure 5. 5 Ultrasound pregnancy scan image of day 38 embryo: Here the allantois volume exceeds that of the yolk sac and the umbilical cord can be seen (U).

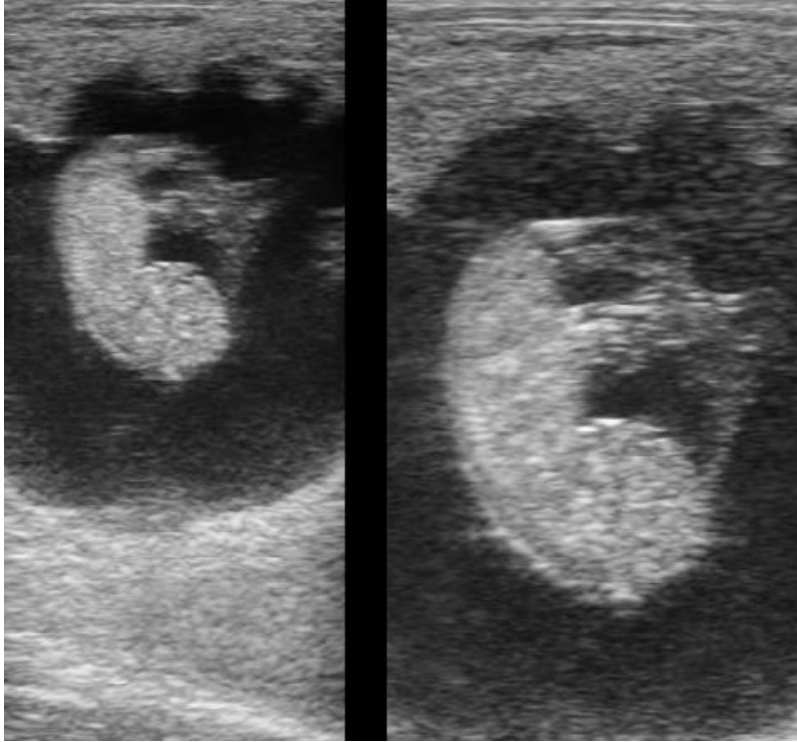


Figure 5. 6 Ultrasound pregnancy scan image of day 45 embryo: embryo shows form.

5.4 DISCUSSION

This experiment evaluated the fertility of frozen-thawed epididymal sperm, which had been frozen in a Botu Crio[®] base diluent and either 2.5% glycerol or 5% glycerol:formamide combination (Botu Crio[®]), to determine the best means to freeze epididymal sperm acquired from the cauda epididymis after routine castration of colts. The effects of the cryoprotectant type on epididymal sperm had a significant effect on conception rate, with the formamide combination resulting in a higher proportion (50%) of pregnancies than epididymal sperm frozen in glycerol alone (10%). Furthermore, these satisfactory conception rates were obtained after a single fixed time insemination using a deep-uterine insemination method, which makes the technique very accessible.

The ability to produce pregnancies is the gold standard for fertility assessment. *In vitro* semen analysis procedures have been used for more than half a century but unfortunately none of the assays developed provide results which accurately predict fertility in stallions, due to the variety of factors which affect results such as: the management of females, age of females and skill of the inseminator (Amann 2005). Furthermore, sperm must have a number of attributes, each above a certain threshold. Therefore, when assessing each sperm attribute it may be possible to predict which samples may have a poor fertilizing capacity, but it is impossible to determine if a particular sample will be fertile. Indeed, the motility of raw epididymal can be very low and its viability difficult to assess using routine microscopic evaluation. For this reason although the numbers of mares in this study were small, the fertility trial was important to clarify factors such as cryoprotectant type which affect the fertility of frozen-thawed epididymal sperm.

5.4.1 Cryoprotectant Type

It has been shown that sperm obtained from the stallion epididymis have progressive motility similar to ejaculated sperm after dilution and incubation. However, after freezing the fertility is much lower when compared to ejaculated sperm. Research on the effects of different cryoprotectants on ejaculated sperm shed some light on the potential beneficial effects of formamides on epididymal sperm.

