DISSERTATION

CHOLESTEROL SUPPLEMENTATION OF BOVINE SPERM

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PHILLIP HAMILTON PURDY ENTITLED "CHOLESTEROL SUPPLEMENTATION OF BOVINE SPERM" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHOLESTEROL SUPPLEMENTATION OF BOVINE SPERM

Bull sperm were treated with a range of cholesterol-loaded cyclodextrin (CLC) concentrations and frozen in egg yolk Tris or sodium citrate diluent. After thawing, the percentages of motile sperm in the 1.5mg CLC treatment (Tris) were higher (60%) than control (0mg CLC) sperm (42%; $P<0.05$) but no differences were detected with the sodium citrate diluent ($P>0.05$). Bull sperm treated with 3.0mg CLC and frozen in egg yolk Tris diluent had more viable cells (66%) compared to the control (48%; $P<0.05$).

Analysis of cholesterol incorporation by spectrofluorometry and HPLC demonstrated that cholesterol content of bull sperm increases with CLC treatment ($R^2=0.824$) and flow cytometry illustrated uniform cholesterol incorporation (CV=12.9% $\pm$ 0.73).

Bull sperm were analyzed to for calcium levels and ability to acrosome react when treated with dilauroylphosphatidylcholine (PC-12), calcium ionophore A23187 (A23187) or heparin. Fresh sperm treated with 1.5mg CLC accumulated calcium at lower rates compared to control sperm when cells were capacitated with PC-12 or A23187 ($P<0.01$). After cryopreservation, CLC treated sperm accumulated calcium at slower when capacitated with PC-12 only. CLC treatment also decreased the rate fresh sperm underwent the acrosome reaction, compared to control sperm, when cells were capacitated with A23187 or heparin ($P<0.05$). No differences were detected in the rate cryopreserved sperm acrosome reacted with any of the compounds tested.

In vitro fertilization using frozen thawed bull sperm treated with either 0 or 1.5mg CLC prior to cryopreservation resulted in no differences in fertility ($P>0.05$).

A breeding trial using dairy heifers resulted in similar pregnancy rates for control (50%) sperm and for sperm treated with 1.5mg CLC (59%; $P>0.05$.

The membrane fluidity of Chinese hamster ovary cells and bull sperm was measured following treatment with CLC. Addition of CLC to CHO cells analyzed at 23°C or at 5°C resulted in decreased membrane fluidity compared to control cells. When CHO cells were cryopreserved (control and CLC treatments) membrane fluidity decreased. Treating CHO cells with PC-12 caused an increase in membrane fluidity. Bull sperm form 2 distinct populations of cells with different membrane fluidity, compared to the single population in CHO cell analyses. At 23°C, bull sperm treated with CLC showed reduced membrane fluidity, in a dose dependent manner. Analysis of percentages of sperm cells in the 2 populations demonstrated that cells from the high fluidity population were converting to the low fluidity population, as CLC concentration increased ($P<0.05$). After cooling to 5°C, control samples had significantly fewer viable cells (59%) than sperm samples treated with 1.5 or 5.0mg CLC (89 and 95%, respectively; $P<0.05$). Furthermore, at 5°C, cells shifted to the more fluid population in a dose dependent manner ($P<0.05$). Following cryopreservation, both populations of bull sperm were significantly less fluid than unfrozen cells ($P<0.05$). Addition of cholesterol resulted in higher percentages of cells surviving cryopreservation (>76%) compared to control sperm (60%; $P<0.05$). Treating bull sperm with PC-12 resulted in a single population of cells that was significantly different from all populations at 23°C ($P<0.05$).

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CHAPTER I

REVIEW OF LITERATURE

Introduction

The goal of cooling or cryopreserving bull spermatozoa is to recover the greatest number of viable cells after rewarming by minimizing cell damage and death. Even with optimal cooling and freezing protocols, sperm cells are still exposed to intracellular and extracellular ice (Mazur, 1977), osmotic injury (Steponkus et al., 1983; Watson, 2000), and cold shock (Watson, 2000); consequently the number of viable cells recovered is reduced.

Cooling and cryopreservation will be considered first in this literature review beginning with an overview of cryo-damage from intracellular ice formation, lipid peroxidation and cold shock. This section will also cover the lipid phase transition of the plasma membrane when cooled, as well as how membrane fluidity is measured. The second section presents information about the lipid composition of sperm membranes, how they were determined and the importance that the lipid ratios in the membranes have on cooling and freezing success. Next, the effects that cooling and cryopreserving sperm have on sperm capacitation and the acrosome reaction will be discussed.

Cooling and/or cryopreserving cells changes the cell membrane dramatically. These changes are due to both the reduced temperatures and the addition of

cryoprotectants. Therefore, membrane permeability and hydraulic conductivity are discussed and the importance of both of these factors in preserving sperm is explained.

This review will also cover the process of sperm capacitation and the acrosome reaction, as well as how several compounds induce capacitation and the acrosome reaction. The ability to assess sperm capacitation and the acrosome reaction will also be discussed, as well as means of inducing and inhibiting these actions.

Finally, a discussion of cyclodextrins is presented, including a description of the molecules, their cellular interactions, and their use in transferring lipids into cell plasma membranes, thereby changing cellular physiology.

The goal of this research was to enable sperm cells to better survive cryopreservation using cholesterol-loaded cyclodextrins. The information presented in this review provides the theoretical basis of why adding cholesterol might enhance cell cryopreservation survival.

Cooling and cryopreservation injury to cells

Ice formation and damage

The processes of cooling and cryopreserving cells cause cellular injury in a variety of ways. Mazur (1977) reported that intracellular ice formation is the principal cause of cell damage and death. Intracellular ice forms from extracellular ice growing through aqueous channels in the membrane and consequently seeding ice formation in the cellular contents (Mazur, 1977). This seeding nucleates ice formation and ice growth by recrystallization, causing cell membrane damage and or cell death (Mazur, 1977). Mazur (1977) also noted that the primary barrier to intracellular ice nucleation is the plasma

membrane. If cells are cooled at sufficiently slow rates to allow equilibration of cryoprotectants and intracellular water across the plasma membrane, intracellular ice formation is reduced and cell survival increases (Mazur, 1977).

Steponkus et al. (1983) agree that a major factor in minimizing cell damage during cooling and cryopreservation is the plasma membrane and its response to intracellular and extracellular ice. In addition, Steponkus et al. (1983) document that during cooling and cryopreservation, cells dehydrate, shrink and lose their osmotic responsiveness. When the cells are rewarmed, rehydration and cellular expansion occur, but because of the lack of osmotic responsiveness, a destabilization of the plasma membrane occurs which can cause cell membrane lysis (Steponkus et al. 1983).

Lipid peroxidation

The previous examples of cell membrane damage are attributed primarily to the formation of intracellular ice, but many other types of injury are the direct result of the membrane destabilization. Lipid peroxidation, enhanced by membrane destabilization during cooling and cryopreservation, produces reactive oxygen species, which in turn, decrease the viability of sperm (Watson, 2000). The production of reactive oxygen species occurs during both the cooling and the rewarming processes, and can prematurely capacitate and induce the acrosome reaction in sperm cells (Chatterjee and Gagnon, 2001).

Cold shock/Phase separation

Rapid cooling of sperm cells causes damage and destabilization of the plasma membrane, as well as intracellular damage and loss of motility, in what is commonly known as "cold shock" (Drobnis et al., 1993). During this damaging process, intracellular calcium increases, potassium decreases and membrane fluidity is reduced (Drobnis et al., 1993; Ladha et al., 1997). This reduction in membrane fluidity is due to membrane reorganization resulting from the decreased temperatures. Temperature reduction causes the plasma membrane to undergo a phase transition in which the membrane lipids change from a liquid-crystalline state to a gel state (Holt and North, 1986; Drobnis et al., 1993). As a result of the phase transition, there is a measurable decrease in the fluidity of the plasma membrane at low temperatures compared to the same membranes at room temperature (Holt and North, 1986; Canvin and Buhr, 1989).

The plasma membrane of a sperm cell is not the same membrane physiologically that it was prior to cooling and cryopreservation. As a result of the decrease in temperature, and the corresponding phase transition, there is a significant loss of total plasma membrane phospholipid (White, 1993). The membranes reorganize during cooling and warming and form nonbilayer structures that cause membrane perturbations resulting in leaky membranes (Drobnis et al., 1993; Parks and Lynch 1992). Furthermore, Ladha et al. (1997) reported that after cryopreservation, sperm cells that have compromised membrane integrity, or are dead, exhibit a decrease in membrane fluidity and large membrane lipid fractions that are immobile. Viable sperm cells exhibit membranes reorganized into aggregations of phospholipids, cholesterol-rich and cholesterol-poor microdomains, and proteins (Ladha et al., 1997). In addition, fatty acyl

chains can be damaged and become cross-linked, both of which promote lipid peroxidation (Johansson and Magnusson, 1990; Ladha et al., 1997) as well as formation of the nonlamellar hexagonal-II phase lipid structures (Quinn, 1985; Ladha et al., 1997), all of which contribute to membrane destabilization.

Holt and North (1986) reported that the lipid phase transition is also responsible for phospholipid hydrolysis and demonstrated that the status of the membrane determines the level of enzymatic activity, particularly of phospholipase A_2. During cooling and cryopreservation, phospholipid hydrolysis causes the release of lysolipids and free fatty acids (Mostafa et al., 1984; Holt and North, 1986). This release stimulates ATPase activity and results in membrane lipid rearrangement, which contributes to the deleterious factors described previously (Holt and North, 1986).

As was stated, following cooling and cryopreservation there is a reorganization of plasma membrane lipids attributed to the phase transition. However, intact cells show very little exchange of phospholipids between the inner and outer leaflets of the plasma membrane following cryopreservation (Muller et al., 1999). On the other hand, damaged cells show membrane reorganization that occurs not just within a particular leaflet, but diffusion of membrane contents also occurs between leaflets. In particular, phosphatidylserine, which normally is present only in the inner leaflet of the plasma membrane, will move into the outer leaflet (Glander and Schaller, 1999; Muller et al., 1999). This implies that an active translocase in sperm cells modifies the asymmetry of the plasma membrane. However, this enzyme has not been specifically identified (Muller et al., 1999). Even though the manner of translocation has not been determined, the presence of phosphatidylserine in the outer leaflet of the sperm cell plasma membrane is

well documented and can be detected using Annexin V and flow cytometry (Glander and Schaller, 1999; Muller et al., 1999).

Inhibition of phase transitions

Cholesterol plays a major role in plasma membrane function at normal temperatures, as well as in how membranes respond to reduced temperatures. Rottem et al. (1973) demonstrated that the action of cholesterol in mycoplasma membranes was temperature dependent. When membranes at 37°C were treated with cholesterol, they became more rigid compared to the untreated (control) samples (Rottem et al., 1973). However, when the membranes were cooled, cholesterol exhibited the opposite effect, and membrane fluidity increased. Control membranes exhibited a phase transition at 25°C in the control samples; however, cholesterol-treated membranes did not exhibit a phase transition even when membranes were cooled to 4°C (Rottem et al., 1973). Rottem et al. (1973) also reported that cholesterol decreased the permeability of the membranes for various substances by causing the phospholipid hydrocarbon chains to pack tightly together in the membrane lipid core. Furthermore, the cholesterol prevented crystallization of the hydrocarbon chains in the plasma membrane, enabling the cells to withstand cold shock and phase transition injury more efficiently (Rottem et al., 1973).

Ladbrooke et al. (1967) reported similar actions for cholesterol in lecithin liposomes. They showed that cholesterol supplementation reduced the phase transition temperature of the membranes (Ladbrooke et al., 1967). More importantly, when cholesterol was added at an equimolar ratio with lecithin, the phase transition was eliminated (Ladbrooke et al., 1967). This observation was also seen when cholesterol

was added to erythrocytes (Ladbrooke et al., 1967). Ladbrooke et al. (1967) explained that these effects were attributed to the packing of the hydrocarbon chains in the membrane. When cholesterol is added, the lipids above their transition temperature will be less fluid because cholesterol limits their hydrocarbon chain motion while membranes that are below their transition temperature will see an increase in fluidity (Ladbrooke et al., 1967). Ladbrooke et al. (1967) explained that cholesterol supplementation orders the hydrocarbon chains of a membrane, but cholesterol will decrease the "cohesive forces" between the hydrocarbon chains when the temperature is decreased and this will increase membrane fluidity. As a result of the effects of cholesterol in these manners, a membrane will have a more stable structure over a wide range of temperatures (Ladbrooke et al., 1967).

A major factor in determining the phase transition is the membrane cholesterol content, but more specifically the cholesterol to phospholipid ratio. Parks and Lynch (1992) reported that boar, rooster, stallion and bull sperm had cholesterol to phospholipid ratios of 0.26, 0.30, 0.36 and 0.45, respectively. Furthermore, human sperm has a cholesterol to phospholipid ratio approaching 1.0; as a result, all of the species respond to temperature fluctuations/phase transitions differently. In addition, Parks and Lynch (1992) demonstrated that integral membrane proteins are also involved in determining the response to cold shock. Analysis of the cholesterol to phospholipid ratios suggests that rooster sperm (0.30) and boar sperm (0.26) should both cool poorly; however, rooster sperm survive cold shock much better that do boar sperm. This difference is likely due to differences in the integral membrane protein to phospholipid ratio. Rooster sperm have protein to phospholipid ratios of 0.46 compared to 0.80 and 0.86 for bull and stallion

sperm, respectively, which indicates that the complex process of the phase transition is dependent upon the interactions of the membrane cholesterol, phospholipids and proteins (Parks and Lynch, 1992).

Another important factor in determining membrane fluidity is the type of lipid present. White (1993) reported that it is not just the presence of cholesterol, phospholipids and proteins, or their ratios, but also the degree of saturation or unsaturation in the fatty acids that comprise the lipids in the membrane affects membrane fluidity. Sperm from a variety of species can be grouped into two general groups based on the ratio of the saturated to unsaturated fatty acids. Bull, ram and boar sperm typically have ratios of 2.5 to 3.0 (saturated vs. unsaturated fatty acids) while rabbit, dog, human and fowl sperm have ratios of about 1.0 (White, 1993). The differences in saturated and unsaturated fatty acids further differentiate sperm from the different species in their ability to withstand cold shock. Sperm having ratios between 2.5 and 3.0 are prone to cold shock while sperm with similar molar ratios of saturated and unsaturated fatty acids seem to be immune to cold shock damage (White, 1993). White (1993) observed that the level of unsaturation of lipids helps determine the fluidity in the sperm cell membrane. If the level of unsaturation is increased, the phase transition will decrease, and the sperm cells will be able to survive temperature fluctuations better (White, 1993). Furthermore, White (1993) stated that interactions of unsaturated fatty acids with cholesterol in sperm cell membranes result in a more cohesive membrane structure, and again, the cell is better prepared to withstand the damage associated with cooling and cryopreservation (White, 1993).

