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The effects of iodixanol present during equine semen cryopreservation

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**THE EFFECTS OF IODIXANOL PRESENT DURING EQUINE SEMEN
CRYOPRESERVATION**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the
Department of Veterinary Clinical Sciences

by

David Paul Beehan

M.V.B., University College Dublin, 2006

December 2012

DEDICATION

The author would like to dedicate this thesis to his family, in recognition of all their support and patience as I pursue my studies a long way from home.

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ABSTRACT

The objectives of this study were to determine what effects iodixanol would have on total and progressive motility, plasma membrane integrity (viability), acrosome integrity and DNA structure when present during cryopreservation of equine spermatozoa. We hypothesized that the addition of iodixanol would improve post-thaw values for measured parameters. Ejaculates from six stallions were collected, centrifuged at 900 x g for ten minutes to remove supernatant, and suspended to 200×10^6 cells/ml with 0%, 2.5% and 5% iodixanol in an egg-yolk based extender and cryopreserved. Before and after cryopreservation sperm motility was assessed by computer assisted semen analysis, and samples were stained with SYBR-14/propidium iodide (PI) for viability, with PI/fluorescent isothiocyanate-PNA (*Arachis Hypogaea*) for acrosome integrity and assessed by flow cytometry. Sperm DNA was evaluated using the sperm chromatin structure assay test and assessed by flow cytometry. The mean (\pm S.E.) percentage pre- and post-thaw total motility, progressive motility, viability, acrosome reactivity, $COMP_{at}$ and $MEAN_{at}$ were analyzed using Shapiro-Wilk test to evaluate if data followed a normal distribution. When results followed a normal distribution an ANOVA was performed and where a significant interaction of treatment was observed ($p=0.05$), a Tukey-Kramer post-hoc test was applied. If results did not follow a normal distribution, a binomial logistical regression was performed. The 5% post-thaw treatment group showed increased viability and decreased DNA damage ($p<0.001$). Although the 0% group showed greater total and progressive motility than the 2.5% group, it was not greater than the 5% group. The 5% post-thaw treatment group had significantly more ($p<0.001$) non-reacted and damaged acrosomes than both the 0% and 2.5% groups. These findings

suggest that the presence of iodixanol during cryopreservation may have a beneficial effect by protecting plasma membrane and sperm DNA, but the exact mechanism of action is unknown.

CHAPTER 1

INTRODUCTION

The goal of spermatozoal cryopreservation is the ability to preserve sperm with no negative effects of processing; and the sperm would perform, when thawed, just as well as it would have if it was never frozen. The first reported pregnancy using cryopreserved stallion epididymal spermatozoa was in 1957, using epididymal sperm, used whole milk with 10% glycerol as a freezing extender (Barker & Gandier, 1957). In this study, seven mares were artificially inseminated and one subsequently foaled: a 14.29% pregnancy rate. Since then, the expected pregnancy rate of frozen semen now ranges from 38% to 73% (Metcalf, 1995, Nielsen et al., 2008), with a mean of 40% to 45% (Vidament, 2005). This is not a large improvement for such a long time span, when compared to equine oocyte collection and transfer, which had its first reported pregnancy to term in 1988 by McKinnon (1/15, 7% foaling rate). By 1995 a pregnancy to term rate of up to 95% has been reported (Carnevale and Ginther, 1998).

This slow advancement in the technique of spermatozoal cryopreservation is surprising, since breeding with frozen semen is now a routine insemination technique practiced worldwide. This may be due to underfunding, as many breed registries prohibit its use, and also to the low pregnancy rates obtained. However by 1985, up to 32,000 mares were bred in three provinces in China, with a pregnancy rate of 68% (Amann & Pickett, 1987). In recent years, large breed registries, particularly the American Quarter, American Paint Horse and the Arabian, have allowed the use of frozen semen based on its advantages including not requiring the shipping of mares to breeding farms; the coordination of chilled semen request in anticipation

of ovulation; the long-term storage of semen of competing, injured or dead stallions; and the ability to transport semen internationally.

In stallions, sperm motion characteristics are lowered after storage with high concentrations of seminal plasma, and the lowering of seminal plasma concentrations is beneficial to motility (Jasko et al., 1991). An important step in cryopreservation is reducing seminal plasma concentration, and is performed by the centrifugation of spermatozoa and the subsequent removal of the seminal plasma supernatant. Industry standard centrifugation forces are typically 400-600 x g. A sperm cushion can be added to allow increased centrifugation forces and to obtain increased spermatozoal recovery rates. Reported recovery rates with an iodixanol based cushion medium ranged from 81% to 107% (Ecot et al., 2005) after centrifugation for 20 minutes at 1000 x g. The complete recovery of the entire iodixanol cushion is a difficult procedure; the cushion media must be removed without disrupting or losing any of the soft pellet. As a result, erring on the side of caution, the cushion is frequently incompletely removed and iodixanol residues are left remaining with the pellet. Sargusty et al., (2009) examined what the effects of these residues could be if iodixanol was added to a bovine freezing extender (Andromed®). The presence of 1.25%, 2.5% and 5% iodixanol (Optiprep™, v/v) during freezing was associated with improved semen longevity and acrosomal viability. To date no similar study has been performed with equine spermatozoa.

The objective of this study was to evaluate the effect of 0%, 2.5% and 5% iodixanol (Optiprep™ (v/v)), in an egg yolk and glycerol based freezing extender (EZ Freezin LE, Animal Reproduction Services, Chino, CA), on equine spermatozoal total and progressive motility, viability, acrosomal integrity and chromatin integrity.

CHAPTER 2 LITERATURE REVIEW

2.1. Stallion Sperm Structure

The equine spermatozoon can be divided into a head, neck, middle piece, principal piece and end piece, and is completely surrounded by a continuous and fluid plasma membrane. The spermatozoal head contains the nucleus, acrosome and cytoplasm. The nucleus occupies the majority of the spermatozoal head space, and contains highly condensed chromatin. This tight condensation of nuclear material is due to establishment of intra- and inter-molecular disulfide linkages that result in compaction and stabilization of the DNA (Varner and Johnson, 2007). These bonds are formed during passage of the spermatozoa through the epididymis, and are believed to occur as the compacted state offers protection during spermatozoal movement prior to fertilization. The nucleus is surrounded by a nuclear envelope, which is a double membrane separating the nucleus from the surrounding cytoplasm.

The acrosome is an exocytotic organelle, covering the rostral two-thirds of the nucleus. It is a membrane bound structure, with connected inner and outer acrosomal membranes, enclosing an acrosomal matrix composed of hydrolytic enzymes (hyaluronidase, proacrosin/acrosin and lipases) (Varner and Johnson, 2007). During the acrosome reaction, the outer acrosomal membrane and the spermatozoal plasma membrane fuse, resulting in exocytotic release of the enzymes and exposure of the binding receptors. The remainder of the spermatozoal head is composed of cytoplasm, containing few organelles and mostly contains cytoskeletal proteins that are important for spermatozoal form and function (e.g. anchoring the acrosome to the underlying nuclear envelope; Varner and Johnson, 2007).

The neck and tail (composed of the middle piece, principal piece and end piece) comprise the rest of the sperm. The neck is the connecting piece between the head and the middle piece and consists of the capitulum, segmented columns and the proximal and distal centrioles (Varner and Johnson, 2007). The proximal centriole remains present in the mature spermatozoa and is the site where the tail beat is initiated. The distal centriole develops into the axoneme during spermatozoal tail development. The middle piece contains numerous mitochondria overlying the dense fibers and the axoneme. Mitochondria are helicoidally arranged in a continuous double spiral, and produce ATP required for flagellar movement (Varner and Johnson, 2007).

The principal piece is composed of the axoneme, which is continued from the middle piece and terminates in the caudal principal piece, and the fibrous sheath. The axoneme is a cylindrical array of nine doublet microtubules that surround two singlet microtubules (termed the central pair) connected by regularly spaced bridges, thus forming the characteristic '9+2' configuration. The outer fibrous sheath provides structural support and flexibility to the flagellum (Varner and Johnson, 2007).

The equine spermatozoal plasma membrane is the outer most component and completely encompasses the entire cell and is composed of lipids and proteins (Flesch and Gadalla, 2000), and how spermatozoa respond to a decrease in environmental temperature is a direct measure of how the lipid and protein components of the membrane alter in response to decreasing temperatures. As temperature decreases, sperm membrane destabilization occurs when the membrane undergoes a phase transition from the fluid phase to the gel phase. Due to the loss of most cell organelles and DNA transcription ability during spermatogenesis,

spermatozoa lack vesicular transport. As a result the plasma membrane is a stable and inert structure, and plasma membrane components (protein, phospholipids and cholesterol) cannot be synthesized de novo (Flesch and Gadella, 2000). The plasma membrane is composed of three zones: lipid bilayer, phospholipid-water interface, and glycocalyx (Amann and Graham, 2010).

The lipid bilayer is composed of polar phospholipids, with hydrophobic fatty acyl chains directed internally and the hydrophilic, charged polar head groups directed externally. The plasma membrane lipids mostly consist of phospholipids and cholesterol; other lipids present include neutral lipids and glycolipids; however, phospholipids comprise up to 70% of all lipids present (Flesch and Gadella, 2000). Also present in the plasma membrane are proteins that are classified as either integral or peripheral proteins. Proteins account for up to 50% of the plasma membrane weight (Amann and Pickett, 1987). The integral proteins serve as pores or channels through the membranes or as receptors for ligands. The peripheral proteins are attached to the plasma membrane by electrostatic forces. Many of the integral and peripheral proteins contain carbohydrate side chains and extend some distance from the lipid bilayer (Amann and Pickett, 1987). The carbohydrate side chains have a net negative charge and attract and loosely bind other proteins or glycoproteins, resulting in the outer surface of the plasma membrane having a transient covering of material that is dependent on its surrounding environment. The cholesterol to phospholipid molar ratio in the plasma membrane of stallion spermatozoa is 0.36, which is midway between the values for boar (0.26) and bull (0.45) spermatozoa (Parks and Lynch, 1992). The plasma membrane is in a fluid state at body temperature, allowing phospholipids to move freely, resulting in differences in phospholipid distribution throughout

the membrane. The ratio of cholesterol to phospholipids with polyunsaturated acyl side chains will determine the fluidity of the membrane, with higher levels of cholesterol associated with better membrane decreased fluidity but better stability during cooling (Amann and Pickett, 1987).

2.2. Stallion Sperm Cryopreservation

It is estimated that 20% of stallions produce semen that freezes well, 60% freeze acceptably and 20% freeze badly (Vidament et al., 1997). When compared to the bull, stallions are considered poor freezers, and the reason for this is not completely understood. Bulls have been selected by the AI industry for many years based on the ability of their spermatozoa to withstand the stress of standard cryopreservation protocols; no such selection process exists for stallions (Loomis & Graham, 2008). Some reasons postulated to explain the poor freezability of equine spermatozoa include the unique cell size and shape of equine spermatozoa, the osmotic tolerance of sperm, the chemical composition of sperm, and the selection of stallions based on athletic traits and not reproductive parameters (Amann & Pickett 1987, Squires 2005, Loomis & Graham 2008).

There is no single standardized equine semen cryopreservation protocol. Samper and Morris (1998) surveyed 21 world-wide laboratories that were involved in the freezing of semen for commercial purposes. Their survey included many questions covering all aspects of cryopreservation, including; the number of ejaculates collected to deplete extra-gonadal reserves prior to the first freeze; the number of test-freeze extenders used; dilution ratio and centrifugation protocol after collection; type of freezing extender used; type of antibiotics used; equilibration of semen before cryopreservation; freezing concentration; freezing and thawing

procedure; post-thaw evaluation criteria; insemination recommendations; acceptable first cycle pregnancy rate; and what the laboratory thought was the single most important factor when freezing stallion spermatozoa. Each of the questions had more than one answer, demonstrating that none of the researchers agreed on any single parameter. It has been suggested a standard protocol should consist of two-step dilution procedure, in which the gel-free ejaculate is either diluted 1:1 or to 50×10^6 spermatozoa/ml with a centrifugation extender, extended semen centrifuged for 10-15 minutes at 400-600g, the supernatant (consisting of the centrifugation extender and seminal plasma) removed, re-extension of the spermatozoal pellet with freezing extender to the desired freezing concentration, packaging into 0.5-ml or 5-ml straws, cooling from room temperature to $+5^\circ\text{C}$, freezing in either liquid nitrogen vapor or in an automated freezing chamber, and storage in liquid nitrogen (Sieme 2010).