Since Baker and Gandier produced a foal after insemination with epididymal sperm frozen with glycerol in 1957 no further pregnancies were obtained until Morris et al (2002) observed that by diluting the cryoprotectants by washing the sperm after thawing, contributed to improved pregnancy rates with epididymal sperm frozen in glycerol (5%). Following this study, Papa et al (2008) experimented with alternative freezing extenders for epididymal sperm. They compared EDTA-Lactose, INRA-82 and Botu Crio[®] and concluded that Botu Crio[®] extender maintained motility (71.4% total motility) and fertility (66.6%) of epididymal sperm better than others. These studies suggest that it is the base diluent which alters the fertilizing ability of epididymal sperm, however the present study suggests otherwise as the base diluent was kept the same.

In agreement with the present study, experiments by Mederios et al. (2002) have demonstrated that amides protect stallion ejaculated sperm from cryodamage better than glycerol and the combination of dimethylformamide with glycerol is superior to glycerol alone. Further studies on ejaculated sperm by Samper & Garcia (2008), found that in their group categorized as 'good freezers' there was no difference when Botu Crio[®] extender was compared with other extenders. The fertility however of ejaculated sperm frozen with Botu Crio[®] was significantly better ($p < 0.01$) than that of ejaculated sperm frozen with glycerol for the 'poor freezing' group. This enhanced fertility may be due to the fact that the freezing extender Botu Crio[®] has a low concentration of glycerol and uses methylformamide as the principle cryoprotectant (Melo et al. 2007). Glycerol sensitivity, observed in 'poor freezing' stallions, has been said to be able to be remedied by combining glycerol with amides (Gomes et al. 2002). These results suggest that the ejaculated sperm from bad freezers and the sperm from the epididymis may have a similar response to freezing and the effects of glycerol toxicity.

5.4.2 Concentration

Pregnancy rates are optimised in mares and sperm survival rates *in vitro* are best when equine sperm are chilled at a concentration of 25×10^6 /ml (Varner et al. 1987). For frozen ejaculated sperm, the recommended minimum dose is 250×10^6 motile sperm. However, as previously discussed, satisfactory pregnancy rates

are possible with 50×10^6 ejaculated sperm contained within a 0.5 ml insemination volume. In the current study sperm were frozen at a concentration of 600×10^6 /ml to ensure that a total of 300×10^6 epididymal sperm were available for insemination when one 0.5 ml straw was used. Katila et al. (2000) established that most of the ejaculate is effectively and quickly evacuated from the uterus during oestrus through the very relaxed cervix. Therefore, only a small fraction of the ejaculate remains on the uterine side of the papillae of each UTJ (Scott et al. 2000). So perhaps in future, a further reduction of epididymal sperm number per dose would be possible for some stallions to increase the total doses available without compromising fertility.

5.4.3 Methods of Insemination

The advantages of reducing the distance the sperm have to travel in the uterus by depositing them closer to the oviduct are well documented. Methods which allow this include hysteroscopic insemination (Manning et al. 1998; Vazquez et al. 1998; Lindsey 2000; Morris et al. 2000b) and deep intra-uterine insemination (Buchanan et al. 2000; Woods et al. 2000; Brinsko et al. 2003; Wongtawan et al. 2006). Other methods of insemination include gamete intrafallopian transfer (McCue et al., 2000) and ultrasound guided deep uterine insemination (Buchanan et al. 2000). Hysteroscopic insemination has been proven to enhance the fertility of frozen-thawed epididymal sperm by 10% when inseminating 200×10^6 sperm (Morris et al. 2002a). The present study confirmed that the less laborious deep intrauterine insemination method produced good pregnancy results with epididymal sperm frozen in a glycerol/ formamide combination. Furthermore this method is cost effective, safe and an effective technique. It is not harmful to the uterine luminal environment and does not increase the incidence of intrauterine fluid accumulation (Martin & Danzinger 2008). Therefore, although it may have been of interest to compare these insemination techniques in this study, it can be concluded that deep-horn insemination was a successful method of choice.