Fluidity measurements and interpretations

Measurement of membrane fluidity has been performed using a variety of fluorescent probes and procedures. Rana and Majumder (1990) used the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and spectrophotometry to assess differences in the fluidity of the plasma membranes from goat sperm obtained from different regions of the epididymis. It was previously known that there is a general decrease in membrane lipids and in sterols, particularly cholesterol, as the sperm cells progress through the epididymis. Rana and Majumder (1990) demonstrated that in accordance with these changes in membrane content, there is also a change in the fluidity of the plasma membranes as well. Caput sperm have the most fluid plasma membranes with a fluorescence polarization value (FPV) of 0.268. Corpus sperm have an intermediate value of 0.279 and cauda sperm have the highest FPV (0.290), indicating that there is a steady decrease in plasma membrane fluidity associated with epididymal maturation.

Using DPH and microviscometry, Holt and North (1986) demonstrated the effects of temperature on the phase transition and fluidity of ram sperm. They detected that ram sperm experience phase transitions that are directly related to temperature (Holt and North, 1986). Furthermore, this work illustrated that the addition of cholesterol to ram sperm using liposome technology decreased the phase transition temperature (Holt and North, 1986). By decreasing the phase transition temperature of ram sperm plasma membranes in this manner, Holt and North (1986) concluded that it was possible to avoid a phase transition that normally would have occurred in the cooling process, and therefore less damage to the sperm cells will occur.

Canvin and Buhr (1989) also demonstrated that temperature has a direct effect on sperm membranes. When boar sperm were stained with the trans-parinaric acid (tPNA) probe, spectrofluorometry revealed differences in membrane fluidity, in membranes from the head of the sperm (more fluid) and membranes from the midpiece and tail (body) of the sperm cell (Canvin and Buhr, 1989). Furthermore, the head became less fluid compared to the body of the sperm cell as the temperature was decreased (Canvin and Buhr, 1989). This work demonstrated that distinct membrane compartments exist in the different regions of a sperm cell.

The effect of temperature on membrane fluidity can also be evaluated using flow cytometry. Fox and Delohery (1987) reported that the membrane fluidity of Chinese hamster ovary cells decreased as temperature was reduced and also when the cells were incubated in a serum free medium. Flow cytometric methods use a lipid probe 1,6-diphenyl-1,3,5-hexatriene (DPH) along with the viability stain propidium iodide (PI). For this analysis, a polarized laser is used, and the amount of rotation of the fluorescent DPH molecule that has intercalated into the membrane of a cell, detected by the degree of depolarization of fluorescent light from the probe, is indicative of membrane fluidity (Fox and Delohery, 1987). If the membrane is fairly rigid, the polarization of the light emitted from the DPH molecules will not differ significantly from the polarization of light used to excite the probe. However, for a fluid membrane, the DPH molecules will rotate within the membrane between the time of excitation and emission, and the emitted fluorescence will have a polarization that is different from that of the light used to excite the probe (Fox and Delohery, 1987). The use of PI, in combination with DPH, enables

dead cells to be excluded from the analysis; therefore, data are collected only on the membranes of viable cells (Fox and Delohery, 1987).

The membrane fluidity of sperm in an ejaculate, prior to cryopreservation may also be indicative of the ability of sperm to survive cryopreservation. Giraud et al. (2000), stained sperm with DPH and analyzed the cells using spectrofluorometry. They reported a high correlation with pre-freeze sperm membrane fluidity and post thaw cell motility and viability. In particular, ejaculates exhibiting high membrane fluidity prior to freezing resulted in greater recovery of motile and viable cells after freezing and thawing (Giraud et al., 2000). In addition, there was a decrease in fluidity in the plasma membranes of sperm cells after freezing as measured by an increase in fluorescence polarization (Giraud et al., 2000). This observation demonstrates that cryopreservation results in plasma membrane reorganization as cryopreserved membranes exhibit much lower membrane fluidity than fresh membranes.

Sperm membrane fluidity also changes during capacitation. When sperm were stained with the fluorescent probe Merocyanine 540 and analyzed by flow cytometry, two distinct populations of cells, based on membrane fluidity, were found within an ejaculate (Flesch et al., 2001). When these two populations were separated by flow cytometry and analyzed using HPLC for both phospholipid and cholesterol content, no difference in phospholipid content was detected between the two populations; however, cholesterol content was significantly different (Flesch et al., 2001). Specifically, the population of cells that had high membrane fluidity had significantly less cholesterol than the population of cells with less fluid membranes (Flesch et al., 2001). In addition, when the cells were treated with bicarbonate to induce the acrosome reaction, only the fluid

population of cells containing low levels of cholesterol was capable of acrosome reacting (Harrison et al., 1996; Flesch et al., 2001). Together these results illustrate that cholesterol directly affects membrane fluidity in general, that cholesterol is lost from the cell during capacitation, and that sufficient cholesterol must be lost from the membrane during capacitation in order for the sperm to acrosome react.

Sperm membrane lipid composition

The composition of sperm membranes is affected by the epididymis. As sperm travel through the epididymis, the composition of membrane lipids and proteins is modified, permitting the sperm to become motile (caput) and fertile (cauda) (Rana and Majumder, 1990). Depending on the species, both lipid and protein composition may be significantly changed. Boar sperm exhibit a decrease in cholesterol concentration as the sperm traverse the epididymis, but an increase in desmosterol and cholesterol sulfate concentrations, resulting in an overall sterol concentration that does not change significantly (Ladha, 1998). In ram sperm, the overall sterol concentration decreases during passage through the epididymis, whereas in goat sperm the opposite occurs; however, for both of these species the overall sterol to phospholipid ratio increases (Parks and Hammerstedt, 1985; Rana et al., 1991; Ladha, 1998). These changes that occur during epididymal maturation result in an overall decrease in the plasma membrane fluidity as sperm traverse from the caput to the cauda epididymis (Rana and Majumder, 1990).

Parks et al. (1987) also reported differences between the outer acrosomal membrane, the plasma membrane and the total membrane components of whole sperm in

the protein, phospholipid and cholesterol content of bull sperm. In general, there are uniform distributions of each class of molecule throughout the plasma membrane (Parks et al., 1987). Analysis across regions shows a significant accumulation of protein, phospholipid and cholesterol in the outer acrosomal membrane compared with the plasma membrane (Parks et al., 1987). Parks et al. (1987) explained the rationale behind these different distributions in membrane constituents and concluded that the membrane must maximize stability during environmental challenges and also to minimize premature capacitation and acrosome reaction (Parks et al., 1987).

Effects of cooling and cryopreservation on sperm capacitation and the acrosome reaction

Cooling and cryopreservation destabilize plasma membranes and result in lipid loss and increased membrane fluidity, both of which make the membrane leaky to several ions (Green and Watson, 2001). Capacitation and the acrosome reaction induce similar membrane rearrangements (Harrison and Miller, 2000; Green and Watson, 2001). While the end results appear similar, the effects that cooling and cryopreservation have on membranes are not identical to true capacitation (Green and Watson, 2001). In true capacitation, the changes in membrane organization and the ionic effects are reversible to a great extent, which is not the case for membranes that have been cooled or cryopreserved (Green and Watson, 2001). Furthermore, cooling and cryopreserving porcine sperm cells enables an immediate acrosomal exocytosis, bypassing the need for capacitation (Green and Watson, 2001). The membrane rearrangements seen in capacitation are necessary in order to have a cell that is fusiogenic and capable of acrosome reacting, but the premature destabilization of membranes that occurs as a result

of cooling or freezing decreases viability and fertility (Nolan and Hammerstedt, 1997; Muller et al., 1999).

Many scientists have increased membrane fluidity by incorporating lipids into the membranes of sperm cells to counter the negative effects on membranes during cryopreservation. Incorporation of lipids into sperm cells can also inhibit capacitation (Carlini et al., 1997). Using several different types of liposomes, He et al. (2001) reported that lipid supplementation to boar sperm decreased the sensitivity of the cells to cold shock, but did not decrease the ability of the cells to capacitate. Liposomes composed of lipids from the plasma membrane of the sperm head or a combination of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and lysophosphatidylcholine, both resulted in higher percentages of viable and motile cells following cryopreservation (He et al., 2001). In addition, treatment of sperm with these lipid combinations did not inhibit capacitation or the acrosome reaction following cryopreservation when the cells were treated with ionophore A23187 to induce the acrosome reaction (He et al., 2001). On the other hand, liposomes containing phospholipids and cholesterol inhibit membrane fusion (Chaudhury and Ohki, 1981), an event similar to the acrosome reaction. This is very important because it demonstrates that the detrimental effects of cryopreservation on membrane structure and function can be ameliorated with lipid supplementation, and that lipid treatments do not necessarily affect the ability of sperm to capacitate and undergo an acrosome reaction (He et al., 2001).

Membrane permeability and hydraulic conductivity

Avoiding intracellular ice during cryopreservation is highly desirable. Controlling the cooling rate is important to minimize the intracellular ice and its resulting cell damage when cells are cryopreserved. As the environmental temperature decreases below 0°C extracellular water will freeze and form ice crystals. The ice crystals are pure water and consequently channels of extracellular fluid form that contain high concentrations of salts and the cells that are to be cryopreserved (Amann and Pickett, 1987). The intracellular water at this point does not freeze but supercools and a chemical potential develops that causes an exosmosis of water, resulting in cell dehydration (Amann and Pickett, 1987; Noiles et al., 1993). If the cooling rate is sufficiently slow, intracellular water will move out of the cell and a water-solute equilibrium will be maintained. If the cooling rate is too slow, the high concentration of salts may damage the cells upon rehydration due to excessive water influx resulting in membrane rupture (Amann and Pickett, 1987). Furthermore, if the cooling rate is too fast, sufficient dehydration will not occur, and intracellular ice will form (Mazur, 1977; Noiles et al., 1993).

If cryoprotective agents are added to the cells, the cooling rate will need to be changed due to changes in the hydraulic conductivity and solute permeability of the plasma membrane. When sperm are treated with penetrating cryoprotectants the cells shrink as water leaves due to the osmotic potential of the cryoprotectant in the extracellular compartment (Gilmore et al., 1997). The cell will then swell to regain its original volume as the cryoprotectant permeates the cell (Gilmore et al., 1997). As cooling occurs, the hydraulic conductivity and solute permeability decrease due to

membrane reorganization or blockage of water channels by the cryoprotectants (Gilmore et al., 2000). The opposite occurs when cryoprotectants are removed. The cells initially swell and then return to their normal state, provided that no membrane damage has occurred (Gilmore et al., 1997).

Membrane permeability coefficients can be determined using flow cytometry to measure critical osmolality (lysis of 50% of cells) and permeation time (Watson et al., 1992). From these values both the hydraulic conductivity and activation energy of sperm are determined and an optimal cooling rate for cryopreservation can be calculated. One of the problems using these data is that these optimal cooling rates do not increase cell survival over the empirical freeze rates developed in the past. In fact, quite frequently, these theoretically determined optimal rates are drastically different from the empirical rates presently being used, and result in poorer cell viability after thawing.

Capacitation and the acrosome reaction

The process known as capacitation is a series of maturation events that occurs in a sperm cell to enable the cell to acrosome react and ultimately fertilize an oocyte.

Capacitation

Visconti and Kopf (1998) reported that both media constituents and intracellular second messengers are important in sperm capacitation. Their discussion of media however, was confined to serum albumin, calcium and bicarbonate. Evidence suggests that serum albumin is responsible for removal of cholesterol from the plasma membrane of sperm cells (Visconti and Kopf, 1998; Visconti et al., 1999). In particular, bovine serum albumin (BSA) has been used as an acceptor of cholesterol in capacitation, where

it functions as a "sink" for the removal of cholesterol from sperm (Visconti and Kopf, 1998). Additional work demonstrated that high density lipoproteins (HDL) act in the same manner as BSA for somatic cell and oviductal fluids, documenting the concept of cholesterol acceptor molecules in an in vivo setting (Therien et al., 1998; Therien et al., 2001; Visconti et al., 1999). The mechanism of action by which BSA and HDL remove cholesterol is not known. It is known that cholesterol is removed from the sperm plasma membrane and either BSA or HDL from semen capture the lipid (Therien et al., 1998; Therien et al., 2001). The fluidity of the plasma membrane increases after cholesterol is lost and second messenger activity and ion transport associated with capacitation and the acrosome reaction increase (Therien et al., 1997; Therien et al., 2001; Visconti and Kopf, 1998).

Calcium is essential for sperm capacitation, but not all researchers agree on the functions of this chemical. Breitbart (2002) reported that the first event of capacitation is an increase in intracellular calcium, and observed that capacitation does not occur in calcium free media. Others (Nolan et al., 1992; Bailey and Buhr, 1993) indicate that calcium is a rate-limiting step but rank the activity as one of the first indicators that capacitation has started.

Parrish et al. (1999) reported that calcium regulates sperm intracellular pH, cAMP, and tyrosine phosphorylation, and that removing calcium suppresses these signaling cascades. This work also showed a time delay between the initial uptake of calcium and the molecular events associated with sperm capacitation. Since there is a delay, Parrish et al. (1999) hypothesized that sperm must somehow store calcium, most

likely within the acrosome, and that this is responsible for the regulation of capacitation through calcium sequestering.

Other research (Bailey and Buhr, 1993; Zhao and Buhr, 1995) agrees with this proposal and demonstrates that calcium increases the percentages of capacitated, hyperactive, and acrosome-reacted sperm cells in a dose dependent manner. Zhao and Buhr (1995) also reported an effect of temperature and diluents on intracellular calcium levels and state of capacitation and the acrosome reaction. They reported that measuring calcium levels was a very sensitive way of determining the true capacitation status of sperm cells. Calcium activity was strongly affected by temperature fluctuations and strongly affected both membrane fluidity and acrosomal status (Zhao and Buhr, 1995). The changes in intracellular calcium in all of these instances were attributed to alterations in plasma membrane ATPase activity, which in turn affected the intracellular calcium concentration (Zhao and Buhr, 1995).

Bicarbonate also plays a role in capacitation, although the actions of bicarbonate are less clear across species. In mice and hamsters there are anion exchangers that are responsible for controlling the intracellular pH, and it is believed that bicarbonate is capable of entering the cells using these exchangers (Visconti and Kopf, 1998). Once inside the cell, bicarbonate increases intracellular pH and regulates sperm cAMP metabolism, both of which affect sperm capacitation (Visconti and Kopf, 1998).

