NOTE TO USERS

DISSERTATION

EFFECTS OF CHOLESTEROL SUPPLEMENTATION ON THE CRYOSURVIVAL OF EQUINE SPERMATOZOA

Submitted by

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WE HERBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY AMANDA I. MOORE ENTITLED "EFFECTS OF CHOLESTEROL SUPPLEMENTATION ON THE CRYOSURVIVAL OF EQUINE SPERMATOZOA" BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

EFFECTS OF CHOLESTEROL SUPPLEMENTATION ON THE CRYOSURVIVAL OF EQUINE SPERMATOZOA

Different concentrations of cholesterol-loaded-cyclodextrins (CLC) were added to stallion spermatozoa to determine which concentration of CLC optimizes cryosurvival. Maximum percentages of motile spermatozoa were maintained after thawing when 1.5 mg CLC was added to sperm. Addition of CLC's also increased the percentages of viable spermatozoa surviving cryopreservation compared to non-treated spermatozoa ($P<0.05$). The amount of cholesterol that incorporated into the membranes of spermatozoa increased in a polynomial fashion ($R^2=0.9978$) and incorporated into all spermatozoal membranes. In addition, there was significant cholesterol loss from spermatozoal membranes after cryopreservation, however, CLC's addition to spermatozoa prior to cryopreservation resulted in higher cholesterol levels in the spermatozoa after cryopreservation than untreated spermatozoa ($P<0.05$). Addition of CLC's also resulted in more spermatozoa binding to bovine zona pellucida after cryopreservation than control spermatozoa (48 vs. 15; $P<0.05$).

In addition, when stallion spermatozoa were subjected to anisosmotic solutions, or after spermatozoa were returned to isotonic conditions, CLC treatment increased the osmotic tolerance limits as measured by spermatozoal motility ($P<0.05$). Additional experiments utilized an electronic particle counter to determine the plasma membrane characteristics of stallion spermatozoa. Stallion spermatozoa were determined to have a

volume of 18.61 $\pm$ 0.51 μm^3, and behaved as linear osmometers when incubated in anisosmotic conditions and exhibited an osmotically inactive volume of 83%. When spermatozoa were treated with CLC's and incubated with one of three cryoprotectants (glycerol, ethylene glycol or dimethyl formamide) and volume excursions measured during cryoprotectant removal at 5° and 22°C, stallion spermatozoa were less permeable to the cryoprotectants at 5°C than 22°C and glycerol was the least permeable cryoprotectant in control cells ($P<0.05$). The addition of CLC's to spermatozoa increased the permeability of stallion spermatozoa to the cryoprotectants ($P<0.05$).

A final study was conducted to determine the optimal cooling rate for stallion spermatozoa frozen in the presence of one of three cryoprotectants. Spermatozoa were frozen in a diluent containing 4% glycerol, ethylene glycol or dimethyl formamide at 10 cooling rates ranging from -5°C/min to -50°C/min. The percentages of viable spermatozoa were higher for spermatozoa cooled at -10°C/min compared to spermatozoa cooled at -50°C/min ($P<0.05$). Spermatozoa frozen using glycerol as the cryoprotectant had higher percentages of motile and progressively motile spermatozoa compared to those spermatozoa frozen using the other two cryoprotectants ($P<0.05$). In conclusion, the cryosurvival of stallion spermatozoa was similar when cooling rates of -5°C/min to -45°C/min were used and when 4% cryoprotectant was used. Glycerol was a more effective cryoprotectant, than ethylene glycol or dimethyl formamide at this concentration.

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Chapter I

Review of Literature

Introduction

The first foal produced from cryopreserved stallion spermatozoa was born in 1957 (Barker and Gandier, 1957) and since that time, research to improve the fertility of cryopreserved spermatozoa has increased with increased acceptance from large breed organizations to register foals produced using frozen semen. An optimal cooling or cryopreservation protocol is one that eliminates cold shock (Watson, 2000), intracellular and extracellular ice (Mazur, 1984), and osmotic injury (Steponkus et al., 1983; Watson, 2000) while recovering the greatest number of viable cells after warming.

Events during cooling and cryopreservation and how these events affect/damage spermatozoa make up the majority of this literature review. Sections in this review will discuss cooling rate and the formation of intracellular ice, cold shock and the membrane phase transition, osmotic damage to cells, and the importance of cryoprotectants. Other sections present information about the lipid composition of spermatozoa, the importance that lipid ratios within the membranes have on cryopreservation success, and the techniques used to measure membrane permeability to water and cryoprotectants. The use of cyclodextrins to transfer lipids into cellular membranes and their ability to alter

membrane physiology will also be discussed. Finally, the use of *in vitro* laboratory assays to measure spermatozoa quality and potential fertility is discussed.

The goal of the research presented in subsequent chapters is to improve the cryosurvival of stallion spermatozoa as they pass through damaging temperature fluctuations. The use of cholesterol-loaded-cyclodextrins to alter membrane lipid physiology has proven successful for bovine spermatozoa (Purdy and Graham, 2004) and the use of this technology for the cryopreservation of stallion spermatozoa is presented here. The information presented in this literature review provides the scientific basis on why this technology might enhance cryosurvival of the extremely sensitive stallion spermatozoon.

Cooling & Warming Rates/Intracellular Ice Formation

The development of optimal cooling and warming rates for successful cryopreservation of spermatozoa is essential because the challenge of cryopreservation is not the spermatozoon's ability to survive storage in liquid nitrogen (-196°C), but rather its ability to survive during the transition temperatures during cooling and warming (Mazur, 1984). The greatest occurrence of cell injury and death occurs between -15°C to -60°C, whereas at very low temperatures (-196°C) no thermally driven or chemical reactions occur.

There are two main causes of cell damage and death that occurs during cryopreservation. The first occurs during cooling of cells from room temperature (~22°C) to around 5°C; this damage is known as cold-shock and will be discussed in more detail in subsequent chapters. Other cellular damage occurs during the freezing

process as cells are cooled below 0°C. Spermatozoa and surrounding diluent remain unfrozen to a temperature of ~-5°C due to supercooling and the presence of cryoprotectants and other solutes in the freezing diluent which reduces the freezing point of the medium. Between -5°C and -15°C, extracellular ice crystals begin to form, however the intracellular components remain unfrozen and supercooled. This is in part due to the fact that the plasma membrane retards the growth of ice crystals in the cytoplasm (Mazur, 1984). At these temperatures water from inside the cell begins to move into the extracellular spaces and freezes externally because the intracellular water is supercooled and has a higher chemical potential and in response an efflux of water occurs (Mazur, 1984). This temperature range (down to ~-60°C) is also the critical zone for optimal cooling rate as cellular death and damage can occur if the cooling rate is sub-optimal or supra-optimal. Changes in membrane composition that lead to cell damage and/or death can include: lipid segregation and altering of head group spacing; hexagonal II phase formation; changes in pore structures; displacement of proteins; and changes to the inner leaflet of the membrane bilayer as cell surface area adjusts (Hammerstedt et al., 1990).

As the extracellular water begins to freeze, the remaining salts and sugars in the freezing diluent are excluded from these ice crystals and accumulate in the unfrozen water channels. The increase in solutes within the unfrozen water channels increases the osmolality of this solution. This increase in osmolality causes an osmotic shift across the plasma membrane and an efflux of intracellular water. Exposure of spermatozoa to the nearly 20-fold increase in osmolality has been shown to produce "solution effects" when in contact with this solution for too long before reaching -196°C (Watson, 1995).

Changes in cellular mechanisms due to this effect are thought to be caused by the increase in salt concentration, increased osmolality, changes in pH and changes in solute composition as the salts reach saturation (Watson, 1995). It is also observed that spermatozoa are more sensitive to these osmotic changes than erythrocytes (Lovelock and Polge, 1954). If the rate of cooling is sufficiently slow, complete cellular dehydration will occur, which prevents intracellular water from freezing.

In the opposite direction, cellular damage can occur if cooling rates occur too rapidly. During rapid cooling, water efflux is insufficient to maintain equilibrium and the cell becomes increasingly supercooled (Amann and Pickett, 1987). To restore equilibrium in the chemical potential intracellular freezing of water occurs. Moderate to rapid cooling rates result in large intracellular ice crystals leading to cellular damage and death, however, if the cooling rate is extremely rapid only microcrystals will form and damage to cellular components is reduced. Formation of intracellular ice crystals has been shown to be a leading cause of cellular death following cryopreservation (Mazur, 1984; Steponkus et al., 1983). The rate of cooling also determines the amount of water that will remain inside the cells and become intracellular ice crystals. Mazur (1990) reported a loss in cell viability when >10-15% of the intracellular water remained inside the cells during cooling, mainly due to the large amount of intracellular ice crystals that formed. Also as the cells are cooled the size of the unfrozen water channels decreases with decreasing temperature. This is critical as only cells that reside in the unfrozen water channels will be able to survive cryopreservation (Amann and Pickett, 1987). About 15% of the extracellular water needs to remain unfrozen for cell viability to be sufficient (Mazur, 1984).

Cell warming is another major area of cellular damage that can occur following cryopreservation. The rate at which cells are warmed should match the rate at which those cells were cooled. Problems in osmotic shock arise when cells that were cooled slowly are warmed rapidly. If the extracellular water melts rapidly then this water will cause a major influx of water into the intracellular compartments possibly leading to cell swelling greater than the volume limits for that cell and lysis will occur (Amann and Pickett, 1987; Mazur, 1984). However, if the cells were cooled rapidly (and intracellular ice crystals have formed) a phenomenon of recrystalization can occur. When cooling rates are extremely rapid and microcrystals have formed within the intracellular compartments, these microcrystals have been shown to be thermodynamically unstable compared to large ice crystals (Mazur, 1984). Therefore, at the slow warming rate, once these small crystals thaw they will tend to aggregate to form larger ice crystals before complete thawing has occurred. The formation of these large ice crystals can then be damaging to the composition of the cell membranes (Mazur, 1984). Warming of spermatozoa (1,000-2,000°C/min) generally occurs at a rate 20-100 times greater than the cooling rates to eliminate the possibility of recrystalization (Watson, 1995).

Most research conducted on the cryosurvival of spermatozoa has been empirical in nature because of constraints on accurately measuring cell parameters due to the spermatozoa's unique size and morphology. Calculation of optimal cooling rates that prevent intracellular ice formation from occurring has been described for most round cells. To obtain this calculation one must be able to measure the permeability of the cell to water (L_p), its activation energy (E), the osmoles of solute initially in the cell and the ratio of the cell surface area to volume (Mazur, 1984). The last requirement of cell

surface area to volume is what makes determining optimal cooling rates for spermatozoa difficult because their unique shape makes accurate measurements complicated. When spermatozoal survival is plotted against cooling rate an inverted "U" shaped curve is achieved, indicating that optimal spermatozoa survival exists at some intermediate cooling rate. Devireddy et al. (2002) was able to measure the optimal cooling rate of stallion spermatozoa utilizing a differential scanning calorimeter. This study concluded that the optimal cooling rate for stallion spermatozoa in glycerol is ~60°C/min with a range from 20-100°C/min. They were also able to show the benefits of cryoprotectants, in particular that the addition of glycerol to stallion spermatozoa was able to broaden the optimal cooling rates (only 27-31°C/min in the absence of glycerol; Deveriddy et al., 2002). The amount of water remaining within the intracellular compartments of spermatozoa frozen in the presence of glycerol cooled at rates of 20°C/min to 100°C/min was ~3.4% to ~7.1%, lower than the required 10-15% as reported by Mazur (1990). While a few studies have been conducted to determine optimal cooling rates of spermatozoa, little consideration has been taken into account when investigating other cryoprotectants or other components within the freezing diluent.

Membrane Phase Transition/Cold Shock

Rapid changes in temperature from 37°C to 1°C can cause damage and destabilization to the plasma membrane, leading to changes in intracellular compartments and a loss of motility. This phenomenon, the damages occurring during rapid cooling of spermatozoa, is known as "cold-shock" (Amann and Pickett, 1987). Signs of cold-shock

include: loss of progressive motility, decreased energy production, increased membrane permeability and loss of intracellular molecules and ions (Amann and Pickett, 1987).

Many of the changes that occur in the plasma membrane are due to its physiology. The plasma membranes of spermatozoa consist of a lamellar bilayer including phospholipids, cholesterol and proteins. The phospholipids contain hydrophilic head groups facing externally with hydrophobic fatty acyl chains internally. This allows the plasma membrane to be impermeable to ions and other molecules in the extracellular matrix, while still maintaining the membrane in a fluid and flexible state at elevated temperatures (Amann and Pickett, 1987). As the spermatozoa are cooled below 18°C, the plasma membrane begins to undergo a membrane phase transition in which the membrane transforms from a liquid to crystalline state. This phase change causes alterations in the shape of the phospholipids. The fatty acyl chains straighten and lengthen, the head groups become closely packed and the proteins become clustered into areas rich in cholesterol. The phospholipids may also rearrange into hexagonal-II micelles with their fatty acyl chains facing externally and head groups internally. These changes to the plasma membrane cause the membrane to become "leaky" to extracellular molecules and fail to maintain metabolism and ion gradients (Amann and Pickett, 1987). Many of these insults are not reversed when returned to room temperatures.

The sensitivity of spermatozoa to cold-shock develops during maturation through the epididymis. White (1993) determined that in the proximal corpus of the epididymis, spermatozoa become susceptible to cold-shock, most likely due to the loss of cholesterol from the plasma membranes. The amount of cholesterol within the plasma membranes determines membrane fluidity and the ability of the spermatozoa to withstand cold-shock

(Darin-Bennett and White, 1977). Rottem et al. (1973) determined cholesterol's role during membrane temperature phase transitions. They found that membranes treated with cholesterol became more rigid than control samples at 37°C; however, when these membranes were cooled, cholesterol treated membranes had increased membrane fluidity. Control samples were reported to undergo a phase transition at 25°C where as the cholesterol treated membranes did not exhibit a phase transition at any temperature tested down to 4°C (Rottem et al., 1973). Cholesterol was also shown to prevent crystallization of the hydrocarbon chains in the plasma membranes as well as causing the phospholipid hydrocarbons chains to pack tightly together in the membrane lipid core. This enabled the cells to withstand cold-shock and phase transition damage more efficiently (Rottem et al., 1973). The ability of cholesterol to reduce or eliminate the phase transition was also observed in lecithin liposomes and erythrocytes (Ladbrooke et al, 1967).

Although the amount of cholesterol in the membrane is important for determining a cell's ability to withstand cold-shock, more specifically it is the cholesterol to phospholipid ratio of the plasma membrane. Species can be placed into two distinct groups, species whose spermatozoa plasma membrane have cholesterol to phospholipid ratio greater than 0.5 and those with less than 0.5. Species in group one whose spermatozoa have a cholesterol to phospholipid ratio of 0.99 and 0.88, include human and rabbit respectively, whereas boar, rooster, stallion, ram and bull have cholesterol to phospholipid ratios of 0.26, 0.30, 0.36, 0.38 and 0.45, placing them into group two. Spermatozoa from group one is resistant to cold-shock damage while the majority of spermatozoa from group two are highly susceptible to cold-shock (Darin-Bennett and

White, 1977; Parks and Lynch, 1992). Therefore, if the cholesterol to phospholipid ratio can be increased in the plasma membranes of spermatozoa from species in group two, then stability to the plasma membrane can be increased and cold-shock damage may be reduced, when sperm are cooled.

Cholesterol has been added to the spermatozoa of bull, ram, boar and stallions to help improve cryosurvival (Combes et al., 2000, Galantino-Homer et al., 2005; Morrier and Bailey, 2004; Purdy and Graham, 2004). Purdy and Graham (2004) reported an increase in both the percentage of motile and viable spermatozoa after cryopreservation when cholesterol that had been loaded into cyclodextrins was added to fresh bull semen. They were able to increase the cholesterol to phospholipid ratio of bull spermatozoa from 0.45 to 0.9, similar to that of human and rabbit spermatozoa. A similar increase in the percentage of motile and viable spermatozoa after cryopreservation was also observed in stallion spermatozoa treated with cholesterol (Combes et al., 2000).

Due to the complexity of the plasma membrane of spermatozoa, the cholesterol to phospholipid ratio is not the sole determinant in cold-shock and cryopreservation success. To illustrate this, the cholesterol to phospholipid ratios of boar and rooster spermatozoa are similar (0.26 and 0.30, respectively), suggesting that spermatozoa from both species should be sensitive to cold-shock, however, rooster spermatozoa are much more resistant to cold-shock damage then boar spermatozoa. Parks and Lynch (1992) hypothesized that the different responses to cold-shock from these two species are due to differences in the integral membrane protein to phospholipid ratio. The membrane protein to phospholipid ratio of boar spermatozoa is 1.26 compared to 0.46 for rooster spermatozoa, indicating that the membrane phase transition is dependent upon the interactions of all membrane

components (Parks and Lynch, 1992). White (1993) also reported that the degree of saturation or unsaturation of the fatty acids that comprise the lipids in the membrane affects membrane fluidity. Again, spermatozoa from different species can be grouped into two populations based on the ratio of the saturated vs. unsaturated fatty acids found within the plasma membrane. Ratios of 2.5 to 3.0 (saturated vs. unsaturated) are found in bull, ram and boar spermatozoa while rabbit, human and rooster spermatozoa have ratios around 1.0 (White, 1993). The concentration of unsaturated fatty acids within the plasma membrane helps determine the fluidity of the plasma membrane as well as their resistance to cold-shock damage. Species with an equal molar ratio of saturated to unsaturated fatty acids are more resistant to cold-shock (White, 1993). The increased level of unsaturation within the plasma membrane will also decrease the temperature at which the phase transition occurs as well as the interaction of these fatty acids with cholesterol results in plasma membranes with a more cohesive membrane structure and improvement in cooling and cryopreservation (White, 1993).

Osmotic Stress

Osmotic stress and intracellular ice formation are the two major causes of spermatozoa damage and death during cryopreservation. Osmotic damage can occur in two forms during cryopreservation 1) addition and removal of permeating cryoprotectants and 2) freezing and thawing of extracellular water. The main focus of this section will be on the osmotic stresses placed upon spermatozoa during the addition and removal of cryoprotectants.

During addition of permeating cryoprotectants to the media, spermatozoa will initially shrink due to efflux of water to equilibrate the hypertonic solution. Cell shrinking will continue until the efflux of water is balanced with the influx of cryoprotectant. Volume excursions will cease once the internal and external osmolalities of the permeating and non-permeating solutes are equal (Levin, 1982). Conversely, when spermatozoa that have been loaded with cryoprotectants are placed into isotonic solutions (e.g. media or female reproductive tract fluids) an initial increase in cell volume will occur as there is an influx of extracellular water to balance the osmolality. Cells will then decrease in volume to normal size as the permeating cryoprotectant diffuses out of the cells (Levin, 1982). If the cell volume increases beyond its membrane tolerance, cell lysis will occur. Various groups have proposed several possibilities for osmotically induced injury to spermatozoa including 1) mechanical rupture of cell membranes in hypo-osmotic solutions; 2) frictional force between water and potential membrane pores; 3) cell shrinkage in hyperosmotic solutions is resisted by cytoskeleton components; 4) cell shrinkage induces irreversible membrane fusion/change, effective area of cell membrane is reduced and cells lyse before normal volume is recovered and 5) hyperosmotic stress causes a net leak/influx of non-permeating solutes and cells lyse when returned to isotonic conditions (Gao et al., 1995; Mazur et al., 1972; Meryman, 1970; Muldrew and McGann, 1994; Steponkus and Wiest, 1979). A spermatozoon's ability to withstand osmotic stress has been shown to be dependent upon an ouabain-sensitive Na^+/K^+ ATPase and an amiloride-sensitive Na^+/H^+ anitporter (Caiza de la Cueva et al., 1997). It appears that these ion pumps are important in maintaining normal intracellular osmolality. In addition to ion pumps, regulation of water movement may be

due to selective water channels. Spermatozoa's high water permeability indicates the necessity of water channels (Liu et al., 1995). An aquaporin has been identified in rat testis and spermatozoa and may help facilitate movement of water and glycerol across the plasma membrane (Ishibashi et al., 1997), but has yet to be identified in other species.