5.4.4 The possibility of using Seminal Plasma and Tyrodes medium

It is known that large volumes of seminal plasma in cooled-stored semen is detrimental to motility and fertility (Jasko et al. 1992b). However, in the case of most extenders the complete removal of seminal plasma does not augment sperm longevity, and it has been proposed that 5- 20 % seminal plasma should be

retained after centrifugation (Jasko et al. 1992b; Loomis 2006). Nevertheless other possible injurious effects on membrane integrity and motility have been illustrated (Akçay et al. 2006). In support of this Morris et al. (2002a) found that exposure of fresh or frozen-thawed epididymal sperm to seminal plasma did not significantly improve fertility after hysteroscopically insemination (Morris et al. 2002c). However when the insemination dose was reduced and the epididymal sperm were washed with Tyrode's medium prior to hysteroscopic insemination, pregnancy rates significantly increased from zero in the absence of the washing step to 29% when sperm were washed with Tyrode's. No published studies have reported if exposure to Tyrode's media exposure significantly enhances the fertility of epididymal sperm after deep-intrauterine insemination and this warrants future research in conjunction with the Botu Crio ® semen freezing and insemination described in the present study.

5.4.5 Shipping

The results for this study support the results reported by Papa et al., (2008) who used the same procedures to ship testicles indicating that sperm obtained from stallion epididymis can be shipped at 5°C for 24 hours post-castration before cryopreservation, maintaining sperm viability and fertility.

5.4.6 Future Advances

Although pregnancy itself is confirmed to be the best means to measure fertility, our ability to determine 'fertility' accurately is dependent upon having adequate numbers of inseminations from a particular sperm sample (Amann 2005). Since fertility is a binomial variable a fairly large number of females must be inseminated in order to achieve accurate pregnancy data (Graham & Mocé 2005). As stated by Graham and Mocé (2005) if only 10 mares are inseminated, the variation in the fertility data will be roughly $\pm 25\%$. In order to calculate a true fertility percentage, with variation of approximately $\pm 10\%$, near 75 females per treatment group must be inseminated, and to lessen the variation to $\pm 7\%$ at least 100 females must be inseminated. Few studies are conducted that inseminate an adequate number of females to produce dependable fertility data, and this also poses difficulties when trying to correlate semen analysis procedures with fertility data. In this study only 10 mares were used in each treatment group due to time

and financial limitations and idealistically more would yield results with greater statistical significance and credibility.

Vidament et al., (2009) found that in Jennies the replacement of glycerol by 2% DMF increased fertility with cooled donkey semen, but not with frozen-thawed donkey semen. Squires et al. (2004) found that DMF protected stallion sperm from cryodamage as effectively as glycerol did. Like the Botu Crio[®] glycerol/formamide combination cryoprotectant, these compounds permeate the plasma membrane more effectively than glycerol does, thus they should cause less osmotic damage to stallion sperm than glycerol does and thus an insemination trial using epididymal sperm frozen with DMF alone would be of interest.

Control samples of ejaculated semen frozen the same way and inseminated into the same number of mares would also benefit this research. Preferably if the semen came from the same stallions of known fertility prior to castration this would reduce the potential bias of stallion variation. Nevertheless it can be concluded that with the freezing and insemination method described good pregnancy rates are possible under good veterinary management.