Similarly, there is no single standard equine freezing extender. Samper and Morris (1998) reported four different freezing extenders that were being used by the surveyed laboratories. Despite the difference in extenders being used, all laboratories combined a source of energy (sugars), a source of lipoproteins to facilitate protection against rapid cooling (e.g. milk, egg yolk, or a combination of both), antibiotics to inhibit bacterial growth, buffers to balance pH, and a cryoprotectant. Cryoprotectants can be divided into permeable and impermeable categories (Sieme et al., 2008). The permeable agents act as both a solvent and solute and their presence will result in a higher concentration of water remaining unfrozen at lower temperatures, which protects the cells by minimizing the time of exposure to critical salt concentrations (Amann and Pickett, 1987, Sieme et al., 2008). Permeable cryoprotectants

include dimethyl sulfoxide, amides (methyl formamide and dimethyl formamide) and alcohols (ethylene glycol and glycerol). Impermeable agents act solely as a solute (Loomis and Graham, 2008). Their mode of action includes protecting cells against rapid cooling, stabilizing the plasma membrane, and by raising the percentage of unfrozen water at a given temperature (Amann and Pickett, 1987, Sieme et al., 2008). Impermeable protectants include sugars (lactose, mannose, raffinose), amino acids (glutamine and proline), proteins and lipids.

Glycerol is the most widely used permeating cryoprotectant used for the cryopreservation of equine spermatozoa, and recommended concentrations are 2.5% to 3.5%, although concentrations up to 10% glycerol have been reported (Samper and Morris, 1998). Glycerol can exert both osmotic and chemical toxic effects. Spermatozoa are subjected to a transient osmotic stress, as glycerol and extender ingredients equilibrates across the plasma membrane, resulting in temporary spermatozoal dehydration and reduced cell volume. The chemical toxic effects of glycerol exposure include protein denaturation, alteration of actin interactions and induction of protein free membrane blisters (Alvaranga et al., 2005).

Alternatives to glycerol that possess a shorter period of equilibration have been investigated (Squires et al., 2004). In this study a 0.55 M glycerol was compared to 0.3, 0.6 and 0.9 M methyl formamide and dimethyl formamide, smaller amide molecules, and it was demonstrated that 0.6 and 0.9 M preparations of both methyl formamide and dimethyl formamide had a post-thaw total motility statistically similar to the glycerol treatment ($P < 0.05$). The results of this study suggest that these amides are acceptable alternatives to glycerol.

Lipid based cryoprotectants, sourced from egg-yolk and milk, are a common component of freezing extenders, although the exact mechanism of cryoprotective action of lipids during

cryopreservation is unclear; it is most likely caused by the low density lipoproteins (LDL) they contain; proposed mechanisms of action include reversible binding to extracellular lipids in stallion seminal plasma and fusion to the spermatozoal membrane (Ricker et al., 2006). In the bull, seminal plasma proteins can cause cholesterol and phospholipid efflux from the spermatozoal plasma membrane and the addition of low density lipoproteins resulted in a phospholipid and cholesterol gain by the plasma membrane, which resulted in greater protection of spermatozoa during cooling and cryopreservation (Bergeron et al., 2004). An animal-free source of lipids is soybean lecithin. When used to freeze bull spermatozoa, a soybean lecithin-based extender was shown to have a significantly higher post-thaw motility and non-return rate when compared to an egg yolk-based freezing extender (Aires et al., 2003). A soybean lecithin-based extender has recently become commercially available for the stallion (Andromed-E™; Minitüb, Wisconsin).

2.3. Spermatozoal Response to Cooling

Rapid cooling of equine spermatozoa from 20 °C to 5 °C induces a partially irreversible damage, which is characterized by abnormal motility, loss of motility, damage to the plasma and acrosome membranes and reduced metabolism, and is aptly referred to as cold-shock (Amann and Pickett, 1987). Watson et al., (1987) demonstrated that cold shock damaged both the acrosomal and plasma membranes after rapidly lowering spermatozoal temperature from 30 °C to 0 to 4 °C (Table 2.1). Ideally, the cooling rate of animal spermatozoa should 0.5 to 1 °C/min (Barbas & Mascarenhas, 2009).

Table 2.1 Percentage of spermatozoa with damaged head membranes after various degrees of cold shock from 30 °C (Adapted from Watson et al., 1987).

Membrane categories	Cold-shock temperature				
	30 °C	16 °C	8 °C	4 °C	0 °C
Intact Plasma & Acrosome Membrane (%)	81	66	63	44	29
Damaged Plasma & Intact Acrosome Membrane (%)	9	10	12	23	41
Damaged Plasma & Acrosome Membrane (%)	11	16	20	25	42

When spermatozoa are cooled, the lipids undergo a phase transition from a liquid state into a gel or solid state, as cooling changes the shape of phospholipids. During membrane transition there is straightening and lengthening of fatty acyl chains which allow phospholipids to become tightly packed, and this process results in the aggregation or exclusion of proteins (Quinn, 1985). Each phospholipid undergoes this phase transition at a different temperature because there are many different acyl fatty chains present, with longer chains transitioning first at a higher temperature, which occurs at 19 °C for stallion spermatozoa (Hammerstedt et al., 1990). As the temperature drops further, more lipids undergo transition and further coalescing occurs. The range of temperature over which phospholipid transition occurs is 20 or 30 °C, and after the change to the gel or crystalline state phospholipids can no longer move in a random fashion (Amann and Pickett, 1987). Coalescence of membrane phospholipids is a change from the lamellar arrangement to a hexagonal-II form, which is a circular arrangement of phospholipids, with the hydrophilic head groups facing out. This change in arrangement is due to a change in shape of the fatty acyl chains due to cold exposure. Coalescence of membrane

lipids causes increased membrane permeability, as hydrophilic channels are created between the outer surface of the hexagonal-II forms and the surrounding phospholipid lamellar arrangement (Amann and Pickett, 1987). Finally, upon reaching the lowest transitioning temperature the entire membrane is in a gel state, and the membrane has lost its fluidity. The formation of the hexagonal-II form is reversible, with return to the normal bi-lamellar arrangement occurring upon warming; however this re-arrangement is an irreversible event for certain phospholipids leading to a loss of normal plasma membrane form and function (Quinn, 1985).

2.4. Spermatozoal Changes in Response to Cryopreservation

Cryopreservation has been reported to cause further changes in spermatozoal plasma membrane structure as a response to decreasing temperatures, and those changes are similar to changes seen at capacitation, including elevated intra-cellular calcium, in preparation for acrosomal exocytosis (Watson, 2000). During capacitation there is an efflux of cholesterol from the plasma membrane. A similar cholesterol efflux has been reported in spermatozoa of boars (Maldjian et al., 2005) and bucks (Chakrabarty et al., 2007) after cryopreservation, both studies showed a reduction in plasma membrane cholesterol content and a positive correlation between decreasing plasma membrane cholesterol levels and an increased proportion of acrosome-reacted spermatozoa present after thawing, the latter being measure using fluorescent lectin PNA and propidium iodide (Maldjian et al., 2005). The presence of intramembranous cholesterol has a protective effect, as its presence during decreasing environmental temperature will block phospholipid coalescence, thus maintaining membrane fluidity. The addition of 1.4 mg/ml of cholesterol-loaded cyclodextrins (CLC) to egg-yolk diluents

has resulted in higher percentages of motile and viable cells of bull semen (Purdy and Graham, 2004). In the stallion, the addition of 1.5 mg/ml to a freezing extender had a beneficial effect (Moore et al., 2005). In addition to the loss of cholesterol, both studies demonstrated a significant change from long chained polyunsaturated fatty acids to saturated fatty acids (Maldjian et al., 2005, Chakrabarty et al., 2007). This change may not be reversible, making the membrane more hydrophobic, which may be a protective mechanism for the cell. Chakrabarty et al., (2007) suggested that the presence of egg yolk and its close adherence to the plasma membrane may facilitate the uptake of exogenous saturated fatty acids by the spermatozoal plasma membrane.

The cryopreservation process can cause irreversible damage to spermatozoa. There are four main causes of spermatozoal damage that occur during the cryopreservation process: ice formation; reactive oxygen species; osmotic stress; and apoptotic-like changes.

2.4.1 Ice Crystal Formation

The “two factor hypothesis”, proposed by Mazur (1984) states that intracellular ice, formed when cells are cooled rapidly, kills cells by either chemical toxicity or osmotic stress, and by mechanical damage caused by the phase separation of solution and crystal growth. However, more recent spermatozoal-specific studies have demonstrated that intracellular ice formation is unlikely to occur at currently used cooling rates during cryopreservation, and that spermatozoal damage is not as a result of intracellular ice crystal formation (Morris, (2006); Morris et al., (2007)). Using cryo-scanning electron microscopy Morris et al., (2007) demonstrated no apparent ice crystal formation in stallion spermatozoa cooled at rates from 0.3 to 3000 °C/min in the presence of a glycerol-based freezing extender. Similar studies in men

(Morris, 2006) have demonstrated that intracellular ice formation is not present when spermatozoa are rapidly cryopreserved at rates of up to 3000 °C/min. For semen packaged in 0.5-ml straws, using a conventional extender, the cooling rate is approximately 60 °C/ min, when straws are suspended 4 cm above liquid nitrogen, in an enclosed container with a vapor temperature of 160 °C (Amann and Pickett, 1987, Watson, 2000). Previously mentioned studies concluded that the loss of spermatozoal viability during cryopreservation is most likely associated with accompanying extra-cellular osmotic pressure changes, during cooling and thawing, and not direct damage caused by intra-cellular ice crystal formation.

2.4.2. Osmotic Stress

Specific steps of cryopreservation expose spermatozoa to periods of hypo- and hyperosmotic extra-cellular environments. Initially the addition of spermatozoa to a hyper-osmotic freezing extender will result in a period of cellular dehydration as water and permeable cryoprotectants (e.g. glycerol) equilibrate across the plasma membrane. The length of time required for equilibration is associated with the permeability of the intra-cellular cryoprotectant to equilibrate. A second period of osmotic stress occurs during thawing, spermatozoa still in thawed freezing extender are initially exposed to a hypo-osmotic environment created by melting ice crystals and as a result there is an increase in cell volume until solute concentration finally equilibrates.

Stallion spermatozoal cell volume, motility and viability change in response to extracellular osmolality (Pommer et al., 2002). Spermatozoal cell volumes change after exposure to hypo-osmotic fluid (75 and 150 mOsm/kg) and hyper-osmotic fluid (450, 600 and 900 mOsm/kg), and all spermatozoa were able to recover initial cell volume upon return to iso-

osmotic conditions (300 mOsm/kg). A change in spermatozoal total motility was observed, with a significant decrease in all hypo- and hyper-osmotic conditions, and only spermatozoa exposed to 450 mOsm/kg conditions were able to regain initial total motility. In addition it was demonstrated that spermatozoa returned to the control solution (300 mOsm/kg) from 75, 150 and 900 mOsm/kg treatments showed significantly lower cell viability. It has been suggested that this dramatic change in cell volume may produce damage in a number of sperm compartments, including plasma membrane and mitochondria (Pommer et al., 2002).

Altering the glycerol in the spermatozoal environment to improve cryoprotection will have detrimental effects on spermatozoal motility and viability. Ball & Vo, (2001) showed that when increasing concentrations of glycerol were used, there was a decrease in equine sperm motility. In addition they demonstrated that spermatozoa equilibrated with glycerol and then abruptly returned to isometric conditions suffered a decrease in motility. When glycerol was used to increase osmolality, there was a decrease in viability above 1200 mOsm/kg. They also showed a significant decline in mitochondrial membrane potential after an abrupt return to isosmotic conditions. Interestingly there was no change in acrosomal-intact spermatozoa, despite the decrease in the number of viable spermatozoa. If after thawing, the glycerol-loaded spermatozoa are abruptly transferred to conditions exceeding their osmotic tolerance, loss of motility, viability and potentially cell lysis may result (Ball, 2008). Attempts to minimize osmotic stress during cryopreservation have included step-wise dilution of cryoprotectants (Wessel and Ball, 2004), evaluation of alternative more-permeable cryoprotectants to glycerol (Squires et al., 2004) and increasing membrane stability by incorporating cholesterol-loaded cyclodextrins (CLC) in freezing diluents (Squires, 2005).