Intracellular second messengers

Visconti and Kopf (1998) describe how various second messenger signaling cascades result in capacitation. They explain that the cascades start either from

cholesterol efflux or as the result of chemical induction (Visconti and Kopf, 1998). Cholesterol efflux due to the extracellular influence of BSA causes membrane reorganization which in turn permits bicarbonate and calcium to enter a sperm cell (Visconti et al., 1995; Visconti and Kopf, 1998). Once inside the sperm cell, both molecules activate adenylate cyclase, which in turn causes an increase in cAMP activity (Visconti and Kopf, 1998). Cyclic AMP activates protein kinase A (PKA) to stimulate protein tyrosine kinases (PTK) which increases protein tyrosine phosphorylation, resulting in sperm capacitation (Visconti and Kopf, 1998). There is evidence that free radicals can stimulate protein phosphorylation and trigger capacitation as well (Leclerc et al., 1997; Visconti and Kopf, 1998). It has also been demonstrated that nitric oxide is a potential initiator of capacitation (Lamirande and Gagnon, 1993; Visconti and Kopf, 1998).

Chemical induction of capacitation may be accomplished using heparin, which binds to membrane receptors and causes increases in intracellular calcium, pH and cAMP (Parrish et al, 1994; Visconti and Kopf, 1998). The increase in intracellular calcium, pH and cAMP directly increases the rate of capacitation through modulation of PKA and the downstream signaling cascades associated with it (Parrish et al, 1994; Visconti and Kopf, 1998).

Glucose may also be involved in capacitation through regulation of pyruvate and its metabolites, but this appears to be species dependent, and the data are controversial (Parrish et al, 1994; Visconti and Kopf, 1998).

A final chemical of importance is potassium. Potassium entry results in hyperpolarization of the plasma membrane (Zeng et al., 1995; Visconti and Kopf, 1998).

Membrane potential changes similar to this in other cells have been associated with fluctuations in calcium flow and channel activity but effects of potassium and potassium channel activity in sperm cells are not well understood (Zeng et al., 1995; Visconti and Kopf, 1998).

Acrosome reaction

The acrosome reaction is not possible in a sperm cell unless capacitation, or extreme damage to the cell, has occurred. Under in vivo conditions, the acrosome reaction is initiated by the sperm binding to zona pellucida glycoprotein 3 (ZP3) (Breitbart and Spungin, 1997). There are three different theories for the processes of capacitation inducing the acrosome reaction (Parrish et al., 1999). The first possibility described by Breitbart and Spungin (1997) states that following zona binding, an increase in cAMP occurs and this triggers the calcium channels in the cytoplasm to open and calcium enters the acrosome. The influx of calcium increases pH and triggers the formation of DAG, by the cleavage of PIP_2 from PLCγ (Breitbart and Spungin, 1997). DAG formation increases protein kinase C (PKC) activity and activates the plasma membrane calcium channels so that a second increase in calcium will occur (Breitbart and Spungin, 1997). The PKC will also activate PLA_2 which releases arachadonic acid from membrane phospholipids, and this, along with the increases in calcium and pH, leads to the fusion of the outer acrosomal and the plasma membranes resulting in acrosomal exocytosis (Breitbart and Spungin, 1997; Breitbart, 2002).

A second theory involves an increase in G protein second messengers due to the sperm cell binding ZP3 (Florman et al., 1989; Parrish et al., 1999). These second

messengers cause PLCγ to release IP_3 or activate adenylate cyclase, both of which will cause an increase in intracellular cAMP (Florman et al., 1989). This cAMP releases the calcium stored in the acrosome, which opens a store operated calcium channel, resulting in an additional spike in intracellular calcium (Florman et al., 1989, Breitbart, 2002). This calcium then triggers fusion of the outer acrosomal and plasma membranes (Florman et al., 1989).

The third theory very simply identifies the acrosome as a calcium store that opens only during fusion of the plasma and acrosomal membranes (Parrish et al., 1999). Regardless of the theory, once fusion has occurred, fenestrations in the membranes enable the sperm cell to release the acrosomal contents (Breitbart, 2002). The acrosomal hydrolytic enzymes erode the hyaluronic matrix of the oocyte and potentially allow zona penetration and completion of the acrosome reaction (Nolan et al., 1992; Breitbart, 2002).

Inhibition of capacitation and the acrosome reaction

Capacitation and the acrosome reaction may be inhibited using pharmacological agents that manipulate membrane activity or by altering media composition.

Nolan et al. (1992) initiated the acrosome reaction using dilauroylphosphatidylcholine liposomes and observed that the acrosomal exocytosis could be inhibited with N-ethylmaleimide (NEM). NEM is a sulfhydryl-modifying reagent that inhibits the aminophospholipid translocase responsible for sperm plasma and mitochondrial membrane lipid asymmetry (Nolan et al., 1992). Treatment with this chemical maintains membrane stability and integrity by arresting any membrane reorganization occurring in the sperm membranes (Nolan et al., 1992). Samples not

treated with NEM underwent an acrosome reaction 2.5 to 10 times more effectively than cells that had been treated (Nolan et al., 1992).

Cross (2000) commented that sterol loss, and particularly cholesterol, was the initial event in the capacitation process. More importantly, he demonstrated that cholesterol and desmosterol treatment inhibited sperm capacitation (Cross, 1998; Cross, 2000). The cholesterol effect also appears to rely on an interaction with the phospholipid sphingomyelin. In studies where sphingomyelin from somatic cells was used, a variety of results were observed, but in particular, this triggered cholesterol efflux (Cross, 2000). While the cholesterol efflux in these instances may be an artifact of membrane degradation, cholesterol in conjunction with sphingolipids produce plasma membrane rafts that limit the lateral mobility of membrane proteins (Simons and Ikonen, 1997; Cross, 2000). Therefore, one of the primary effects of cholesterol and sphingolipids on the plasma membrane is to maintain homogeneity and inhibit capacitation and the acrosome reaction.

Maintaining plasma membrane integrity using cholesterol also inhibits capacitation and the acrosome reaction by suppressing the second messenger signaling cascades. Capacitation and the acrosome reaction involve signaling pathways that activate tyrosine kinases and protein tyrosine phosphorylation via plasma membrane tyrosine kinase or tyrosine-associated kinase receptors (Visconti et al., 1998). When these kinases are activated during capacitation there is a corresponding increase in PKA activity, but Visconti et al. (1998) demonstrated that this increase in PKA activity only follows treatment with BSA and an efflux of plasma membrane cholesterol. Visconti et al. (1998) commented that the true action of cholesterol in this process is not known, but

speculated that cholesterol efflux increases the permeability of the plasma membrane and therefore bicarbonate, and calcium entry will activate the adenylyl cyclase second messenger cascade.

Work to determine if cholesterol could inhibit the acrosome reaction in human sperm following treatment with progesterone was performed by Khorasani et al. (2000), similar to that of Visconti et al., (1998). This work demonstrated that the presence of cholesterol in the plasma membrane suppressed sperm capacitation and the acrosome reaction. Khorasani et al. (2000) concluded that cholesterol has an effect on the plasma membrane by decreasing its fluidity, but also speculated that cholesterol may compete with or block progesterone receptors on the plasma membrane and inhibit ligand activity, thus inhibiting capacitation and the acrosome reaction.

Assessment of capacitation and the acrosome reaction

Intracellular calcium measurements

Intracellular calcium levels may be used as an indicator of sperm capacitation and the acrosome reaction because increases in intracellular calcium concentrations are correlated with the processes. Nolan et al. (1992) demonstrated that an increase in intracellular calcium occurs prior to the acrosome reaction, and that the acrosome reaction is prevented in calcium-free media. These authors were able to measure a concentration dependent increase in calcium levels in acrosome reacted bull sperm using the fluorescent probe Fluo3 and flow cytometry (Nolan et al., 1992). The authors cautioned, however, that use of the fluorescent probe in this manner provides an estimate of calcium in the entire cell and not specific regions (Nolan et al., 1992). Therefore,

measuring calcium using this technology may be measuring calcium in the mitochondria or cytoplasm, and not specifically that of the acrosome itself (Nolan et al., 1992).

Calcium levels have also been measured in bull (Bailey and Buhr, 1993; Zhao and Buhr, 1995) and human (Brewis et al., 2000) sperm using the ratiometric fluorescent stain Indo-1/AM and flow cytometry. Because this stain is ratiometric, it can be used to measure increases in intracellular calcium concentrations, or show shifts in populations exhibiting "low or high" calcium concentrations (Brewis et al., 2000).

Acrosome reaction measurement

A variety of staining methods in combination with microscopic evaluation may be used to assess both sperm capacitation and the acrosome reaction, but flow cytometric analysis of lectins conjugated with fluorescent molecules enables speed, objectivity and evaluation of a large number of sperm cells in a very short time. Typically, the fluorescent molecule is fluorescein or a similar molecule that excites at 488nm and emits at approximately 525nm to allow ease of use with visible lasers. A variety of lectins may be used for analysis, but the most common is peanut lectin, which is very specific for terminal beta-galactose (Molecular Probes Product Information, 2002). Lectins have been used successfully to assess acrosomal integrity in bull (Nolan et al., 1992), human (Khorasani et al., 2000) and stallion sperm (Rathi et al., 2001). The lectins only bind to the terminal beta-galactose residues of the acrosomal contents and therefore, will only bind to cells that have undergone an acrosome reaction.

Induction of in vitro capacitation and the acrosome reaction

Heparin

Heparin is a glycosaminoglycan that can induce capacitation. Manjunath and Therien (2002) report that we do not have a complete understanding of heparin activity, but hypothesized that there may be an interaction with bovine seminal plasma proteins. Binding of bovine seminal plasma proteins to sperm cells causes an increase in the number of heparin binding sites on the sperm (Manjunath and Therien, 2002). Heparin binds to these sites and interacts synergistically with the bovine seminal plasma proteins on the sperm surface (Manjunath and Therien, 2002). It is believed that because the bovine seminal plasma proteins normally interact with HDLs and cause capacitation, heparin catalyzes this reaction and increases the number and rate of cells undergoing capacitation (Parrish and First, 1991; Manjunath and Therien, 2002).

First and Parrish (1987) also explained that heparin may displace sperm surface proteins that inhibit capacitation, reorganize membrane domains, or directly cause an increase in intracellular calcium levels (Varner et al., 1993). In addition, heparin may also activate phospholipase A_2 or PKA, all of which will result in the capacitation of a sperm cell (First and Parrish, 1987; Varner et al., 1993).

Calcium ionophore A23187

Calcium ionophores have been used to induce the acrosome reaction in bull (Januskauskas et al., 2000), stallion (Alm et al., 2001) and goat sperm (Pereira et al., 2000). The ionophore A23187 is an "ion carrier that transports cations (Ca^{2+}) from an aqueous medium into an organic phase" (Januskauskas et al., 2000). Administration of

this chemical causes calcium entry down the electrochemical gradient (Januskauskas et al., 2000). Because of the nature of the acrosome reaction, calcium is required to be present in the incubation media or the acrosome reaction will not occur (Januskauskas et al., 2000). The ionophore results in a massive influx of calcium that may be above physiological levels and could potentially damage the sperm cells (Watson et al., 1992; Alm et al., 2001). Aside from experimental uses for assessing the ability of a sperm cell to acrosome react, calcium ionophores have also been used to improve in vitro fertilizing potential by forcing an acrosome reaction (Pereira et al., 2000).

Dilauroylphosphatidylcholine (PC-12)

PC-12 liposomes induce a rapid acrosome reaction in sperm cells. The PC-12 liposomes do not fuse with sperm cells, but rather "spontaneously transfer short-chain phospholipids from vesicles to cells" (Nolan et al., 1992). The transfer is dependent on the "solubility of the aqueous phase and the acyl chain length of the transferring lipid species" (Nolan et al., 1992). Membrane reorganization occurs once the sperm cell has been treated with PC-12 because incorporation causes accumulation of the short chain lipids in the outer leaflet of the plasma membrane (Nolan et al., 1992). PC-12 incorporation causes an asymmetry between the inner and outer leaflets of the plasma membrane, and when calcium is added, results in membrane fusion and enables a sperm cell to acrosome react (Nolan et al., 1992).

Cyclodextrins

Cyclodextrins are cyclic saccharides of glucose monomers that increase the solubility of nonpolar substances through their incorporation into the hydrophobic center of the molecule, thus forming inclusion complexes (Klein et al., 1995). To date, a variety of nonpolar substances have been used to form inclusion complexes, including cholesterol, pregnenolone and cholestene (Klein et al., 1995).

Three basic types of cyclodextrins exist (α, β and γ) and may be modified through addition of methyl or hydroxyl groups. The cyclodextrins have been used to remove lipids from a variety of cell plasma membranes, but the β cyclodextrins preferentially extract cholesterol over other membrane lipids (Klein et al., 1995). The cyclodextrins are also capable of inserting cholesterol into the plasma membrane of cells. The methyl-β-cyclodextrin inclusion complex alters plasma membrane cholesterol content by forming a "cholesterol-containing pool in the aqueous phase by means of the cyclodextrin" (Klein et al., 1995). The pool then establishes an equilibrium between the membrane bound cholesterol and that of the inclusion complex (Klein et al., 1995).

Use of cyclodextrin inclusion complexes is beneficial to cryopreservation of stallion sperm. Combes et al. (1998) demonstrated that incorporation of cholesterol into the plasma membrane of stallion sperm prior to freezing, resulted in higher motility (67%), and higher percentages of viability (61%) compared to controls that were not treated with cholesterol (52% and 40%, respectively). The rationale for the use of these complexes was to increase the cholesterol to phospholipid ratio of the plasma membrane and decrease the effects of the phase transition (Combes et al., 1998).

Zeng and Terada (2001) reported contradictory results with boar sperm. In this study, Zeng and Terada (2001) used methyl-β-cyclodextrin that were either empty or pre-loaded with cholesterol-3-sulfate. The authors believe that fluidity plays a prominent role in determining survival of cryopreservation and should therefore be increased by removing cholesterol from the plasma membrane using empty methyl-β-cyclodextrin (Zeng and Terada, 2001). In these experiments, removal of cholesterol did in fact provide greater percentages of viable, motile cells (50%) compared to either the control (no cyclodextrin or cholesterol, 15%) or the samples treated with cholesterol inclusion complexes (25% and 20% for 10 and 20mM cholesterol, respectively, Zeng and Terada, 2001). The results of this research must be interpreted cautiously, as the number of ejaculates was not disclosed, although it was noted that all ejaculates came from a single boar (Zeng and Terada, 2001). Furthermore, these results are viewed skeptically because the boar has one of the lowest cholesterol to phospholipid ratios in plasma membranes of mammalian sperm. It is known that species with ratios approaching 1 do not cold shock readily, and thus survive freezing more readily.