CHAPTER 6

OVERALL CONCLUSIONS

The primary aim of the current study was to examine the effects on epididymal sperm motility, morphology, acrosome status, plasma membrane integrity, DNA structure, chromatin structure and fertilising ability, after the exposure to different cryoprotectants, freezing and thawing. The results of this study reflect that the cryoprotectant type does not significantly alter stallion sperm functional integrity as tested. However, the type of cryoprotectant used did appear to have an effect on morphology, altering the occurrence of distal droplets and more importantly the fertility rate. Therefore, which cryoprotectant was used had very little effect at the extracellular level or on the intracellular DNA and mitochondria but it did affect fertility, with epididymal sperm frozen in a glycerol and formamide combination having significantly higher pregnancy rates than epididymal sperm frozen in glycerol alone. Because the base diluent was the same for all treatment groups the results of this study suggest that it is the cryoprotectant and not the components of the base diluent which affects fertility. Unfortunately no *in-vitro* tests appeared to correlate with the fertility rates found in this study. Overall these results do, however, provide a basis upon which further detailed studies can be built. These results are encouraging as after freeze-thawing, normal motile sperm were able to capacitate, undergo the acrosome reaction and those frozen in the combination cryoprotectant could penetrate oocytes *in vivo*.

Comparisons *in vitro* of epididymal sperm and ejaculated sperm illustrated that ejaculated sperm displayed greater total motility immediately after thawing and at 30 minutes after thawing. Furthermore ejaculated sperm displayed significantly less intact plasma membranes after thawing compared to epididymal sperm (37% and 61% respectively). This suggests that perhaps the later combination with seminal plasma and the process of ejaculation has harmful effects on the plasma membrane integrity of spermatozoa, yet this has known stimulating effects on motility. It may also be that the morphological and functional differences which

exist between epididymal sperm and ejaculated sperm influence their membrane stability, cold shock susceptibility and the resistance to osmotic stress.

The results of this study also illustrate that if epididymal sperm was frozen with glycerol as the cryoprotectant, then there was a beneficial effect on motility of adding SW3 medium (JEL Media, NZ) to the thawed epididymal sperm. However results illustrate good pregnancy rates without the addition of SW3 medium containing complex amino acids and sugars, thus supporting the conclusion that the cryoprotectant is the primary factor altering the fertilizing ability of epididymal sperm. Indeed, simple dilution of epididymal sperm with SW3 medium after thawing may improve the fertility of epididymal sperm by reducing the effects of glycerol toxicity.

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APPENDIX 1: MEDIA AND STAINS

MEDIA FOR CRYOPRESERVATION

Kenney Extender (Kenney et al., 1975)

Ingredient	Amount
Glucose	49 g
Non fat dried skim milk powder	24 g
Streptomycin Sulphate	1 g
Penicillin G	0.8 g
Sterile water	Up to 1000 ml

Botu Crio® Semen Extender

Biotech-Botucatu, Brazil

MEDIA AND STAINS FOR VISUAL SPERM ASSESSMENT

Eosin-Nigrosin Stain (Barth & Oko, 1989)

Ingredient	Amount
Eosin (water soluble)	1.67 g
Nigrosin (water soluble)	10 g
Sodium Citrate	2.9 g
Pure Water	Up to 100 ml

Filter prior to use

FITC-PNA Stain (Cheng et al., 1996)

Ingredient	Amount
Peanut agglutinin FITC conjugated	1000 µg
PBS (NaCl - 8 g, KCl - 0.2 g, KH ₂ PO ₄ - 0.2 g, Na ₂ HPO ₄ - 1.15 g)	10 ml

UCD Mounting Media (Weston, 2005)

Ingredient	Amount
Sodium Azide	0.005 g
p-Phenylenediamine	0.005 g
PBS	0.5 ml
Glycerol	4.5 ml

Adjust pH to 8.0

MEDIA FOR EMBRYO TRANSFER AND FREEZING

Media	Source and quantity
Vigro™ complete flush media	Containing surfactant and antibiotic; Bioniche, Animal Health, USA -1000 ml
Vigro™ holding medium	Containing surfactant and antibiotic; Bioniche, Animal Health, USA – 1 ml of each
S1, S2, S3 and dilution solution	ICPbio LTD, Auckland, NZ