2.4.3. Reactive Oxygen Species

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the cellular capacity to detoxify ROS or to repair the resulting damage. Reactive oxygen species are produced during normal oxidative metabolism (Ball, 2008), and cryopreservation will result in an excess production of ROS. Equine spermatozoa are more susceptible to oxidative stress than other species due to the high concentration of unsaturated fatty acids present in cellular membranes (Squires, 2005). Reactive oxygen species are products derived from the reduction of diatomic oxygen (O_2), and include radicals and other reactive products (Ball et al., 2001). Radicals are atomic or molecular species that have unpaired electrons in their orbits, making them unstable as they will be involved in cellular reactions in order to displace or share these electrons. Reactive oxygen species have an important role in spermatozoal capacitation, and an imbalance in either the production or degradation of ROS can adversely affect spermatozoal survival and function (Baumber et al. 2003, Ball, 2008).

The superoxide anion (O_2^-), the most common ROS produced by spermatozoa, is rapidly converted to hydrogen peroxide (H_2O_2) by the scavenger superoxide dismutase ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) (Ball et al. 2001). The superoxide anion increases in equine spermatozoa exposed to hypo and hyperosmotic extracellular conditions (Burnaugh et al. 2010). Hydrogen peroxide is more stable than O_2^- and can more readily cross plasma membranes, and has been shown to be the major ROS responsible for human and equine spermatozoal injury (Aitken et al. 1992, Baumber et al. 2000). The other two common ROS scavengers described in semen are catalase (which catabolizes H_2O_2) and glutathione peroxidase system, which reduces H_2O_2 (Ball, 2008). Spermatozoa have limited endogenous sources of these scavengers and rely predominantly on

extracellular sources, namely seminal plasma (Varner and Johnson 2007). Since the presence of excess seminal plasma can be detrimental to spermatozoal longevity and cryopreservation and is commonly removed prior to cryopreservation (Moore et al., 2005), the removal of ROS scavengers leaves spermatozoa particularly vulnerable to ROS during cryopreservation.

The production of ROS by equine spermatozoa is from either sperm-specific NADPH oxidase (NOX 5) present in the plasma membrane of the spermatozoal head or from spermatozoal mitochondria (Sabeur & Ball 2006, 2007). The generation of ROS by NOX 5 in the spermatozoal head may be a necessary step in the induction of capacitation, resulting in tyrosine phosphorylation (Baumber et al. 2003). In cryopreserved equine spermatozoa the principle source of ROS generation is due to electron leakage from the mitochondrial electron transport chain (Sabeur and Ball 2006). The leakage of electrons results in the formation of the superoxide ion, which is rapidly converted to H_2O_2 by superoxide dismutase.

The generation of H_2O_2 by equine spermatozoa is five-fold greater in amount by cryo-damaged spermatozoa compared to control spermatozoa (Ball et al., 2001). The iatrogenic generation of ROS has been demonstrated in spermatozoal samples, resulting in a decrease in spermatozoal motility, but not viability, acrosomal integrity or mitochondrial membrane potential (Baumber et al. 2000). The presence of catalase, but not superoxide dismutase, appeared to maintain normal motility when spermatozoa were exposed to oxidative stress, indicating that H_2O_2 was the main ROS responsible for the observed changes. This effect on motility by H_2O_2 has also been demonstrated in human spermatozoa, possibly caused by a decrease in ATP without a significant decrease in mitochondrial membrane potential (Armstrong et al. 1999). In a follow-up equine study, the presence of H_2O_2 and not superoxide

anion appears to be the ROS responsible for DNA fragmentation, but the exact mechanism of damage is undetermined (Baumber et al. 2003). Cryopreservation causes oxidative stress, and spermatozoal damage is caused by H₂O₂, whether spermatozoal damage is morphologically apparent or not.

2.4.4. Apoptotic-Like Changes

Apoptosis is defined as the process of programmed cell death that affects single cells and induces a series of cellular, morphological and biochemical alterations, leading to cell suicide, without any related inflammation in the surrounding tissue (Ameison 1996). The initiation of apoptosis is caused by two distinctly different pathways, the intrinsic and extrinsic pathways. The intrinsic pathway in spermatozoa is activated by stress induced cell changes; these stresses include oxidative stress, osmotic stress, heat, hypoxia, DNA damage or lack of pro-survival factors (Ball et al., 2008, Glazer et al., 2009, Peña et al., 2011). These stressors result in activation of the mitochondrial-dependent pathway of apoptosis and subsequent mitochondrial release of cytochrome c, which activates caspases 3, 7, and 9, resulting in cell death. The extrinsic pathway is activated by plasma membrane receptor-ligand binding leading to activation of caspases.

Cryopreservation can cause apoptotic like changes to equine spermatozoa. A number of apoptotic like changes have been demonstrated in equine spermatozoa after cryopreservation including decreased mitochondrial membrane potential, altered membrane structure, caspase activation (Brum et al., 2008), and increased DNA fragmentation (Brum et al., 2008, Baumber et al., 2003). Active caspases 3, 7, and 9 have been detected in sperm and are considered a measure of mitochondrial apoptotic changes (Ortega-Ferrusola et al., 2008).

2.5. Spermatozoal Evaluation

2.5.1. Motility

The evaluation of spermatozoal motility is considered one of the most important measures of spermatozoal quality. Poorly motile or immotile spermatozoa are considered incapable of fertilization without the help of assisted reproductive techniques. However spermatozoal motility alone should never be used to predict fertility, as there are many other requirements of spermatozoa for the fertilization of an oocyte.

Motility evaluation can be performed using light microscopy. A drop (5 to 10 μ l) of raw or extended semen is placed on a pre-warmed microscope slide, covered with a coverslip, and the total and progressive motility at 100x, 200x or 400x magnification evaluated in four to six fields. To overcome the subjectivity of microscopic evaluation, computer assisted sperm analysis (CASA) systems have been developed. This technique offers an objective and rapid method of motility analysis by recording 40 to 60 frames/second of spermatozoal movement (Baumber-Skaife, 2010). The recommended evaluation concentration is 25 to 40 x 10⁶/ml (Varner et al., 1991). Characterization of motility is based on a number of defined parameters, including total motility (TM), progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), lateral head displacement (LHD), beat cross frequency (BCF), linearity (LIN), straightness (STR), wobble (WOB), and circularly motile spermatozoa (CIR). Using this technique variability is not totally eliminated as there is currently no universally accepted definitions or values for the above parameters, with some variation between laboratories reported (Hoogewijs, 2011).

2.5.2. Concentration

Concentration evaluation of ejaculates is a fundamental part of semen evaluation and allows a calculation of total-sperm number. The current gold standard method of concentration measurement is the hemacytometer (Baimber-Skaife, 2010). The most commonly used design is the Neubauer hemacytometer. Other commonly used methods of concentration measurement include spectrophotometric analysis, and more recently developed NucleoCounter®SP-100™ (ChemoMetec A/S, Denmark), which utilizes the nucleic stain propidium iodide.

To calculate concentration using a hemacytometer, raw semen a 1:100 dilution with formal buffered saline (FBS). The chamber space between the hemacytometer and coverslip is loaded on both sides of the hemocytometer, and then given time for the spermatozoa to settle. Using light microscopy at 400x, all the spermatozoal heads are counted within the 25 central squares. The counting of spermatozoa is repeated on the second grid, and the average numbers of spermatozoa present is calculated, which represents the sperm concentration in millions/ml. Possible sources of variation include difference in hemacytometer design and operator error. At least two chambers should be counted, with no greater than a 10% difference in spermatozoa counted between chambers. It is suggested that up to four chambers should be counted to help reduce error (Christensen et al., 2005).

2.5.3. Morphology

Spermatozoal morphology is an important component of semen evaluation. Abnormalities in spermatozoal shape can reflect abnormal spermatogenesis; these abnormalities may potentially affect spermatozoal fertilizing capacity (Baumber-Skaife, 2010).

Morphology is examined by phase contrast or differential interference contrast (DIC) microscopy, at 1000x oil immersion. This can be done by placing a drop (5 to 10µl) of raw semen diluted with formalin buffered saline (FBS) on a microscope slide, covered with a cover slip, and then examined under by microscopy. Alternative methods of evaluation include using background or cellular stains. At least 100 to 200 spermatozoa should be evaluated and morphological defects noted. Typical abnormalities noted include head shape, acrosome form, proximal or distal droplets, mid-piece abnormality, coiled and kinked tail, detached heads and round cells. Spermatozoa can have more than one defect. It is also possible to induce morphological defects through poor sample preparation; iatrogenic abnormalities include reflected mid-pieces (cold-shock) and coiled tails (hypo-osmotic shock) (Baumber-Skaife, 2010).

2.5.4. Plasma Membrane & Acrosome Integrity

The assessment of spermatozoal viability using fluorescent probes uses a combination of two stains, one that will identify living cells and one that identifies non-viable (dead) cells. The level of staining can be assessed by fluorescent microscopy or flow cytometry. The combination of nucleic acid stains SYBR-14 and propidium iodide (PI) to assess spermatozoal viability was developed by Garner and Johnson (1995). These nucleic acid stains consist of one that is membrane permeate (SYBR-14) and the other that is impermeate to the cell membrane (PI). It has been used to assess viability in bull, boar, ram, rabbit, mouse, stallion and human spermatozoa. Spermatozoa that stain with SYBR-14 (fluoresce green) are viable, and those stained by PI fluoresce red and are non-viable.

Evaluation of acrosome status can be performed using flow cytometry on cells stained with fluorescein isothiocyanate (FITC) conjugated with either peanut agglutinin (PNA) or

Pisum sativum agglutinin (PSA). The PNA lectin is preferred over PSA because its binding is more specific, being limited to the outer acrosomal membrane (Cheng et al., 1996). The PNA lectin binds to β -galactose moieties of the outer acrosomal membrane of reacted or damaged acrosomes; therefore, spermatozoa with non-reacted acrosome will not bind the FITC-PNA conjugate (no fluorescence), whereas spermatozoa with a reacted or damaged acrosome will bind FITC-PNA conjugate and fluoresce green (Rathi et al., 2001).

2.5.5. Spermatozoal Chromatin Analysis

The evaluation of spermatozoal DNA has now become an important test of fertility in the stallion. Per cycle pregnancy rates range from 45 to 75%, and despite this very wide range in fertility, semen parameters (e.g. motility) can be quite similar between stallions with different per-cycle pregnancy rates. The sperm chromatin structure assay (SCSA) test was first proposed by Evanson et al., (1980), to measure DNA quality. The SCSA test only evaluates the DNA and not the nucleoproteins. The principal of the test uses a metachromatic fluorochrome, acridine orange (AO), to evaluate the proportion of single to double stranded DNA present in spermatozoa. Acridine orange will fluoresce either red (single-stranded DNA) or green (double-stranded DNA). Single-stranded DNA is associated with spermatozoa unable to maintain a double helix. Either heat or an acid can be used to test the DNA's ability to maintain a double helix. Susceptibility to denaturation is correlated with the level of DNA strand breaks, which can be caused by a multitude of factors including idiopathic apoptosis, oxidative stress, heat stress, radiation injury, or protamine deficiency. Spermatozoa affected with this type of damage can appear normal based on other frequently used laboratory tests, including motility, morphology and membrane integrity.