Spermatozoa volume excursions can be used to determine potential osmotic injury during cryoprotectant addition and removal, as well as determine optimal procedures to reduce osmotic injury of cryoprotectants. Analysis of the percentage of motile spermatozoa placed into anisosmotic solutions of non-permeating cryoprotectants can determine the cell volume excursion limits. Motility appears to be more sensitive to osmotic injury than plasma membrane integrity, probably due to mitochondrial damage, and thus serves as the assay of choice for these studies (Gao et al., 1995). The osmotic tolerance limits have been reported for various species (Figure 1.1; Ball and Vo, 2001; Gao et al., 1995; Gilmore et al., 1998; Guthrie et al., 2002). From these reports it can be seen that boar spermatozoa are the most sensitive to osmotic injury, human spermatozoa appear to be the most resistant and bull and stallion spermatozoa fall in between human and boar spermatozoa. However, it has been reported that stallion spermatozoa can only maintain $\geq$ 50% motility at isosmolal $\pm$ 100 mOsm suggesting a very narrow range of osmotic tolerance (Ball and Vo, 2001). The osmotic tolerance limits of these species coincide with their ability to survive cryopreservation. Failure of spermatozoa to return to normal parameters after osmotic stress and return to isosmotic conditions indicates that the damage occurring during the osmotic insult is not reversible (Ball and Vo, 2001).

Methods have been investigated to reduce the amount of osmotic stress imposed on spermatozoa during cryopreservation. It is known that due to molecular weight,

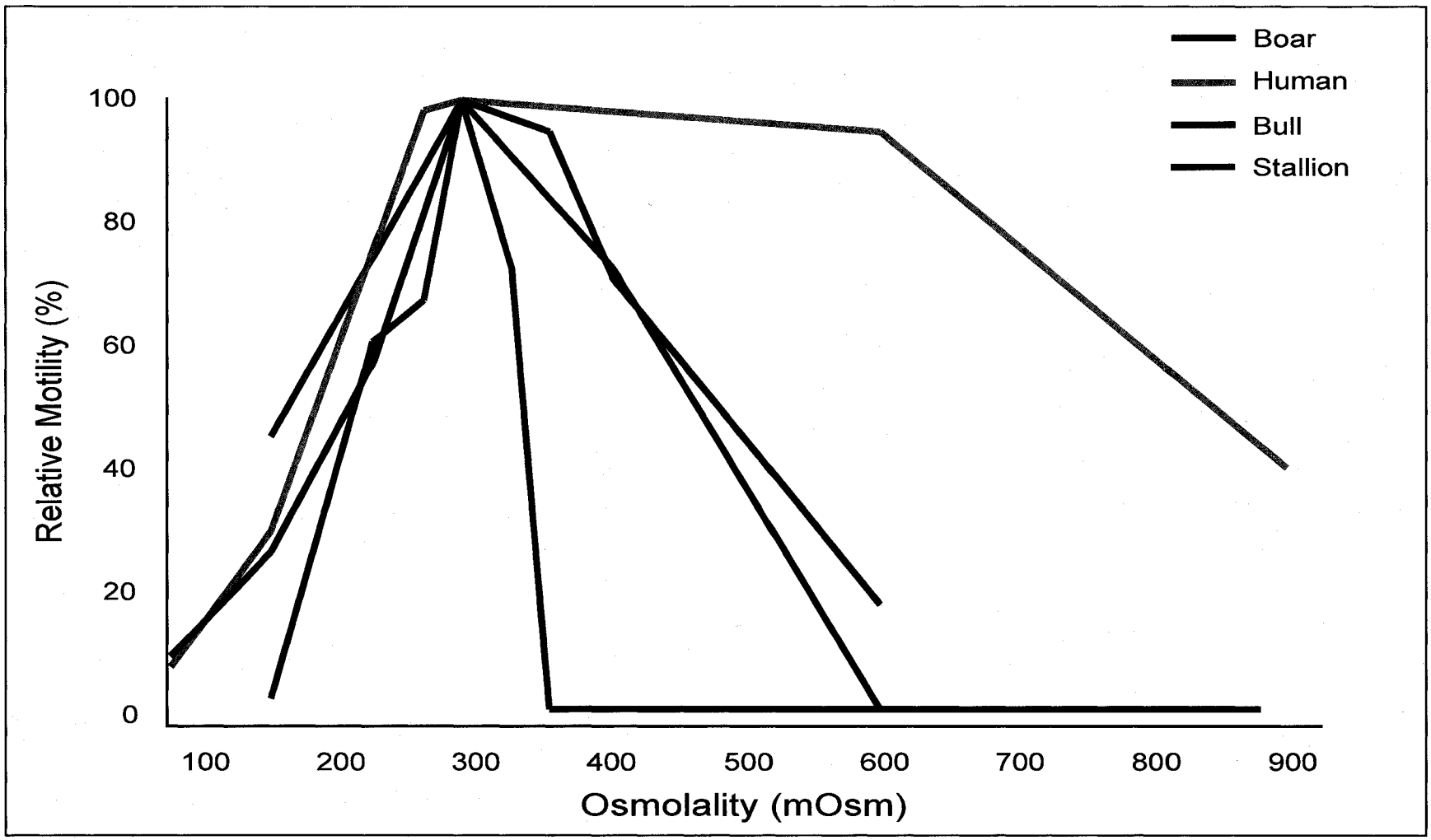

Figure 1.1. Osmotic tolerance limits of Boar (Gilmore et al., 1998), Human (Gao et al., 1995), Bull (Guthrie et al., 2002) and Stallion spermatozoa (Ball and Vo, 2001) after exposure to anisosmotic solutions and returned to isosmotic conditions. Data are presented as the percentage of motile spermatozoa normalized to the control value (290-300 mOsm).

solubility in water and various other factors that various cryoprotectants differ in their permeability across the plasma membrane. Although glycerol is the most widely used cryoprotectant for spermatozoa, its permeability across the plasma membrane is much slower than several other cryoprotectants (Ball and Vo, 2001; Gilmore et al., 1998). Ball and Vo (2001) reported that glycerol caused the greatest amount of osmotic damage, ethylene glycol produced the least and dimethylsulfoxide and propylene glycol fell in between the other two cryoprotectants. Gilmore et al. (1998) also reported higher plasma membrane permeability for ethylene glycol than glycerol at several different temperatures. The use of different cryoprotectants in the cryopreservation of spermatozoa has been discussed in a previous section, but it does appear that utilizing a cryoprotectant that is more permeable to the plasma membrane than glycerol should reduce the osmotic stress imposed on those cells. Other methods of reducing the osmotic stress on spermatozoa during cryopreservation include addition of cryoprotectants at a temperature in which permeability is greatest, addition of extenders containing egg yolk or milk proteins to modify the plasma membrane protein components, or by altering the cooling/warming rates to reduce cellular dehydration and lysis (Gilmore et al., 1998). It has also been shown that a multi-step dilution of the cryoprotectant may maintain the spermatozoa within their volume excursion limits and therefore protect the cells from cell lysis (Ball and Vo, 2001). This procedure maintains spermatozoa integrity in fresh cells loaded with cryoprotectants; however, when a multi-step dilution procedure was used with cryopreserved stallion spermatozoa no benefit was observed (Wessel and Ball, 2004). Knowledge of the biological properties of spermatozoa from a given species including water and cryoprotectant permeabilities and the osmotic tolerance limits will

permit one to calculate the optimal procedures for addition and removal of any cryoprotectant at any concentration at a given temperature. Alteration of the plasma membrane itself may also be another way of reducing the osmotic stress placed on spermatozoa during cryopreservation.

Cryoprotectants

In order for spermatozoa to survive cryopreservation, cryoprotective agents (CPA) must be added to spermatozoa prior to cooling and these remain with the spermatozoa during the warming phase. Cryoprotectants, which were first recognized in 1949, are any agent that specifically maintains the viability of living cells after cryopreservation (Karow, 1969; Polge et al., 1949). Various chemical compounds have been investigated as CPA's including alcohols, sugars, inorganic cations and amino acids (Karow, 1969). Cryoprotectants can exert their protective functions in at least two ways, by acting in the extracellular matrix of the spermatozoa or within the intracellular compartments of the spermatozoa. The ability of the cryoprotectants to penetrate the plasma membrane also enhances their protective function.

The physical properties of cryoprotectants allow these solutes to lower the temperature at which freezing first occurs, to reduce both extracellular and intracellular salt concentrations, as well as increase the size of unfrozen water channels available for the spermatozoa to reside (Amann and Pickett, 1987). In the absence of a cryoprotectant, the molality of a physiological saline solution (0.15M) rises 20-fold in the unfrozen water channels of this solution as it is cooled below freezing. However, if 0.5M glycerol is present the rise in molality only increases around 7-fold, greatly reducing the salt

concentration in the extracellular media (Amman and Pickett, 1987). The addition of glycerol also increases the amount of unfrozen water in these channels from 5% to 13%, and only spermatozoa residing in these unfrozen water channels will remain viable upon warming (Amann and Pickett, 1987). Along with changes in osmotic and freezing properties of the cryopreservation diluent, direct effects of cryoprotectants on the plasma membranes of cells have been observed (Hammerstedt et al., 1990). Effects of cryoprotectants on cellular membranes include direct alteration of membrane bilayers, membrane interactions with bound proteins and glycoproteins, induction of increased bioenergetic demand and increased interdigitation of the non-polar regions of the bilayer (Hammerstedt et al., 1990). Cryoprotectants can therefore cause an indirect effect on plasma membrane permeability to water as well as transmembrane ion movement. The addition of glycerol to cells causes formation of gap-junction like structures and a loss of proteins from the plasma membrane (Ballas, 1981; Kachara and Reese, 1985). Metabolism of the cryoprotectant and increases in ATP demand are ways that cryoprotectants can affect bioenergetic imbalances. Phosphotases and ubiquitous glycerol kinases can phosphorylate or dephosphorylate glycerol and other cryoprotectants and the reduced ATP synthetic capacity at low temperatures can make spermatozoa susceptible to increased ATP demands (Hammerstedt et al., 1990).

While cryoprotectants are absolutely necessary for the survival of spermatozoa during cryopreservation, addition of these solutes is not without significant damage occurring to the cells. The two major types of cellular insult that occurs during exposure to a cryoprotectant are osmotic damage and toxicity. The effect of osmotic damage to spermatozoa is discussed in previous sections of this review. Toxicity of the

cryoprotectant to spermatozoa is another concern to consider when choosing a solute and concentration. The optimal concentration of a cryoprotectant is one that provides the maximal amount of protection during cryopreservation but produces the least amount of toxic effects on the spermatozoa or is least detrimental to fertility. Virtually all solutes determined to be good cryoprotectants can have toxic effects if concentration and exposure times are not regulated. Major occurrences related to cryoprotectant toxicity of cells were reviewed by Fahy et al. (1990) and are summarized in table 1.1.

Table 1.1. Cellular damage occurring due to cryoprotectant toxicity (Fahy et al., 1990).

Polymerizes tubulin, alters meiotic spindle
Large amounts glycerol accumulate as L-glycerol-3-phosphate
Induces protein-free membrane blisters
Permeabilizes or lyses plasma membranes
Induces cell fusion
Alters genetic expression
Alters RNA polymerase
Weakens DNA-nucleosome binding
Destabilizes nucleic acid duplexes
Enhances solubility of DNA bases (destabilizes DNA)
Impairs ribosomal subunit reassociation after removal
Activates low molecular weight acid phosphatase

The single most important characteristic of a solute required to be a successful cryoprotectant is its affinity for water. This depends on the number of lone pair electrons the solute contains and the spherical symmetry of the lone pair electrons (Nash, 1996). The number of lone pair electrons for some of the most commonly used cryoprotectants: glycerol – 3, ethylene glycol – 2, dimethyl formamide – 1, and dimethyl sulfoxide – 2. The difference in the number of lone pair electrons between these cryoprotectants determines its solubility in water as well as its optimal concentration for cryopreserving spermatozoa. Several studies have investigated the cryoprotective capabilities of several solutes in the cryopreservation of stallion spermatozoa. Glycerol has been the

cryoprotectant of choice for cryopreservation of stallion spermatozoa; however, it is known to produce osmotic damage to the cells due to its large molecular weight and has been shown to be contraceptive in mares (Amann and Pickett, 1987). For this reason, dimethyl formamide and ethylene glycol have been studied because of their low molecular weight (dimethyl formamide=73 and ethylene glycol=43) compared to glycerol (formula weight=92). Medeiros et al. (2002) observed an increase in the percentage of motile spermatozoa when dimethyl formamide replaced glycerol as the cryoprotectant. This difference was greatest when used with spermatozoa from stallions whose semen does not freeze well. Ethylene glycol is widely used in the cryopreservation of embryos (Seidel, 1996), but very few studies have looked at its cryoprotective effects on spermatozoa. Squires et al. (2004) recently compared glycerol to ethylene glycol and dimethyl formamide. Results concluded that ethylene glycol and dimethyl formamide protected spermatozoa similar to glycerol when increased concentrations of ethylene glycol or dimethyl formamide were used. This is in agreement with the number of lone pair electrons for each cryoprotectant mentioned above. Because of the reduced number of lone pair electrons compared to glycerol, both ethylene glycol and dimethyl formamide must be added at higher concentrations to provide the same protective qualities as glycerol. These studies indicate that there may be other cryoprotectants that can reduce the osmotic damage and toxicity to stallion spermatozoa compared to glycerol.

Membrane Permeability Characteristics

Successful cryopreservation of spermatozoa requires knowledge of certain physical and biophysical characteristics of these cells. There are five principal

characteristics which are significant to the cryopreservation of spermatozoa: 1) the plasma membrane's permeability to water, referred to as hydraulic conductivity (L_p); 2) plasma membrane permeability to cryoprotectants (P_{CPA}); 3) the temperature dependence of L_p, referred to as the activation energy (E_a); 4) the osmotically inactive fraction of cell volume (V_b); and 5) the isosmotic cell volume (V_{iso}), which is the volume of the cell existing in osmotic equilibrium with an isosmotic solution (Gilmore et al., 1995). Using these characteristics, a maximum cooling rate at which spermatozoa can be cooled while avoiding intracellular ice crystals can be determined if hydraulic conductivity and activation energy are known (Mazur, 1984). Concurrently, it is important to determine the permeability of the spermatozoa plasma membrane to cryoprotectants and their effects on osmotically driven volume excursions because cryoprotectants are maximally effective after equilibration across the plasma membrane (Gao et al., 1995). The osmotically inactive fraction and isosmotic cell volume help to determine the linear behavior and the amount of swelling and shrinking that occurs during cryopreservation (Gilmore et al., 1995).

To calculate the osmotic response of spermatozoa it is hypothesized that spermatozoa behave as linear osmometers over a range of 145-900 mOsm. A Boyle van't Hoff (BVH) relationship is used to describe that the spermatozoa equilibrium volume is a linear function of the reciprocal of the extracellular osmolality of solutes:

$$\frac{V}{V_{iso}} = \frac{M_{iso}}{M}\left(1-\frac{V_b}{V_{iso}}\right)+\frac{V_b}{V_{iso}}$$

The linear behavior of the spermatozoa can then be analyzed by plotting the experimentally measured, normalized cell equilibrium volume (V/V_{iso}) vs. the reciprocal

of the normalized osmolality (M_{iso}/M). The activation energy of the hydraulic conductivity can be calculated from the following Arrhenius relationship:

$$L_p(T) = L_p(T_o) \exp\left[-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_o}\right)\right]$$

or by plotting the natural log of L_p as a function of the reciprocal temperature in °K, with the slope equaling the activation energy (Gilmore et al., 1995). Description of all symbols used in the equations is described in table 1.2.

The Kedem-Katchalsky membrane permeability model is used to characterize the cell volume changes in response to anisosmotic conditions and therefore determine hydraulic conductivity and cryoprotectant permeability (Kedem and Katchalsky, 1958). The equations used to calculate these properties are two coupled first-order nonlinear ordinary differential equations that describe total transmembrane volume flux and transmembrane permeable solute flux (Gilmore et al., 1995) as follows:

$$J_v = \frac{1dV(t)}{A_c dt} = -L_p \{(C^e_{salt} - C^i_{salt}) + \sigma (C^e_{CPA} - C^i_{CPA})\} RT$$

$$J_{CPA} = \frac{1dN_{CPA}}{A_c dt} = \overline{C}_{CPA} (1 - \sigma) J_v + P_{CPA} (C^e_{CPA} - C^i_{CPA})$$

Table 1.2. Description of symbols used in equations (Gilmore et al., 1995)

Symbol	Description	Units
L_p	water permeability	$\mu m \cdot min^{-1} \cdot atm^{-1}$
P_{CPA}	CPA permeability	cm/min
A_c	surface area of spermatozoa	μm^2
V	total cell volume	μm^3
V_b	osmotically inactive cell volume	μm^3
M	osmolality	Osm/kg H_2O
σ	reflection coefficient	-
N	moles of solute inside cell	moles
C	concentrations	molal
iso	subscripts (iso=isosmolality,	-
salt	salt=impermeable salts,	-
CPA	CPA=cryoprotectant)	-
e,i	superscripts (e=external, i=internal)	-
J	volume flux	
R	universal gas constant	L atm/mol K
T	absolute temperature	°K
t	time	
C_{CPA}	average of external and internal CPA concentrations	molal

Many of these characteristics have been described for bull, fowl and human spermatozoa using the "time-to-lyses" technique (Gao et al., 1992; Noiles et al., 1993; Watson et al., 1992). This technique utilizes the critical osmolality of spermatozoa at which 50% of the cells lyse to calculate hydraulic conductivity, activation energy and cryoprotectant permeability. Another method of measuring these properties is to measure actual volume changes in spermatozoa over time. Electronic particle counters have been used to measure cell volume changes in a wide variety of cell types (McGann et al., 1982) including spermatozoa (Gilmore et al., 1995, 1998). The basis behind electronic particle counters (Coulter Counters) is that there is an alteration of electrical resistance produced at the aperture by the cell, which displaces a volume of the conducting fluid. This produces an electrical pulse which is proportional to the volume of the cell that passes through the aperture. The most common analysis procedures include having the Coulter Counter interfaced to a microcomputer with Cell Size Analyzer-1s interface software (The Great Canadian Computer Company, Alberta, Canada). The Cell Size Analyzer software produces the data in two acquisition modes- cell volume vs. count histogram (Appendix I, Figure 1) and cell volume vs. time (Appendix I, Figure 2). The first mode is used to measure static cell volume (for analysis of isosmotic cell volume and osmotically inactive fraction while the second mode measures cell volume over a given time (for analysis of hydraulic conductivity and cryoprotectant permeability; Gilmore et al., 1995). These data are then transferred to a computer program (Coulter Counter Fitting; Liu, personal communication) where a line is fit to the experimental data (Appendix I, Figures 3,4) and then membrane transport parameters are established using MLAB software to find the best fit parameters (Appendix I, Figure 5). This data analysis

procedure has been validated for membrane permeability characteristics of human and boar spermatozoa (Gilmore et al., 1995).