MEDIA AND STAINS FOR DNA ANALYSIS

Comet assay

PBS-SDS (2%)

Ingredient	Amount
PBS	50 ml
SDS	1 g

PBS-SDS- Proteinase K solution

Ingredient	Amount
PBS-SDS (2%)	1 ml
Proteinase K	100 µl

Normal Melting Point (NMP) Agarose – 1%

Ingredient	Amount
PBS	60 ml
NMP agarose	0.6 g

Store at 60°C before use

Low Melting Point (LMP) Agarose – 0.5%

Ingredient	Amount
PBS	20 ml
LMP agarose	0.1 g

Store at 37°C before use

Lysis Buffer (200ml)

Ingredient	Amount
NaCl - 2.5M	29.22 g
EDTA – 100 mM	7.4 g
Tris – 10 mM	0.2422g
Pure water	to 200 ml
1% Triton X-100	Added just prior to use (see methods)

Adjust pH to 10

Heat in a microwave to dissolve

2 Merca-captoethanol (2ME) solution

Ingredient	Amount
Distilled Water	75ml
2 ME	5 µl

Make in the laminar flow cabinet as 2 ME inhalation can be dangerous

Lithium diiodosalicylate (LD) solution – 4 mM

Ingredient	Amount
Distilled Water	100ml
LD	0.158g

NaOH-EDTA Neutralization Buffer

Ingredient	Amount
NaOH – 300 mM	6 g
EDTA – 1 mM	0.1791 g
Distilled Water	to 500 mls

Adjust pH to above 13

Tris Buffer 0.4M

Ingredient	Amount
Tris	48 g
Distilled Water	1000 ml

Adjust pH to 7.5

Chromatin Integrity Test

PBS-SDS (2%)

Ingredient	Amount
PBS	50ml
SDS	1g

Carnoy's Solution

Ingredient	Amount
Methanol	50 ml
Acetic Acid	25 ml

Citric Acid/PO₄ Buffer to make Acridine Orange stain (200 ml)

Ingredient	Amount
Citric Acid	4.2g
Na ₂ H ₂ PO ₄	5.66g

Adjust pH to 2.5

Prior to use add AO 1% stain 4:1 (Buffer: stain) (amount depends on number of slides, with 400 µl required per slide)

Mounting Medium

Ingredient	Amount
NaHCO ₃	0.036g
Na ₂ CO ₃	0.008g
H ₂ O	5.0ml
Glycerol	45ml

MTT assay***MTT***

Ingredient	Amount
PBS	10 ml
MTT	0.5 g

Lysis Buffer

Ingredient	Amount
DMF	20mls
Demineralised water	20ml
SDS	10g
INHCl	1.0ml
HAc	1.0ml

Adjust ph to 4.7

APPENDIX 2: INSTRUMENTS, MATERIALS, CHEMICALS AND DRUGS

Table 1: Instruments, Materials, Chemicals and Drugs

<i>Item</i>	<i>Company</i>	<i>Specifications</i>
Instruments and Materials		
Beakers	BIOLAB NZ	250 ml, 500 ml
Rotating wheel	Bellco orbital	
Centrifuge	BIOLAB NZ	Centra CL2
Cover slips	BIOLAB NZ	22x22 mm
Disposable pipets	BIOLAB NZ	3 ml
Centrifuge	BIOLAB NZ	
Centrifuge tube/microcentrifuge tube (Falcon tube)	BIOLAB NZ	15 ml, 1.7 ml
Centrifuge tube (Falcon tube)	MINITUB	50 ml
Curvets for Spectrophotometer	BRAND GMBH, Germany	2.5ml
Dissection kit		
Electrophoresis unit	Owl tank and Power packs	
Fluorescent microscope	Leica DMRE	50 Watt mercury vapour lamp, 450-490nm block, blue light, dichroic mirror RKP510, Emission filter LP520
Freezing racks	MINITUB, Germany	
Flexible insemination pipette	MINITUB, Germany	
Hemocytometer	MINITUBE, Germany	
Immersion oil	BIOLAB NZ	Type A 4 oz
Liquid Nitrogen tanks	Gasses NZ	
Olympus microscope	Olympus, NZ	BX41
PVC powder	MINITUB, Germany	2.70 mm, glass
MINIFLUSH™ filter	Minitube of America, Inc – Verona. WI	
Microsheild 4 Pipettes	Johnson&Johnson™ BIOLAB NZ	5L Eppendorf 2-20 µl and 200-1000 µl
Pipette Tips	BIOLAB NZ	2-100µl and 50-1000µl
Tupperware® container	Tupperware®	
Water jet pump	BIOLAB NZ	
Semen straws	MINITUB, Germany	0.5cc
Slides	BIOLAB, NZ	Clear and frosted
Straws		0.25, 0.5 ml
Syringe	BIOLAB NZ	20 ml
Test tubes	BIOLAB NZ	Glass, 5 ml