Because cyclodextrins are capable of removing cholesterol from plasma membranes, they have been used for capacitating and acrosome reacting mouse, bull (Visconti et al., 1999), and goat sperm (Iborra et al., 2000). Visconti et al. (1999) reported that both mouse and bull sperm were capable of capacitating and acrosome reacting in defined media devoid of BSA so long as methyl-β-cyclodextrin was present. Furthermore, Parinaud et al. (2000) reported that treating human sperm with 2-hydroxypropyl-β-cyclodextrin increased the rate of zona binding assessed in a hemizona-binding assay. In these analyses, 24% of the sperm treated with the cyclodextrin were

bound to the zona pellucida compared to 13% of sperm in the control samples (Parinaud et al., 2000). Sterol content was also determined for control and 2-hydroxypropyl-β-cyclodextrin treated samples after 4 hours of treatment; this illustrated that control sperm had significantly more membrane lipids (144 pmol/10^6 spermatozoa) compared to the treated samples (75 pmol/10^6 spermatozoa) (Parinaud et al., 2000). Therefore, the increased binding was attributed to the removal of cholesterol and desmosterol from the plasma membranes (Parinaud et al., 2000).

Conclusions

From this review it is obvious that increasing the plasma membrane cholesterol concentration of sperm should result in greater numbers of motile and viable cells after freezing. Treating sperm with cholesterol may be done with liposomes or cyclodextrins and this will increase the fluidity of the plasma membrane at decreased temperatures. Increasing the fluidity of the plasma membrane will decrease the phase transition temperature and enable greater dehydration of the cells, which will result in less cooling and freezing damage.

The studies presented here were performed to determine the feasibility and efficacy of treating bull sperm with cholesterol loaded cyclodextrin (CLC) prior to cryopreservation. Studies were performed to determine the motility and viability of CLC-treated sperm post thaw and to determine the quantity and uniformity of cellular cholesterol incorporation. Experiments were also performed to determine the effect of CLC on fertility using in vitro fertilization and a field trial. In addition, studies were designed to measure the effect of CLC treatment on the membrane fluidity of bull sperm

at various temperatures. Finally, because cholesterol is an inhibitor of capacitation and the acrosome reaction, an analysis of the calcium levels and acrosomal integrity was performed. Together these analyses were done to increase understanding of the role of cholesterol in capacitation and the acrosome reaction as well as in membrane integrity during cryopreservation.

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CHAPTER II

EFFECTS OF CHOLESTEROL LOADED CYCLODEXTRIN ON BULL SPERM DURING CRYOPRESERVATION

INTRODUCTION

Cryopreservation induces many stresses on spermatozoa, including plasma membrane destabilization (Steponkus et al., 1983), which causes intracellular ice formation resulting in cell death (Mazur, 1977). Membrane destabilization occurs when the membrane undergoes a phase transition from the fluid phase to the gel phase as temperature is decreased. At least some of this membrane damage can be diminished by adding lipids to the sperm prior to cooling and freezing (Combes et al., 1998; He et al., 2001). Although the exact mechanism by which egg yolk protects sperm membranes from cooling damage is not known, it may reduce the temperature at which the membranes undergo the phase transition. Adding cholesterol to liposomes lowered the lipid phase transition temperature, and with sufficient cholesterol, the phase transition was completely eliminated (Ladbrooke et al., 1968). Similarly, Rottem et al. (1973) reported that cholesterol supplementation to yeast cells eliminated plasma membrane phase transitions at all of the low temperatures tested.

Incorporation and removal of cholesterol from cell membranes has been reported for many cell types, including sperm. In particular, cyclodextrins (cyclic

heptasaccharides consisting of ß (1-4) glucopyranose units), which have a hydrophobic center, effectively remove cholesterol from cell plasma membranes (Klein et al., 1995). On the other hand, Combes et al. (1998) demonstrated that stallion sperm treated prior to cryopreservation with cyclodextrins that had been pre-loaded with cholesterol, resulted in higher percentages of motile and viable sperm after cryopreservation than untreated (control) sperm.

These experiments were conducted to determine if treating bull sperm with cholesterol-loaded cyclodextrins (CLC) could increase the percentages of motile and viable cells after freezing and also to develop an optimal freezing protocol for bull sperm treated with CLC.

MATERIALS AND METHODS

Chemicals

All chemicals, unless otherwise stated, were reagent grade (Sigma, St. Louis, MO).

Experiment 1: Determining effects of cholesterol incorporation on the motility and viability of bull sperm.

Cyclodextrin preparation

Cholesterol, 200mg, was dissolved in 1ml of chloroform. In a separate test tube 1g of Methyl-β-cyclodextrin was dissolved in 2ml of methanol. A 0.45ml aliquot of the cholesterol solution was added to the cyclodextrin solution, stirred until the solution was clear and then the solution was poured into a glass petri dish. The solvents were then removed using a stream of nitrogen gas and the resulting crystals allowed to dry for an

additional 24 hours, at which time they were removed from the dish and stored in a glass container at room temperature. A CLC working solution was prepared by adding 50mg of the CLC to 1ml TALP (Graham et al., 1986) at 37°C and mixing the solution briefly using a vortexer.

Semen collection and processing, diluent preparation and cryopreservation

Semen was collected from Holstein bulls by artificial vagina. For each ejaculate, the sperm concentration was determined using a spectrophotometer (Hammerstedt, 1975) and the initial percentage of motile sperm was determined visually.

Ejaculates from 9 bulls were split and one portion (control) was diluted to 120 x 10^6 cells in 1ml in diluent A (Table 2.1 containing Tris (T) or sodium citrate (NaC)), and incubated for 15 minutes at 23°C. Control samples were then diluted 1:1 (v:v) with an additional 1ml of diluent A, and the samples were cooled to 5°C over a 2 hour period. Upon reaching 5°C, control samples were diluted 1:1 (v:v) with diluent B, allowed to equilibrate for 15 minutes, and the sperm were then packaged into 0.5ml French straws and frozen in liquid nitrogen vapor (4.5cm above the N_2) for 12 minutes before being plunged into liquid nitrogen for storage.

Cells to be treated with CLC were diluted to 1ml at a concentration of 120 x 10^6 sperm in Tris diluent A1 (Table 2.1) containing either 0.75, 1.5, 3.0, 4.5, 6.0 or 7.5mg CLC or 1ml of the NaC diluent (A1) containing either 0.75, 1.5 or 3.0mg CLC. The sperm were incubated with the CLCs for 15 minutes at 23°C and then diluted 1:1 (v:v) with 1ml of diluent A2 of the respective diluent. The samples were then cooled to 5°C over a 2 hr period, diluted 1:1 (v:v) with the respective B diluent, and allowed to

equilibrate for 15 minutes. The sperm were then packaged into 0.5ml French straws and frozen in liquid nitrogen vapor as described above.

Table 2.1. Composition of egg yolk tris (T) and sodium citrate (NaC) bull sperm cryopreservation diluents used with control (C) and cholesterol-loaded cyclodextrin (CLC) treatments.

Diluent	Contents	Fractions	Egg yolk	Glycerol
T-C	25mM Tris, 8mM citric acid, 7mM glucose	A	20%	0
		B	20%	14%
T-CLC	25mM Tris, 8mM citric acid, 7mM glucose	A1	0%	0%
		A2	40%	0%
		B	20%	14%
NaC-C	1mM Sodium Citrate	A	20%	0%
		B	20%	16%
NaC-CLC	1mM Sodium Citrate	A1	0%	0%
		A2	40%	0%
		B	20%	16%

Motility analysis

Straws were thawed in a 37°C water bath for 30 seconds and the sperm motion characteristics were determined using a Hamilton Thorne Motility Analyser (Beverly, MA). The percentages of total motile and progressively motile sperm for a minimum of 200 cells using at least 5 areas of the slide were measured using the following parameters: frames acquired, 30; minimum contrast, 20; minimum cell size, 6; threshold straightness, 60; medium VAP cut-off, 60; low VAP cut-off, 25; low VSL cut-off, 10; nonmotile head size, 17; nonmotile head intensity, 70; magnification, 1.95; static size limits, 0.46-2.47; static intensity, 1.28; static elongation limits, 5-60.

Viability analysis

Ejaculates from 5 bulls were treated with 0, 0.75, 1.5, 3.0, 4.5, 6.0 or 7.5mg CLC frozen and thawed as described above. The contents of a single straw per treatment were emptied into separate test tubes containing 1ml of Tris A1 diluent. An aliquot (0.5ml) was transferred to a test tube containing 0.5ml Tris A1. The samples were then stained with SYBR-14 (5μl of a 20μM solution in DMSO) and propidium iodide (PI) (10μl of a 2.4mM solution in distilled water) (Molecular Probes, Eugene OR) as described by Garner et al. (1994), and the samples analyzed using an Epics V flow cytometer (Coulter Electronics, Miami, FL) equipped with an argon laser tuned to 488nm at 100mW power. Fluorescence from 50,000 cells was measured using a 515 long pass filter, a 525nm band pass filter to detect SYBR-14, a 590nm dichroic mirror and a 630nm long pass filter to detect PI (Garner et al., 1994). With this protocol all cells will stain with SYBR-14 but only non-viable cells will stain with PI.

Statistics

Data were transformed using arcsine, and treatment differences in the motility and viability of the frozen-thawed samples were determined using analysis of variance (Zar, 1996). Individual treatment means were separated using Student Newman Keuls multiple range test (Zar, 1996).

Experiment 2: Determining cholesterol incorporation into bull spermatozoa.

Semen was collected from 5 Holstein bulls by artificial vagina. For each ejaculate, sperm concentration was determined using a spectrophotometer (Hammerstedt, 1975), and the initial percentage of motile sperm was determined visually.

Spectrofluorometry

Aliquots of semen (240 x 10^6 cells/ml) were diluted in Tris buffered diluent (A1) and incubated for 15 minutes at 23°C with 0 (control), 0.75, 1.5, 3.0 or 6.0mg of CLC per 120 x 10^6 cells to a final volume of 1ml. Of the cholesterol loaded into the cyclodextrin, 1% was labeled with the fluorescent molecule 22-(N-(7-nitrobenz -2-oxa-1,3- diazol-4 - yl) aminl-23,24- bisnor-5- cholen-3B -ol (NBD). After incubation sperm were layered over 1ml of 70% Percoll and centrifuged at 585 x g for 20 minutes. The supernatant of each CLC treatment was removed, and the sperm pellet suspended at 50 x 10^6 cells per ml in 3ml of TALP. The amount of cholesterol in sperm from each treatment was determined by measuring the amount of fluorescence in each sample using a SPEX Fluorolog Tau-3 Lifetime System spectrofluorometer (Miami, FL). The spectrophotometer was set with an excitation wavelength of 468nm and recorded the amount of fluorescence detected at an emission wavelength of 537nm. Counts per second (CPS) of fluorescence were recorded every 10 seconds, for 50 seconds, per sample. A standard curve was determined using serial dilutions of NBD labeled cholesterol to quantify the amount of cholesterol for each CLC treatment.

HPLC

Aliquots of semen (500 x 10^6 cells) from 5 bulls were diluted in separate test tubes containing TALP and 0, 0.5, 1.0, 1.5, 2.0, 2.5 or 5.0mg CLC per 120 x 10^6 cells to a 1ml final volume and incubated at 23°C for 15 minutes. The samples were centrifuged for 18minutes through 70% Percoll and the pellet suspended in 1ml TALP. The samples were then prepared for HPLC analysis using a modification of the method described by Crick and Carroll (1987). Briefly, each centrifuged sample was saponified in a boiling

mixture of 2ml of 90% KOH (w/v) and 1ml methanol. The samples were washed (585 x g for 5 minutes per wash) 3 times with 2ml volumes of ether and the ether extracts were pooled. The pooled ether extracts were washed (585 x g for 5 minutes) with 4ml of distilled water and the ether fraction was recollected. The ether samples were washed with 10ml of 5% acetic acid, put into separate test tubes and the ether was removed using nitrogen gas. The resulting lipids were suspended in 1ml of chloroform/methanol (2:1, v:v) for analysis by HPLC. Samples (20µl injection volume) were analyzed for cholesterol concentration using a Waters HPLC (Milford, MA) equipped with a Hypersil 5µm, 250 x 4.6 mm reverse-phase C_{18} column (Supelco, Inc., Bellefonte, PA, #Z226343). The HPLC was also equipped with a variable wavelength detector set to 210nm. The mobile phase consisted of hexane delivered at 2 ml/minute. The amount of cholesterol in each sample was determined by comparing the area under the collected peaks with those of a standard curve of known amounts of cholesterol.

Statistics

Differences in the cholesterol content of treated bull sperm samples, analyzed by spectrofluorometry and HPLC, were determined by analysis of variance (Zar, 1996). The Student Newman Keuls test was used to separate treatment means for each analysis (Zar, 1996). Linear regression was used to determine the line represented in Figure 2.1 (Zar, 1996).

Flow cytometry

Aliquots of semen (120 x 10^6 cells/ml) from 5 bulls were diluted in Tris buffered diluent (A1) and incubated for 15 minutes at 23°C with 1.5mg of NBD labeled CLC in a final volume of 1ml. Samples were then stained with 5µl of 2.4mM PI (in

distilled/deionized water) for 10 minutes at 23°C. The samples were filtered through a 20μm nylon mesh, and the amount of NBD-labeled cholesterol in each live spermatozoa (PI negative) was measured using an Epics V flow cytometer. PI and NBD were excited using an argon laser tuned to 488nm and a 100mW power source. The flow cytometer configuration included a 515 long pass laser blocking filter and a 560nm dichroic filter. NBD was detected using a 560nm short pass filter and PI detected using a 630nm long pass filter. At least 50,000 cells were analyzed per sample.

Statistics

The coefficient of variation for each sample was determined using the Cyclops software of the Epics V flow cytometer. The mean and standard deviation of the samples were determined using SAS (Princeton, NJ).

RESULTS

Motility analysis

Bull sperm frozen in egg yolk Tris diluent containing 1.5mg CLC exhibited higher percentages of motile sperm (60%) than sperm frozen in egg yolk Tris alone (42%; $P<0.05$; Table 2.2). The addition of 1.5mg CLC to sperm diluted in NaC diluent resulted in maximum cell survival but the percentage of motile sperm (53%) was not significantly different from the control sperm (37%, $P>0.05$; Table 2.2).

Viability analysis

Bull sperm treated with 1.5 or 3.0 mg CLC and frozen in egg yolk Tris diluent had greater post thaw viability (61 and 66%, respectively) than sperm frozen without

CLC (control; 48%) but the treatments were not significantly different (P>0.05) (Table 2.2).