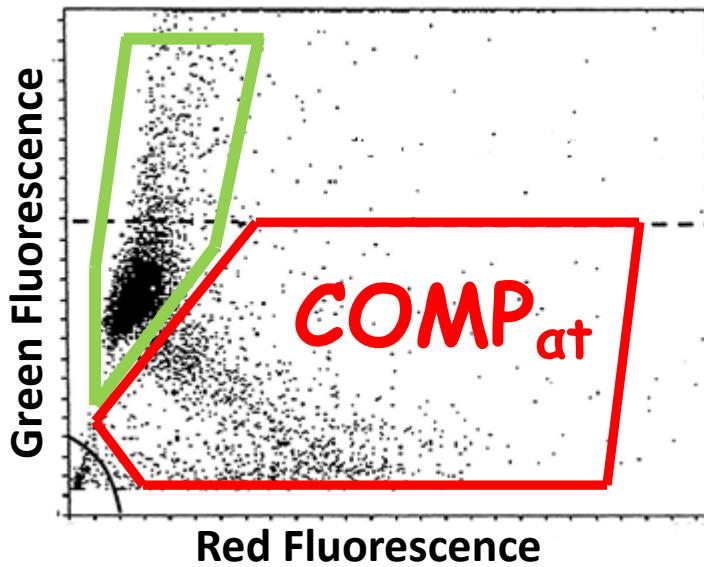


Figure 2.1 Flow cytometric presentation of Sperm Chromatin Structure Assay analysis data. Green fluorescence indicates a spermatozoal population with normal undamaged chromatin. Red fluorescence represents the cells outside the main population that have chromatin damage.

SCSA uses flow cytometry to evaluate fluorescence and the results are depicted in a scatter-gram, with green fluorescence on the y-axis and red fluorescence on the x-axis. The majority of normal spermatozoa will emit a green fluorescence (main population), and are found in the upper left-hand portion of the graph. The damaged spermatozoa will fluoresce red and be located outside the main population and along the x-axis. The terms used to describe this population of abnormal cells, include the percentage of cells outside the main population ($COMP_{\alpha t}$) and the degree of DNA denaturation ($Mean_{\alpha t}$) (Figure 2.1). The αt is calculated as the ratio of red fluorescence to red and green fluorescence, with the $COMP_{\alpha t}$ and $Mean_{\alpha t}$ values calculated from spermatozoal flow cytometric histogram analysis. Love and Kenney (1998) demonstrated a correlation between the $COMP_{\alpha t}$ and the seasonal pregnancy rate (Table 2.2),

in the absence of other overt reproductive abnormalities and apparent disease, thus demonstrating an association between denaturation and reduced fertility.

Table 2.2 The association between DNA denaturation and fertility (Adapted from Love and Kenney, 1998).

Fertility Groups	1	2	3
Seasonal pregnancy rate	93 ^a	86 ^b	73 ^c
% Pregnant/cycle	82 ^a	55 ^b	39 ^c
% Total motile	84 ^a	74 ^b	63 ^c
% Morphologically normal	69 ^a	46 ^b	46 ^b
% COMP α_t	12 ^a	17 ^b	25 ^c

*Superscripts within rows are significantly different (p=0.05)

2.6. Iodixanol

2.6.1. Introduction

Iodixanol (systematic name 5,5'-[(2-hydroxy-1,3-propanediyl)-bis(acetylamino)] bis[N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide] (Figure 2.2) was developed as an imaging contrast agent for radiography.

It has been evaluated as a contrast agent for endoscopic retrograde cholangiopancreatography, cardioangiography, cerebral angiography, urography and aortic femoral angiography. It is now used predominately as density gradient for the separation and isolation of viable cells (Kjeken et al., 2001), and as a cushion medium for centrifugation. There are a number of commercially prepared iodixanol cushion media available (Optiprep™,

Accurate Chemical, Westbury, NY; Eqcellsire® Component B, IMV, L'Aigle, France; Cushion Fluid™ Minitube, Mt Horeb, WI). There is only one preparation with a known percentage of iodixanol (Optiprep™, 60% w/v).

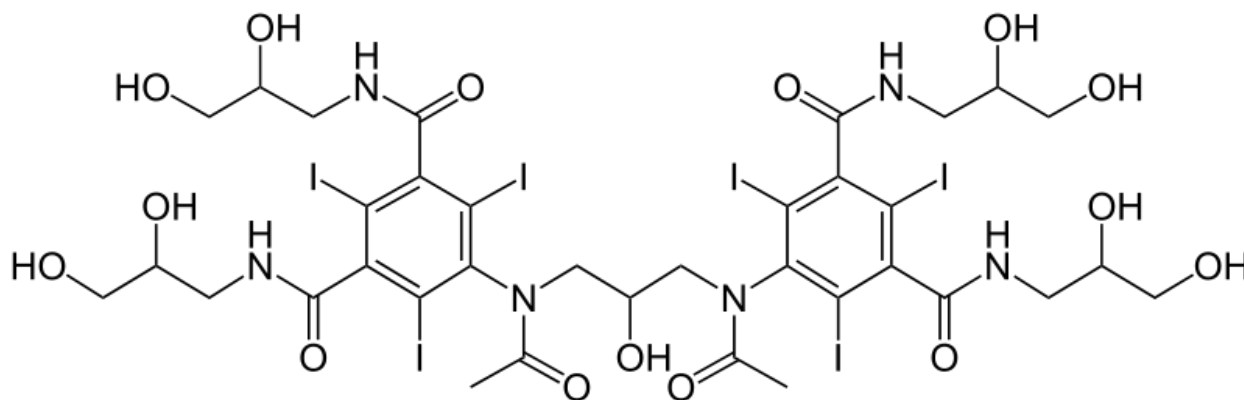


Figure 2.2 The chemical structure of an iodixanol (5,5'-[(2-hydroxy-1,3-propanediyl)-bis(acetylamino)] bis[N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide]) molecule.

2.6.2 Iodixanol as a Contrast Agent

Iodixanol has a number of chemical properties, including high molecular weight and inertness, which suggest it may be safely used with biological material. Iodixanol is a non-ionic dimer, with an osmolarity similar to that of blood (270-320 mOsm/kg). Initial iodinated contrast agents had osmolarities 5 to 8 fold greater than blood. Iodixanol is considered a safe contrast agent. Iodinated contrast agents are commonly used in human medicine, with up to 70 million injections reported worldwide per year (Christiansen, 2004). Iodixanol has become a popular contrast agent because it displays a lower incidence of discomfort (heat and pain) compared to other iodinated contrast agents after injection. Although rare, the reported side effects include

both immediate and delayed reactions to the contrast media in a variety of body systems including; cutaneous (erythema); cardiovascular (hypotension, vasodilation, tachycardia, cardiac arrest); gastro-intestinal (nausea and vomiting); and respiratory (dyspnea, bronchospasm, laryngeal edema). The exact pathogenesis of these reactions is unknown, but they are typical of hypersensitivity-like reactions, including mast cell and basophil dose dependent histamine release (Tumlin et al., 2006). The reported reaction rates for iodixanol range from 0.04% for severe reactions to 3% for delayed reactions (ADIS Data Information, 2006). In a study by Davenport et al., (1999) the toxicity of iodixanol in the lung and its absorption from the peritoneal cavity in rabbits was studied. In the lung it was considered a good and safe contrast agent because of its isotonicity with reduced pulmonary edema compared to iohexol (another iodinated contrast medium, osmolality 920 mOsm/kg), after administration through an endotracheal tube. After intra-peritoneal administration, iodixanol was shown to be rapidly absorbed within 1 hour in 89% of treated rabbits, after intra-peritoneal administration. Iodixanol is eventually excreted in the urine by glomerular filtration. After intravenous injection, 97% of an injected dose is excreted unchanged in the urine after 24 hours in healthy patients, and in patients with renal impairment, 8.2% is reportedly recovered in the feces (Spencer and Goa, 1996).

Iodixanol is a large, water-soluble and nonionic molecule, and therefore is not expected to pass easily into cells. Kjekken et al., (2001) demonstrated iodixanol uptake by hepatic Kupffer cells of rats. Kupffer cells of the liver demonstrate phagocytic activity like macrophage cells, and may be more capable of macro-endocytosis as they play an important part in antigen uptake and antigen presentation. It is unlikely that spermatozoa are able to take up iodixanol into their

intracellular compartment. This is especially unlikely to occur when using freezing extenders that have short or no equilibration times.

2.6.3 Iodixanol and Semen Processing

Iodixanol was first proposed as a density gradient for the separation of spermatozoa by centrifugation, and it has been demonstrated to perform similarly to Percoll (Gellert-Mortimor et al., 1988). McCann and Chantler, (2000) compared Percoll and IxaPrep (a polysucrose and an iodixanol based density centrifugation preparation, respectively), and evaluated motility, spermatozoal longevity, morphology and acrosome reaction. Percoll fractions demonstrated higher spermatozoal recovery and better longevity, but there was no significant difference in morphology or percent acrosome-reacted cells. In contrast Yang et al., (1998), demonstrated no difference in motility and longevity between the two density gradient preparations.

In a study by Ford et al., (1994), bovine spermatozoa were exposed to 20 to 25% iodixanol for one hour without any deterioration in morphology or motility. Harrison, (1997) studied the use of iodixanol as a two layer density gradient for the separation of motile human spermatozoa by centrifugation. Two layers of iodixanol selected from 25, 35, 40, 45, 50, 60, 70, 80 and 90% dilutions, were used to separate 25×10^6 progressively motile spermatozoa by centrifugation at 400 x g for 15 to 20 minutes. In addition to recovery rates, the toxic effects of iodixanol on human spermatozoa and mouse embryos were investigated. Fresh normal spermatozoa, previously separated by 30/45% and 25/40% gradients, were incubated for 72 hr before re-evaluating progressive motility. Murine embryos were incubated for 72 hr at 37°C in a 5% CO₂ environment, after 25 µl of 25, 40 or 100% iodixanol was placed below the embryos and

culture medium. Results showed that greater than 80% of spermatozoa retained their motility and 82 to 86% of embryos developed to the blastocyst stage. There were no controls used in this study, but the author concluded these results were satisfactory.

2.7 Spermatozoal Cushioned Centrifugation

The use of fresh cooled or cryopreserved semen is now a mainstay of equine breeding programs worldwide. One of the techniques used to process semen for both fresh-cooled shipment and cryopreservation is centrifugation. Centrifugation of spermatozoa is performed to remove seminal plasma, and replace it with a chilling or freezing extender, as the retention of seminal plasma may result in a decrease in fertility for the spermatozoa of some stallions (Moore et al., 2005). The properties of an ideal centrifugation protocol are the maximum recovery of spermatozoa after centrifugation with minimal spermatozoal damage. The effects of centrifugation on equine spermatozoa have been intensively studied, with results reaching no definitive conclusion. As a result, centrifugal forces used to process semen are kept conservatively low. Forces from 400 to 600 x g are currently considered industry standards (Hoogewijs et al., 2011), and it is accepted that losses of up to 25% will occur when diluted semen is centrifuged at 400 to 600 x g for 10 to 15 minutes (Loomis & Graham, 2008). Raising centrifugation forces to 900 x g has been shown to have no detrimental effect on total and progressive motility, viability and acrosome integrity, with a 75% recovery rate of spermatozoa reported at 900 x g (Len et al. 2010).

Another use for iodixanol is cushion-centrifugation. This process involves placing an inert, high-molecular weight solution (iodixanol) below the spermatozoa prior to centrifugation

to help protect against the mechanical forces that spermatozoa are subjected to during centrifugation. The first report of using iodixanol as a cushion for centrifugation of equine spermatozoa was by Revell (1997). The use of iodixanol as a centrifugation cushion media has now become a popular technique to improve current centrifugation protocols. The current popularity of centrifugation cushions is based on reports of higher recovery rates of spermatozoa, which subsequently results in more spermatozoa for preservation and larger profits for stallion owners. Significantly higher recovery rates of 99-107% for three of four treatments (Ecot et al., 2005) and 93% for all cushion treatments (Sieme et al., 2006), have been demonstrated when extended spermatozoa are centrifuged at 1000 x g for 20 minutes over a cushion, however only one of these studies had similar centrifugation protocols for the control samples (Sieme et al., 2006). Despite a difference in centrifugation protocols, Ecot et al., (2005) showed a significantly higher number of straws/ejaculate, 72 and 92 straws for 600 x g 10 mins (control) and 1000 x g 20 mins, respectively, with equivalent pregnancy rates for treatments being reported (62.5%).