Zeiss fluorescent light microscope	Carl Zeiss, Inc	Anixostar, 240 volt
21 G and 18 G needles	Percision Glide™, Pasig	
33G Foley Type catheter	MINITUB, Germany	

Chemicals

Liquid Nitrogen	Gases, NZ	
Hydrogen peroxide	Sigma	
Botu crio	Criovital, Botucatu, Brazil	Biotech-
PBS	GIBCO™	Filtered, (0.1 micron) filtered, pH 7.4
Ethanol	BIOLAB	Absolute, analytical grade
Sterile water	Rukura NZ	Filtered, 1L bottles
Formamides	Sigma	
Sucrose	Sigma	100 mosmol/l

Drugs

hCG - Chorulon	Intervet, ScheringPlough Animal Health	1500µl injection IV
BioRelease Deslorelin	Caledonian Holdings LTD NZ	1.25 mg/ml

APPENDIX 3: CALCULATIONS

DILUTING SAMPLE TO DESIRED CONCENTRATION

(Equation 1)

$$\text{Dilution Factor (DF)} = \frac{\text{Concentration of spermatozoa sample}}{\text{Desired Concentration (DC)}}$$

(Equation 2)

$$\text{Final Volume (FV)} = \text{DF} \times (\text{Working Volume}) \text{ WV}$$

(Equation 3)

$$\text{Amount of cryoprotectant (ml) (Gly/DMF/BC)} = \text{FV (final volume)} \times \text{percentage of cryoprotectant (i.e. 2.5\%)}$$

(Equation 4)

$$\text{Volume of Extender needed (ml)} = \text{FV-WV (working volume)} - \text{amount of cryoprotectant}$$

(Equation 5)

$$\% \text{ Total Motility} = \frac{\text{Estimated number of motile spermatozoa}}{\text{Estimated of total number of spermatozoa}} \times 100$$

DETERMINING MOTILITY PARAMETERS

(Equation 6)

$$\% \text{ Progressive Motility} = \frac{\text{Estimate of forward moving spermatozoa}}{\text{Estimate number of motile spermatozoa}} \times 10$$

(Equation 7)

$$\% \text{ Total Progressive Motility} = \frac{\text{Estimated forward moving spermatozoa}}{\text{Estimated total number of sperm}}$$

APPENDIX 4: TABLES

Refer to following pages in landscape view

Table 1 TPM (Total Progressive Motility) at 0 and 30 minutes post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	Glycerol 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Ejaculated
0 minutes					
Untreated post-thaw	4.18 \pm 3.60	8.69 \pm 5.70	8.30 \pm 2.50	9.31 \pm 3.92	28.5 \pm 1.67
SW3 treated post-thaw	22.4 \pm 0.80	20.4 \pm 4.22	19.4 \pm 3.13	25.6 \pm 2.5	21.9 \pm 1.59
<i>Difference between means</i>	18.22	11.71	1.11	16.29	-6.60
30 minutes					
Untreated post-thaw	4.43 \pm 3.46	2.94 \pm 2.97	6 \pm 3.49	3.5 \pm 4.02	14 \pm 4.17
SW3 treated post-thaw	19.74 \pm 1.93	15.6 \pm 2.49	10.5 \pm 4.14	27.5 \pm 0.69	19 \pm 1.17
<i>Difference Between means</i>	15.31	12.66	4.50	24.00	5.0