Cholesterol incorporation

Spectrofluorometry and HPLC: The cholesterol content of bull sperm, determined with spectrofluorometry following treatment with 1.5, 3.0 or 6.0mg NBD-labeled CLC, was 4.91, 5.61 and 6.68 x 10^6 counts per second (CPS), respectively, which were significantly higher than control (0mg) sperm (2.42 x 10^6 CPS; P<0.05). The 0.75mg CLC treatment (4.28 x 10^6 counts per second) was not significantly different from control. The cholesterol concentrations for the 0 (control), 0.75, 1.5, 3.0, and 6.0mg CLC treatments were 47, 81, 113, 145 and 190 μg, respectively, as determined from the standard curve of serial dilutions. When the amount of cholesterol in sperm was determined using HPLC, only sperm treated with 5mg CLC had higher levels of cholesterol compared to control sperm (P<0.05). However, the amount of cholesterol that was detected in sperm increased as the CLC dose increased. Regression analysis of the spectrofluorometric and HPLC values resulted in an R^2 value of 0.82 (Figure 2.1).

Flow cytometry: Flow cytometric analysis determined that cholesterol incorporates uniformly into live sperm resulting in a coefficient of variation of 12.9% ± 0.73 (Figure 2.2).

Table 2.2. Percentage of motile bull sperm treated with different dosages of cholesterol loaded cyclodextrin (CLC) and diluted in 2 different cryopreservation media, post thaw, (n=9) as well as the percentage of viable cells, post thaw, in egg yolk tris media, determined using flow cytometry and SYBR-14/PI analysis (n=5).

CLC dose (mg/ml)	Sodium citrate	Egg yolk tris	Viability
Control (0)	37^{a}	42^{b}	48^{ab}
0.75	45^{a}	58^{ab}	55^{ab}
1.5	53^{a}	60^{a}	61^{a}
3.0	50^{a}	57^{ab}	66^{a}
4.5		54^{ab}	57^{ab}
6.0		53^{ab}	45^{ab}
7.5		48^{ab}	31^{b}
SEM	5	4	6.4

^{ab}Different superscripts indicate treatment differences by column ($P<0.05$).

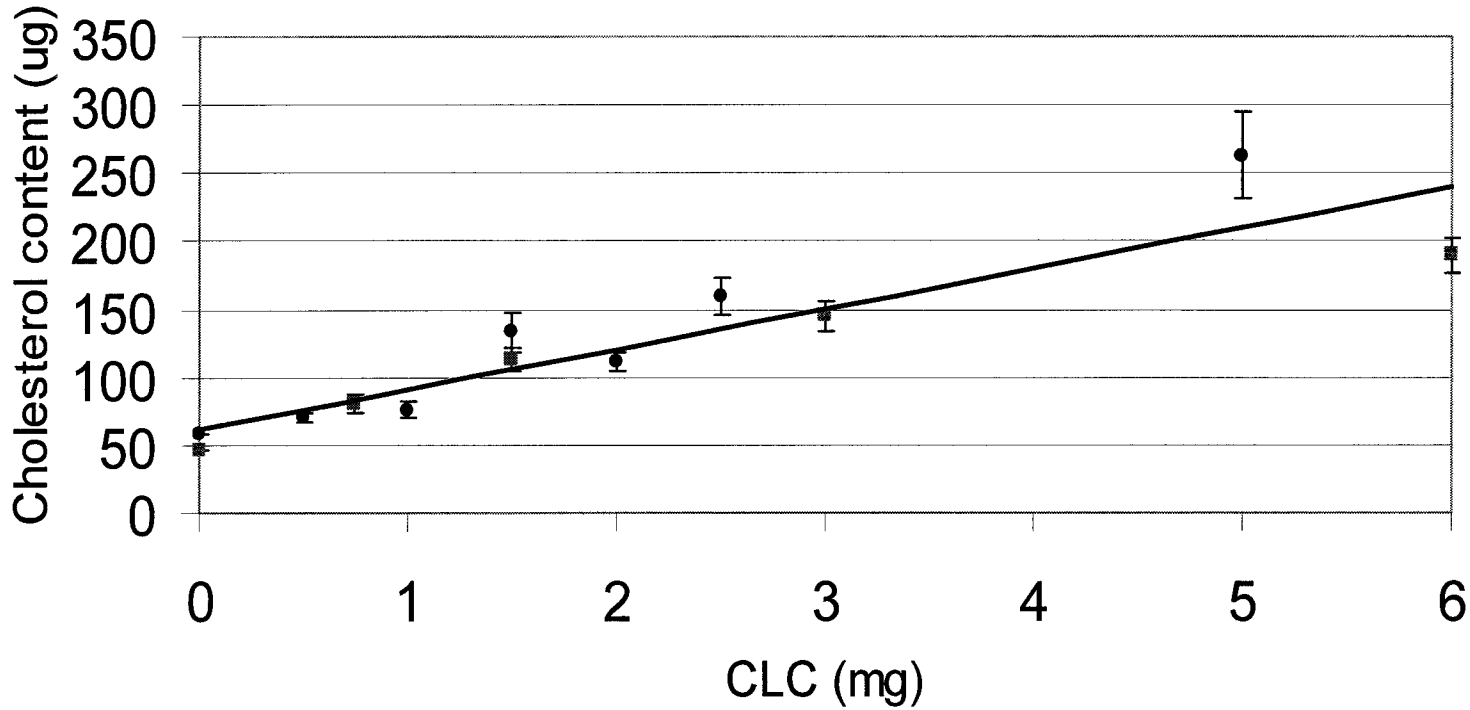


Figure 2.1. Cholesterol content of bull sperm determined with HPLC (circle) and spectrofluorometry (square). Bull sperm were treated with 0, 0.5, 1.0, 1.5, 2.0, 2.5 or 5.0mg cholesterol loaded cyclodextrin (CLC) prior to HPLC analysis and 0, 0.5, 0.75, 1.5, 3.0 or 6.0mg CLC prior to spectrofluorometry analysis. Regression analysis was used to determine the slope of the lines for spectrofluorometry and HPLC analyses ($y=29.8x + 60.9$; $R^2=0.82$).

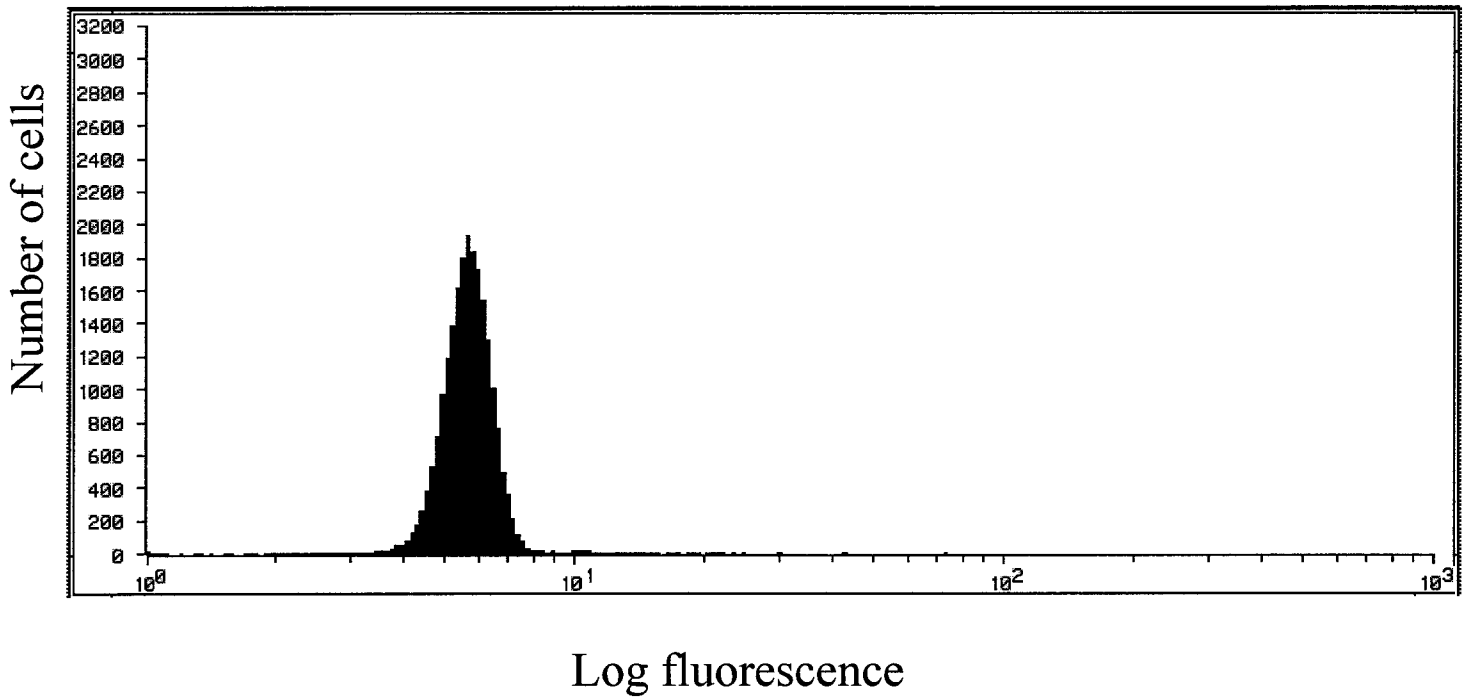


Figure 2.2. Flow cytometric analysis of the incorporation of fluorescently labeled cholesterol in sperm from 5 bulls treated with 1.5mg CLC per 120 x 10^6 cells. The coefficient of variation for these analyses is 12.9% ± 0.73.

DISCUSSION

During the freeze-thaw process, sperm cells experience both intracellular and extracellular stresses that result in membrane perturbations, lipid/protein reorganizations and osmotic injury (Mazur, 1977; Steponkus et al., 1983). These effects can be exacerbated by incorrect freezing and/or cooling rates, which result in sperm cell damage and death. In particular, damage occurs when the cell membranes undergo the phase transition from a liquid crystalline to a gel state and the deleterious effects, previously mentioned, occur. Overcoming these damaging effects, by decreasing the temperature at which this lipid phase transition occurs or by eliminating the phase transition completely, may be accomplished by increasing the fluidity of the plasma membrane. Ladbrooke et al. (1968) demonstrated that membrane fluidity could be increased at low temperatures by increasing the cholesterol content in the membranes to the point that during the cryopreservation process the phase transition was eliminated as was membrane damage. Rottem et al. (1973) produced similar results as Ladbrooke et al. using yeast cells, and suggested that the increased cholesterol concentration in membranes inhibits the crystallization of plasma membrane hydrocarbon chains, thereby eliminating the phase transition.

The results of this study show that treating bull sperm with cholesterol loaded cyclodextrin (CLC) results in the recovery of higher percentages of motile and viable cells after cryopreservation. This is in agreement with results reported by Combes et al. (1998) where stallion sperm was treated with cholesterol-loaded methyl-beta-cyclodextrin at different concentrations, and responded in a similar manner. Graham (1998) also reported that stallion sperm treated with CLCs had similar results. In this

study, stallion sperm was treated with cholesterol-loaded alpha, alpha-hydrate, beta, beta-hydrate or methyl-beta-cyclodextrin and cryopreserved. The cholesterol-loaded methyl-beta cyclodextrin (53%) was the only treatment with post-thaw motility significantly different from the control samples (28%; $P<0.05$) (Graham, 1998). In a separate experiment, Graham (1998) treated bull sperm with cholesterol-loaded methyl-beta-cyclodextrin and found that the post-thaw motility of treated samples (51%) was significantly different from control samples (35%; $P<0.05$).

Important differences exist between this preliminary work and the work presented here. The work by Combes et al. (1998) and Graham (1998) used CLCs that were manufactured according to Klein et al. (1995) in a procedure that required heating the mixtures of cholesterol and the cyclodextrin to 80°C and then evaporating the solvents used in the solutions. We have simplified the CLC preparation procedure so that the heating step is not necessary. The work by Graham (1998) demonstrated the benefit of CLC treatment on post-thaw cell recovery but tested only a single dosage (1.33mg) to cells of a lower concentration (50×10^6) than what was used in these analyses (1.5mg per 120×10^6 cells). We believe we were able to decrease the dosage because of the use of the modified diluent. The 3-part diluent was used in these experiments, because preliminary experiments showed that when CLCs were added to sperm already in media containing egg yolk, no increase in post-thaw motility or viability was detected. Consequently, the CLC dosage needed to be increased to a greater level to overcome the effect of the egg yolk. We hypothesize that in this situation the CLCs preferentially load cholesterol into egg yolk lipid droplets, and not the sperm. The 3-part diluent contains the same constituents as the 2 part diluents but the 3-part diluent allows incubation of

sperm cells with CLCs in egg yolk free media. Furthermore, we believe that it is beneficial to treat sperm cells with CLCs in this manner based on the observation of greater post-thaw motility following incubation in egg yolk-free media (60%) compared to the results obtained from Graham (1998) (51%) where the incubation occurred in a media containing 4% egg yolk.

Adding CLCs to sperm cells results in increased cell survival determined by CASA and flow cytometry, as well as increased cholesterol concentration in the spermatozoa determined by HPLC, flow cytometry and spectrofluorometry. We hypothesized that the CLC treatment would modify the membranes of sperm, reduce the temperature at which the membranes would undergo the phase transition, and consequently result in greater numbers of viable cells after freezing and thawing. However, we did not know whether the cholesterol would "rigidify" the cells (Giraud et al., 2000) and reduce motility at normal physiologic temperatures.

From our analyses using CASA, we determined an optimal dosage for use in cryopreservation and also illustrated the effects of supraoptimal CLC dosages, namely a decrease in motility. Analysis of the viability of the frozen thawed cells by flow cytometry also was used to determine the optimal dosage of CLC for cryopreservation. Likewise, decreases were also observed in post thaw viability associated with increasing CLC doses.