After centrifugation, the cushion media must be removed before the addition of further semen extenders (Figure 2.3); it is difficult to remove the cushion media without disrupting or losing any of the soft spermatozoal pellet. As a result, erring on the side of caution, the cushion is frequently incompletely removed and some unknown percentage of iodixanol residues remains. To date only one study (Waite et al., 2009) has demonstrated that the use of iodixanol had no deleterious effects on equine spermatozoa, with no reported decrease in spermatozoal motility or DNA damage after cushion centrifugation and storage at 4 °C for 24 hour. Saragusty et al., (2009) evaluated the effects of incomplete removal of iodixanol during pre-freezing

centrifugation of bull spermatozoa. The investigators hypothesized that iodixanol residues may have positive cryoprotective effects, as measured by post-thaw motility, plasma membrane functionality and viability, and acrosomal integrity. Their results showed that the presence of iodixanol in fact helped to maintain membrane integrity and longevity.

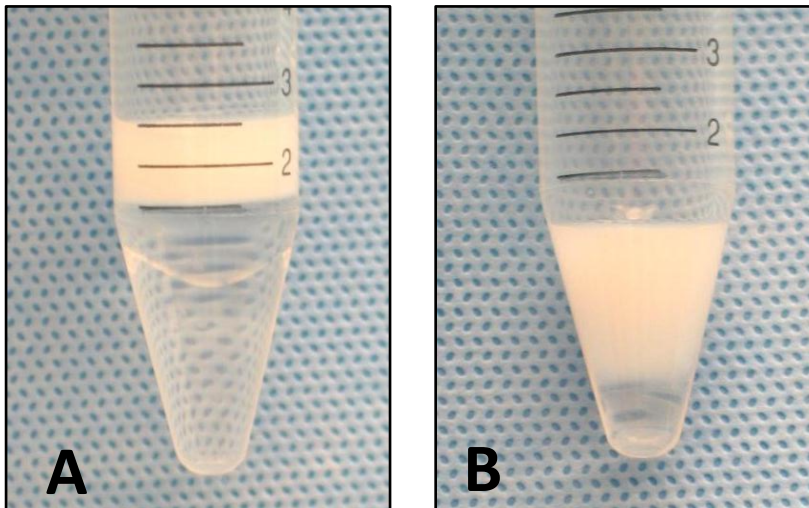


Figure 2.3 Post-centrifugation pellet and cushion. The supernatant has been removed from the conical tubes, and the pellet is the white layer, with the cushion being the clear solution located underneath the pellet. These pictures demonstrate before (A) and after (B) iodixanol removal. A cushion residue can be seen in the bottom of the tube in B, with the overlying spermatozoal pellet.

The objective of this study was to characterize the effects of iodixanol residues present during the cryopreservation of equine spermatozoa. Our hypothesis was that the addition of iodixanol would improve equine spermatozoal post-thaw parameters.

CHAPTER 3
THE INVESTIGATION OF THE EFFECTS OF IODIXANOL DURING EQUINE SEMEN
CRYOPRESERVATION

3.1. Introduction

The objective of this study was to evaluate the effect that 0%, 2.5% and 5% iodixanol (v/v; Opitprep™, Accurate Chemical, Westbury, NY; 60% iodixanol w/v) in an egg yolk glycerol-based freezing extender (EZ Freezin LE, Animal Reproduction Services, Chino, CA) on equine spermatozoal total and progressive motility, viability, acrosomal integrity and DNA damage after cushion free centrifugation. Our hypothesis was that the addition of iodixanol would improve equine spermatozoal post-thaw parameters.

3.2. Materials and Methods

Six stallions were used for this study; four were from the LSU Department of Animal Sciences, one from the LSU School of Veterinary Medicine teaching herd and one from a client of the LSU School of Veterinary Medicine. The stallions ranged from five to 29 years old. Stallion semen was collected between August and November in 2010 and 2011. All procedures were in accordance (or approved by) the Louisiana State University Institutional Animal Care and Use Committee and Clinical Protocols Committee.

3.2.1. Semen Collection

Semen was collected from all stallions at least seven times in an everyday collection schedule to deplete the extra-gonadal spermatozoal reserves. After seven consecutive days of collection, daily sperm output (DSO) for each stallion had been achieved, as total spermatozoal

collected had stabilized, and cryopreservation was performed on the next three ejaculates, collected on 24 h intervals.

Before semen collection the stallion was teased, until the stallion achieved an erection, the penis washed with cotton soaked in warm water to remove smegma, and the excess water was removed with a clean towel. Stallions mounted an estral mare or phantom, and semen was collected into a Hannover artificial vagina (Animal Reproduction Services, CA), temperature 50-51 °C. The artificial vagina was equipped with a disposable plastic liner and an in-line nylon mesh filter to obtain a gel-free semen sample. After the semen was collected, the filtered ejaculate was diluted 1:1 with a milk-based semen extender (EZMix Original Formula, Animal Reproduction Services, CA), which had been warmed to 37° C prior to collection. A 2-ml sample of raw semen was saved for later determination of concentration and morphology, and samples were transported to the laboratory within 30 min of collection. To account for the variable distance of collection facility to the laboratory a mandatory waiting period of 30 minutes was applied to all extended samples before centrifugation. During this holding period the extended samples were maintained at room temperature in a styrofoam shipping box to protect against temperature changes and light exposure. The collected samples were not cooled for transportation in this project, as spermatozoal cooling would have resulted in a temporary spermatozoal stress which may have had unquantifiable effects on spermatozoal response to changes during cryopreservation.

3.2.2. Semen Processing Overview

At the end of the mandatory waiting period, concentration, motility and morphology were evaluated by hemacytometer, CASA and wet-mount preparation respectively. The treatment groups were 0%, 2.5% and 5% iodixanol (v/v) in an egg-yolk based extender (EZ Freezin LE, Animal Reproduction Services, CA). The calculation of percent iodixanol was based on the freezing volume that had a final freezing spermatozoal concentration of $200 \times 10^6/\text{ml}$.

3.2.3. Centrifugation

The extended semen was divided into three equal volumes prior to freezing, and placed in 15-ml conical centrifuge tubes and centrifuged (Eppendorf 5804, Hamburg, Germany) at $900 \times g$ for 10 min without a cushion. After centrifugation, the supernatant was removed from each tube, leaving approximately 0.5 ml of the supernatant containing the spermatozoal pellet in the bottom of the conical tube. Each spermatozoal pellet was then suspended with one of the freezing extender/iodixanol preparations, to a freezing concentration of $200 \times 10^6/\text{ml}$.

3.2.4. Concentration

A 100- μL semen aliquot was diluted into 900 μl of FBS to make a 1:10 dilution. A 100- μl aliquot of the 1:10 dilution was further diluted with 900 μl of FBS, making a 1:100 dilution. The 1:100 dilution was used to fill a Neubauer hemacytometer. The spermatozoal concentration was determined by counting all the spermatozoal cell heads within the 25 squares surrounded by the triple lines on both sides of the hemacytometer using a phase contrast microscope at 400x (Olympus BH-2, Olympus America INC., NC). The concentration was the product of the average of the number of cells counted on both sides, at 10^6 per ml. This procedure was

performed, and repeated if required, until the difference between the cells counted from both sides of the hemacytometer was $\leq 10\%$.

3.2.5. Morphology

Spermatozoal morphology was assessed with a sample from the 1:10 dilution. A 1- μL sample was placed on a plain microscope slide and a coverslip was added. Morphological assessment was performed under 1000x oil immersion using phase contrast microscopy (Olympus BH-2, Olympus America INC., NC). A total of 100 spermatozoa were counted and categorized.

3.2.6. Cryopreservation

After supernatant removal, the spermatozoa were re-suspended with prepared freezing extender (EZ-Freezin LE, Animal Reproduction Services, Chino, CA) and iodixanol (Opitprep™, Accurate Chemical, Westbury, NY) preparations. Iodixanol was added to the extender (which had been thawed at room temperature), with the calculation of the percentage iodixanol being based on a final volume that had a freezing concentration of $200 \times 10^6/\text{ml}$. The mixture was then inverted repeatedly to ensure complete mixing and distribution. The spermatozoal pellet was re-extended to a freezing concentration of $200 \times 10^6/\text{ml}$ with the prepared treatment freezing extenders. The extended semen was loaded into 0.5-ml straws. For five of the six stallions, 0.5-ml straws were used but had been reduced in length to contain 0.25-ml of semen, thus allowing a reduced volume but maintaining the same straw circumference and dead space. Spermatozoa from the sixth stallion were frozen in regular 0.5-ml straws. After the straws were filled, they were placed on a styrofoam float, with the straws kept 3 cm above liquid nitrogen. Straws remained in the vapor in an enclosed styrofoam box (22 cm x 26.5 cm x 34 cm, 0.198 m^3) for

ten minutes before being plunged into liquid nitrogen. After cryopreservation straws were stored in liquid nitrogen for a minimum of 30 days before post-thaw analysis was performed.

3.2.7. Motility

The spermatozoal motility after collection was analyzed by placing 2 μL of 1:1 extended semen in a 20- μm height, four-chambered slide (Leja Products B.V., The Netherlands) over a slide warmer at 37°C. The slide was then placed on an optical microscope with a warming stage at 37°C (Olympus BX41, Olympus America Inc., NC). Using the 20x phase contrast magnification objective, spermatozoal motility was analyzed program with a computer assisted sperm analysis program (Sperm Vision® 3.0, Minitube, Verona, WI). Spermatozoa were identified based on defined parameters (Appendix A), and before final values were stored each recorded clip was edited and all non-spermatozoal events (e.g. egg-yolk particles) were removed. Mean percentages of total and progressive motility were assessed from seven fields with a minimum of 100 spermatozoa in each field.

Motility analysis prior to cryopreservation was performed on each treatment. A 100- μL aliquot of each treatment (0%, 2.5% and 5%) was collected before straw-filling, placed in a micro-centrifuge tube, and diluted with 400 μL of centrifuge extender to a final concentration of $40 \times 10^6/\text{ml}$ for motility analysis. Post-thaw analysis was performed after thawing samples in a 37°C water bath for thirty seconds. A similar dilution from a sample of thawed semen as described above was used. Total and progressive motility analysis was performed at 5 and 30 min of incubation at 37 °C.

3.2.8. Plasma Membrane Integrity

The fluorescent dyes Propidium Iodide (PI) and SYBR-14 (Molecular Probes Inc., Eugene, OR) were used to assess plasma membrane integrity. Both are nucleic acid fluorescent dyes; SYBR-14 is able to cross an intact plasma membrane staining the spermatozoa green, and PI is only able to cross a disrupted membrane causing the spermatozoa to stain red. Therefore green spermatozoa are considered viable, and red-stained spermatozoa are considered non-viable or dead.

A 1- μ L aliquot from each of the treatment groups was stained to assess the plasma membrane integrity, by dilution with 200 μ L of PBS (pH 7.4 and 320 mOsm) to a final concentration of 1×10^6 /ml. Cells were then initially stained with 2- μ L of SYBR-14 and incubated at 37°C for 10 min in the dark. After incubation, 2- μ L of PI was added and incubation continued for a further 10 min before flow cytometric analysis. A triplicate preparation of each treatment was made for flow cytometric analysis.

Stained samples were kept in the dark and analyzed with a flow cytometer (C6 Flow Cytometer, Accuri Cytometer, Ann Arbor, MI) and kept dark until analysis. The flow rate of cells was set at 100 to 200 cells/sec. Cells were gated to exclude debris based on side-scatter and forward-scatter characteristics using a dot plot (Appendix B.1). The gated population was then subjected to green and red light with spermatozoal fluorescence allowing the separation of spermatozoa from non-spermatozoal events, and viable from non-viable spermatozoa, and were analyzed using software (Appendix B.2) (CFlowPlus, Accuri Cytometer, Ann Arbor, MI).

3.2.9. Acrosome Reactivity

A separate 1- μ L aliquot of each treatment was stained to assess the change in acrosome reactivity before and after cryopreservation for each treatment. Acrosome reactivity was measured by dual staining spermatozoa with a combination of fluorescein-conjugated *Arachis Hypogaea* agglutinin (FITC-PNA) and PI. Fluorescein-conjugated *Arachis Hypogaea* agglutinin binds to the β -galactose moieties of the outer acrosomal membrane and fluoresces green. The PI was used to differentiate viable from non-viable spermatozoa.