The importance in obtaining a value for these membrane permeability characteristics is to optimize the cryopreservation protocol for a given species. Permeability of the spermatozoa plasma membrane to cryoprotectants appears to be the most important parameter because this is where the greatest membrane damage is occurring. By knowing the permeability characteristics of various cryoprotectants then an optimal cryoprotectant that reduces volume excursions can be used as well as developing a protocol for adding and removing this cryoprotectant successfully. An understanding of these membrane characteristics is critical in the cryopreservation of spermatozoa from species that are highly sensitive to this process, such as stallions, boars and mice.

Spermatozoa Membrane Lipid Composition

Phospholipids and sterols make up the majority of spermatozoal membranes. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) represent 70-80% of the total phospholipids while the predominant sterol found in the membranes of spermatozoa is cholesterol and to a lesser degree desmosterol (Parks and Hammerstedt, 1985). Differences in the lipid composition of the membranes occur between species resulting in different levels of resistance to cold-shock and cryopreservation damage (Darin-Bennett and White, 1977). Changes in the spermatozoa membrane lipid composition also occurs as a result of maturation in the epididymis. As spermatozoa travel from the caput (head) to cauda (tail) of the epididymis they become motile and fertile due to modifications of

membrane lipids and proteins (Rana and Majumder, 1990). Cholesterol concentration of boar and ram spermatozoa decreases as they are transported through the epididymis, whereas in goat spermatozoa an increase in cholesterol concentration is seen. However, an overall increase in the sterol to phospholipid ratio increases and results in an overall decrease in plasma membrane fluidity (Ladha, 1998; Parks and Hammerstedt, 1985; Rana and Majumder, 1990).

Parks et al. (1987) also reported a difference in the protein, phospholipid and cholesterol content of ejaculated bull sperm between the plasma membrane, outer acrosomal membrane and total membrane components of whole spermatozoa. Analysis across regions showed a significant accumulation of protein, phospholipid and cholesterol in the plasma membrane compared to the outer acrosomal membrane. Conclusions from this report stated that differences in membrane concentrations are necessary to maximize stability during environmental changes and also to minimize premature capacitation and acrosome reaction (Parks et al., 1987).

There have been very few reports discussing the composition of stallion spermatozoa membranes. Komarek et al. (1965) reported that phospholipids and cholesterol make up approximately 90% of the lipids in stallion spermatozoa. Phospholipids accounted for 59% while cholesterol made up 14% of the total dry matter weight. They also noted that stallion spermatozoa had a relatively low percentage of phospholipids compared to spermatozoa from other species and speculated that this lower percentage of phospholipids may be the reason stallion sperm are more sensitive to stress when compared to other species (Komarek et al., 1965; Maule, 1962). Johnson et al. (1980) also reported that stallion spermatozoa recovered from the epididymis are more

resistant to cold-shock damage than ejaculated spermatozoa, although only spermatozoa recovered from the cauda epididymis were motile. This change in cold-shock response from epididymal to ejaculated spermatozoa may be indicative of the loss of cholesterol from the plasma membranes as seen in other species.

Cyclodextrins

Cyclodextrins have been used in the drug industry to solublize compounds having poor aqueous solubility. Cyclodextrins are appropriate for the drug industry because they are amorphous, have good solubility, lack toxicity and form inclusion complexes with drugs that are amorphous (Pitha et al., 1988). These compounds have also proven useful in manipulating lipid content of cellular membranes (Christian et al., 1997; Yancey et al., 1996). Many forms of cyclodextrins exist, of which all are cyclic oligomers of glucose, containing either six (α), seven (β) or eight (γ) glucopyranose units. The external surface is hydrophilic, with the internal cavity lined with C(3)H and C(5)H hydrogen's and ether-like oxygen's that create a hydrophobic environment (Christian et al., 1997). Other modifications to the cyclodextrins, including hydroxylations and methylations, enhance their solubility in water as well as their ability to dissolve amorphous compounds. Compared to other cyclodextrins, β-cyclodextrins have the highest affinity for encapsulating sterols, in particular cholesterol (Irie et al., 1992) and modification of β-cyclodextrin to methyl-β-cyclodextrin also increases its efficiency in accepting cholesterol (Yancey et al., 1996). Cyclodextrins are able to efficiently add and remove sterols from cellular membranes by reducing the activation energy for sterol efflux/influx from ~20 kcal/mol to 7-9 kcal/mol (Yancey et al., 1996).

Cyclodextrins have been used widely to cause cholesterol efflux from various cellular membranes (Christian et al., 1997; Yancey et al., 1996), including spermatozoa (Iborra et al., 2000; Shadan et al., 2004; Visconti et al., 1999). An efflux of cholesterol is thought to be one of the initial events of sperm capacitation, cellular and molecular events necessary for a spermatozoon's ability to fertilize an oocyte (Tulsiani et al., 1997). Goat spermatozoa were able to undergo an acrosome reaction in the presence of 4-8 mM β-cyclodextrin after 150 min of incubation (30-35% acrosome reacted cells) with a 40-50% loss in membrane cholesterol (Iborra et al., 2000). Visconti et al. (1999) also reported the ability of mouse and bull spermatozoa to undergo protein tyrosine phosphorylation indicative of capacitation in the presence of 1 or 3 mM 2-OH-propyl-β-cyclodextrin or methyl-β-cyclodextrin in media devoid of bovine serum albumin. Human spermatozoa incubated with 2-hydroxypropyl-β-cyclodextrin were more likely to bind to the zona pellucida as assessed in a hemizona-binding assay (Parinaud et al., 2000). The increased binding was attributed to an increase in the number of capacitated spermatozoa due to a loss of cholesterol from the plasma membranes.

Klein et al. (1995) showed that cholesterol can also be incorporated into the plasma membrane of somatic cells. Cholesterol that has been incorporated into cyclodextrins is able to alter plasma membrane cholesterol content by forming a "cholesterol-containing pool in the aqueous phase by means of the cyclodextrin." The pool then establishes an equilibrium between the membrane bound cholesterol and that of the inclusion complex (Klein et al., 1995). The cholesterol concentration of Fu5AH rat hepatoma cells and Chinese hamster ovary cells increased in a dose dependant fashion as the concentration of cholesterol within the cyclodextrin was increased (Christian et al.,

1997). This group also showed that the cholesterol that had been loaded into the plasma membranes was metabolically available to the cells, as measured by esterification of cholesterol. A similar increase in cholesterol concentration within bull spermatozoa by increasing the dose of cholesterol-cyclodextrin complex was also seen (Purdy and Graham, 2004).

In Vitro Laboratory Assays

In vitro laboratory analyses of spermatozoa are unable to measure the actual fertilizing capabilities of the spermatozoon. However, progressive motility, normal spermatozoa morphology, the ability to undergo capacitation and the acrosome reaction are traits considered to be indicative of fertile spermatozoa (Graham, 1996). Measuring one attribute does not correlate highly with the fertility of a semen sample, but it is likely that analyzing multiple attributes may better estimate overall fertility (Amann and Hammerstedt, 1993).

MOTILITY

Progressive motility is the most common trait measured, especially on the farm (Pickett et al., 2000). Motility estimates can be performed either visually or with a computer assisted sperm analysis (CASA) system. Initial motility estimates should be performed at 37°C immediately after collection. Visual estimations are most effectively performed using a 200x magnification, phase-contrast microscope and analyzing a minimum of three to five fields on each slide. Spermatozoa are considered progressively

motile if they are moving reasonably rapidly across the field and have a 360° rotation of their head with each movement of their tail (Pickett et al., 2000).

Computer assisted sperm analysis (CASA) systems provide a more objective measurement of the motion characteristics of a large number of spermatozoa (Jasko et al., 1991). The CASA system takes consecutive video frames of the sample and stores the X and Y coordinates of the center of each spermatozoon's head into the computer memory. Algorithms then reconstruct each sperm's path to determine the motion characteristics (Budworth et al., 1988). Total and progressive motility are reported as well as other motion characteristics such as velocity, linearity and amplitude of lateral head displacement although the benefit of these other motion characteristics are not fully understood (Graham, 1996). Although CASA provides a means for an objective analysis of motility parameters, Kirk et al. (2005) reported a high correlation between visual motility estimates and the CASA system for both total and progressive motility ($r=0.95$ and $r=0.80$, respectively). This allows for a less expensive, but still accurate, analysis of motility for a stallion owner.

MORPHOLOGY

Gross morphology of a spermatozoon can be obtained by a simple staining procedure and analysis under a 1000x oil immersion light microscope. Eosin-nigrosin (Hancock's) is the most common stain for these evaluations. Eosin only enters into sperm cells with damaged plasma membranes. Damaged or dead spermatozoa appear pink, while the live, membrane intact spermatozoa are transparent. Nigrosin is a large

molecule that is impermeant to spermatozoa and provides a dark blue-purple background to allow observation of both live and dead sperm populations (Dott and Foster, 1972).

Normal morphology must be present in all aspects of the spermatozoon in order for it to be fertile (Ostermeier and Parrish, 1997). Morphological aberrations are determined at specific locations on the spermatozoon and can be broken down into head, neck, midpiece or tail abnormalities. Common head abnormalities include irregular or enlarged shape, acrosomal defects, double heads or detached heads; while proximal and distal droplets are the most common midpiece abnormality (Dott, 1975). A bend in the neck is considered an abnormal occurrence, however, abaxial placement of the midpiece and tail are considered normal in stallion spermatozoa, a trait that otherwise would be classified as abnormal in other species (Graham, 1996). Finally, abnormalities of the tail can result from looped, coiled, bent, threadlike or double tails (Dott, 1975). In general, stallions are considered to have acceptable morphology when their percentage of primary abnormalities (occurring in the testis) and secondary abnormalities (occurring in epididymis or post-ejaculation) is less than 40% (SFT, Breeding Soundness Exams).

FLOW CYTOMETRY

Flow cytometry allows for accurate and objective measurement of thousands of cells in a very short time period (<1 m). The use of flow cytometry can also measure multiple sperm attributes at one time. Viability, plasma and acrosomal membrane integrity, as well as mitochondrial membrane potential can all be measured with flow cytometry. Cells are stained with specific fluorescent dyes and are individually passed through a laser beam. If the cells have incorporated the dye into them, they are excited

by the laser and an emission of fluorescence is observed. Photomultiplier tubes measure the fluorescence and a computer quantifies these readings into valuable information, such as percentage of live versus dead cells (Garner et al., 1986).

Viability and membrane integrity can be easily measured using fluorescent microscopy or flow cytometry. A dual stain of propidium iodide (PI) and SYBR-14 is added to the cells and when excited by the laser dead cells will fluoresce red from the PI and live, membrane intact cells will fluoresce green from SYBR-14. PI stains the DNA of cells and is impermeant to the plasma membrane and therefore can only enter cells with a damaged membrane. SYBR-14 penetrates into all cells, membrane damaged or intact, and is used to identify cells from other debris in the sample (Garner and Johnson, 1995; Graham, 1996).

The acrosomal integrity of a spermatozoon is very important for its fertilizing potential. The acrosome of many species can be visualized using light or phase contrast microscopy. However, the acrosome of stallion spermatozoa is too small to visualize in this manner. Flow cytometric evaluation of the acrosome has been validated for many species, including the stallion (Cross et al., 1986; Farlin et al., 1992). Cross et al. (1986), reported that lectin from the common pea plant, *Pisum sativum* agglutinin (PSA), binds to the sugar residues on the acrosomal matrix. When this lectin is conjugated with a fluorescent probe, fluorescein isothiocyanate (FITC), the acrosomal integrity can be measured by a flow cytometer. Another common lectin used is the peanut lectin (PNA), *Arachis hypogaea*, and this probe stains the stallion acrosome brighter and with less nonspecific staining than PSA (Graham, 1996).

Visual or CASA aided assessment of spermatozoal motility has been poorly correlated with the estimated fertility of a stallion (Jasko et al, 1991; Graham, 1996). The energy source for motility is produced in the mitochondria of the middle piece of a spermatozoon. Changes in mitochondrial membrane potential may result in changes in sperm motility and be more indicative of spermatozoal function (Gravance et al., 2000; Gravance et al., 2001) Mitochondria function for stallion spermatozoa has been validated by Papaioannoiu et al. (1997) using rhodamine 123, however, this stain cannot differentiate between high and low membrane potential (Gravance et al., 2000). The lipophilic probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) has been reported to distinguish cells with high membrane potential (fluorescing orange) or low membrane potential (fluorescing green) (Reers et al., 1991) and has recently been validated for stallion spermatozoa (Gravance et al., 2000).

OOCYTE PENETRATION/ ZONA BINDING

In vitro penetration assays utilizing heterologous zona-free oocytes can provide information about sperm-oocyte interactions. These assays can evaluate a spermatozoon's ability to undergo the acrosome reaction and penetrate an oocyte. It is suggested that spermatozoa unable to penetrate the ovum in vitro, may indicate an inability of the spermatozoa to undergo capacitation and the acrosome reaction and fertilize an oocyte in vivo (Brackett et al., 1982). The use of zona free hamster oocytes for performing these studies provides accurate results while minimizing the costs of oocyte collection from homologous species (i.e. horse or cow). Brackett et al. (1982)

described this test as an accurate application to distinguish between potentially fertile or potentially infertile animals.

The ability of spermatozoa to bind to and penetrate the zona pellucida (ZP) evaluates spermatozoa function in a more complex manor than just oocyte penetration. In order for a spermatozoon to bind to the ZP, they must be capable of undergoing capacitation, have a functioning/intact acrosome that is able to undergo the acrosome reaction and contain functional ZP receptors along their equatorial segment. The ability of spermatozoa to bind to homologous ZP has been correlated with fertilizing potential of the male (Braundmeier et al., 2003; Fazeli et al., 1993; Meyers et al., 1996; Zhang et al., 1998). While this assay provides valuable information on fertilizing potential, the ability to obtain equine oocytes in the United States is limited. Sinowatz et al. (2003) described a heterologous ZP binding assay for use in stallions. They were able to show that stallion spermatozoa were able to bind to bovine oocytes firmly, undergo an acrosome reaction upon binding, penetrate the ZP of bovine oocytes and even fuse with the oocyte. The use of bovine oocytes for analysis of stallion spermatozoa's binding ability provides increased sources of oocytes at lower cost, compared to equine oocytes.

FERTILITY TRIALS

The true test of a stallion's fertility is the percentage of mares that become pregnant after mating or insemination. This can be described in many ways: 1) percentage of mares from which embryos are flushed after insemination; 2) percentage of mares pregnant after insemination during a single heat cycle; 3) percentage of inseminated mares that produce offspring over many heat cycles; or 4) number of foals

produced per mares inseminated (Graham, 1996). Each of these tests has different levels of maternal input that are not reflected in a semen analysis. In addition, in vivo fertility trials are expensive to conduct and may present low accuracy of semen evaluation, as most fertility trials are performed using only a small number of mares which, decreases the power and accuracy of the experiments (Graham, 1996).

Conclusions

From this review it is evident that stallion spermatozoa are extremely sensitive to cryopreservation processes. Optimal freezing protocols and genetic selection has led to fertility rates of frozen bovine spermatozoa comparable to that of fresh spermatozoa, however, this is not the case with frozen stallion spermatozoa where fertility is markedly reduced compared to that of fresh spermatozoa. Alteration of the lipid profile of the plasma membrane should result in greater numbers of motile and viable spermatozoa. If the cholesterol to phospholipid ratio of stallion spermatozoa can be increased from 0.36 to a value close to human or rabbit spermatozoa, the spermatozoa should be able to tolerate cold shock and cryopreservation better. By increasing the cholesterol content of stallion spermatozoa, these spermatozoa should have increased membrane fluidity at lowered temperatures, undergo the phase transition at lower temperatures, and have increased membrane permeability to cryoprotectants.

The studies presented in subsequent chapters were conducted to determine if the addition of cholesterol will indeed increase the cryosurvival of stallion spermatozoa. Studies were performed to determine an optimal level of cholesterol-loaded-cyclodextrin (CLC) to add to stallion spermatozoa to maximize post-thaw motility and viability, as

well as quantify the amount of cholesterol that incorporates into the membranes of stallion spermatozoa. The effect of CLC's on the loss of cholesterol during cryopreservation was also investigated, as well as how well CLC-treated spermatozoa bind to the zona pellucida. Since osmotic damage causes decreased cryosurvival, CLC-treated spermatozoa were compared to control samples to observe their osmotic tolerance limits and the changes in membrane permeability to various cryoprotectants. In addition, the optimal cooling rate of stallion spermatozoa using a programmable cell freezer was analyzed.

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Chapter II

Adding Cholesterol to the Stallion Sperm Plasma Membrane Improves Cryosurvival

Introduction

The use of cryopreserved equine sperm has increased in the United States since the large breed organizations now permit foals produced from frozen semen to be registered. However, cryopreservation induces partially irreversible damage to sperm that results in reduced fertility for frozen sperm from many stallions compared to fresh or cooled semen. Part of this damage occurs to sperm membranes when the cells are cooled from room temperature (22°C) to 1°C. As stallion sperm are cooled below 18°C the membrane phospholipids undergo a phase transition from a liquid to gel state (Amann and Pickett, 1987). During this phase transition, phospholipids are lost from the plasma membrane leading to increased membrane permeability, membrane disruption and cell death (Darin-Bennett et al., 1973; Watson, 1981). Previous studies indicate that the cholesterol:phospholipid ratio of the plasma membrane is a major determinant in plasma membrane fluidity and stability during cryopreservation (Darin-Bennett and White, 1977; Watson, 1981). Cholesterol reduces the transition temperature of membranes, and maintains them in a fluid state at reduced temperatures thereby reducing the membrane damage that occurs at low temperatures (Amann and Pickett, 1987). Sperm from species that posses very high cholesterol:phospholipid ratios, such as human and rabbit sperm, do not experience this membrane damage when cooled (Darin-Bennett and White, 1977).