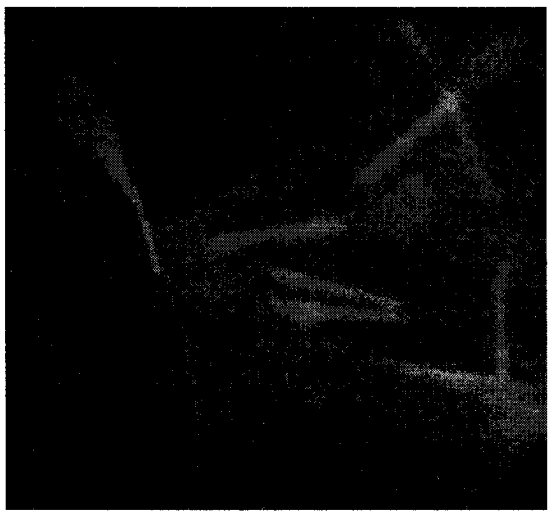
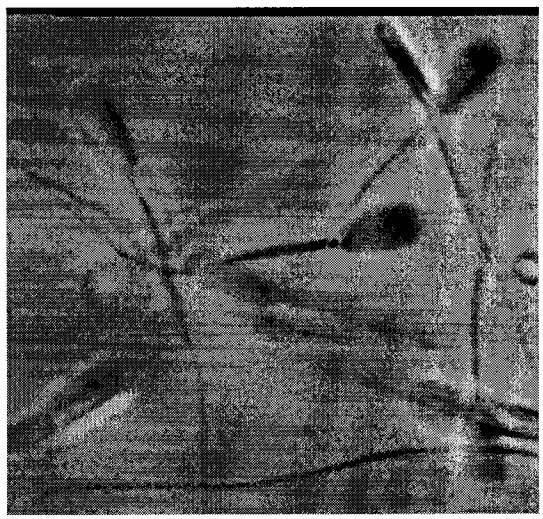
Analyses using spectrofluorometry and HPLC demonstrated that cholesterol incorporated into sperm cells in a dose dependent manner (Figure 2.1). We also determined that cholesterol was incorporated into sperm in a uniform manner within

ejaculates using flow cytometry (Figure 2.2). Furthermore, the fluorescent cholesterol could be visualized using fluorescence microscopy (Figure 2.3).

CONCLUSIONS

The use of cholesterol-loaded cyclodextrin results in greater percentages of motile and viable bull sperm cells post-thaw. We have also demonstrated that cholesterol incorporates into sperm cell plasma membranes in a uniform and dose dependent manner.

Figure 2.3. Photographs of bull sperm using (a) fluorescence and (b) phase contrast illumination after cells were incubated with cholesterol loaded cyclodextrin containing 1% NBD labeled cholesterol (400x magnification). The photographs were created using a Nikon Optishot-2 fluorescent microscope equipped with a Nikon Microflex UFX-DX Photomicrographic Attachment, FX-35DX camera and a filter cube containing a 450-490nm excitation filter, a 505nm dichroic mirror and a 520nm long pass filter.

a.  b. 

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CHAPTER III

EFFECTS OF CHOLESTEROL SUPPLEMENTATION ON BULL SPERM DURING CAPACITATION AND THE ACROSOME REACTION

INTRODUCTION

Capacitation of sperm is initiated by cholesterol efflux (Ehrenwald et al., 1988; Langlais and Roberts, 1985) that results in plasma membrane reorganization and subsequently an increase in membrane permeability to Ca^{2+}, HCO_3^- and K^+ (Visconti and Kopf, 1998 and cited authors). High intracellular concentrations of these ions are required for the acrosome reaction to occur (Parrish et al., 1999) and ultimately for gamete fusion (Brewis and Moore, 1997). A lack of any of these ions in the medium limits the rate of capacitation and the acrosome reaction to the degree that excluding Ca^{2+} from the medium will inhibit these processes (Visconti and Kopf, 1998). Because intracellular Ca^{2+} is so important in the early stages of capacitation, measuring intracellular levels of Ca^{2+} in sperm can indicate the state of capacitation that a spermatozoon is in. Parrish et al. (1993) demonstrated that calcium levels increase in the head of the sperm cells during capacitation. This calcium rise enables intracellular cAMP production, protein phosphorylation and an increase in intracellular pH (Visconti and Kopf, 1998).

The rate at which capacitation and the acrosome reaction occurs depends in large part upon the membrane status of the sperm and in particular on the level of cholesterol in

the membranes. Davis (1978) demonstrated that sperm could be "decapacitated" using a lipid vesicle derived from seminal plasma or with synthetic phospholipid liposomes containing cholesterol that inhibited the capacitation process. Cholesterol does not necessarily have to be actively incorporated into the sperm to affect capacitation; the mere presence of cholesterol in medium can also inhibit the acrosome reaction. Khorasani et al. (2000) inhibited sperm treated with progesterone from undergoing the acrosome reaction when cholesterol was added to the incubation medium. Similarly, when mouse and bull sperm were treated with cholesterol sulfate, sperm protein tyrosine phosphorylation decreased, indicating that capacitation was not occurring (Visconti et al., 1999a). On the other hand, when cholesterol was removed from the sperm plasma membrane, an increase in protein tyrosine phosphorylation occurred (Visconti et al., 1999a).

Altering the lipid composition of sperm plasma membranes not only affects capacitation and the acrosome reaction but also changes the way sperm respond to cryopreservation. When bull sperm were treated with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation, higher percentages of motile and viable cells were achieved after freezing and thawing compared to control sperm (Purdy and Graham, 2001). This added cholesterol most likely benefits cells by decreasing the temperature at which the phase transition occurs during cooling and cryopreservation (Ladbrooke et al., 1968). Similarly, adding liposomes consisting of sperm head plasma membrane lipids or defined phospholipids to boar sperm prior to cryopreservation resulted in significantly more motile and viable sperm cells following cryopreservation compared to the control (He et al., 2001). More importantly, these lipid-treated cells were capable of undergoing

an acrosome reaction in the presence or absence of calcium ionophore A23187 treatment (He et al., 2001). The acrosome reaction results in membrane instability and loss of membrane integrity due to cholesterol efflux and membrane lipid reorganization (Nolan et al., 1992). Therefore, the true significance of the work by He et al. (2001) is that the boar sperm were able to acrosome react in spite of the fact that the liposome treatment incorporated lipids into the plasma membranes, an event that normally suppresses the acrosome reaction.

Parrish et al. (1999) reported that calcium is essential for sperm capacitation and observed that without calcium, the intracellular activities that characterize capacitation, increases in intracellular pH, cAMP and tyrosine phosphorylation, will not occur. Nolan et al. (1992) demonstrated that intracellular calcium levels are indicators of sperm capacitation and the acrosome reaction. Furthermore, increases in sperm intracellular calcium during capacitation occur to enable the sperm cell to acrosome react (Nolan et al., 1992; Parrish et al., 1999). Nolan et al. (1992) demonstrated that the value of calcium cannot be overstated because sperm could not be induced to acrosome react when calcium free media were used. Similar results were also obtained by Parrish et al. (1999).

Cyclodextrins are cyclic oligosaccharides of glucose that contain a hydrophobic center capable of incorporating lipids (Klein et al., 1995). Treating bull sperm with CLCs results in increased plasma membrane cholesterol content, and consequently higher sperm survival after cryopreservation (Purdy and Graham, 2001b). Because we are adding cholesterol to sperm, it is likely that the ability of bull sperm to capacitate and acrosome react will be affected. Therefore, this research was conducted to determine if CLCs will effect bull sperm capacitation and subsequent acrosome reaction.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade and were purchased from Sigma Aldrich (St. Louis, MO). Fluorescent stains were purchased from Molecular Probes, Eugene OR.

Experiment 1: Determining the effects of cholesterol-loaded cyclodextrin on intracellular calcium and the acrosome reaction of bull sperm.

Sample preparation

Aliquots (1ml neat semen) of ejaculates from 5 bulls were diluted in separate 15ml conical tubes containing 1ml TALP (Nolan et al., 1992) and centrifuged at 585 x g for 8 minutes. The supernatant was removed and the sperm pellet suspended with 1ml TALP containing 10mM calcium chloride (Nolan et al., 1992). Sperm concentration of each sample was then determined spectrophotometrically (Hammerstedt, 1975). Samples were then split and frozen or diluted to 120 x 10^6 cell in 10mM Ca^{2+} TALP containing either 0 or 1.5mg cholesterol loaded cyclodextrin (CLC) to a final volume of 1ml, and incubated at 23°C for 10 minutes.

Samples (120 x 10^6 cells) from 5 bulls were treated with 0 (control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) in 1ml Tris buffered diluent (25mM Tris, 8mM citric acid, 7mM glucose) and frozen according to the protocol described in Chapter II. (Purdy and Graham, 2001). Briefly, control or CLC treated samples were diluted 1:1 (v:v) with egg yolk tris diluent (25mM Tris, 8mM citric acid, 7mMglucose, 40% egg yolk) and cooled to 5°C over a 2 hour period. After reaching 5°C, the samples were diluted 1:1 (v:v) with egg yolk tris glycerol diluent (25mM Tris, 8mM citric acid, 7mM

glucose, 20% egg yolk, 14% glycerol) and allowed to equilibrate for 15 minutes. Following equilibration, the samples were packaged into 0.5ml French straws and frozen in liquid nitrogen vapor (4.5cm above the N_2) for 12 minutes before being plunged into liquid nitrogen for storage. The straws (2 per treatment/bull) were thawed in a 37°C water bath for 30 seconds. The samples were then diluted with 1ml of 10mM Ca^{2+} TALP and centrifuged at 585 x g for 8 minutes. The supernatant was removed, and the sperm pellet was suspended at 120 x 10^6 cells in 10mM Ca^{2+} TALP.

Aliquots (fresh or frozen-thawed) from each sample (100µl) were then stained with 41.5µl of Indo-1 AM (40µM) and 5µl of FITC/PNA (1mg/ml) for 10 minutes. The samples were then treated, in separate analyses, with PC-12 liposomes, calcium ionophore A23187 or heparin/LPC.

PC-12 liposomes were prepared according to Nolan et al. (1992). Briefly, phospholipids were evaporated under nitrogen gas and suspended in TALP (Nolan et al., 1992). The solution was extruded through a 0.1µm polycarbonate membrane and the concentration was determined using ammonium ferrothiocyanate (Stewart, 1980). The PC-12 liposomes were then diluted to 60µM in TALP.

Calcium ionophore A23187 was diluted to a concentration of 2.5mg/ml in DMSO (2.375mM). From this a 4.75µM working solution in TALP was prepared. Heparin and lysophosphatidylcholine were diluted to 2mg/ml and 5mg/ml, respectively in TALP.

Following staining, the samples were diluted, in separate analyses, a) 1:1 (v:v) with the 60µM solution of PC12; b) with 30µl of the working solution of A23187 (142.5nM final concentration) and TALP (870µl) to a 1ml final volume or c) with the heparin (5µl) and lysophosphatidylcholine (24µl) solutions.

After treatment with PC-12, A23187 or heparin/LPC, samples were incubated at 39°C for the duration of the analyses. At designated times, 5 minute increments for PC-12 and A23187 up to 30 minutes, and 30 minute increments up to 3 hours for heparin, aliquots of the treated samples (1.0 x 10^6 cells) were removed and diluted to 0.5ml in TALP for flow cytometric analysis.

Flow cytometry

Calcium analysis was conducted using an Epics V flow cytometer (Coulter Electronics, Miami, FL) equipped with an ultraviolet laser tuned to 351nm (100mW) and a visible laser tuned to 488nm (50mW) with a 7μsec delay between the beams. The flow cytometer set-up included a 408nm laser blocking filter, a 440nm short pass filter to detect calcium-bound dye (Figure 3.1A), and a 490nm long pass filter to detect calcium-free dye (Figure 3.1B. The cells fluorescing positive for the calcium-bound dye (Figure 3.1A) were considered the viable population because a membrane-damaged cell does not contain the enzymes necessary to perform this reaction (Brewis et al., 2000). The ratios of the fluorescence signals (bound dye: free dye) were calculated with the Cyclops software (Cytomation, Fort Collins, CO) to create histograms that illustrate populations of cells with low (Figure 3.1C) and high (figure 3.1D) intracellular calcium concentrations as described by Brewis et al. (2000). Simultaneous detection of the acrosome reaction was performed with a 488nm dichroic mirror, a 500nm long pass filter and a 525nm bandpass filter to detect the population of cells that fluoresced positive for FITC-PNA (Figure 3.1E and F).

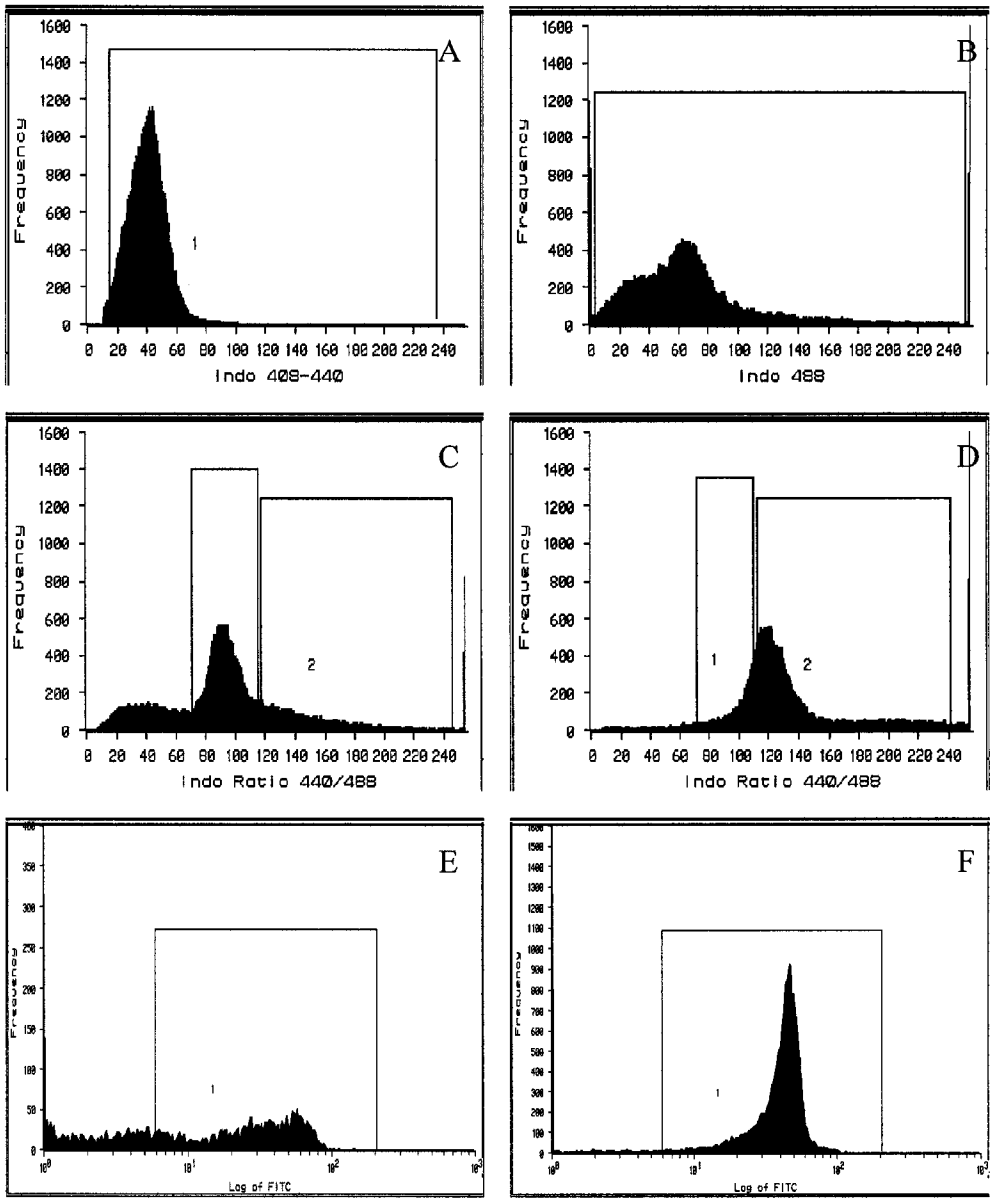

Figure 3.1. Histograms of flow cytometric calcium and acrosome reaction analyses using the fluorescent stains Indo-1 AM and FITC PNA. Histogram A, Region 1, represents the viable sperm cells containing the calcium bound dye that fluoresces within the range of 408-440nm. Histogram B represents the cells fluorescing positive for calcium free dye at 488nm. Histogram C represents the ratio of Histogram A to Histogram B at time 0 and illustrates the population of cells exhibiting low calcium levels (Region 1). Following 30 minutes of incubation with PC-12 the samples exhibit high levels of calcium as illustrated in Histogram D , Region 2. Time gating of cells allows analysis of viable cells at time 0 (Histogram E) that fluoresce positive for an acrosome reaction (FITC-PNA) and at 30 minutes after PC-12 treatment (Histogram F).