The 1- μ L aliquot was initially diluted with 200 μ l of PBS to a concentration of 1×10^6 /ml. Cells were stained first with 2 μ L FITC-PNA, incubated at 37°C for 10 min in the dark, then 2 μ L of PI was added, and incubated for a further 10 minutes before analysis by flow cytometry, at a flow rate of 100 to 200 cells/sec. A triplicate preparation of each treatment was made for flow cytometric analysis, and all samples were stored in the dark until analysis.

Stained samples were then analyzed with a flow cytometer (C6 Flow Cytometer, Accuri Cytometer, Ann Arbor, MI) using quadratic gating (Appendix B.3). Cells were initially gated to exclude debris based on side-scatter and forward-scatter characteristics using a dot plot, and fluorescence analysis was subsequently performed on eleven thousand events, which were quadrant gated. Spermatozoa were analyzed as four groups, viable spermatozoa non-reacted acrosome, viable spermatozoa reacted acrosome, non-viable spermatozoa non-reacted acrosome and non-viable spermatozoa reacted acrosome (CFlowPlus software, Accuri Cytometer, Ann Arbor, MI).

3.2.10. Sperm Chromatin Structure Assay

Sample collection for SCSA was performed immediately after the addition of the prepared freezing treatments. A 0.5-ml aliquot from each of the freezing extender treatments was placed in 1.0-ml cryovials (Sigma-Aldrich[®], St. Louis, MO), and snap-frozen in liquid nitrogen (LN). Samples were stored in LN for at least 30 days before analysis.

Individual samples were thawed in a 37°C water bath. Aliquots of approx 2- μ l of thawed semen were mixed with 198 μ l of a buffered isotonic solution (0.186 g disodium EDTA, 0.79 g Tris-HCL, 4.38 g NaCl, in 500 ml deionized water, pH 7.4). Samples were then combined with 400 μ l of a detergent solution (pH 1.2; 2.19 g NaCl, 1.0 ml of 2 M HCL, 0.25 ml Triton-X, qs. 250 ml deionized water) for 30 sec, and then 1.2 ml of acridine orange solution (3.8869 g citric acid monohydrate, 8.9429 Na₂HPO₄, 4.385 g NaCl, 0.17 disodium EDTA, 4.0 μ g/ml acridine orange stock solution (1 mg/ml), qs. 500 ml water, pH 6.0) was added to the sample, and processed immediately (30 sec) on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). After 30 sec, 11000 events were recorded for analysis, at a flow rate of 100 to 200 cells/sec. During preparation and evaluation of individual samples, the buffered solution, detergent solution and acridine orange solutions were all kept in an ice bath, and all sample handling was performed in a darkened room.

3.2.11. Extender Osmolality

The extender osmolality after addition of iodixanol was measured using a vapor pressure osmometer (VAPRO 5520, VAPRO[®] WESCOR, Logan, UT) Solutions of 0%, 2.5% and 5% iodixanol in freezing extender (v/v) solutions were prepared. A 10- μ L sample of each preparation to be tested was aspirated and inoculated onto a solute free paper disc. The

inoculated disc was then inserted and locked into the sample chamber and the osmolality measured. Samples were measured in duplicate, with the mean value recorded.

3.2.12. Statistical Analyses

The mean (\pm S.E.) percentage pre- and post-thaw total motility, progressive motility, viability, acrosome reactivity, $COMP_{at}$ and $MEAN_{at}$ were analyzed using the Shapiro Wilk's test to evaluate if data followed a normal distribution. The only parameter that followed a normal distribution was $MEAN_{at}$ and an ANOVA was performed and where a significant interaction of treatment was observed ($p < 0.05$), a Tukey-Kramer post-hoc test was applied. For measured parameters that did not follow a normal distribution, a binomial logistical regression was performed using spermatozoal (events and trials) numbers for each measured parameter (Appendix C), and results are presented in percentage. Where a significant interaction of treatment was observed ($p < 0.05$), a Tukey-Kramer post-hoc test was applied. All statistical analysis was performed using statistical analysis software (SAS 9.3, SAS Institute Inc, Cary, NC).

3.3. Results

Total motility results did not follow a normal distribution and therefore a binomial logistic regression was performed (Appendix C.1). The 0% post-thaw TM was found to be significantly higher than that of 2.5% iodixanol treatment, and neither 0% or 2.5% treatment groups were significantly different from the 5% treatment group. There was a significant difference between pre- and post-cryopreservation TM for all treatment groups (Table 3.1).

Table 3.1 Mean (\pm SE) spermatozoal total motility (TM) percentage (%) pre- and post-cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze TM (%)	Post-Thaw TM (%)
0 % Iodixanol	65.16 \pm 3.28% ^{a1}	31.93 \pm 3.1% ^{a2}
2.5 % Iodixanol	64.65 \pm 3.3% ^{a1}	28.1 \pm 2.9 % ^{b2}
5 % Iodixanol	64.14 \pm 3.3% ^{a1}	30.4 \pm 3.1% ^{ab2}

Values within column with different letter subscript are significantly different (P<0.001). Values within rows with different number subscript are significantly different (P<0.001).

Progressive motility results did not follow a normal distribution and was analyzed using a binomial logistic regression (Appendix C.2). The 0% post-thaw PM was found to be significantly higher than 2.5% iodixanol treatment, and neither 0% or 2.5% treatment groups were significantly different from the 5% treatment group. There was a significant difference between pre- and post-cryopreservation PM. There was a significant difference between pre- and post-cryopreservation PM for all treatment groups (Table 3.2).

Table 3.2 Mean (\pm SE) spermatozoal progressive motility (PM) percentage (%) before and after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze PM (%)	Post-Thaw PM (%)
0% Iodixanol	53.75 \pm 4.44% ^{a1}	23.72 \pm 3.23% ^{a2}
2.5% Iodixanol	53.44 \pm 4.44% ^{a1}	20.19 \pm 2.87% ^{b2}
5% Iodixanol	52.5 \pm 4.44% ^{a1}	21.94 \pm 3.04% ^{ab2}

Values within column with different letter subscript are significantly different (P<0.001). Values within rows with different number subscript are significantly different (P<0.001).

Thirty minute post-thaw TM results did not follow a normal distribution and was analyzed by a binomial logistic regression (Appendix C.3). The 2.5% post-thaw TM was found to be significantly lower than the 0% and 5% iodixanol treatment groups, and neither 0% nor 5% treatment groups were significantly different (Table 3.3).

Table 3.3 Mean (\pm SE) spermatozoal thirty minute post-thaw TM percentage (%) after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Thirty Minute Post-thaw Total Motility (%)
0% Iodixanol	40.25 \pm 3.52% ^a
2.5% Iodixanol	36.77 \pm 3.41% ^b
5% Iodixanol	39.53 \pm 3.49% ^a

Values within column with different letter subscript are significantly different (P<0.001).

Thirty minute post-thaw PM results did not follow a normal distribution and was analyzed by a binomial logistic regression (Appendix C.4). The 2.5% post-thaw PM was found to be significantly lower than the 0% and 5% iodixanol treatment groups, and neither 0% nor 5% treatment groups were significantly different (Table 3.4).

Table 3.4 Mean (\pm SE) spermatozoal thirty minute post-thaw PM percentage (%) after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Thirty Minute Post-thaw Progressive Motility (%)
0% Iodixanol	30.41 \pm 3.87% ^a
2.5% Iodixanol	27.17 \pm 3.62% ^b
5% Iodixanol	28.94 \pm 3.76% ^a

Values within column with different letter subscript are significantly different (P<0.001).

Post-thaw spermatozoal viability results did not follow a normal distribution and was analyzed using a binomial logistic regression (Appendix C.5). The 5% post-thaw viability was significantly greater than both the 0% and 2.5%, with the 2.5% being significantly higher than the 0% treatment group in post-thaw analysis; however, the pre-freeze 2.5% treatment group had significantly less spermatozoal viability prior to freezing than the 0% and the 5% treatment groups. There was a significant difference between pre- and post-cryopreservation spermatozoal viability for all treatment groups (Table 3.5).

Table 3.5 Mean (\pm SE) spermatozoal viability percentage (%) before and after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze Viability (%)	Post-Thaw Viability (%)
0% Iodixanol	59.88 \pm 6.72% ^{a1}	22.97 \pm 4.95% ^{a2}
2.5% Iodixanol	59.07 \pm 6.77% ^{b1}	24.14 \pm 5.13% ^{b2}
5% Iodixanol	62.24 \pm 6.57% ^{c1}	24.81 \pm 5.22% ^{c2}

Values within column with different letter subscript are significantly different (P<0.001). Values within rows with different number subscript are significantly different (P<0.001).

Post-thaw results for damaged or reacted acrosomes did not follow a normal distribution and was analyzed using a binomial logistic regression (Appendix C.6). Results showed significantly less reacted or damaged acrosomes for the 0% post-thaw treatment group compared to the 2.5% and 5% groups, with the 2.5% group having significantly less reacted or damaged acrosome than the 5% group. There was a significant difference between pre- and post-cryopreservation acrosome reactivity for all treatment groups, with the 5% pre-freeze having significantly fewer reacted or damaged spermatozoa than 0% or 2.5%, and then having a post-thaw value showing significantly more reacted or damaged acrosomes than the 0% or 2.5% treatment groups (Table 3.6).

Table 3.6 Mean (\pm SE) acrosome reactivity percentage (%) before and after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze Reacted (%)	Post-Thaw Reacted (%)
0% Iodixanol	8.92 \pm 1.27% ^{a1}	12.88 \pm 1.75% ^{a2}
2.5% Iodixanol	8.12 \pm 1.17% ^{b1}	13.5 \pm 1.83% ^{b2}
5% Iodixanol	7.8 \pm 1.13% ^{c1}	14 \pm 1.13% ^{c2}

Values within column with different letter subscript are significantly different (P<0.001). Values within rows with different number subscript are significantly different (P<0.001).

The post-thaw COMP_{at} results did not follow a normal distribution and was analyzed using a binomial logistic regression (Appendix C.7). Pre- and post-thaw results showed significantly less COMP_{at} as iodixanol was added. Post-thaw results showed 5% iodixanol treatment group having significantly better COMP_{at} than both the 0% and 2.5% groups, with the 2.5% group having significantly better COMP_{at} than the 0% group post-cryopreservation. Except for the 0% treatment group, there was a significant difference between pre- and post-cryopreservation chromatin denaturation (Table 3.7).

Table 3.7 Mean (\pm SE) COMP_{at} (%) before and after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze COMP _{at} (%)	Post-Thaw COMP _{at} (%)
0% Iodixanol	16.24 \pm 1.05 ^{a1}	16.75 \pm 1.07 ^{a1}
2.5% Iodixanol	13.28 \pm 0.88 ^{b1}	15.02 \pm 0.98 ^{b2}
5% Iodixanol	14.6 \pm 0.96 ^{c1}	13.8 \pm 0.92 ^{c2}

Values within column with different letter subscript are significantly different (P<0.001). Values within rows with different number subscripts are significantly different (P<0.001).

The MEAN_{ot} results were normally distributed and an ANOVA was performed. There was no significant difference either between pre- and post-cryopreservation, or between treatment groups (Table 3.8).

Table 3.8 Mean (\pm SE) MEAN_{ot} (%) before and after cryopreservation (n=6) with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

Treatment	Pre-Freeze MEAN _{ot} (%)	Post-Thaw MEAN _{ot} (%)
0% Iodixanol	220.91 \pm 4.77% ^{a1}	223.12 \pm 2.41% ^{a2}
2.5% Iodixanol	218.08 \pm 4.77% ^{a1}	224.7 \pm 2.52% ^{a2}
5% Iodixanol	218.89 \pm 4.77% ^{a1}	223.6 \pm 2.42% ^{a2}

Values within column with different letter subscripts are significantly different (P=0.05). Values within rows with different number subscripts are significantly different (P=0.05).

Measurement of the osmolality for each treatment group freezing extender combination showed that with increasing concentrations of iodixanol, extender osmolality decreased (Figure 3.1).