To prevent phospholipid rearrangement and to increase membrane fluidity at low temperatures, cholesterol can be added to the plasma membrane of several cell types (Klein et al., 1995; Ladbrooke et al., 1968; Rottem et al., 1973), including sperm (Combes et al., 2000; Purdy and Graham, 2004a). When cholesterol is added to synthetic liposomal membranes, it reduces the temperature at which the phase transition occurs and at high concentrations eliminates the phase transition altogether (Ladbrooke et al., 1968; Rottem et al., 1973). Cholesterol can easily be incorporated into or extracted from the plasma membranes of cells using cyclodextrins. Cyclodextrins, cyclic heptasaccharides consisting of β (1-4) glucopyranose units, are water soluble but have a hydrophobic center (Purdy and Graham, 2004a) and can transport cholesterol into or out of membranes down a concentration gradient (Klein et al., 1995). When cholesterol is loaded in cyclodextrins (CLC) and the CLC's added to bovine sperm prior to cryopreservation, higher percentages of motile and viable sperm were recovered after thawing compared to untreated sperm (Purdy and Graham, 2004a). Similar results have also been reported for stallion sperm treated with a single concentration of CLC's (Combes et al., 2000). Whereas this procedure has been optimized for bull sperm (Purdy and Graham, 2004a) it has not been optimized for stallion sperm.

The objectives of these experiments were to determine 1) the concentration of cholesterol-loaded-cyclodextrins that needs to be added to stallion sperm prior to centrifugation and cryopreservation to optimize sperm cryosurvival; 2) the amount of cholesterol in the sperm plasma membrane after the addition of CLC's both prior to and after cryopreservation; and 3) the ability of equine sperm treated with CLC's to bind to the zona pellucida.

Materials and Methods

Unless otherwise specified all chemicals were of reagent grade (Sigma Chemical Co., St. Louis, MO).

Preparation of CLC's:

Cyclodextrins were prepared as described by Purdy and Graham (2004a). Briefly, to load cholesterol into the cyclodextrins, 1g of methyl-β-cyclodextrin was dissolved into 2 ml of methanol in a glass test tube. In a second glass test tube, 200mg of cholesterol was dissolved into 1 ml of chloroform and a 450 µl aliquot of this solution added to the methyl-β-cyclodextrin solution. The combined cyclodextrin/cholesterol solution was thoroughly mixed, and the solvents then removed using nitrogen gas. The resulting crystals were stored in a glass vial at 22°C until use. To add cholesterol to sperm, a working solution of CLC's was made by adding 50 mg of CLC to 1 ml of a modified Tyrode's medium (TALP; Moore et al., 2005), mixed vigorously with a vortex and incubated in a 37°C water bath until use. The CLC working solution was mixed again, using a vortex mixer, prior to any aliquot being removed for addition to sperm.

Semen Collection:

A total of 17 stallions were collected for the following experiments. Stallions ranged from 5-22 years of age; were of Thoroughbred, Quarter Horse, Arabian and mixed breeds; and were on a regular semen collection schedule (3 times/week). Semen was collected from each stallion using a Colorado model artificial vagina with an in-line gel filter (Animal Reproduction Systems, Chino, CA) and the semen was used within 45 min

of collection. All stallions were maintained under the guidelines presented by the Colorado State University's Animal Care and Use Committee.

Experiment 1: Effect of CLC's on Frozen-Thawed Stallion Sperm

Ejaculates from 15 stallions were used for this experiment. Single ejaculates from stallions with > 40% post-thaw progressive motility ("Good" freezers, n=8) and two ejaculates from stallions with < 40% post-thaw progressive motility ("Poor" freezers, n=7) were used. Sperm from each ejaculate were diluted to a concentration of 120 x 10^6 sperm/mL in TALP and divided into seven aliquots of 600 x 10^6 total sperm. Sperm were treated with varying levels of CLC's (0, 0.5, 1.5, 3.0, 4.5, 6.0 or 7.5 mg CLC/ 120 x 10^6 sperm) and the cells incubated at room temperature (~22°C) for 15 minutes. Following incubation the cells were diluted 1:1 (v:v) with a skim milk, glucose diluent (EZ-Mixin; "BF", Animal Reproduction Systems, Chino, CA) and the samples centrifuged at 600g for 10 minutes. The supernatant was removed, sperm concentration in the remaining pellet determined, and the cells resuspended to a final concentration of 200 x 10^6 sperm/ml in Lactose-EDTA freezing extender containing 5% glycerol (EZ-Freezin' "LE", Animal Reproduction Systems, Chino, CA). The sperm were then packaged into 0.5 cc straws and frozen in a programmable freezer (Kryo 10 Series III, Planer, Middlesex, UK) at a rate of -10°C/min from 20°C to -15°C and then -15°C/min from -15°C to -120°C. At -120°C, straws were plunged into liquid nitrogen and stored until analyzed for motility and viability.

One straw from each treatment was thawed in a 37°C water bath for 30 seconds for assessment of motility using a computer-assisted sperm analysis system (CASA) (HTM

IVOS; Hamilton-Thorne Biosciences, Beverly, MA). The contents of each straw was diluted 1:4 (v:v) in EZ-Mixin' BF to give a final concentration of 20 x 10^6 sperm/ml and each sample maintained at room temperature for approximately 10 minutes before analysis. System parameters for these analyses were 45 frames acquired at 60 frames per second; minimum contrast 70, minimum cell size 4 pixels; lower VAP cut-off 20 μm/s; lower VSL cut-off 0 μm/s; VAP cut-off for progressive cells 50 μm/s and straightness 75%. A 5-μl drop of sperm from each sample was placed on a preheated (37°C) slide and a minimum of 200 sperm per sample analyzed.

One straw from each treatment was thawed as previously described to assess the percentage of viable sperm, using flow cytometry. Cells were diluted to 40 x 10^6 sperm/ml in TALP and 100 μl (4 x 10^6 cells) was placed into 500 μl of TALP containing 10 μl of propidium iodine (PI, 1 mg/ml in H_2O) and 20 μl of SYBR-14 (Molecular Probes, Eugene, OR; 10μM in Me_2SO). Samples were incubated at room temperature for 10 minutes prior to analysis. A minimum of 50,000 sperm per sample were analyzed using an EPICS V flow cytometer (Coulter Electronics, Miami, FL) equipped with Cicero computer software (Cytomation, Fort Collins, CO) as described by Purdy and Graham (2004a). Briefly, the fluorescent probes were excited with a 488 nm argon laser and the fluorescence of PI and SYBR-14 detected using a filter setup including a 515 nm long-pass filter to block laser light, a 590 nm dicroic beam splitting filter, a 530 nm short-pass filter to detect SYBR-14, and a 630 nm long-pass filter to detect PI.

Experiment 2: Amount of Cholesterol Incorporating into Stallion Sperm

Semen from 12 stallions was collected and a total of 3.5 x 10^9 sperm from each ejaculate was diluted to 200 x 10^6 sperm/ml with TALP. The sperm sample was then divided into 7 aliquots and each aliquot treated with 0, 0.5, 1.5, 3.0, 4.5, 6.0, or 7.5 mg CLC/120 x 10^6 sperm. The samples were incubated as described previously prior to being centrifuged through a 45% Percoll gradient at 600g for 25 minutes and the resulting sperm pellet suspended in 3 ml of phosphate buffered saline (PBS; 171 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The sperm were washed a second time (600g for 10 min), resuspended in 0.5 ml of PBS and the sperm concentration determined. The samples were stored at -20°C until cholesterol analysis was performed.

The amount of cholesterol in each sample was determined using the Cholesterol Liquicolor enzymatic assay (Stanbio, Boerne, TX) as described by Navratil et al. (2003). Briefly, cells were diluted 1:1 (v:v) with lysate buffer (0.4% Triton X-100 in PBS) for 1h to solublize the plasma membranes. Samples were diluted 1:5 (v:v) with reagent and allowed to incubate for 25 min at 37°C. Samples were then centrifuged to remove the cells and the supernatant was analyzed for cholesterol content via spectrophotomer (Spectronic Genesys 5, Phoenix, AZ) with wavelength set to 500 nm. Cholesterol concentrations of the unknown samples were compared to a standard curve from known amounts of cholesterol.

Experiment 3: Cholesterol Concentration in the Sperm Plasma Membrane Before and After Cryopreservation

Semen from 14 stallions was extended to 120 x 10^6 in TALP and each ejaculate divided into 2 sub-groups (fresh and frozen). Fresh samples were again divided into 2 aliquots and treated with 0 or 1.5 mg CLC/ 120 x 10^6 sperm, as described previously. After incubation, the cells were centrifuged through a 45% Percoll gradient at 600g for 25 min and then washed free of Percoll, as described above and the total number of sperm in each sample determined. Samples to be frozen were similarly divided into 2 aliquots and treated with 0 or 1.5 mg CLC/120 x 10^6 sperm, as described. The samples were then processed for cryopreservation as described in experiment one, with the exception that the sperm were frozen at a final concentration of 400 x 10^6 sperm/ml. Five straws (1 x 10^9 sperm) were thawed, the contents pooled and then centrifuged through 45% Percoll and prepared as the fresh samples. The cholesterol content of the samples was determined as described above.

Experiment 4: Fluorescent Imaging of Cholesterol Incorporation into Stallion Sperm

Semen from 3 stallions was extended to 60 x 10^6 sperm/ml in TALP and incubated with 3.0 mg CLC/ 120 x 10^6 sperm in which the cyclodextrin had been pre-loaded with cholesterol in which 20% of the cholesterol was labeled with NBD (N-1148; Molecular Probes, Eugene, OR). After incubation, 5-µl drops of sample were placed onto pre-heated glass slides (37°C) and examined using a Nikon Optishot-2 fluorescent microscope equipped with a 450-490 nm excitation filter, a 505 nm dichroic mirror and a 520 nm long pass filter, to determine which sperm membranes contained fluorescence.

Experiment 5: Zona Binding Assay of CLC Treated Stallion Sperm

Sperm Preparation

Straws of cryopreserved sperm for six of the stallions in experiment one were used to determine the ability of cryopreserved stallion sperm treated with CLC's to bind to the zona pellucida of bovine oocytes.

For each replicate, a single straw from each of two different stallions was thawed as described above and the contents of each straw diluted 1:3 (v:v) with TALP. The sperm were then washed by centrifugation at 400g for 5 min, suspended in 1 ml of TALP containing 35 μg/ml Hoechst 33342 (ICN Biomedical, Inc., Aurora, OH) and incubated for 15 min at 37°C. The sperm were then washed again (400g for 5 min) and suspended to a final concentration of 2 x 10^6 sperm/ml in TALP. Five μl aliquots (10,000 sperm) from each sperm suspension were added to the droplets containing 10 oocytes.

Oocyte Preparation

Immature bovine oocytes were recovered from ovaries obtained from a local abattoir and transported to the laboratory in saline at 37°C within 4-5 h of collection. Follicles ranging from 2-6 mm were aspirated with a 20g needle and the follicular fluid searched for oocytes under a stereomicroscope and oocytes placed into TALP after which the cumulus cells were removed using a vortex mixer at maximum speed for 2 min. The denuded oocytes were washed in TALP and stored in a hyperosmotic salt solution until use (Coutinho da Silva et al., 2005).

Zona Binding Assay

Prior to adding sperm, the oocytes were washed several times in TALP and then incubated in TALP for approximately 1h at 38.5°C in an atmosphere of 5% CO_2 in air. After incubation, 10 oocytes were randomly placed into 45µl droplets of TALP (1 droplet/treatment) to which sperm were added. Oocytes and sperm were incubated together at 38.5°C in an atmosphere of 5% CO_2 in air for 2h after which the oocytes were washed four times in TALP, using a small-bore fire polished glass pipette to remove loosely bound sperm. Groups of five oocytes were placed onto glass slides and covered with a cover slip supported by a mix of paraffin wax and petroleum jelly. Oocytes were viewed using an epifluorescence microscope (Eclipse E800, Nikon Instruments, Inc., Melville, NY) equipped with a 360/40 nm band pass excitation filter and a 460/50 nm band pass emission filter. The total number of sperm bound to each zona pellucida was determined at 400x magnifications.

Statistical Analysis:

Data for experiments 1-4 were analyzed by analysis of variance (ANOVA) and treatment differences separated by Student-Newman-Keuls (SNK) mean separation technique (SAS Institute Inc., 1985). Treatments were considered different if $P<0.05$. Data from experiment one was transformed to minimize variation due to stallions. Data were transformed within each ejaculate by subtracting each CLC treatment from its own control value. A regression analysis was preformed on data from experiment three to determine the fit of the regression line. In experiment five, data were transformed by square root to adjust for unequal variances. To find the binding potential of an individual

sperm cell data was also normalized to take into account the percentage of motile sperm added to each droplet of oocytes. To find the number of motile sperm bound to the zona pellucida the average of the total number of sperm bound for each stallion was divided by the percent motile sperm. Data were then analyzed by paired-t test for the pair-wise comparison between treatments (SAS Institute Inc., 1985). For all experiments, untransformed means are reported.

Results

Experiment 1:

The percentages of total and progressively motile sperm were similar for control samples and samples containing 0.5, 1.5, 3.0 or 4.5 mg CLC's ($P>0.05$; Table 2.1). When data for stallions were grouped into "poor" and "good" categories, the percentages of motile sperm from "poor freezer" stallions after cryopreservation, was significantly higher when the sperm were treated with 1.5 mg CLC (67%) compared to control samples (50%; $P<0.05$; Table 2.1).

Sperm frozen at each concentration of CLC's exhibited significantly higher percentages of viable sperm compared to sperm frozen in the absence of CLC's for all stallions regardless of freezing category ($P < 0.05$; Table 2.1).

Table 2.1: The percentage of total and progressively motile stallion sperm treated with varying levels of cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation. Stallions were divided into "Good" freezers (n=8) and "Poor" freezers (n=7) based on post-thaw progressive motility, with two ejaculates being collected from "Poor" freezers and the average mean reported.

	CLC Dose (mg/120 x 10^6 cells)							
	0	0.5	1.5	3.0	4.5	6.0	7.5	SEM
All Stallions (n=15)								
Total	62^{ab}	70^{a}	72^{a}	64^{ab}	55^{b}	43^{c}	38^{c}	4
Progressive	45^{ab}	49^{a}	50^{a}	39^{b}	29^{c}	20^{d}	16^{d}	3
Viability	47^{a}	55^{b}	56^{b}	55^{b}	56^{b}	55^{b}	52^{b}	2
"Poor" Freezers								
Total	50^{b}	64^{ab}	67^{a}	65^{ab}	58^{ab}	51^{ab}	48^{b}	4
Progressive	32^{abc}	42^{ab}	45^{a}	37^{ab}	29^{bc}	22^{c}	19^{c}	4
Viability	43^{a}	52^{b}	53^{b}	52^{b}	55^{b}	54^{b}	52^{b}	2

^{a,b,c,d} Different superscripts within rows indicate treatment differences ($P < 0.05$).

Experiment 2:

The amount of cholesterol that became incorporated into the stallion sperm increased in a polynomial fashion (Figure 2.1; μg Cholesterol = -0.0149 (mg CLC)^2 + 0.246 (mg CLC) + 0.2291; $R^2 = 0.9978$). The amount of cholesterol within the sperm when 0, 0.5, 1.5, 3.0, 4.5, 6.0, or 7.5 mg CLC are added was 0.23, 0.34, 0.56, 0.86, 1.03, 1.14 and 1.26 μg cholesterol/10^6 sperm. When ≥ 1.5 mg CLC was added, the amount of cholesterol in the sperm was higher than control sperm (Figure 2.1; $P<0.05$).

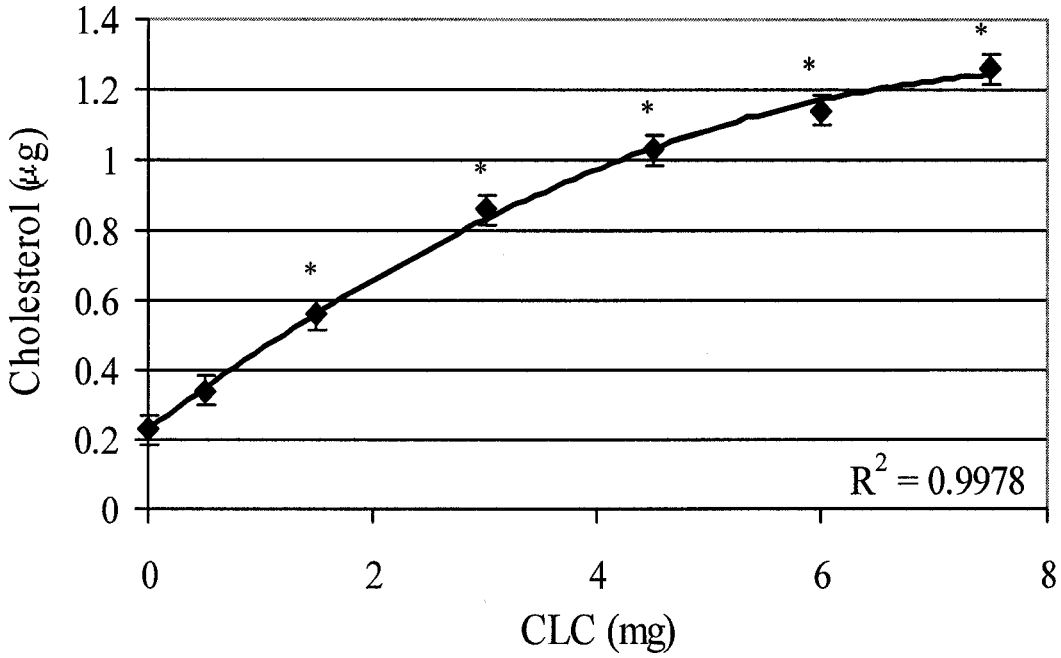

Figure 2.1. The concentration of cholesterol (µg/10^6) within the sperm plasma membrane when increasing levels of CLC's (mg/120 x 10^6 sperm) were added to fresh cells (n=12). * Denotes difference from control value (*P<0.05*).

Experiment 3:

The concentration of cholesterol in control and CLC treated cells decreased after cryopreservation (0.28 vs. 0.20 µg cholesterol/10^6; Figure 2.2; $P < 0.05$). However, the addition of CLC's to the sperm increased the cholesterol in sperm both before and after cryopreservation (0.63 vs. 0.52 µg cholesterol/ 10^6; Figure 2.2; $P<0.05$).

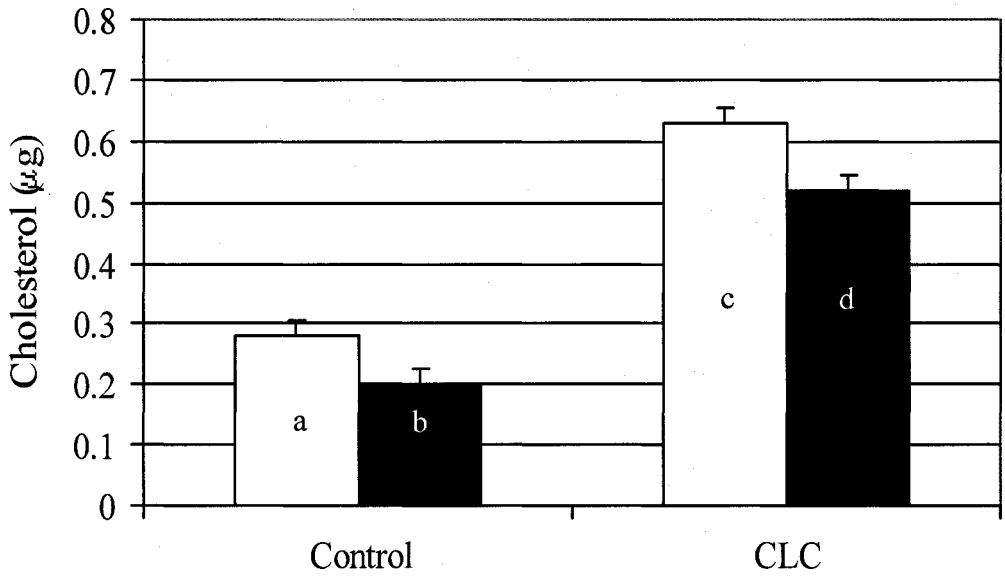

Figure 2.2. The concentration of cholesterol (µg/10^6 sperm) in the sperm plasma membrane of cells treated with 0 or 1.5 mg CLC/120 x 10^6 sperm. The concentration of cholesterol was determined both before (□) and after (■) cryopreservation (n=14). [a,b,c,d] Different labels denote treatment differences ($P<0.05$).