Statistics

Calcium data and percentages of acrosome reacted cells were transformed using arcsine (Zar, 1996). Regression analysis of the calcium and acrosome reaction means was performed, and the paired t-test was used to determine differences in slopes between the control (0mg) and CLC (1.5mg) for the PC-12, A23187 and heparin/LPC treatments, exclusively (Zar, 1996).

Slopes of lines that resulted in no differences in either the calcium or acrosome reaction analyses were analyzed using the general linear model procedure of arcsine-transformed data to determine differences at individual time points (Zar, 1996). Treatment means were separated using Student Newman Keuls multiple range test (Zar, 1996).

Viability data were transformed using arcsine, and treatment differences were determined using analysis of variance. Individual treatment means were separated using Student Newman Keuls multiple range test (Zar, 1996).

Experiment 2: Determining effects of cholesterol loaded cyclodextrin treatment on bull sperm fertility

In vitro fertilization

Sperm cryopreservation: Aliquots of semen, 120 x 10^6 cells, were diluted in TALP to a final volume of 1ml. Samples were treated with either 0 or 1.5 mg of CLC, incubated at 25°C for 30 minutes and diluted with 1 ml of egg-yolk-TALP extender (5% egg yolk). The samples were cooled to 5°C over 2hr, diluted with a 2ml aliquot of egg-yolk-TALP diluent (2.5% egg yolk), supplemented with glycerol (14%), and allowed to

equilibrate for 15 minutes. The samples were then packaged into 0.5ml straws and frozen as described previously.

In vitro fertilization, In vitro maturation: Oocytes from slaughterhouse ovaries were aspirated from 2 to 6mm follicles. The acceptable oocytes were washed twice in H-199 medium, placed in wells containing 0.5ml Hepes-buffered TCM-199 (#2520, Sigma Chemical Co., St. Louis, MO) with 10% heat-treated estrous cow serum, 1μg/ml of estradiol 17-β, 1μg/ml of LH (NIH-oLH-26) and .05μg/ml of FSH (NIH-FSH-S-17), and matured in an incubator at 39EC (5% CO_2) for 21 to 24 hours (Olson and Seidel, 2000). Prior to fertilization a control and a treatment straw of semen were thawed for 30 sec in a 37EC water bath, layered on top of separate 90/45% Percoll columns and centrifuged for 20 minutes at 700 x g. The supernatant was removed and the pellet washed in TCM-199. The concentration of the resulting pellet was determined and aliquot of semen, per treatment, was diluted in CDM-1 (Olson and Seidel, 2000) to a final concentration of 200 x 10^6 motile cells per ml. Oocytes, (20 per treatment), were removed from the maturation medium, washed twice in H-CDM and placed in 45μl drops of CDM-1 (Olson and Seidel, 2000) under paraffin oil. Aliquots (5μl containing 1.0 x 10^6 motile sperm cells) of frozen-thawed sperm (0 or 1.5mg CLC treatments) from 5 bulls were used for insemination (5 x 2 factorial). Insemination was done at time 0 hours, meaning immediately after Percoll filtration and establishment of the sperm cell concentration, and at time 0 plus 1 hour. After 18 hours of co-incubation at 39°C (5% CO_2), the oocytes were pipetted from the drops, placed in microcentrifuge tubes containing 50μl H-CDM, by treatment, and vortexed for 60 seconds to remove cumulus cells. The sides of the microcentrifuge tube were rinsed with H-CDM and the total volume of solution was

placed in a petri dish for retrieval of embryos. Repeated washing of the microcentrifuge tube was performed in order to retrieve all known embryos. Upon retrieval, the embryos were washed 3 times in H-CDM, and placed in freshly prepared 50μl drops of CDM-2 under paraffin oil for culture at 39EC, (5% CO_2, 5% O_2, and 90% N_2). The embryos were placed in fresh drops of CDM-2 every 48 hours (Olson and Seidel, 2000). Percentages of cells demonstrating cleavage were recorded at 72 hours post fertilization. Resulting blastocysts were evaluated at 8 days post fertilization and graded as to degree of development (early, mature, expanded, expanding, hatched or hatching).

Statistics

The SAS GLM procedure was used to compare effects of treatment and replicate by cleavage, blastocyst rates and time. The Student-Newman-Keuls test was used to separate treatment and replicate differences (Zar, 1996).

Breeding trial

Semen preparation: Bull sperm from 3 bulls was cryopreserved as described in Experiment 1 except in this experiment the sperm was treated with either 0 or 1.5mg CLC and frozen at a concentration of 3.0×10^6 cells per ml in 0.25 ml straws.

Estrus synchronization and insemination: Estrus was synchronized in 77 Holstein heifers using 2 injections of prostaglandin $F_{2\alpha}$ (5 ml per treatment), 12 days apart. Estrus checks were performed starting 36 hours after the second prostaglandin treatment and continued every 12 hours for 4 days. Artificial insemination of heifers in estrus was performed using a single 0.25 ml straw per animal. A total of 64 inseminations (32 animals per CLC treatment) were performed. Pregnancy rates were determined by

ultrasound through the rectal wall at day 50 post-insemination. The same technician performed all of the inseminations and palpations blindly.

Statistics

Chi square analysis was used to determine differences between the percentages of pregnant heifers in the control and CLC treated groups (Zar, 1996).

RESULTS

Calcium activity

Addition of CLC decreased the rate that fresh CLC-treated sperm accumulated high levels of calcium compared to control sperm, when cells were induced to capacitate using PC-12 or A23187 ($P<0.01$; Tables 3.1 and 3.2, respectively). Addition of CLC had no effect on intracellular calcium accumulation following heparin treatment (Table 3.3). After cryopreservation, accumulation of high levels of calcium was enhanced for CLC-treated sperm when induced to capacitate with PC-12 (Table 3.1) but no effect was observed on cells following A23187 or heparin treatments (Tables 3.2 and 3.3, respectively).

Acrosome reaction

Addition of CLC decreased the rate fresh CLC-treated sperm underwent an acrosome reaction compared to control sperm when cells were induced to capacitate using PC-12, A23187 or heparin ($P<0.05$; Tables 3.1, 3.2 and 3.3, respectively). No differences in the rate of acrosome reactions were detected for cells that had been cryopreserved following treatment with any of the capacitation inducers.

Table 3.1. Mean percentages of fresh and frozen thawed (Cryo) bull sperm samples (n=5) treated with 0 (Control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) exhibiting high calcium levels (Ca^{2+}) and the mean percentages of cells that acrosome reacted (AR) following treatment with dilauroylphosphatidylcholine (PC-12). The slopes, determined using regression analysis, of the calcium and acrosome reaction trends are presented as well as the results of ANOVA of treatments with similar slopes. The percentages were determined using flow cytometry and the stains Indo-1 AM (calcium) and FITC-PNA (acrosome reaction).

		Time (minutes)							
PC-12		0	5	10	15	20	25	30	Slope
Fresh Ca^{2+}	Control	27	35	41	47	52	49	44	1.23 ± 0.19
	CLC	23	32	34	37	39	41	40	0.66 ± 0.19**
	SEM	3	2	3	4	3	4	4	
Cryo Ca^{2+}	Control	36	50	52	56	60	58	56	1.12 ± 0.21
	CLC	27	50	57	62	65	64	62	1.76 ± 0.21**
	SEM	2	4	3	2	2	3	3	
Fresh-AR	Control	9	13	45	48	56	62	66	1.99 ± 0.21
	CLC	7	44*	69*	71*	79*	80*	81*	2.17 ± 0.21
	SEM	1	6	4	5	5	5	4	
Cryo-AR	Control	4	2	3	4	6	7	8	0.17 ± 0.09
	CLC	3	2	3	4	6	8	9	0.23 ±0.09
	SEM	1	1	1	1	1	1	2	

*Indicates significant difference of means at $P<0.05$ by treatment, within a column.
**Indicates significant difference of slopes at $P<0.05$, by treatment.

Table 3.2. Mean percentages of fresh and frozen thawed (Cryo) bull sperm samples (n=5) treated with 0 (Control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) exhibiting high calcium levels (Ca^{2+}) and the mean percentages of cells that acrosome reacted (AR) following treatment with calcium ionophore A23187 (A23187). The slopes, determined using regression analysis, of the calcium and acrosome reaction trends are presented as well as the results of ANOVA of treatments with similar slopes. The percentages were determined using flow cytometry and the stains Indo-1 AM (calcium) and FITC-PNA (acrosome reaction).

		Time (minutes)							
A23187		0	5	10	15	20	25	30	Slope
Fresh Ca^{2+}	Control	18	21	25	42	58	55	45	2.03 ± 0.19
	CLC	19	19	19	22	33	48	54	1.27 ± 0.19**
	SEM	4	2	3	5	5	5	6	
Cryo Ca^{2+}	Control	17	22	21	30	31	32	32	0.68 ± 0.28
	CLC	16	16	12	26	26	26	27	0.53 ± 0.28
	SEM	4	4	5	5	5	5	6	
Fresh-AR	Control	8	11	10	11	11	12	18	0.1 ± 0.01
	CLC	8	11	9	9	9	8	9	-0.03 ± 0.01**
	SEM	1	2	1	1	1	1	4	
Cryo-AR	Control	3	2	3	4	7	8	8	0.22 ± 0.19
	CLC	4	4	6	12	17	20	21	0.65 ± 0.19
	SEM	2	1	1	3	3	4	4	

**Indicates significant difference of slopes at $P<0.05$, by treatment.

Table 3.3. Mean percentages of fresh and frozen thawed (Cryo) bull sperm samples (n=5) treated with 0 (Control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) exhibiting high calcium levels (Ca^{2+}) and the mean percentages of cells that acrosome reacted (AR) following treatment with heparin and lysophosphatidylcholine (LPC). The slopes, determined using regression analysis, of the calcium and acrosome reaction trends are presented as well as the results of ANOVA of treatments with similar slopes. The percentages were determined using flow cytometry and the stains Indo-1 AM (calcium) and FITC-PNA (acrosome reaction).

		Time (hr)							
Heparin + LPC		0	0.5	1.0	1.5	2.0	2.5	3.0	Slope
Fresh Ca^{2+}	Control	34	56	46	39	33	26	23	12.3 ± 0.81
	CLC	34	54	51	45	41	39	33	17.6 ± 0.81
	SEM	6	3	4	2	2	1	3	
Cryo Ca^{2+}	Control	19	16	16	17	15	15	14	-1.2 ± 0.3
	CLC	19	16	13	13	12	13	13	-1.8 ± 0.3
	SEM	3	1	1	2	1	1	1	
Fresh-AR	Control	6	6	11	18	24	45	45	14.9 ± 0.47
	CLC	6	6	7	9	11	13	14	3.1 ± 0.47**
	SEM	1	1	2	2	3	2	2	
Cryo-AR	Control	10	13	16	18	21	25	24	5.8 ± 0.73
	CLC	12	12	15	18	24	24	26	5.3 ± 0.73
	SEM	3	3	4	6	7	10	10	

**Indicates significant difference of slopes at $P<0.05$, by treatment.

Viability

Differences in viability at specific time points for PC-12 are reported in Table 3.4. For the fresh PC-12 analysis only one significant difference was detected at 15 minutes with the CLC treatment having a greater percentage of viable cells (96%) compared to the control (92%; $P<0.05$) (Table 3.4). Analysis of the frozen thawed PC-12 treated cells resulted in significant differences at the 0, 5, 10 and 15 minute time points with the CLC treatments (78, 78, 79 and 78%, respectively) having greater percentages of viable cells compared to the corresponding controls (70, 74, 73 and 73, respectively; $P<0.05$) (Table 3.4).

Analysis of cells treated with A23187 resulted in one statistically significant difference at 10 minutes for the frozen thawed treatment with the CLC treated cells having a greater percentage of viable cells (81%) compared to the control (72%; $P<0.05$) (Table 3.5).

The only significant difference observed for the heparin/LPC treated samples was for the frozen thawed cells at 1 hour, where the control had a greater percentage of viable cells (73%) compared to the CLC treatment (63%; $P<0.05$) (Table 3.6).

Table 3.4. Viability of fresh and frozen-thawed (Cryo) bull sperm samples (n=5) following treatment with either 0 (Control) or 1.5mg cholesterol loaded cyclodextrin (CLC) and induced to acrosome react with dialuroylphosphatidylcholine (PC-12) (30μM). The percentages of live cells were determined at 5minute intervals up to 30minutes using flow cytometry.

		Time (minutes)						
PC-12		0	5	10	15	20	25	30
Fresh	Control	95	89	91	92	92	93	93
	CLC	95	93	95	96*	96	95	94
	SEM	1	2	2	1	2	1	1
Cryo	Control	70	74	73	73	72	67	63
	CLC	78*	78*	79*	78*	76	71	66
	SEM	2	1	1	1	2	2	3

*Indicates significant difference at $P<0.05$ within a column, within either fresh or cryopreserved.

Table 3.5. Viability of fresh and frozen-thawed (Cryo) bull sperm samples (n=5) following treatment with either 0 (Control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) and induced to acrosome react with calcium ionophore A23187 (A23187) (142.5nM). The percentages of live cells were determined at 5minute increments up to 30 minutes using flow cytometry.

		Time (minutes)						
A23187		0	5	10	15	20	25	30
Fresh	Control	98	98	98	98	97	95	96
	CLC	98	98	97	96	95	94	94
	SEM	1	1	1	1	1	8	1
Cryo	Control	73	71	72	72	58	72	71
	CLC	74	77	81*	74	60	76	75
	SEM	3	2	2	1	1	1	1

*Indicates significant difference at $P<0.05$ within a column, within either fresh or cryopreserved.