Table 2 The mean percentage of intact acrosomes, post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Control
Post-thaw					
Untreated post-thaw	66.20 \pm 12.26	80.2 \pm 6.72	78.25 \pm 10.14	80.6 \pm 7.87	72.50 \pm 19.44
SW3 treated post-thaw	66.20 \pm 13.35	67.40 \pm 18.62	78.50 \pm 10.14	77.33 \pm 7.09	50 \pm 24.06
Difference Between means	0.00	-12.80	0.25	-3.27	-22.50

Table 3. The mean percentage of patchy acrosomes, post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Ejaculated
Post-thaw					
Untreated post-thaw	24 \pm 14.44	11.6 \pm 4.88	14.25 \pm 9.14	15.50 \pm 9.18	21.17 \pm 18.90
SW3 treated post-thaw	23.40 \pm 13.33	24 \pm 14.71	13.25 \pm 9.14	12.33 \pm 9.71	32.75 \pm 19.98
Difference Between means	-0.60	12.40	-1	-3.17	11.58

Table 4 The mean percentage of &75% reacted acrosomes, post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Ejaculated
Post-thaw					
Untreated post-thaw					
	4.4 \pm 1.44	2.80 \pm 2.68	4.25 \pm 0.96	1.75 \pm 2.06	2.33 \pm 2.16
SW3 treated post-thaw					
	6.80 \pm 3.19	4.60 \pm 3.05	4.25 \pm 0.96	5.67 \pm 4.04	7.75 \pm 5.56
Difference Between means	2.40	1.80	0.00	3.92	5.42

Table 5 the mean percentage of reacted acrosomes, post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Ejaculated
Post-thaw					
Untreated post-thaw					
	4.8 \pm 4.15	3.8 \pm 1.92	2.5 \pm 1.29	2.25 \pm 2.63	3.33 \pm 1.97
SW3 treated post-thaw					
	2.60 \pm 0.89	3.40 \pm 2.07	3.50 \pm 1.29	1.67 \pm 1.15	8.00 \pm 2.94
Difference Between means	-2.20	-0.4	1	-0.58	4.67

Table 6 The mean percentage of equatorial segments, post-thaw with and without the treatment of SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	Glycerol/ Formamide combination 5%	Ejaculated
Post-thaw					
Untreated post-thaw	0.60 \pm 1.34	1.6 \pm 1.67	1.0 \pm 1.41	0.50 \pm 1.00	0.83 \pm 0.75
SW3 treated post-thaw	1.20 \pm 1.64	0.60 \pm 0.55	1.0 \pm 1.41	0.3 \pm 0.3	1.75 \pm 1.71
Difference Between means	0.6	-1.00	0.00	2.50	0.92

Table 7 Percentage of coiled tail sperm in HOS analysis post-thaw with and without the treatment with SW3 medium. The values (%) are the means \pm s.d. of the sperm samples. Differences between the means = SW3 treated post-thaw - Untreated post-thaw (%).

Treatment Group	GLY 2.5%	DMF 2.5%	DMF 5%	BC 5%	Control
Post-thaw					
Untreated post-thaw					
	71.25 \pm 5.12	63.50 \pm 13.03	76.67 \pm 1.53	61.00 \pm 22.65	36.75 \pm 6.6
SW3 treated post-thaw					
	60.33 \pm 6.43	65.00 \pm 18.68	72.67 \pm 10.02	63.67 \pm 9.29	35.25 \pm 8.18
Difference Between means	-10.92	1.5	-4	2	-1.5