Experiment 4:

The cholesterol incorporates into all sperm membranes. However, the mitochondria and acrosomal compartments appear to be more heavily labeled (Figure 2.3).

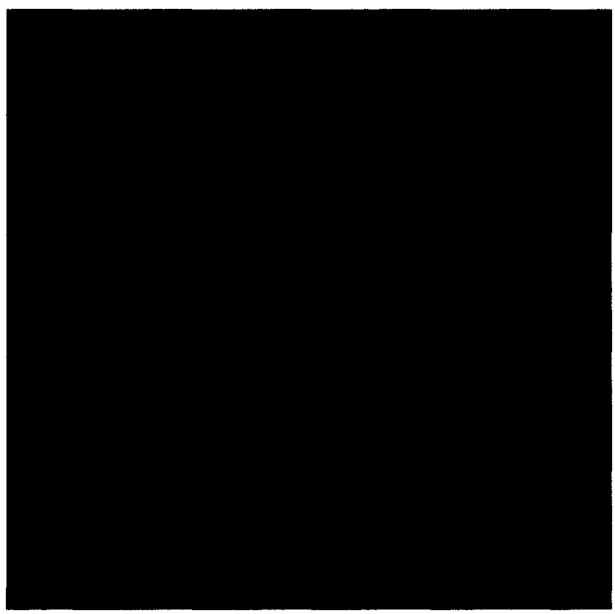

Figure 2.3. Stallion sperm incubated with 3.0 mg CLC/ 120 x 10^6 sperm with 20% of the cholesterol loaded into the cyclodextrin labeled with NBD.

Experiment 5:

Treatment with CLC's prior to cryopreservation resulted in more sperm binding to the zona pellucida of bovine oocytes compared to control cells (48 vs. 15; $P<0.05$; Table 2.2). When data were normalized to determine the binding potential of an individual sperm, CLC treated sperm were 2.76 times more likely to bind to the zona pellucida than control sperm (Table 2.2).

Table 2.2. The total number of stallion sperm bound (#SB) and total number of motile stallion sperm bound (#MSB) to bovine zona pellucida. Sperm were cryopreserved with 0 or 1.5 mg CLC/ 120 x 10^6 sperm for analysis (n=6). Data presented as mean ± SEM.

	Sperm Treatment	
	Control	CLC
# SB	15 ± 3^a	48 ± 18^b
# MSB	0.231 ± 0.023^a	0.638 ± 0.205^b

[a,b] Different superscripts within rows indicate treatment differences ($P<0.05$).

Discussion

Equine sperm are damaged during the cryopreservation process by formation of intracellular ice crystals and plasma membrane damage (Mazur, 1977; Steponkus et al., 1983). The damage to the sperm membranes occurs as these membranes undergo a phase transition from a liquid to crystalline state resulting in the membrane becoming rigid and porous to extracellular molecules (Amann and Pickett, 1987). It has been shown that increasing the cholesterol:phospholipid ratio within the plasma membrane of many cell types can reduce the temperature at which the phase transition occurs as well as maintain a more fluid membrane at lower temperatures (Klein et al., 1995; Ladbrooke et al., 1968; Rottem et al., 1973).

This study reports similar findings to Combes et al. (2000) in that cholesterol loaded into methyl-β-cyclodextrin improves both the percentages of motile and viable sperm after cryopreservation. When individual stallions were analyzed, stallion's considered to be "poor" freezers and treated with CLC's had an increase in the percentage of motile cells after cryopreservation, while all stallions exhibited an increase in the percentage of viable cells after freezing indicating a protective effect of cholesterol on the plasma membrane during cryopreservation. The optimal level of CLC for cryopreservation of equine sperm was 1.5 mg CLC/120 x 10^6 sperm, which is similar to that reported for bull sperm (Purdy and Graham, 2004a).

The concentration of cholesterol that incorporated into stallion sperm increased in a polynomial fashion with CLC level ($R^2 = 0.9978$). It is likely that as CLC concentration increased beyond 3.0 mg, the sperm became saturated with cholesterol. A similar phenomenon is seen for bull sperm treated with CLC's (Purdy and Graham, 2004a). This indicates that there is a finite amount of cholesterol that can incorporate into the sperm. The addition of 1.5 mg CLC increases the cholesterol concentration within the stallion sperm membranes approximately 2.5 times. This increase in cholesterol, therefore, increases the cholesterol:phospholipid ratio from 0.36 (Parks and Lynch, 1992) to approximately 0.82-0.89. This increase in cholesterol increases the cholesterol:phospholipid ratio of stallion sperm to values reported for human and rabbit sperm, both of which are resistant to cold-shock damage (Darin-Bennett and White, 1977). The actual concentration of cholesterol within the membranes of stallion sperm have not been reported, however, the values of cholesterol reported in this study lie between reported values of boar and ram sperm (0.16 and 0.28 μg/10^6 sperm;

respectively) (Darin-Bennett and White, 1977; Cerolini et al., 2001). This also coincides with the cholesterol:phospholipid ratios of 0.26, 0.36 and 0.38 for the boar, stallion and ram (Parks and Lynch, 1992). When fluorescent microscopy was used to visual the incorporation of cholesterol into the stallion sperm, cholesterol could be visualized throughout the entire sperm. Areas containing more membranous structures (acrosomal cap and mitochondria) appeared to fluoresce brighter.

Cerolini et al. (2001) reported that boar sperm lose approximately 50% of the cholesterol from the plasma membranes after the cells have been cryopreserved. This may be one of the primary causes of a "pre-mature" capacitation phenotype seen in cryopreserved sperm cells. It is known that a loss of cholesterol from the plasma membrane is one of the first stages of capacitation which decreases the stability of the membrane (Tulsiani et al., 1997). This "pre-mature" capacitation state of cryopreserved sperm is thought to be one major reason why longevity and viability in these cells are decreased and why they must be inseminated closer to the time of ovulation (Ashworth et al., 1994; Watson, 1995). Data from experiment three shows that stallion sperm also lose a significant amount (28%) of cholesterol from the plasma membranes during the cryopreservation process. When 1.5 mg CLC is added to the sperm prior to freezing, the cholesterol concentration is maintained at a higher level after cryopreservation. This higher cholesterol concentration during cryopreservation may inhibit these cells from undergoing "pre-mature" capacitation and allow the sperm to remain viable for a longer period of time. This may reduce the need for intense mare management when frozen semen is being used.

The zona binding assay determined if sperm treated with CLC's prior to cryopreservation could undergo capacitation and bind to the zona pellucida (ZP). Previous studies have shown that equine sperm bind equally well to equine as bovine ZP (Coutinho da Silva et al., 2005; Sinowatz et al., 2003). Sinowatz et. al. (2003) reported that equine sperm bound tightly to bovine ZP, underwent the acrosome reaction and penetrated the ZP after 4h of incubation. This suggests that there are similar ZP protein ligands and sperm receptors between bovine and equine species. A sperm's ability to bind to the ZP has been correlated with fertility of several species, including stallions (Braundmeier et al., 2004; Fazeli et al., 1993a,b; Fazeli et al., 1995; Meyers et al., 1996). In this study, there was a significant increase in the total number of sperm bound to the ZP when sperm were treated with CLC's. This increase was also observed when the data were normalized to account for the number of motile sperm co-incubated with the oocytes. It is thought that sperm that have been treated with CLC's are protected from the acute membrane damage and/or loss of sperm/oocyte receptors that occurs during cryopreservation. This may explain why more CLC-treated sperm were able to bind to the ZP than control sperm. Although it was not specifically observed in this experiment, previous reports suggest that the sperm that have bound to the ZP will undergo an acrosome reaction and are capable of penetration (Sinowatz et al., 2003). When CLC-treated bovine sperm were used for *in vitro* fertilization, no differences were observed between the ability of CLC-treated or control sperm to fertilize oocytes (Purdy and Graham, 2004b). However, there was a slight increase in pregnancy rates of CLC treated sperm (59% vs. 50%) (Purdy and Graham, 2004b). *In vitro* fertilization assays are not

practical using equine gametes and further experiments including a fertility trial need to be preformed to determine the actual fertilizing potential of CLC treated equine sperm.

Conclusions

Adding cholesterol to stallion sperm prior to cryopreservation increases the cryosurvival rates of stallion sperm. This is a simple procedure that alters the cholesterol:phospholipid ratio of sperm that are affected by cold shock and cryopreservation damage. The data reported in this study shows the positive results of CLC addition to stallion sperm by using several *in vitro* laboratory assays including increasing the percentages of motile and viable sperm, to maintaining higher cholesterol levels within the sperm after cryopreservation, as well as increasing the ability of the sperm to bind to the zona pellucida. This technique may prove beneficial to cryopreserving sperm from all stallions in general, but more importantly from stallions whose sperm routinely do not freeze in acceptable condition.

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Chapter III

Osmotic Tolerance Limits and Membrane Permeability Characteristics of Stallion Spermatozoa Treated with Cholesterol

Introduction

Cryopreservation of spermatozoa causes irreversible cell damage in which only 30-40% of the spermatozoa are viable upon thawing (Watson, 2000). This leads to reduced fertility for cryopreserved spermatozoa when compared with non-cryopreserved samples. Formation of intracellular ice crystals due to improper freeze rate and/or destabilization of the plasma membrane are thought to be the major source of damage to spermatozoa (Mazur, 1984; Steponkus et. al., 1983). Other significant sources of cellular damage during cryopreservation include osmotic and oxidative stresses (Watson, 2000). Many of these damaging events have been characterized in spermatozoa from several species, but little data are reported on equine spermatozoa. By increasing our knowledge of the damage that occurs to equine spermatozoa during cryopreservation, we will then be able to optimize the freezing/thawing process to maximize the survival of spermatozoa from individual stallions.

Osmotic stress to the spermatozoa occurs when cryoprotectants are added to and removed from the spermatozoa. Addition of permeating cryoprotectants creates an anisosmotic environment for the spermatozoa, that when removed causes large increases in cell volume leading to membrane damage or lysis (Gilmore et. al., 1995; Gilmore et.

al., 1998; Guthrie et. al., 2002). Equine spermatozoa have been reported to have a very narrow osmotic window of survival compared to human or bull spermatozoa and are similar to that of boar spermatozoa (Ball and Vo, 2001; Guthrie et. al., 2002). To reduce the osmotic stress imposed on spermatozoa, an increase in the permeability of the plasma membrane is necessary.

Glycerol is the most commonly used cryoprotectant for cryopreservation of equine spermatozoa; however, glycerol may also be the cryoprotectant that causes the greatest osmotic damage because its permeability to the plasma membrane is much lower than that of water and many other cryoprotectants (Guthrie et. al., 2002). Alternatively, low molecular weight (MW) cryoprotectants may prove less damaging to sperm, as they are more permeable to the plasma membrane than glycerol and will equilibrate across the plasma membrane more rapidly decreasing the abrupt volume changes that occur to spermatozoa during cryoprotectant removal. For this reason, dimethyl formamide, a low MW cryoprotectant, resulted in higher percentages of motile stallion spermatozoa after freezing than spermatozoa cryopreserved with glycerol (Medeiros et. al., 2002). Ethylene glycol, another low MW cryoprotectant, has been used to freeze equine embryos (Seidel, 1996), but few studies have evaluated its effect on cryopreserving stallion spermatozoa and results have varied (Squires et al., 2004).

Changing the permeability of the plasma membrane is another way to reduce the effects of osmotic stress placed on spermatozoa during cryopreservation. Cholesterol has been shown to reduce the temperature at which the membrane phase transition occurs in cells, thereby maintaining a more fluid plasma membrane at low temperatures (Ladbrooke et. al., 1968; Rottem et. al., 1973). Cholesterol can easily be incorporated

into or extracted from the plasma membrane of cells using cyclodextrins (Klein et al., 1995). Cyclodextrins, cyclic heptasaccharides consisting of β (1-4) glucopyranose units, are water soluble but have a hydrophobic center (Purdy and Graham, 2004) and can transport cholesterol into or out of membranes down a concentration gradient (Klein et. al., 1995). Cholesterol that has been loaded into cyclodextrins (CLC) and added to spermatozoa prior to cryopreservation resulted in higher percentages of motile and viable spermatozoa after thawing of several species, including stallions (Combes et. al., 2000; Moore et al., 2005a; Morrier and Bailey, 2004; Purdy and Graham, 2004). If cholesterol benefits spermatozoa by increasing membrane fluidity, then it should also increase the permeability of cryoprotectants across the plasma membrane, and this could have additional benefits by minimizing osmotic damage.

Most of the studies that have been conducted on freezing and thawing of stallion spermatozoa have been based on empirical methods. These generally have involved various cooling rates and various cryoprotectants without knowledge of their mobility through the stallion plasma membrane. If one could determine the permeability of stallion sperm to water as well as to various cryoprotectants, it would then be possible to design an ideal protocol for freezing stallion spermatozoa that would minimize cell damage. Therefore, the objectives of this study were to determine: 1) the osmotic tolerance limits of equine spermatozoa in the presence of cholesterol; 2) the isosmotic cell volume (V_{iso}), osmotic response and osmotically inactive cell volume (V_b) for equine spermatozoa; and 3) the hydraulic conductivity (L_p) and permeability coefficients (P_{cpa}) for three different cryoprotectants of equine spermatozoa in the presence of cholesterol.

Materials and Methods

Preparation of CLC's:

Cyclodextrins were prepared as described by Purdy and Graham (2004). Briefly, to load cholesterol into the cyclodextrins, 1g of methyl-β-cyclodextrin was dissolved into 2 ml of methanol in a glass test tube. In a second glass test tube, 200mg of cholesterol was dissolved into 1 ml of chloroform and a 450 µl aliquot of this solution added to the methyl-β-cyclodextrin solution. The combined cyclodextrin/cholesterol solution was thoroughly mixed, and the solvents then removed using nitrogen gas. The resulting crystals were stored in a glass vial at 22°C until use. To add cholesterol to sperm, a working solution of CLC's was made by adding 50 mg of CLC to 1 ml of a modified Tyrode's media (TALP) (Moore et al., 2005b), mixed vigorously with a vortex and incubated in a 37°C water bath until use. The CLC working solution was mixed again, using a vortex mixer, prior to any aliquot being removed for addition to sperm.

Experiment 1: Osmotic Tolerance Limits of Spermatozoa Treated with Cholesterol

Semen was collected from 9 light horse stallions (aged 5 to 22 years old; Quarter Horse, Arabian and mixed breeds) using a Colorado model artificial vagina with an in-line gel filter (Animal Reproduction Systems, Chino, CA). All stallions were on a routine collection schedule (3 times/week). Semen was diluted to 120 x 10^6 spermatozoa/ml TALP and divided into three treatment groups containing 0, 1.5 or 6.0 mg CLC/120 x 10^6 cells. Spermatozoa were allowed to incubate for 15 min at room temperature (~22°C) before being diluted 1:1 (v:v) with TALP and centrifuged at 600g for 9 min. The supernatant was removed and the spermatozoa resuspended to a concentration of 1 x 10^9 spermatozoa/ml in TALP. Spermatozoa from each CLC

treatment were placed into anisosmotic solutions of 75, 150, 225, 270, 300, 350, 370, 425, 600, 1200 or 2400 mOsm in STALP (90 x 10^6 spermatozoa/ml) and incubated at room temperature for 5 min. After incubation the percentage of motile spermatozoa were analyzed using a computer assisted sperm analysis (CASA) system (HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). The same spermatozoa were then returned to isosmotic conditions (280-325 mOsm) by diluting spermatozoa in TALP or deionized water (20 x 10^6 spermatozoa/ml), incubated as before and percentage of motile spermatozoa analyzed.

Motility Analysis

To assess the percentage of motile spermatozoa using CASA, a 5 µl drop of spermatozoa from each treatment was placed on a preheated (37°C) slide and a minimum of 200 spermatozoa per sample analyzed. System parameters for these analyses were 45 frames acquired at 60 frames per second; minimum contrast 70, minimum cell size 4 pixels; lower VAP cut-off 20 µm/s; lower VSL cut-off 0 µm/s; VAP cut-off for progressive cells 50 µm/s and straightness 75%.

Statistical Analysis

The percentage of motile spermatozoa was transformed to minimize stallion variation. Data were transformed within each ejaculate by dividing each anisosmotic motility value by the control value at 300 mOsm within each CLC treatment. Data were blocked by osmolality and analyzed by analysis of variance (ANOVA) and treatment differences separated by Student-Newman-Keuls (SNK) mean separation technique

(SAS). Treatments were considered different at $P<0.05$. Untransformed data is presented.

Experiment 2: Determination of Spermatozoa Isosmotic Volume (V_{iso}), Osmotic Response and Inactive Volume (V_b)

Semen was collect from six stallions and diluted to 40 x 10^6 spermatozoa/ml in TALP (300 mOsm). The volume of equine spermatozoa was determined at isosmotic and anisosmotic conditions to determine isosmotic volume, osmotic response and inactive volume. A sample of the spermatozoal suspension (150 µl) was added to 20 ml of PBS (160, 300, 580, and 870 mOsm) and cell volumes were measured in triplicate using an electronic particle counter at room temperature (~22°C).

Electronic Particle Counter

A Coulter counter (ZM model; Coulter Electronics, Hialeah, FL) with a 50 µm aperture tube was used to determine cell volume as described previously by Gilmore, et. al. (1995). Stallion spermatozoa volumes were calibrated in each solution using spherical styrene beads (Duke Scientific Corporation, Palo Alto, CA) with a diameter of 3 µm and a volume of 14.14 μm^3. The Coulter counter was interfaced with a microcomputer using a CSA-1S interface (The Great Canadian Computer Company, Alberta, CA). Changes in cell volume were measured over time using a computer software program (Gilmore et al, 1995).

Experiment 3: Permeability Characteristics of Spermatozoa Treated with Cholesterol

Semen from 8 stallions was diluted to 120 x 10^6 sperm/ml in TALP and divided into 3 treatment groups containing 0, 1.5 or 6.0 mg CLC/120 x 10^6 cells and incubated as described in experiment 1. Samples were then centrifuged through 50% Percoll for 15 min at 600g and resuspended to 40 x 10^6 spermatozoa/ml in TALP (300 mOsm). Spermatozoa samples (1 ml) were then further divided into three sub-samples containing glycerol, ethylene glycol or dimethyl formamide in TALP as the cryoprotective agent. One milliliter of 2 M cryoprotectant (CPA) solution was added drop wise over 60 sec to 1 ml of sperm suspension at 22°C and allowed to equilibrate for 3 min (final CPA concentration = 1M). One hundred and fifty microliters of the cell suspension was then abruptly diluted in 20 ml of isosmotic PBS (285-310 mOsm) and the cell volume changes recorded using an electronic particle counter. For each treatment, data was replicated three times and analyzed at room temperature (~22°C) and 5°C. For samples analyzed at 5°C, medium, vials and aperture were pre-equilibrated to the desired temperature and maintained within $\pm$ 2°C during each run.