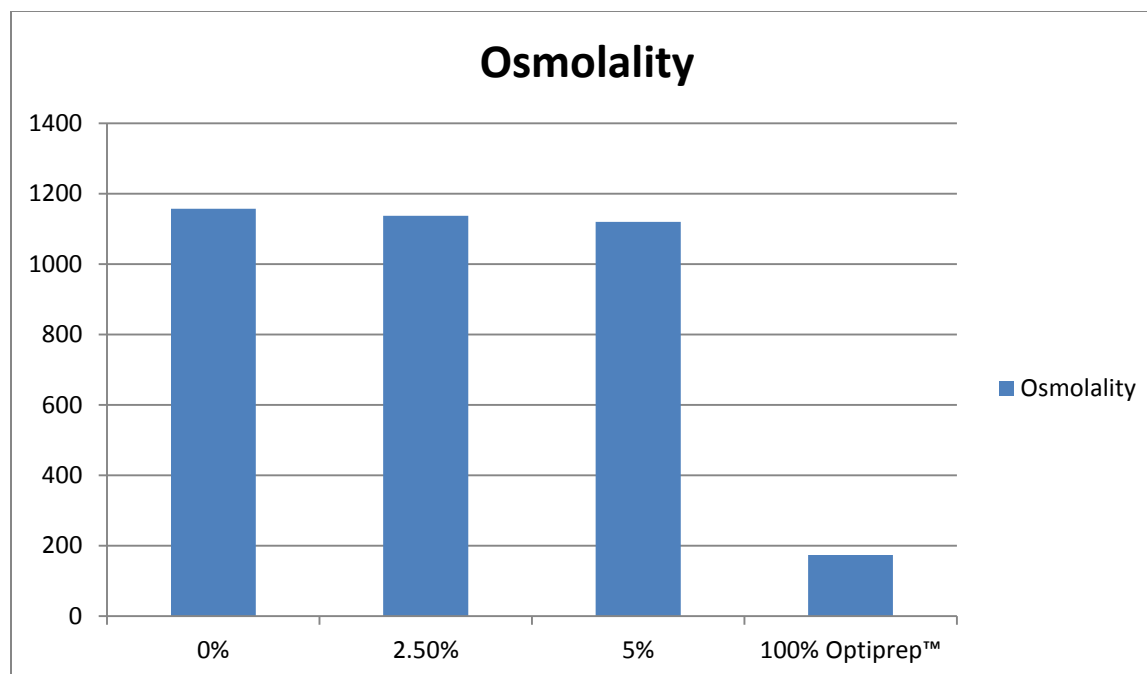


Figure 3.1 Mean treatment iodixanol in extender osmolality measurement with 0%, 2.5% and 5% iodixanol (v/v) in freezing extender.

3.4. Discussion

In this experiment, the semen from five of the six stallions was cryopreserved in 0.5-ml straws that had been shortened to a capacity of 0.25-ml. This reduction in straw length was performed because the stallions at DSO were not producing enough spermatozoa to allow sufficient samples to be collected in 0.5-ml straws. Whether such low sperm output was due to normal stallion production, or if there was a seasonal effect could not be determined as the DSO for these stallions during the physiological breeding season had not been previously determined. Based on a constant external surface area, the thermodynamic changes of either a 0.5-ml straw or a 0.5-ml straw reduced in length should not differ as both types have a similar straw circumference. Both 0.5-ml and 0.25-ml straws have been used for cryopreservation in horses (Love 1989 and Volkman 1987). No significant difference in total and progressive

motility, intact plasma membranes or intact acrosomal membrane has been demonstrated when semen was frozen in 0.25-ml or 0.5-ml straws (Nascimento et al., 2008). Additionally the freezing concentration of 200×10^6 /ml (used in this study) has been demonstrated to have superior total and progressive post-thaw motility than semen frozen and stored in 0.5-ml straws at 400×10^6 or 800×10^6 /ml when cryopreserved in an egg-yolk based extender (Loomis and Clark 1998). As a result, if the freezing concentration for each stallion in this experiment was maintained at 200×10^6 /ml in 0.5-ml straws, it is unlikely that there would have been an effect of straw length exerted on spermatozoa during cryopreservation.

A lactose-egg yolk-glycerol extender (EZ-Freezin LE), was selected for this study because a period of refrigeration to allow spermatozoa to equilibrate at 4 °C prior to freezing is not required. As per the manufacturer's instructions the extender is kept frozen, and allowed to thaw to 22 °C before extension of the semen. Heitland et al., (1996) demonstrated that a prolonged pre-freeze cooling was not required for lactose egg-yolk extended spermatozoa. Stallion spermatozoa are particularly susceptible to damage from 20°C to 5°C when a rapid cooling rate is used, which can result in disruption of the plasma membrane integrity and function due to phase transition of phospholipids, alterations in water transport properties (Devridy et al., 2002), and physical disruption of spermatozoal plasma and acrosome membranes (Watson et al., 1987). The exact mechanism of action of egg-yolk is still unknown; however, it is proposed that the low density lipoproteins (LDL) stabilize the plasma membrane and forming a protective film around the sperm membrane (Sieme, 2010). Our results demonstrate that as the percentage of iodixanol increased spermatozoal viability increased. Iodixanol is a large inert molecule that unlike the egg-yolk LDLs, may not interact with the

plasma membrane, but the presence of iodixanol may alter the shape of unfrozen channels present as water crystalizes with falling temperatures. The main disadvantage of using an egg-yolk diluent is the opaqueness of egg-yolk particles, which results in significant debris requiring manual removal from CASA analysis. Egg-yolk particles were also responsible for a large proportion of the recorded events using flow cytometry due to similar side-scatter and forward-scatter characteristics as spermatozoa.

Reducing the effects of osmotic stress is an important method of improving both post-thaw values and also fertilization rates. Osmolality measurement in this study showed that as Optiprep™ concentration increased, total freezing extender osmolality decreased. This may partially explain the significantly higher spermatozoal plasma membrane integrity as shown in this study. Pommer et al., (2002) demonstrated that when spermatozoa were exposed to 900 mOsm/kg, and subsequently returned to isosmolal conditions (300 mOsm/kg), there was a lower number of viable spermatozoa present than control spermatozoa maintained at 300 mOsm/kg; and that spermatozoa failed to return to pre-treatment levels of motility if incubated in a solution in excess of 450 mOsm/kg. Although all treatment extenders were in excess of 900 mOsm/kg in our study, the addition of Optiprep™ resulted in a decrease in osmolality, this slight decrease in osmolality may explain why there were significantly more viable spermatozoa present after cryopreservation in the 2.5% and 5% treatment groups compared to the 0% treatment group.

The presence of iodixanol may alter the formation of extracellular ice crystals during cryopreservation. As temperatures drop, water crystalizes, leaving solutes and spermatozoa in

unfrozen channels. The presence of large iodixanol molecules may alter the shape of these channels. Saragusty et al., (2009) showed a positive effect of iodixanol on both spermatozoal motility and acrosomal reactivity, and increasing the percentage of iodixanol altered extra-cellular ice crystal shape and organization (Figure 5). It was noted that a 60% iodixanol (w/v; Optiprep™) produced fine grainy crystals, while a 2.5 and 5% iodixanol in Andromed® preparation produced ice crystal dendrites that were tight and well organized. The 2.5%

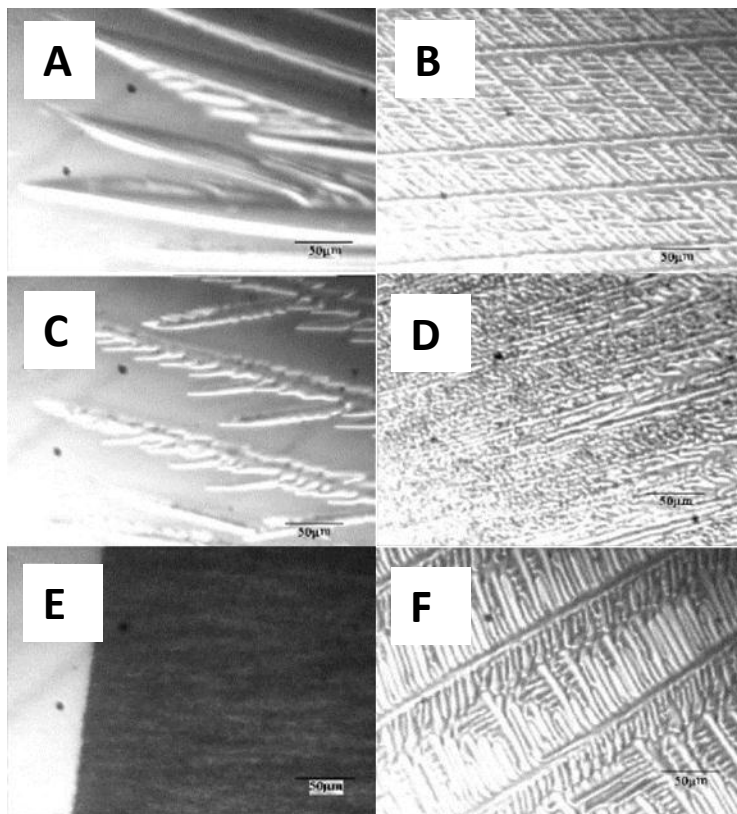


Figure 3.2 From Saragusty et al., (2009). Directional cryo-microscopy of A) Phosphate Buffered Saline B) 2.5% Optiprep™ in Andromed® C) Andromed® D) 5% Optiprep™ in Andromed® E) Optiprep™ F) 50% Optiprep™ in Andromed®.

iodixanol had an average intra-dendritic width of $8.71 \pm 4.03 \mu\text{m}$. The authors suggest that these iodixanol concentrations produce “cell-friendly” ice crystal dendrites, as the typical

equine spermatozoal head length is 3 to 4 μm . Below 2.5% iodixanol it was noted the ice crystal dendrites were looser and less well organized. This hypothesis may help to explain the higher percentage of viable spermatozoa when 5% iodixanol was used in the present study.

We showed that there was significantly less damaged DNA when iodixanol was added. It is unlikely that iodixanol is membrane permeable or acquired by endocytosis; therefore the protective effect of iodixanol on DNA must come from either alteration of ice crystal dendrites (Saragusty et al., 2009), or protection of plasma membranes by reducing osmotic stress. Reduced plasma membrane damage, as shown in our study, may prevent entry of calcium ions that have been shown to be associated with activation of the intrinsic apoptotic pathways in spermatozoa (Watson 2000). In addition, an overall decrease in non-viable cells should result in decreased ROS production; ROS has been shown to cause increase DNA fragmentation (Baumber et al., 2003). It was noted during analysis that the 5% treatment group COMP_{ot} pre-freeze value was $14.6 \pm 0.96\%$ and post-thaw was $13.8 \pm 0.92\%$. This would suggest that chromatin integrity improved with cryopreservation. The analysis and gating of flow cytometry results were performed by a single experienced operator, to reduce operator-induced gating differences. The presence of egg-yolk particles being counted during analysis may have falsely increased COMP_{ot} results.

If iodixanol has a protective effect, before it can be included in commercially available freezing extenders in vivo studies need to be performed to examine what effects iodixanol might have on the endometrium and potential pregnancy in the horse. To date no such studies have been performed in the horse. There are limited in vitro studies examining the effect of

iodixanol on reproductive systems in any species. A study by Heglund et al., (1995) examined the reproductive toxicology of the daily administration of intravenous iodixanol to both male and female mice. Female rats (n=30) received 0, 1 or 2 grams iodixanol/kg, beginning 15 days before initiation of a 14-day cohabitation period with untreated males, with treatments continuing until either euthanasia at day 20 of presumed gestation or until day 21 post-partum. Male mice were treated with similar dosages from nine weeks prior to mating, through mating, and then until day 20 of presumed gestation. In both experiments treatment with iodixanol showed no effect on cycle length, mating performance, pregnancy rate, copulation, litter size and mean fetal weight. The only abnormalities noted were some transient changes in body weight and urine consistency in the iodixanol treated groups. Similar studies in the horse would be cost-prohibitive.