Statistical Analysis

Analysis of variance was performed on the water permeability (L_P) and cryoprotectant permeability (P_{CPA}) of stallion spermatozoa to analyze the main effects of temperature, CLC concentration and CPA as well as their interactions. Treatment differences were separated by Student-Newman-Keuls (SNK) mean separation technique (SAS). Further analysis included blocking control spermatozoa by temperature and CPA and analyzing by ANOVA and determining treatment differences by SNK. To analyze

the effects of CLC treatment, data were compared by orthogonal contrast and blocked by CPA with treatment differences separated by SNK (SAS). Treatments were considered different at $P<0.05$.

Results

Experiment 1: Osmotic Tolerance Limits of Sperm Treated with Cholesterol

The percentages of motile spermatozoa were higher than controls for samples treated with 1.5 mg CLC when cells were exposed to 150 mOsm diluent and for samples treated with 6.0 mg CLC exposed to 75, 150, 225, 370, 425, 600 and 1200 mOsm (Figure 1; $P<0.05$). In addition, the percentages of motile spermatozoa were significantly higher for samples treated with 1.5 mg CLC at 75, 150, 225 and 600 mOsm and with 6.0 mg CLC at 75, 150, 225, 600 and 1200 mOsm when the spermatozoa were returned to isosmolality compared to control samples (Figure 2; $P<0.05$).

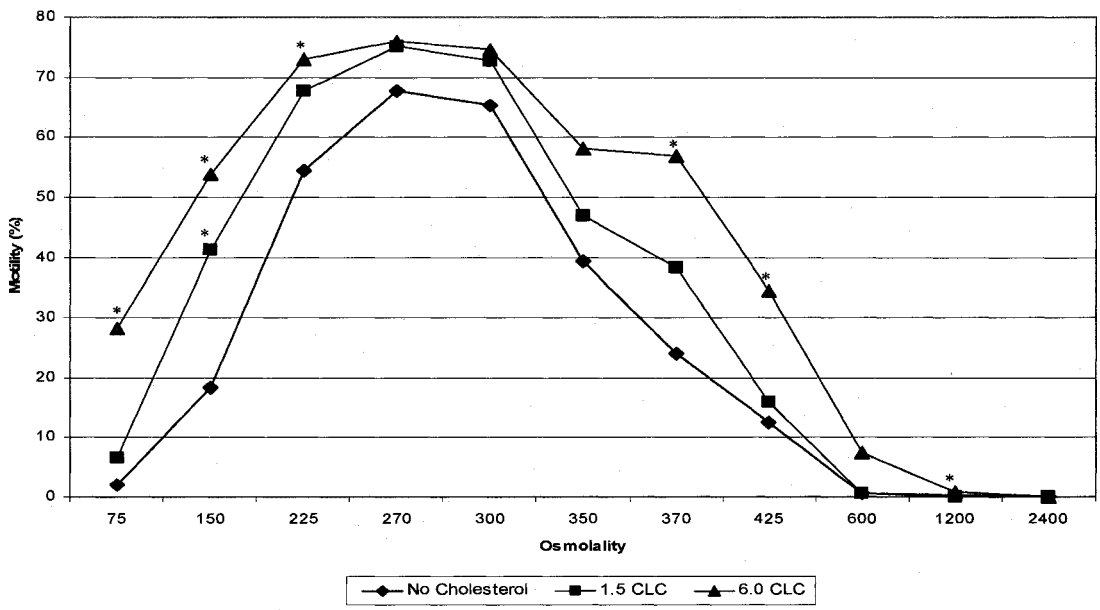

Figure 1: The percentage of motile spermatozoa placed into anisosmotic solutions. Treatments included spermatozoa that contained 0, 1.5 or 6.0 mg CLC/120 x 10^6 sperm. * Indicates difference compared to control values (no cholesterol; $P<0.05$). (n=9)

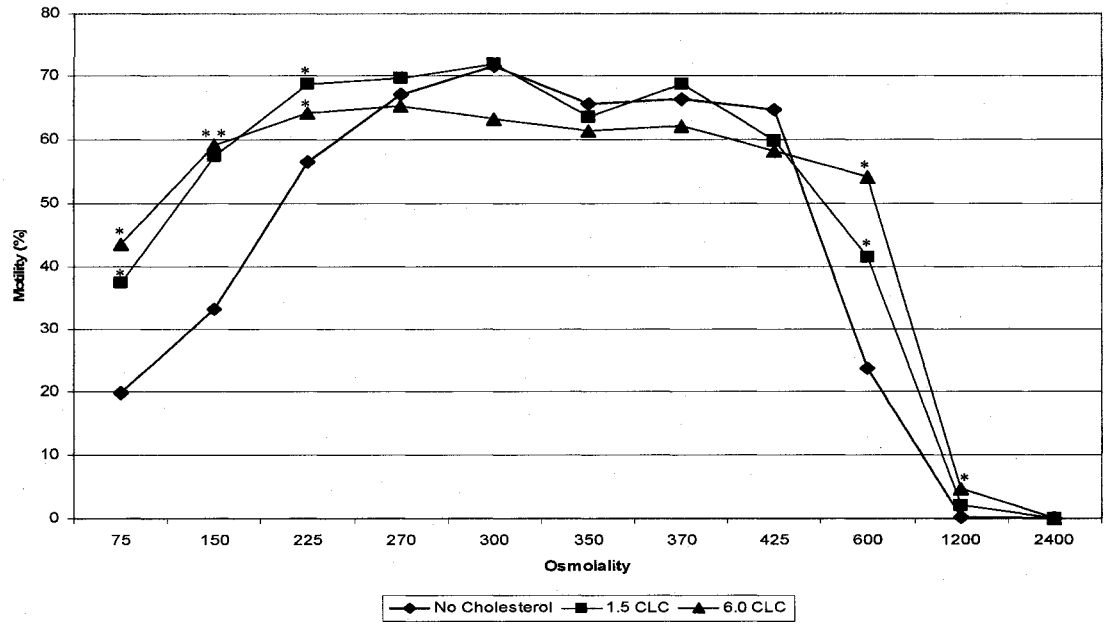

Figure 2: The percentage of motile spermatozoa after return to isosmotic conditions. Treatments included spermatozoa that contained 0, 1.5 or 6.0 mg CLC/120 x 10^6 sperm. * Indicates difference compared to control values (no cholesterol; $P<0.05$). (n=9)

Experiment 2: Determination of Spermatozoal Isosmotic Volume (V_{iso}), Osmotic Response and Inactive Volume (V_b)

The isotonic volume of stallion spermatozoa determined at room temperature was 18.61 $\pm$ 0.51 μm^3. The variability within donors was small, having a coefficient of variation (CV) $\leq$ 1.1%, while the CV among donors was 7.7%. To determine if equine spermatozoa behave as linear osmometers, the osmotic response of cells was measured over a range of 160 -871 mOsm. The osmotic response is presented as a BVH plot (Fig. 3). The osmotically inactive volume (V_b) was determined to be 83% with a CV among donors of 4.6%.

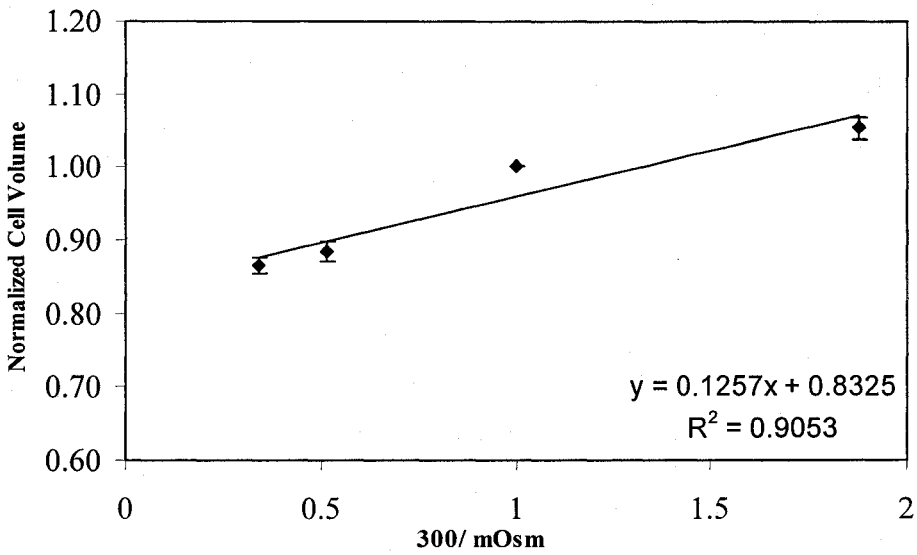

Figure 3: BVH plot of equine sperm at room temperature (22°C). The cells were incubated in solutions at four different osmolalities (160, 300, 580 and 871 mOsm). The y-intercept indicates a V_b of 83%. Data is presented as mean ± SEM (n=6).

Experiment 3: Permeability Characteristics of Spermatozoa Treated with Cholesterol

The permeability of stallion spermatozoa to water (L_p) was similar (P>0.05) regardless of temperature, CLC treatment or CPA. When all data were pooled to evaluate the main effects of temperature, CLC concentration, cryoprotectant and their interactions on cryoprotectant permeability (P_{cpa}), there were permeability differences due to temperature, CLC concentration, and cryoprotectant as well as an interaction between temperature and CPA (Table 1; P<0.05). The P_{cpa} of control spermatozoa was lower at 5°C than at 22°C (P<0.05) and at 22°C glycerol was less permeable than ethylene glycol or dimethyl formamide (Table 2; P<0.05). The P_{cpa} were similar (P>0.05) at all CLC treatments at 22°C. However, at 5°C spermatozoa treated with CLC's were more permeable to CPA's than untreated controls (Table 2; P<0.05) but cells treated with either 1.5 or 6 mg CLC had similar permeability coefficients to the cryoprotectants (P>0.05).

The permeability of spermatozoa to DMF was similar regardless of CLC treatment at 5°C, but spermatozoa treated with CLC's were more permeable to GLY or EG at that temperature (Table 2; $P<0.05$).

Table 1. Main effects of temperature, CLC concentration and CPA on cryoprotectant permeability of stallion spermatozoa. (n=8)

Main Effect		$P_{cpa}(10^{-3}cm/min)$
Temperature (°C)	**5**	0.3018^{b}
	22	0.6232^{a}
	SEM	0.03
CLC	**0**	0.3972^{b}
(mg/120x10^6 sperm)	**1.5**	0.4853^{ab}
	6.0	0.5379^{a}
	SEM	0.04
CPA	**GLY**	0.3570^{b}
	EG	0.5475^{a}
	DMF	0.5071^{a}
	SEM	0.04

[a,b] Different superscripts within main effect indicate treatment differences ($P<0.05$).

Table 2. Permeability of stallion spermatozoa to water (L_p; μm/min/atm) and cryoprotectants (P_{cpa}; 10^{-3}cm/min) at 5° and 22°C in the presence of CLC's (mg/120x10^6 sperm). Cryoprotectants (CPA) evaluated were glycerol (GLY), ethylene glycol (EG) and dimethyl formamide (DMF). (n=8)

		5°C		22°C	
CLC	**CPA**	**L_p**	**P_{cpa}**	**L_p**	**P_{cpa}**
0	**GLY**	0.3510	0.1523^{B}	0.7403	0.3831*
	EG	1.2866	0.1838^{B}	1.1877	0.7280**
	DMF	0.8131	0.2374^{A}	0.7373	0.6944**
	Mean	**0.8169**	**0.1912^{b}**	**0.8884**	**0.6018**
	SEM	1.13	0.03	0.59	0.07
1.5	**GLY**	1.0789	0.2737^{AB}	0.4591	0.4112
	EG	1.4488	0.4491^{A}	1.6335	0.7649
	DMF	0.7375	0.2934^{A}	0.5951	0.6463
	Mean	**1.0884**	**0.3387^{a}**	**0.8959**	**0.6075**
	SEM	0.83	0.07	0.56	0.12
6.0	**GLY**	0.8410	0.4483^{A}	1.8006	0.4823
	EG	1.4373	0.3020^{AB}	1.0556	0.7734
	DMF	1.5188	0.4051^{A}	0.9611	0.7159
	Mean	**1.2657**	**0.3851^{a}**	**1.2724**	**0.6572**
	SEM	1.17	0.11	0.82	0.09

^{a,b} Different superscripts among mean values within temperature are different ($P<0.05$)
*,** Different superscripts within temperature and CLC treatment are different ($P<0.05$)
^{A,B} Different superscripts within temperature and CPA are different ($P<0.05$)

Discussion

Spermatozoa undergo osmotic stresses during addition and removal of CPA and during the actual process of cryopreservation. As a CPA is added to spermatozoa, cells will initially shrink because of the hypertonicity of the extracellular solution and then return to normal cell volume once water and CPA have equilibrated across the plasma membrane. Conversely, after warming and deposition into isotonic media, spermatozoa will swell due to a large influx of water to equilibrate the osmotic pressure across the plasma membrane, and cells will only return to their original volume once the CPA has left the spermatozoa. Equine spermatozoa have a very narrow osmotic tolerance

compared to human or mouse spermatozoa (Ball and Vo, 2001; Gao et al., 1995; Willoughby et al., 1996) and have an osmotic tolerance similar to that of boar spermatozoa (Gilmore et al., 1998). Ball and Vo (2001) reported a loss in total motility to less than 50% when stallion spermatozoa were incubated in diluents ± 100 mOsm from that of isosmolal. They also reported lower percentages of viable spermatozoa under hyposmotic conditions, suggesting that stallion spermatozoa are more sensitive to osmotic damage induced during the thawing process of cryopreservation rather than the cooling process (Ball and Vo, 2001). Compared to other species, equine spermatozoa are less able to recover from osmotic damage once returned to isotonic conditions than spermatozoa from other species and this is reflected in the differences in osmotic tolerances of spermatozoa from these species.

The addition of CLC to equine spermatozoa increased the osmotic tolerance limits of spermatozoa placed into anisosmotic solutions as well as their return to isotonic conditions. This additional cholesterol in the plasma membrane appears to increase the volume changes that stallion spermatozoa can endure. Both concentrations of CLC used improved the osmotic tolerance limits of equine spermatozoa, however, in a previous study, 1.5 mg CLC/120 x 10^6 spermatozoa provided the greatest protection against cryodamage (Moore et al., 2005a). Treatment with CLC increased stallion spermatozoa osmotic tolerance from isosmolal ± 100 mOsm too approximately ± 140 mOsm with 1.5 mg CLC and ± 225 mOsm when 6 mg CLC was added. Treatment of spermatozoa with CLC's provides confirmation that alteration of the plasma membrane composition of the spermatozoon can enhance cell cryosurvival, by reducing osmotic stress.

Since very few studies have reported the osmotic behavior of equine spermatozoa (Ball and Vo, 2001; Pommer et al., 2002) we wanted to verify that equine spermatozoa behave as linear osmometers. The results of our study showed a mean volume of equine spermatozoa in isotonic medium to be 18.61 ± 0.51 μm^3 at room temperature. This value is slightly lower than what has been previously reported for equine spermatozoa using an electronic particle counter (24.4 μm^3; Pommer et al., 2002) or optical microscopy (37 μm^3; Gravance et al., 1997). It has been described that estimates of cell volume obtained from electronic particle counters are less than those obtained by microscopy although the reason for this is not well understood (Gilmore et al., 1995; Jeyendran et al., 1987; Willoughby et al., 1996). One reason for the disagreement in size between this study and that reported by Pommer et al. (2002), which also utilized an electronic particle counter, is the low number of stallions used by Pommer et al. (2002). With the use of spermatozoa from several stallions of several breeds these data may better represent the horse population as a whole. This study also reports that equine spermatozoa do behave as linear osmometers (r^2=0.91) within a range of 160-871 mOsm and have an osmotically inactive volume (V_b) of 83%. This volume is also higher than that reported by Pommer et al. (2002), 70.7%; however, the CV in the current study was 4.6% among stallions indicating again that the difference in sample size may be the reason for this discrepancy. The V_b of equine spermatozoa reported in this study is much higher than what has been reported for human (50%; Gao et al., 1995); bull (61%; Guthrie et al., 2002) and boar spermatozoa (67.4%; Gilmore et al., 1996) and may indicate why equine spermatozoa are more sensitive to osmotic stress and the cryopreservation process, than spermatozoa from these other species.

Knowing the plasma membrane permeability characteristics permits determination of optimal cooling rates for a chosen CPA. These permeability characteristics have been characterized for human and boar spermatozoa (Gao et al., 1995; Gilmore et al., 1998), but there has only been one report in equine spermatozoa (Noiles et al., 1992). Noiles et al. (1992) reported very high water and glycerol permeabilities at 22°C (26 $\pm$ 12 μm/min/atm and 2.5 x 10^{-3} $\pm$ 0.4 cm/min, respectively) for equine spermatozoa by using the time-to-lysis technique. These values are much higher than those reported values for boar and bull spermatozoa, which are species that respond similarly in osmotic tolerance and cryosurvival (Chaveiro et al., 2004; Gilmore et al., 1998). The values reported in the present study, using an electronic particle counter, are more similar to what has been reported for boar spermatozoa with the exception that we saw no difference in L_p at different temperatures or with different CPA's. Glycerol has lower permeability across the plasma membrane than ethylene glycol or dimethyl formamide, suggesting the low molecular weight cryoprotectants may be able to improve cryosurvival of equine spermatozoa by producing smaller volume changes during addition and removal. Spermatozoa treated with CLC had higher P_{cpa} than untreated controls and this difference was greatest at 5°C. Although CLC treatment did not affect the membrane permeability of dimethyl formamide, it did increase the membrane permeability of glycerol and ethylene glycol. These data are in agreement with the osmotic tolerance studies of experiment one and show that cholesterol is affecting the plasma membrane in a positive manner by reducing the amount of osmotic stress that is imposed on the spermatozoa during cryopreservation.

Cholesterol supplementation has been shown to increase the percentage of motile and viable spermatozoa in stallion, ram and bull spermatozoa (Moore et al., 2005a; Morrier and Bailey, 2004; Purdy and Graham, 2004) as well as increase the number of equine spermatozoa binding to the zona pellucida (Moore et al., 2005a). From this study we have been able to show that cholesterol can improve the cryosurvival of equine spermatozoa by increasing the osmotic tolerance limits of spermatozoa and by increasing the permeability of the spermatozoa to the cryoprotectants. This is extremely valuable information that leads to a greater understanding of the cryobiology of equine spermatozoa and further explains the role of cholesterol within the plasma membrane.