CHAPTER 4

CONCLUSIONS

The utilization of centrifugation cushions has become a popular technique used in equine practice for the handling and preparation of semen. Its use has been promoted by numerous positive studies demonstrating increased spermatozoal recovery rates, without any increase in spermatozoal damage, thereby making semen centrifugation a more efficient process. Centrifugation cushions are marketed as allowing increased numbers of mares to be bred, with resulting increased profits for stallion owners. As a result there are now a number of iodixanol-based products commercially available, with only one product (Optiprep™) that has a known iodixanol percentage included in the manufacturer's data sheet. Despite the increasing use of iodixanol, to date there has been no equine safety study examining the effect iodixanol will have on the outcome of semen quality when present during cryopreservation. The amount of cushion media required varies from 30 μ L to 3 ml, depending on what centrifugation vessel is used and manufacturer's instructions, but all techniques will result in an iodixanol residue being retained with the semen during further processing. The attempted recovery of all iodixanol is hindered by the resultant soft spermatozoal pellet that rests on the cushion after centrifugation. During the recovery process there is a point where retrieving the iodixanol must be stopped as it may compromise spermatozoa recovery, with a result that not all iodixanol is recovered.

There were a number of problems encountered in this study. These included stallion number and location, and the collection of samples outside the normal physiological breeding

season for a stallion. There were limited numbers of stallion available for this study and the stallions used were located within a 15 mile radius of the laboratory. To account for the handling time, a standard 30 min period was established when the semen was extended and kept in a styrofoam box to help negate the effects of storage temperature. The age range of the stallions used was from 5 to 29 years, with an age difference of 16 years between the oldest and next oldest stallion. Ideally this study should have been performed during the normal physiological breeding season. Whether there was an effect of season is difficult to establish as there were no previous semen analysis records available for five of the stallions in this study.

The results of significantly higher plasma membrane integrity as demonstrated in this study, before and after freezing, with the addition of iodixanol are consistent with the decrease in freezing extender osmolality as iodixanol percentage is increased. This would suggest a possible protective mechanism of iodixanol is prevention of osmotic damage to spermatozoal plasma membranes during cryopreservation. This protective effect may occur either after the addition of the freezing extender to the spermatozoa, as the glycerol equilibrates across the plasma membrane or during thawing, when spermatozoa are exposed to a transient hypo-osmotic environment. The subsequent retention of an intact plasma membrane will result in maintaining DNA integrity, as the intact membrane will help prevent apoptotic changes and formation of ROS which are associated with increased DNA damage. Whether the presence of large iodixanol molecules create similar spermatozoal-friendly channels for spermatozoa during freezing, and alter ice crystal formation as shown in a study of bovine cryopreservation (Saragusty et al. 2009) needs to be demonstrated. A positive cryoprotective effect is suggested as the 2.5% treatment group had significantly lower pre-freeze plasma membrane integrity, but

after cryopreservation it had a significantly higher integrity than the 0% treatment group. In contrast acrosome integrity after cryopreservation deteriorated with the addition of iodixanol, suggesting that the presence of iodixanol is detrimental to the acrosome's ability to survive freezing. This is an unusual finding as the anatomic location of the acrosome in the spermatozoal head means it is surrounded by a cellular cytoskeleton which would make the acrosome better protected from the effects of osmotic stress, and suggests whatever plasma membrane damage that is occurring is regionally located to the spermatozoal head, and the underlying acrosome.

In conclusion, although this study showed some significant differences of treatment for total and progressive motility, plasma membrane integrity, acrosomal integrity and DNA damage, it may be difficult to demonstrate any clinical importance of these changes. A fertility trial would be the ultimate test to evaluate the effects of iodixanol. The hypothesis of this study was that the presence of iodixanol would have positive cryoprotective effects, and these results show that the addition of 2.5% or 5% iodixanol may have some significant cryoprotective properties. In addition, this study has answered an important question posed frequently by clinicians regarding what the implications of iodixanol residue inclusion would be.

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APPENDIX A

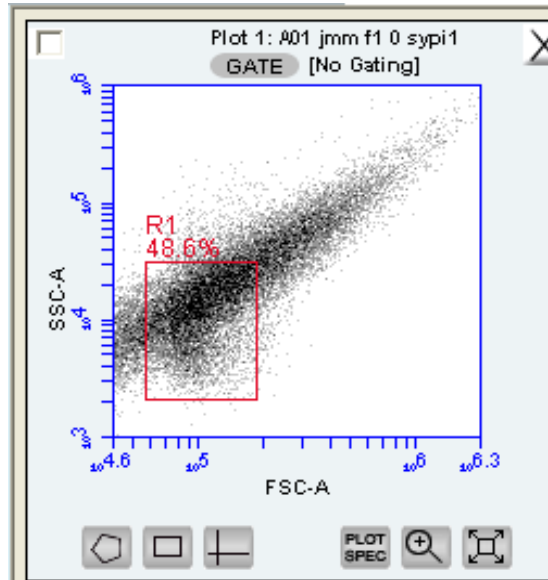
THE CASA SPERM VISION® 3.0. PARAMETERS USED FOR EQUINE SPERMATOZOAL ANALYSIS

- 20 μ M field of view depth
- Cell identification: 14 to 80 μ M²
- 500 cells or 7 fields
- Immotile: AOC < 9.5
- Local motile: DSL < 6
 - Hyper-active= VCL > 80, LIN < 0.65, ALH > 6.5
 - Linear= STR > 0.9, LIN > 0.5
 - Non-linear= STR < 0.9, LIN < 0.5
 - Curvilinear= DAP/radius \geq 3 & LIN < 0.5

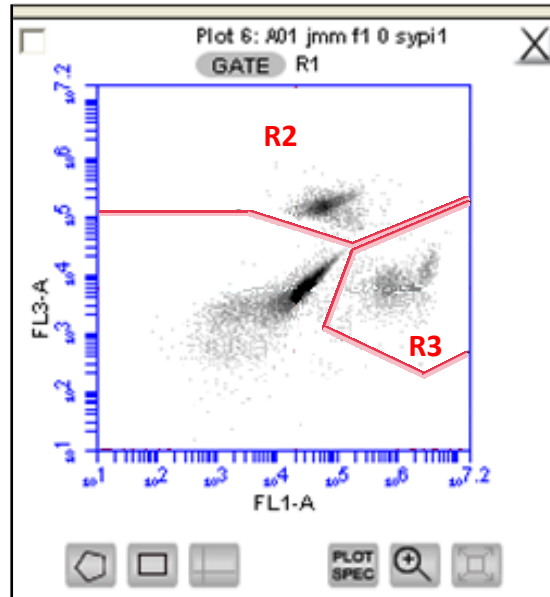
APPENDIX B

FLOW CYTOMETRIC GATING

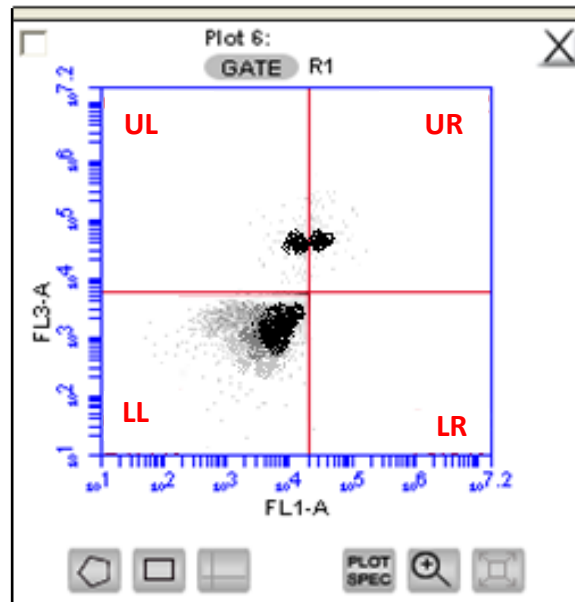
Appendix B.1 An example of flow cytometric analysis (CFlowPlus Software , Accuri Cytometer, Ann Arbor, MI). The distribution of recorded events based on side-scatter and forward-scatter characteristics is shown, with the population R1 (red) being the location of spermatozoa.



Appendix B.2 Scatter dot plot of a viability stained equine semen sample analyzed with a flow cytometer (CFlowPlus Software , Accuri Cytometer, Ann Arbor, MI). Axis are described as FL3-A for red fluorescence and FL1-A for green fluorescence. R2, non-viable cells; R3, viable cells.



APPENDIX B.3 Scatter plot of an acrosome integrity stained equine semen sample analyzed with a flow cytometer. Quadratic gating was used to analyze the cell population (CFlowPlus Software, Accuri Cytometer, Ann Arbor, MI). Upper left (UL), dead-nonreacted acrosome; upper right (UR), dead-reacted acrosome; lower left (LL), viable-nonreacted acrosome; lower right (LR), viable-reacted acrosome.



APPENDIX C

EVENTS AND TRIALS USED FOR BINOMIAL LOGISTIC REGRESSION

Appendix C.1 The total events and trials used for the binomial logistic regression for total motility pre-cryopreservation.

	0% Iodixanol	2.50% Iodixanol	5% Iodixanol
Events	4695	4342	4096
Trials	7263	6792	6466

Appendix C.2 The total events and trials used for the binomial logistic regression for progressive motility pre-cryopreservation.

	0% Iodixanol	2.50% Iodixanol	5% Iodixanol
Events	3594	3594	3375
Trials	7263	6792	6466

Appendix C.3 The total events and trials used for the binomial logistic regression for total motility at 5 and 30 mins post-thaw.

		0% Iodixanol	2.50% Iodixanol	5% Iodixanol
5 min Post-thaw	Events	1900	1742	1913
	Trials	5911	6160	6256
30 min Post-thaw	Events	1648	1464	1614
	Trials	5911	6160	6256

Appendix C.4 The total events and trials used for the binomial logistic regression for progressive motility at 5 and 30 mins post-thaw.

		0% Iodixanol	2.50% Iodixanol	5% Iodixanol
5 min Post-thaw	Events	1453	1287	1425
	Trials	5911	6160	6256
30 min Post-thaw	Events	1208	1045	1183
	Trials	5911	6160	6256

Appendix C.5 The total events and trials used for the binomial logistic regression for spermatozoal viability pre-cryopreservation and post-thaw.

		0% Iodixanol	2.50% Iodixanol	5% Iodixanol
Pre-cryopreservation	Viable	209410	209078	233270
	Non-viable	139426	141610	137165
	Trials	348836	350688	370435
Post-thaw	Viable	69698	71336	70203
	Non-viable	203295	195152	188800
	Trials	272973	266488	259003

Appendix C.6 The total events and trials used for the binomial logistic regression for spermatozoal acrosomal integrity pre- and post-cryopreservation.

		0% Iodixanol	2.50% Iodixanol	5% Iodixanol
Pre-cryopreservation	Non-viable & intact acrosome	90247	91710	85410
	Non-viable & non-viable acrosome	48119	43778	42076
	Trials	501467	499313	499795
Post-thaw	Non-viable & intact acrosome	121910	115944	111210
	Non-viable & non-viable acrosome	72558	75916	79260
	Trials	540939	540652	540910

Appendix C.7 The total events and trials used for the binomial logistic regression of $COMP_{\alpha}$.

		0% Iodixanol	2.50% Iodixanol	5% Iodixanol
Pre-cryopreservation	Events	12736	10300	11573
	Trials	77174	76522	78159
Post-thaw	Events	13057	11729	10724
	Trials	76778	76790	76774

VITA

David Paul Beehan was born in Dublin, Republic of Ireland, in 1982. He studied veterinary medicine at the School of Veterinary Medicine, University College Dublin, Dublin, Ireland, and obtained his veterinary degree (Hons) in 2006. He then completed a 12 month equine internship at Goulburn Valley Equine Hospital, Shepparton, Victoria, Australia. After completing his internship he became an in-house reproductive veterinarian at Goulburn Valley Equine Hospital, and upon completing a southern hemisphere breeding season he commenced work at Grey Abbey Veterinary Hospital, Kildare, Co. Kildare, Ireland as a Thoroughbred stud veterinarian in January 2008. He then completed a second breeding season at both Goulburn Valley Equine Hospital and Grey Abbey Veterinary Hospital. In July 2009, he started a combined residency/master of science program in Theriogenology at the School of Veterinary Medicine, Louisiana State University. He became a diplomat of the American College of Theriogenology 2011. His main area of interest is equine reproduction.