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Chapter IV

Effect of Freeze Rate and Cryoprotectant on the Cryosurvival of Equine Spermatozoa

Introduction

There has been a dramatic increase in the use of cryopreserved stallion spermatozoa during the past five years due to many large breed organizations permitting registration of foals produced from frozen semen. A recent study demonstrated that the fertility of frozen semen is nearly that of cooled semen, however, there are tremendous differences in the fertility, of cryopreserved spermatozoa, among individual stallions (Squires et al., 2003). Therefore, certain stallions may require customized procedures to be used in order to obtain acceptable fertility with their cryopreserved spermatozoa.

The rate at which cells are frozen affects the motility and viability of the spermatozoa upon re-warming (Amann and Pickett, 1987). As spermatozoa are cooled from room temperature (~22°C) to below 0°C water in the extracellular media begins to freeze creating ice crystals of pure water and unfrozen water channels. These unfrozen channels create a hyperosmotic environment in which the spermatozoa reside and induce the cells to undergo cellular dehydration. The rate at which the cells are cooled determines the extent of dehydration the cells will undergo before sufficiently low temperatures are reached to induce vitrification of the unfrozen channels. Optimal cell cryosurvival requires a specific cooling rate for each cell type and cryopreservation diluent. If the

cooling rate of stallion spermatozoa is too slow, the cells will dehydrate too much causing irreversible cell damage. However, if cooling rate is too rapid, intracellular water does not have sufficient time to move out of the cell and damaging intracellular ice crystals will form (Amann and Pickett, 1987).

Cryoprotectants added to the freezing diluent protect cells during cryopreservation. Cryoprotectants increase the volume of the unfrozen solution in which the cells reside and reduce the salt concentration of that solution, thereby reducing damage occurring due to dehydration. However, since cryoprotectants are less permeable to cell membranes than water, and since they are added at molar concentrations, they induce large although transient osmotic gradients across the cell plasma membranes when they are added to the cells prior to freezing and when removed from the cells after thawing which can damage or lyse the cells (Gilmore et al., 1995; Gilmore et al., 1998; Guthrie et al., 2002).

Glycerol is the most commonly used cryoprotectant for cryopreserving equine spermatozoa. However, glycerol may also be the cryoprotectant that causes the greatest osmotic damage to spermatozoa because its permeability across the sperm plasma membrane is much lower than that of water and lower than that of many other cryoprotectants (Guthrie et al., 2002). Therefore glycerol induces large cell volume changes when it is added to or removed from spermatozoa. Alternative, low molecular weight (MW) cryoprotectants may prove less damaging to spermatozoa, as they equilibrate across the plasma membrane more rapidly than glycerol and will therefore induce much smaller and potentially less damaging cell excursions, than glycerol. Squires et al. (2004) found that stallion spermatozoa cryopreserved using the low MW cryoprotectants dimethyl formamide or ethylene glycol, resulted in similar post-thaw

spermatozoal attributes as spermatozoa cryopreserved with glycerol. Dimethyl formamide also maintained higher percentages of motile spermatozoa after cryopreservation than glycerol for spermatozoa from stallions whose semen normally do not cryopreserve well (Medeiros et al., 2002).

Although some benefit of these low MW cryoprotectants have been observed, there have been no reports investigating the interactions between these cryoprotectants and freeze rate. The objective of this experiment was to determine the optimal cooling rate for equine spermatozoa when cryopreserved with glycerol, ethylene glycol or dimethyl formamide as the cryoprotectant.

Materials and Methods

Three ejaculates were collected from each of six light-horse stallions, using a Colorado model artificial vagina (Animal Reproduction Systems, Chino, CA). Due to the large number of treatments each ejaculate, from an individual stallion, was used to evaluate only one specific cryoprotectant at 10 different cooling rates. Each ejaculate was diluted to a concentration of 50 x 10^6 sperm/ml in a skim milk, glucose diluent (EZ-Mixin' "BF", Animal Reproduction Systems, Chino, CA) and centrifuged at 600g for 10 min. The spermatozoa were resuspended to a final concentration of 200 x 10^6 sperm/ml in a skim milk, egg yolk freezing diluent (EZ-Freezin' "MFR5", Animal Reproduction Systems, Chino, CA) containing 4% of the desired cryoprotectant (0.55 M glycerol, 0.71 M ethylene glycol or 0.52 M dimethyl formamide) and the spermatozoa packaged into 0.5 cc PVC straws. The straws were placed into a 100 ml water bath which was placed in a cold room at 5°C, which permitted the straws to cool to 5°C over 2h. Upon reaching

5°C, the straws were divided into 10 groups and frozen at different cooling rates using a programmable freezer (Kryo 10 Series III, Planer, Middlesex, UK). All samples started at 5°C and straws were cooled either at -5°C/min; -10°C/min; -15°C/min; -20°C/min; -25°C/min; -30°C/min; -35°C/min; -40°C/min; -45°C/min; or -50°C/min to -120°C before being plunged into LN_2. Samples were thawed in a 37°C water bath for 30s before analysis of the percentage of motile and viable spermatozoa.

Motility analysis

One straw from each treatment was thawed and the percentage of motile spermatozoa in each sample determined using a computer-assisted spermatozoa analysis system (CASA) (HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). Prior to analysis, the contents of each straw was diluted 1:4 (v:v) in EZ-Mixin' BF (a final concentration of 20 x 10^6 sperm/ml) and maintained at room temperature for 10 minutes before analysis. System parameters for these analyses were 45 frames acquired at 60 frames per second; minimum contrast 70, minimum cell size 4 pixels; lower VAP cut-off 20 μm/s; lower VSL cut-off 0 μm/s; VAP cut-off for progressive cells 50 μm/s and straightness 75%. A 5-μl drop of spermatozoa from each sample was placed on a preheated (37°C) slide and a minimum of 200 spermatozoa per sample analyzed.

Viability analysis

The percentage of viable spermatozoa was determined using flow cytometry, as described by Squires et al. (2004). Briefly, one straw from each treatment was thawed and the cells diluted to 40 x 10^6 sperm/ml in a modified Tyrode's medium (TALP)

(Moore et al., 2005). A 100 µl volume of cells (4 x 10^6 cells) was placed into 500 µl of TALP containing 10 µl of propidium iodine (PI; 1 mg/ml in H_2O) and 20 µl of SYBR-14 (10µM in Me_2SO) and the samples incubated at room temperature for 10 minutes prior to analysis. A minimum of 50,000 spermatozoa per sample were analyzed using an EPICS V flow cytometer (Coulter Electronics, Miami, FL) equipped with Cicero computer software (Cytomation, Fort Collins, CO). The fluorescent probes were excited with a 488 nm argon laser and the fluorescence of PI and SYBR-14 was detected using a filter setup including a 515 nm long-pass filter to block laser light, a 590 nm dicroic beam splitting filter, a 530 nm short-pass filter to detect SYBR-14, and a 630 nm long-pass filter to detect PI.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) for the main effects of cooling rate, cryoprotectants and for an interaction between cooling rate and cryoprotectant. Treatment differences were separated by Student-Newman-Keuls (SNK) mean separation technique (SAS Institute Inc., 1985). Treatments were considered different if $P<0.05$.

Results

Within each cryoprotectant, all cooling rates were similar ($P>0.05$) neither was there an interaction between cooling rates and cryoprotectants, therefore data were combined for further analysis. Spermatozoa cryopreserved in the presence of glycerol had higher percentages of total and progressively motile spermatozoa than spermatozoa cryopreserved in the other two cryoprotectants (Table 4.1; $P<0.05$).

Table 4.1. The percentage of total, progressively motile and viable stallion spermatozoa frozen among three different cryoprotectants (glycerol, ethylene glycol, or dimethyl formamide). (n=6)

	Cryoprotectant			
	Glycerol	**Ethylene Glycol**	**Dimethyl Formamide**	**SEM**
Total	65^{a}	55^{b}	48^{c}	2
Progressive	37^{a}	31^{b}	30^{b}	2
Viable	56^{a}	55^{a}	55^{a}	2

[a,b,c] Different superscripts within rows denotes treatment differences ($P<0.05$).

The only factor affecting the percentage of viable spermatozoa was freeze rate. Samples frozen at -10°C/min had a higher percentage of viable cells compared to samples frozen at -50°C/min (Figure 4.1; $P<0.05$).

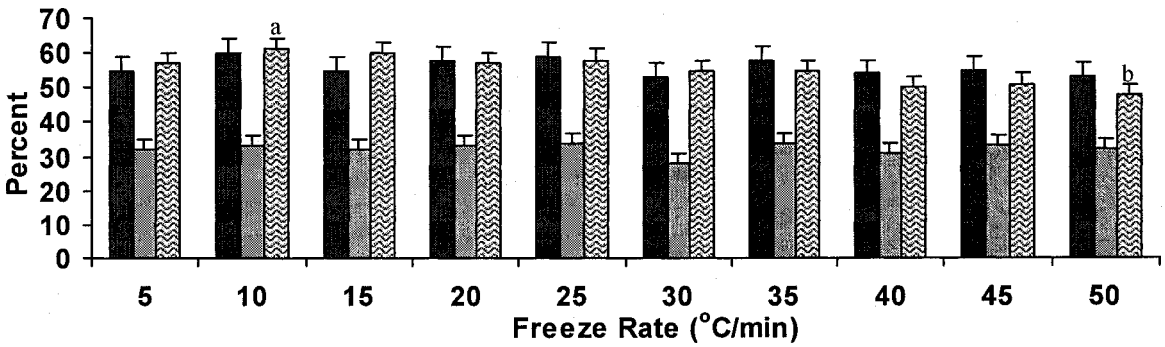

Figure 4.1. The percentage of total (■), progressively motile (▩) and viable (▨) stallion spermatozoa frozen among various freeze rate cycles (5-50°C/min). (n=6)
[a,b] Different superscripts denotes treatment differences ($P<0.05$).

Discussion

The results from this study indicate that stallion spermatozoa can be cooled over a wide range of cooling rates, 5-45°C/min, while maintaining optimal percentages of motile and viable spermatozoa. These data are similar to a study conducted by Devireddy et al. (2002) who using differential scanning calorimetry determined that

stallion spermatozoa survive similarly at cooling rates between 20-100°C/min. This group also reported that the addition of glycerol to the cryopreservation diluent protected the cells over a broad range of cooling rates compared to spermatozoa in media containing no cryoprotectant and that the amount of osmotically active water that remains inside of the spermatozoa once a low temperature is reached (-30°C), determines the fatality of the cells (Devireddy et al., 2002). Mazur (1990) reported a loss in cell viability when > 10-15% of water remains inside the spermatozoa since at this high concentration the water forms intracellular ice crystals when cooled. The trapped water volume inside of spermatozoa cooled at 5°C/min, 20°C/min and 50°C/min was 1.6%, 3.4% and 4.6%, far less than what has been proposed to be lethal (Devireddy et al., 2002). Higher cooling rates were not examined in this study because they exceeded the maximum cooling rate capable of our programmable freezer.

Glycerol was the most beneficial cryoprotectant utilized in this study and this is most likely due to the concentration of cryoprotectants evaluated. Squires et al. (2004) observed similar benefits when dimethyl formamide or ethylene glycol was compared to glycerol when the concentrations of dimethyl formamide or ethylene glycol were increased from 0.55M to 0.6M or 0.9M. This required increase in concentration of dimethyl formamide and ethylene glycol compared to glycerol is likely due to the fewer lone electron pairs that dimethyl formamide and ethylene glycol possess (Nash, 1996). Possessing fewer lone electron pairs should therefore, require higher concentrations of the cryoprotectant to equal the protective properties of glycerol. Since only one cryoprotectant concentration was evaluated in this study, the protective effects of dimethyl formamide and ethylene glycol may not have been optimal (Nash, 1996).

Several studies have reported that both dimethyl formamide and ethylene glycol may provide greater benefit to spermatozoa from stallions whose semen does not cryopreserve well (Medeiros et al., 2002; Squires et al., 2004) and only one stallion in the current study was classified as a "poor" freezing stallion.

Only one freezing diluent was examined in this experiment due to the length of time required to run each cryopreservation cycle. Straws were randomly held for up to three hours before cryopreservation and it has been reported that stallion spermatozoa diluted into a SMEY freezing diluent can be held for at least 6-12 h with no detriment in the percentage of motile spermatozoa after cryopreservation (Crockett et al., 2001; Moore et al, 2005). Other freezing diluents that do not have to be cooled to 5°C prior to cryopreservation may require a different cooling rate.

In summary, stallion spermatozoa can be successfully cryopreserved when cooling rates between 5-45°C/min are utilized. When the freezing diluent contains 4% cryoprotectant, glycerol is the most effective cryoprotectant to maintain cryosurvival of stallion spermatozoa.

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CHAPTER V

SUMMARY

Cryopreservation of stallion spermatozoa induces partially irreversible cell damage that results in reduced cryosurvival. Destabilization of and damage to the plasma membrane occurs as the cells are cooled as the membrane undergoes the phase transition leading to membranes that become "leaky" to extracellular solutes. Other damage occurs during cryopreservation from the formation of intracellular ice crystals, decreased membrane fluidity and osmotic stress (Levin, 1982; Mazur, 1984; Steponkus et al., 1983). The addition of cholesterol to cell membranes has been reported to decrease and/or eliminate the membrane phase transition, increase membrane fluidity at lowered temperatures (Rottem et al., 1973) and increase cryosurvival of spermatozoa (Purdy and Graham, 2004).

In the present study, the addition of cholesterol loaded cyclodextrin (CLC) to stallion spermatozoa increased the percentage of motile and viable spermatozoa after cryopreservation. This improvement in cryosurvival was especially noted for spermatozoa from stallions whose spermatozoa do not survive the cryopreservation process well ("poor" freezers). Stallion spermatozoa treated with CLC's also had increased cholesterol in their membranes in a dose dependent fashion until saturation occurred and the addition of CLC's resulted in less cholesterol being lost from the membranes as a result of cryopreservation. The addition of CLC to stallion spermatozoa

prior to cryopreservation also resulted in more spermatozoa binding to the zona pellucida of bovine oocytes than untreated samples.

The effects of CLC treatment on the osmotic damage of stallion spermatozoa were also evaluated. The addition of CLC to stallion spermatozoa increased the osmotic tolerance limits of spermatozoa in both anisosmotic solutions and upon their return to isosmolality. An electronic particle counter was used to observe volume excursions in stallion spermatozoa upon abrupt removal of three different cryoprotectants. Membrane permeability to all cryoprotectants was lower at 5°C than 22°C and at room temperature glycerol had lower membrane permeability than ethylene glycol or dimethyl formamide in control spermatozoa. The addition of CLC to spermatozoa prior to exposure to the cryoprotectants increased membrane permeability for glycerol and ethylene glycol at 5°C. Membrane permeability was similar for all cryoprotectants at 22°C and for dimethyl formamide at 5°C regardless of CLC treatment.

Stallion spermatozoa were also exposed to various cooling rates and cryoprotectants during cryopreservation to determine the cooling rate that optimizes cryosurvival. The percentage of post-thaw spermatozoa motility and viability were similar for glycerol, ethylene glycol and dimethyl formamide. Cooling rates between 5°C and 45°C/min resulted in similar numbers of motile and viable spermatozoa after cryopreservation. Cooling rates higher than 50°C/min may result in increased spermatozoal damage due to intracellular ice crystal formation but was beyond the ability of this study.

This research reports similar findings to those observed for bull spermatozoa (Purdy and Graham, 2004) and provides a better understanding of the cryobiology and

actions of cholesterol in the stallion spermatozoa membranes during cryopreservation. This research presents optimism for improving the cryosurvival of spermatozoa from species who are traditionally known as poor freezers such as the ram and boar.

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Steponkus PL, Dowgert MF and Gordon-Kamm WJ. Destabilization of the plasma membrane of isolated plant protoplasts during a freeze-thaw cycle: the influence of cold acclimation. Cryobiology 1983; 20: 448-465.

Appendix I

Figures from Coulter Counter Analysis

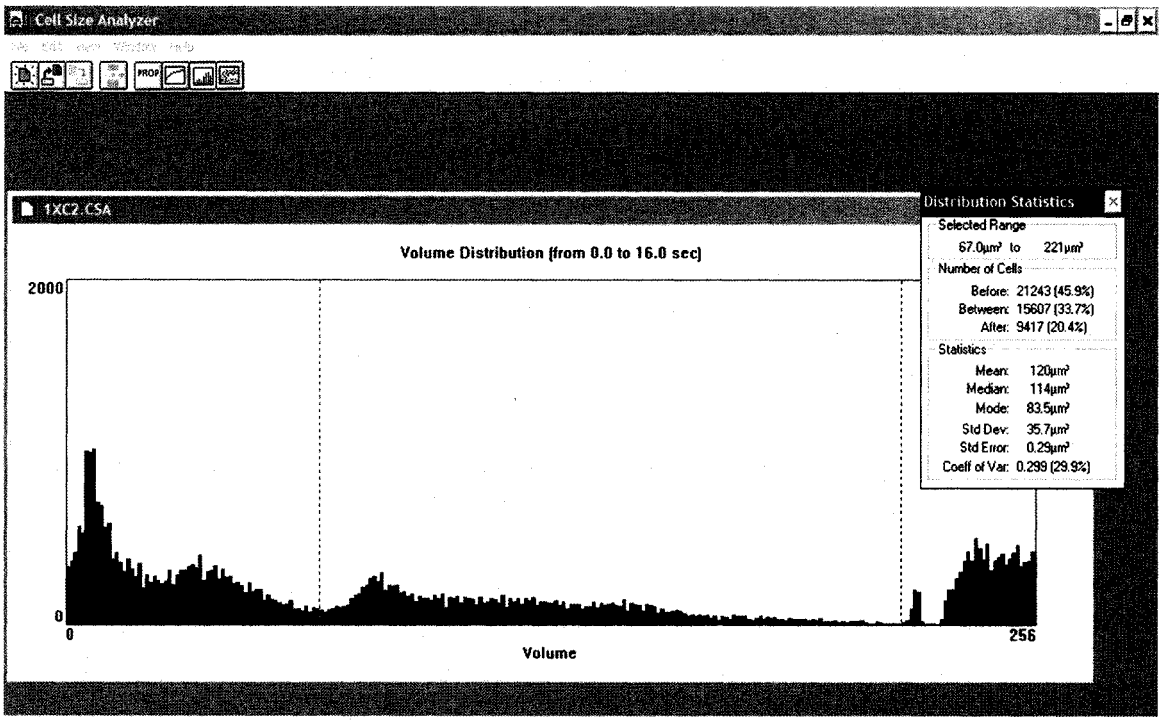

Figure 1. Cell volume versus count histogram of spermatozoa in isosmolality from Cell Size Analyzer software.

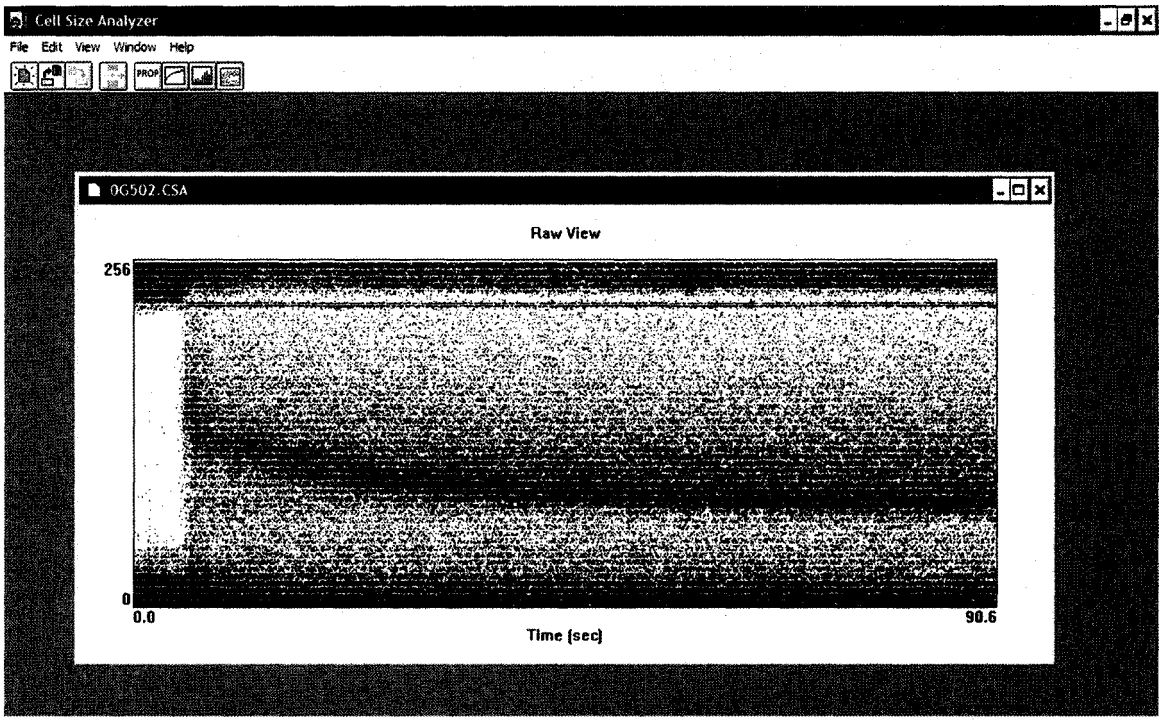

Figure 2. Volume versus time plot of removal of glycerol from spermatozoa at 5°C.

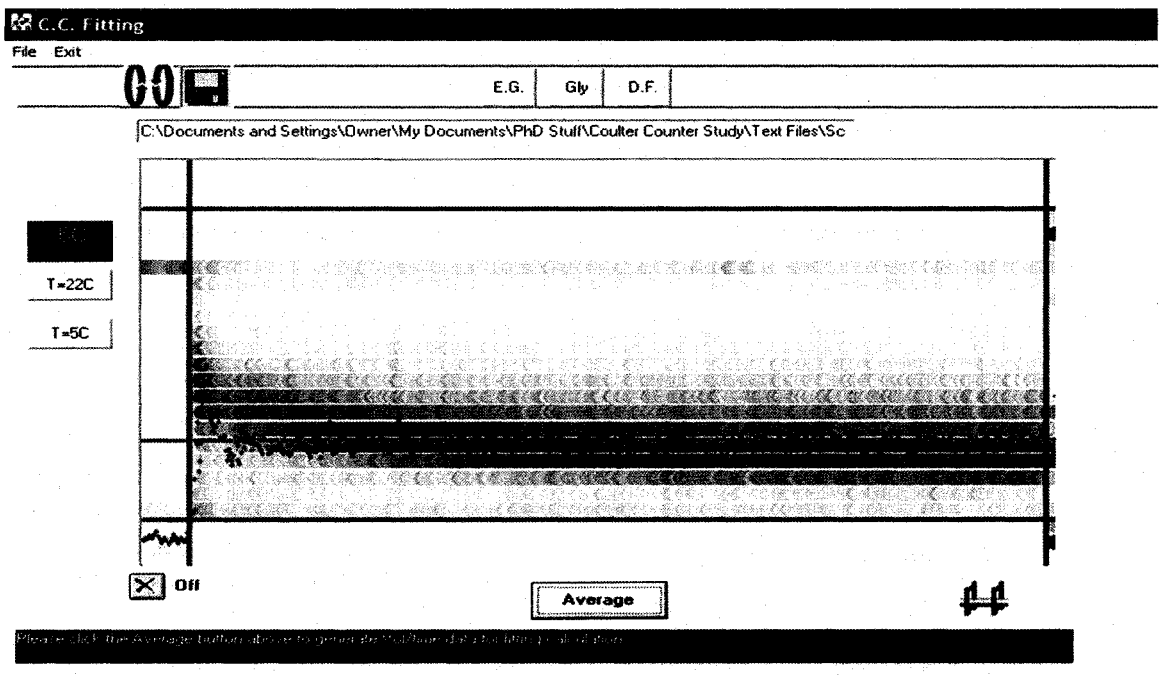

Figure 3. Above data imported into the Coulter Counter Fitting program. Purple dots represent hypothetical best fit line.

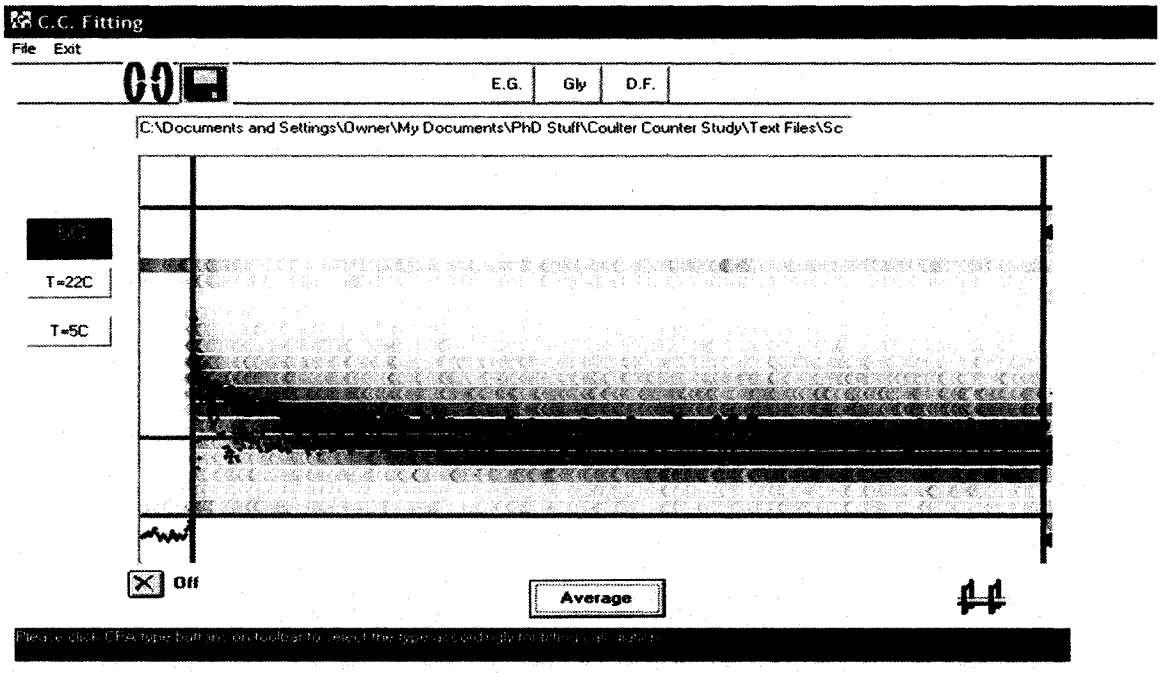

Figure 4. Representation of averaged cell volume over time. Blue dots represent actual best fit line.

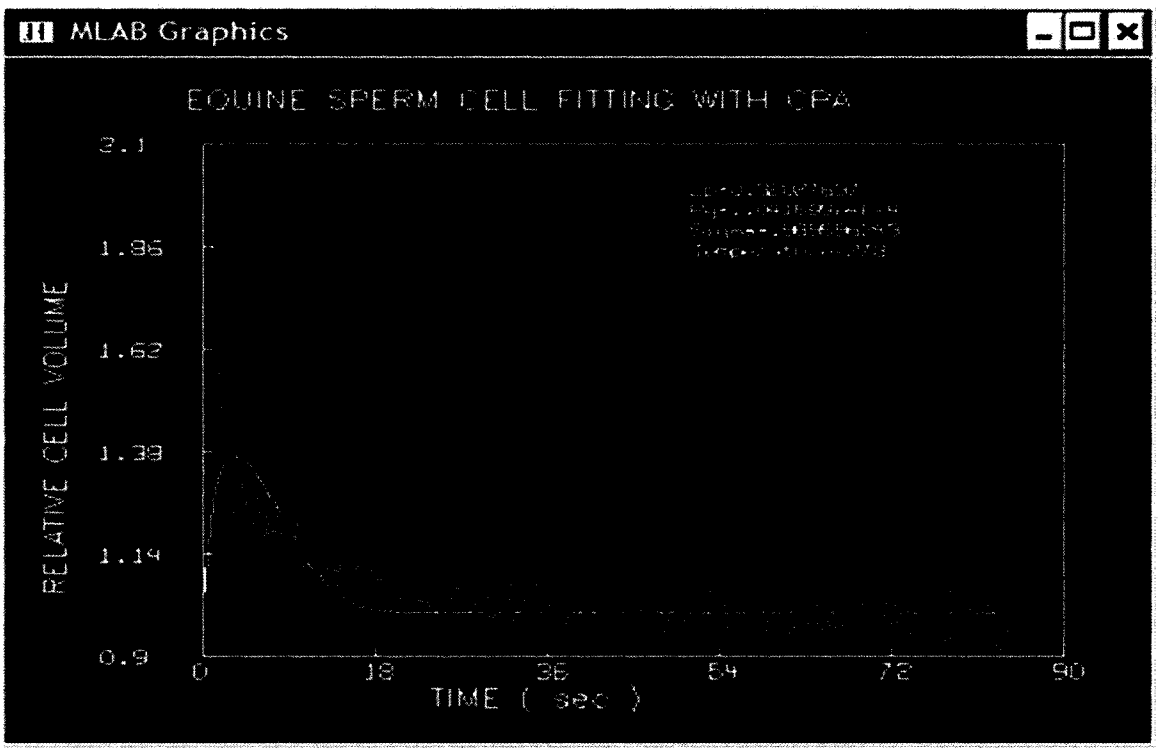

Figure 5. Final fitting parameters for removal of glycerol from spermatozoa at 5°C using MLAB software.

APPENDIX II

Analysis of Cholesterol Concentration Using the Cholesterol LiquiColor® Assay

- Cholesterol LiquiColor® Assay (Stanbio, Boerne, TX; Product # 1010-430)
- For use in whole cells

Protocol

- Warm reagent up to room temperature before use
- Standards
  - 1, 0.5, 0.25, 0.125 & 0.0625 mg CHO/ml
  - No need to centrifuge
- Dilute cells 1:1 with lysate buffer (0.4% Triton-X in PBS)
- Incubate cells at room temperature for at least 1h
- Can sonicate for 15 min during incubation
- Add 100 µl of cell suspension to 500 µl reagent (in glass test tubes)
- Incubate at 37°C for 25 min
- Place samples in micro-centrifuge tubes and centrifuge for 2 min
- Remove supernatant and add to cuvettes
- Run on spectrophotometer (wavelength set to 500 nm)
- Plot unknown sample values against standard curve
- REMEMBER: Cell concentration is ½ of original due to 1:1 dilution

APPENDIX III

Analysis of Variance Tables for Following Experiments

Table 2.1. Total Motility for All Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	14466.132	2411.022	8.10	<0.0001
Error	97	28858.308	297.508		
Total	103	43324.440			

Table 2.1. Progressive Motility for All Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	16679.092	2779.849	13.45	<0.0001
Error	97	20047.437	206.675		
Total	103	36726.529			

Table 2.1. Viability for All Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	973.077	162.179	3.94	0.0014
Error	97	3990.632	41.141		
Total	103	4963.709			

Table 2.1. Total Motility for "Poor" Freezing Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	2687.531	447.922	3.81	0.004
Error	42	4938.786	117.590		
Total	48	7626.316			

Table 2.1. Progressive Motility for "Poor" Freezing Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	3947.918	657.986	6.73	<0.0001
Error	42	4108.143	97.813		
Total	48	8056.061			

Table 2.1. Viability for "Poor" Freezing Stallions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	632.551	105.425	3.70	0.0049
Error	42	1197.929	28.522		
Total	48	1830.480			

Figure 2.1. Cholesterol Concentrations

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	6	11.627	1.938	88.16	<0.0001
Error	77	1.692	0.022		
Total	83	13.320			

Figure 2.2. Cholesterol Concentrations Before and After Cryopreservation

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	3.381	1.127	65.80	<0.0001
Error	107	1.832	0.017		
Total	110	5.213			

Table 2.2. Zona Binding (Paired T-Test)

Analysis Variable: Diff			
Mean	Std Error	t Value	Pr > |t|
2.769	0.890	3.11	0.027

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (75 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	3512.963	1756.481	19.33	<0.0001
Error	24	2180.667	90.861		
Total	26	5693.630			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (150 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	5852.519	2926.259	9.79	0.0008
Error	24	7176.889	299.037		
Total	26	13029.407			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (225 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	1632.296	816.148	3.44	0.0485
Error	24	5692.667	237.194		
Total	26	7324.963			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (270 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	381.407	190.704	1.14	0.3375
Error	24	4025.556	167.731		
Total	26	4406.963			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (300 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	438.296	219.148	1.24	0.3086
Error	24	4257.778	177.407		
Total	26	4696.074			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (350 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	1604.519	802.259	2.11	0.1432
Error	24	9124.889	380.204		
Total	26	10729.407			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (370 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	5940.571	2970.286	10	0.0008
Error	24	6533.429	296.974		
Total	26	12474			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (425 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	2530.667	1265.333	6.67	0.005
Error	24	4553.333	189.722		
Total	26	7084			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (600 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	294	147	9.17	0.0011
Error	24	384.667	16.028		
Total	26	678.667			

Figure 3.1. Osmotic Tolerance Limits of Sperm Placed in Anisosmotic Solutions (1200 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	4.222	2.111	4.30	0.0253
Error	24	11.778	0.491		
Total	26	16			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (75 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	2697.185	1348.593	6.72	0.0048
Error	24	4815.333	200.639		
Total	26	7512.519			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (150 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	3708.598	1854.299	11.24	0.0004
Error	23	3793.556	164.937		
Total	25	7502.154			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (225 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	682.889	341.444	5.33	0.0121
Error	24	5913.778	246.407		
Total	26	6596.667			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (270 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	89.556	44.776	0.32	0.7297
Error	24	3366.444	140.269		
Total	26	3456			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (300 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	445.407	222.704	1.03	0.3714
Error	24	5176	215.667		
Total	26	5621.407			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (350 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	80.222	40.111	0.24	0.7908
Error	24	4060.444	169.185		
Total	26	4140.667			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (370 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	199.406	99.701	0.57	0.5738
Error	23	4029.056	175.176		
Total	25	4228.462			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (425 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	194	97	0.42	0.6617
Error	24	5540.667	230.861		
Total	26	5734.667			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (600 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	4149.407	2074.704	18.91	<0.0001
Error	24	2633.778	109.741		
Total	26	6783.185			

Figure 3.2. Osmotic Tolerance Limits of Spermatozoa Returned to Isosmolality (1200 mOsm)

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	93.852	46.926	4.17	0.0278
Error	24	269.778	11.241		
Total	26	363.630			

Table 3.2 L_P for Stallion Spermatozoa

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	17	51.319	3.019	0.97	0.611
CLC	2	7.669	3.835	1.10	0.3328
Temp	1	0.168	0.168	0.05	0.8263
CPA	2	16.898	8.449	2.43	0.0894
CLC*Temp	2	0.773	0.386	0.11	0.8947
CLC*CPA	4	10.447	2.612	0.75	0.5572
Temp*CPA	2	2.960	1.480	0.43	0.6533
CLC*Temp*CPA	4	2.404	3.101	0.89	0.4683
Error	305	1059.042	3.472		
Total	322	1110.361			

Table 3.1 P_{CPA} for Stallion Spermatozoa

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	17	15.836	0.932	6.05	<0.0001
CLC	2	1.244	0.622	4.04	0.0185
Temp	1	9.483	9.483	61.55	<0.0001
CPA	2	2.391	1.196	7.77	0.0005
CLC*Temp	2	0.352	0.176	1.14	0.3198
CLC*CPA	4	0.541	0.135	0.88	0.4774
Temp*CPA	2	1.627	0.813	5.28	0.0055
CLC*Temp*CPA	4	0.197	0.049	0.32	0.8650
Error	357	55.002	0.154		
Total	374	70.838			

Table 3.2. P_{CPA} of Control Stallion Spermatozoa at 22°C with Various Cryoprotectants

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	1.656	0.828	8.07	0.0008
Error	63	6.462	0.103		
Total	65	8.119			

Table 3.2. P_{CPA} of Control Stallion Spermatozoa between 5° and 22°C

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	1	5.165	5.165	71.97	<0.0001
Error	126	9.042	0.072		
Total	127	14.207			

Table 3.2. P_{CPA} of Stallion Spermatozoa Comparing Control vs. CLC Treatment

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	1.244	0.622	3.32	0.0371
Error	372	69.594	0.187		
Total	374	70.838			

Parameter	Estimate	Std Error	t Value	Pr > |t|
Con vs. CLC	-0.114	0.047	-2.43	0.0157
CLC1 vs. CLC2	-0.053	0.055	-0.95	0.3405

Table 3.2. P_{CPA} of Stallion Spermatozoa at 5°C and DMF Comparing CLC Treatments

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	0.234	0.117	1.62	0.208
Error	53	3.833	0.072		
Total	55	4.067			

Table 3.2. P_{CPA} of Stallion Spermatozoa at 5°C and EG Comparing CLC Treatments

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	0.761	0.380	3.68	0.0312
Error	59	6.100	0.103		
Total	61	6.861			

Table 3.2. P_{CPA} of Stallion Spermatozoa at 5°C and GLY Comparing CLC Treatments

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	0.896	0.448	4.09	0.0219
Error	56	6.134	0.110		
Total	58	7.031			

Table 4.1. Total Motility for Spermatozoa Frozen in Three Cryoprotectants

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	8551.011	4275.506	17.76	<0.0001
Error	177	42613.983	240.757		
Total	179	51164.994			

Table 4.1. Progressive Motility for Spermatozoa Frozen in Three Cryoprotectants

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	1777.811	888.906	4.99	0.0078
Error	177	31546.167	178.227		
Total	179	33323.978			

Table 4.1. Viability for Spermatozoa Frozen in Three Cryoprotectants

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	2	70.902	35.451	0.25	0.7827
Error	176	25432.226	144.501		
Total	178	25503.129			

Figure 4.1. Total Motility for Spermatozoa Frozen 10 Cooling Rates

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	9	1042.717	115.857	0.39	0.9373
Error	170	50122.278	294.837		
Total	179	51164.994			

Figure 4.1. Progressive Motility for Spermatozoa Frozen 10 Cooling Rates

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	9	499.867	55.541	0.29	0.9774
Error	170	32824.111	193.083		
Total	179	33323.978			

Figure 4.1. Viability for Spermatozoa Frozen 10 Cooling Rates

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	9	3012.733	334.748	2.52	0.010
Error	169	22490.395	133.079		
Total	178	25503.128			