Table 3.6. Viability of fresh and frozen-thawed (Cryo) bull sperm samples (n=5) following treatment with either 0 (Control) or 1.5mg cholesterol-loaded cyclodextrin (CLC) and induced to acrosome react with heparin and lysophosphatidylcholine (LPC). The percentages of live cells were determined at 30 minute increments up to 3 hours using flow cytometry.

		Time (hr)						
Heparin + LPC		0	0.5	1.0	1.5	2.0	2.5	3.0
Fresh	Control	94	96	96	96	94	92	93
	CLC	94	95	95	95	94	94	93
	SEM	2	1	1	1	1	1	2
Cryo	Control	75	76	73	73	75	74	76
	CLC	75	67	63*	65	66	67	68
	SEM	2	3	3	3	4	3	3

*Indicates significant difference at $P<0.05$ within a column, within either fresh or cryopreserved.

Fertility estimates

IVF/IVM: No differences were detected ($P>0.05$) in percentages of cleavage or percentages of blastocysts per cleavage at either 0 or 60 minutes for either control or CLC treated bull sperm (Table 3.7). There were also no differences detected when the combined results for control and CLC treatment were compared at each of the analysis times (Table 3.7).

Breeding trial: No differences were detected ($P>0.05$) when frozen thawed control and 1.5mg CLC treated sperm were inseminated into dairy heifers (Table 3.8). The breeding trial resulted in 50% of the heifers in the control group pregnant at day 50 while 59% of the heifers in the treatment group were pregnant (Table 3.8).

Table 3.7. Percentage of oocytes that cleaved and matured to blastocysts, by insemination time using frozen thawed sperm from 5 bulls. Prior to freezing sperm had been treated with either 0 (control) or 1.5mg cholesterol loaded cyclodextrin (CLC) per 120 x 10^6 cells. Inseminations were performed at either 0 or 60 minutes following post-thaw processing.

Treatment	Insemination time	Mean % Cleavage	Mean % blastocyst per cleavage
Control	0 min	48	25
CLC	0 min	46	26
Combined	0 min	47	25
Control	60 min	48	13
CLC	60 min	34	18
Combined	60 min	41	16

Table 3.8. Results of breeding trial using bull sperm treated with either 0 (control) or 1.5mg cholesterol loaded cyclodextrin (CLC) per 120 x 10^6 cells and frozen in egg yolk tris diluent. Single inseminations were performed using 1 straw that contained 750,000 total cells. (n=3 bulls).

	Number of heifers pregnant/total	
Bull Number	Control	CLC Treatment
1	3/11	7/11
2	7/11	8/11
3	6/10	4/10
Total	16/32 (50% bred)	19/32 (59% bred)

DISCUSSION

The results presented demonstrate that each of the acrosome reaction inducers affects bull sperm differently. This conclusion is reasonable since PC12, A23187 and heparin function very differently.

When cells were treated with PC12, CLC-treated fresh sperm resulted in fewer cells with high levels of calcium, but at the same time had significantly higher percentages of acrosome reacted sperm. We predicted that CLC treatment would inhibit both intracellular calcium and the acrosome reaction since cholesterol would decrease membrane fluidity at 37°C, and thereby stabilize the membrane. Although intracellular calcium uptake was suppressed, it was surprising to see that PC12 resulted in increased percentages of acrosome reacted cells. PC12 rapidly induces acrosome reactions by remodeling the outer leaflet of the plasma membrane of a sperm cell through incorporation of short-chain phospholipids (Nolan et al., 1992). Therefore, cholesterol may reorganize the plasma membrane in such a manner that PC12 is better able to intercalate into the plasma membrane. Analysis of cryopreserved cells treated with PC12 showed that calcium uptake was higher for CLC treated cells although the percentages of acrosome reacted cells were similar.

Cells treated with A23187 responded in the predicted manner for calcium uptake for both the fresh and frozen thawed treatments. In both analyses the control samples accumulated greater amounts of calcium compared to the CLC treated samples. The calcium ionophore A23187 is an ion carrier that can transport calcium down its established electrochemical concentration gradient and into a cell (Januskauskas et al., 2000). This results in a massive influx of calcium into cells, which initiates capacitation,

and subsequently the acrosome reaction. Cholesterol suppressed calcium accumulation by maintaining membrane integrity, which consequently inhibited membrane transport mechanisms (Visconti et al., 1999a).

Sperm treated with heparin had similar levels of calcium regardless of CLC treatment for both the fresh and frozen thawed analyses. The acrosome reaction analyses demonstrated that CLC treatment suppressed the percentage of cells that could acrosome react in the fresh treatment, but no difference was detected in the frozen thawed treatment. First and Parrish (1987) hypothesized that heparin acts through a variety of mechanisms such as displacement of sperm surface proteins, reorganization of membrane domains, or causes an increase in intracellular capacitation but the actual mechanism is not known (Varner et al., 1993). Therefore, we postulate that CLC treatment may inhibit the interaction of sperm surface proteins with heparin and the reorganization of membrane domains, as discussed by Parrish and First (1987); consequently, fewer acrosome reacting cells were observed.

In our previous research, we demonstrated that greater percentages of motile and viable bull sperm cells could be recovered following cryopreservation by using CLC treatment (Purdy and Graham, 2001). This research demonstrates that in one instance, CLC use suppresses capacitation and the acrosome reaction (fresh heparin treatment) but in general, the treatments either allow or enhance capacitation and the acrosome reaction. Our results are in agreement with research by He et al. (2001) in which boar sperm survived cryopreservation better when the cells were treated with exogenous lipids and more importantly, the cells were still able to capacitate and acrosome react (He et al., 2001).

Of further concern to our research was the potential contraceptive effect of CLC on sperm cells by either inhibiting or promoting premature capacitation and the acrosome reaction. Capacitation is characterized by plasma membrane cholesterol loss (Cross, 1998; Visconti and Kopf, 1998), which results in membrane reorganization, and increased intracellular ion concentrations that activate second messenger signaling cascades (Visconti and Kopf, 1998). The acrosome reaction, which *in vivo* proceeds from capacitation, is further characterized by membrane reorganization and increases in intracellular calcium (Khorasani et al., 2000). Perhaps the most potent regulator of these processes is endogenous plasma membrane cholesterol. Increases in intracellular calcium and second messenger activity as well as the acrosome reaction have only been shown to occur in mammalian sperm following treatment with cholesterol acceptors, such as bovine serum albumin or cyclodextrins, which remove cholesterol from the plasma membrane (Visconti et al., 1999a; Visconti et al., 1999b). Therefore, our concern was that the CLCs would accept cholesterol from the sperm plasma membrane and cause premature capacitation and acrosome reactions. Our second concern was that the addition of cholesterol from the CLCs, while it may result in greater percentages of motile/viable cells post thaw, was beyond physiologic levels, and could not be removed which would cause cell death or missed fertilization. Analysis of the IVF experiments resulted in no differences between the control and CLC treatments; inseminations were performed with equal numbers of live/motile cells post thaw. This indicates there is no contraceptive effect of the CLC treatment, and because there is no difference between insemination treatment times, we believe that any increase in time to capacitate for CLC treated cells is negligible. Furthermore, results of the breeding trial demonstrate that

there may be a beneficial effect to using the CLC treatment. Low numbers of cells (750,000 per insemination dose) were inseminated, which resulted in no statistical difference between control (50% bred) and CLC treatment (59% bred). This further demonstrates that the treatment is not contraceptive. Obviously, a larger breeding trial would need to be performed to determine if a true statistical difference exists.

In conclusion, supplementing sperm cells with cholesterol alters the processes of sperm capacitation and the acrosome reaction. However, cholesterol supplementation does not completely inhibit these processes, particularly in regard to the beneficial effects of CLCs in the cryopreservation process. Consequently, further investigation is needed to understand the complex interactions of cholesterol and calcium in vivo and in vitro.

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CHAPTER IV

EFFECTS OF CHOLESTEROL TREATMENT ON THE MEMBRANE FLUIDITY OF CHINESE HAMSTER OVARY CELLS AND BULL SPERM

INTRODUCTION

Cryopreserved semen has been successfully used for artificial insemination in a number of species, but the success across males of many species is inconsistent. The cryopreservation process causes a variety of deleterious factors to sperm cells such as membrane lipid/protein rearrangement (Parks and Graham, 1992) osmotic stress, and intra/extracellular ice damage (Mazur, 1977; Steponkus et al., 1983) that result in reduced cell viability, and hence fertility. Overcoming these effects may be achieved by manipulating the fluidity of the plasma membrane. Giraud et al. (2000) reported that the membrane fluidity of cells prior to cryopreservation was correlated with the ability of human sperm to survive freezing and thawing, as monitored by both sperm motility and viability. The fluidity of a membrane at physiological conditions is determined by membrane sterol, phospholipid and protein concentration as well as the degree of fatty acyl chain unsaturation of the phospholipids (Graham and Foote, 1987; Giraud et al., 2000). Furthermore, mammalian sperm from various species differ widely in their cholesterol/phospholipid ratios, and in the molar ratios of saturated/unsaturated fatty acyl chains in the phospholipids (Giraud et al., 2000; White, 1993). The composition of the membrane, including the types of lipid, level of unsaturated fatty acyl chains and sterol

levels, determine overall membrane fluidity of a cell, and consequently the cells' response to environmental insults, such as at what temperature the lipid phase transition of the membrane occurs during the cryopreservation process.

Membrane fluidity can be measured using spectrofluorometry after sperm have been stained with the fluorescent probe trans-parinaric acid (Canvin and Buhr, 1989), 1,6-diphenyl-1,3,5-hexatriene (DPH) (Holt and North, 1986; Rana and Majumder, 1990) or its more membrane permeable form 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Varga et al., 1999). These spectrofluorometric analyses show that differences occur in fluorescence polarization due to temperature (Canvin and Buhr, 1989; Holt and North, 1986) and state of epididymal maturation (Rana and Majumder, 1990). Furthermore, Giraud et al. (2000) compared the membrane fluidity of human sperm prior to and after cryopreservation, and found that the population of cells surviving cryopreservation exhibited a decreased overall mean membrane fluidity compared to the pre-freeze population. Using Chinese hamster ovary (CHO) cells, Fox and Delohery (1987) showed membrane fluidity changes if cells were exposed to serum in the incubation medium, as well as when cells were exposed to reduced temperature. These actions were demonstrated by measuring the polarization of fluorescence of the TMA-DPH molecule after excitation with a polarized light (Fox and Delohery, 1987). TMA-DPH and TMAP-DPH are cationic derivatives of the DPH molecule that incorporate into plasma membranes parallel to the lipid acyl chain axes and shift in response to the changes in fluidity attributed to reorientation of the lipid acyl chains (Molecular Probes, 2002). Polarization is defined as $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ (Fox and Delohery, 1987). In this equation $I_{\parallel}$ represents the intensity of the fluorescent light that is

parallel to the excitation light (vertical) and $I_{\perp}$ represents the intensity of the fluorescent light that is perpendicular to the excitation light (horizontal) (Fox and Delohery, 1987). Fox and Delohery (1987) further explain that "if the molecule of interest is in a fluid environment where it can freely rotate with a rotational lifetime substantially less than the fluorescent decay lifetime, then the fluorescent light will have a different polarization than the incident light". Consequently, increases and decreases in membrane fluidity can be documented in accordance with shifts in fluorescence polarization values.

The purpose of this research was to determine how cholesterol addition to or removal from cell plasma membranes affects membrane fluidity, and how this, in turn, affects the ability of cells to survive cryopreservation. This research should provide a better understanding of membrane fluidity in CHO cells and bull sperm plasma membranes during the cryopreservation process, resulting in an increased knowledge of membrane lipid actions and interactions.

MATERIALS AND METHODS

Sample preparation and CLC treatment

Neat bull semen (1ml) from 5 bulls was diluted in 1ml Tris buffered diluent (25mM Tris, 8mM citric acid, 7mMglucose) and centrifuged at 575 x g for 8 minutes to remove seminal plasma. The supernatant was removed, and the sperm pellet was suspended in 1ml tris buffered diluent. The sperm concentration was then determined spectrophotometrically (Hammerstedt, 1975), and aliquots (7 per bull) were prepared at a concentration of 120×10^6 cells per ml in Tris buffered diluent.

CHO cells (3 separate preparations) were thawed in a 37°C water bath, mixed with 5ml of F12 media, and centrifuged for 3 minutes at 1500 x g to remove DMSO. The supernatant was removed and the cell concentration was determined using a Coulter counter (Miami, FL). The cells were diluted with PBS to 0.5 x 10^6 cells per ml and plated to flasks 24 hours before the membrane fluidity analyses. Immediately prior to the analyses, the cells were trypsinized (.03% solution in PBS for 4 minutes), collected into a 15ml conical tube, and centrifuged at 1500 x g for 3 minutes. Following centrifugation, the cell concentration was determined using a Coulter counter, and the cells were diluted to 1 x 10^6 per ml in PBS.

Samples of both cell types were treated with 0, 1.5 or 5.0mg cholesterol-loaded cyclodextrin (CLC) from a stock solution of 50mg/ml CLC (Purdy and Graham, 2001) in TALP (Graham et al., 1986) for 15 minutes at 23°C. The membrane fluidity of cells was then assessed using flow cytometry for cells at 23°C, after cooling to 5°C over 2 hours and at 23°C following cryopreservation. In a separate analysis, both cell types were treated with dilauroylphosphatidylcholine (PC-12) (30μM final concentration), incubated at 39°C for 15minutes and analyzed at 23°C.

Cooling and cryopreservation

Following incubation with CLCs, CHO cells and sperm were cooled over a 2 hr period by placing the test tubes containing the samples into 100ml water baths at 23°C and placing the samples into a cold room (5°C). Sperm samples were cryopreserved as described by Purdy and Graham (2001). Briefly, control and CLC treated samples were diluted 1:1 (v:v) with egg yolk Tris diluent (25mM Tris, 8mM citric acid, 7mM glucose, 40% egg yolk) and cooled to 5°C over a 2 hr period, as described previously. After

reaching 5°C, the samples were diluted 1:1 (v:v) with egg yolk tris glycerol diluent (25mM Tris, 8mM citric acid, 7mM glucose, 20% egg yolk, 14% glycerol) and allowed to equilibrate for 15 minutes. Following equilibration, the samples were packaged into 0.5ml French straws and frozen in liquid nitrogen vapor (4.5cm above the N_2) for 12 minutes before being plunged into liquid nitrogen for storage.

CHO cells were cryopreserved using a Nalgene Cryo 1°C Freezing Container (Rochester, NY). Briefly, the cells (0.5×10^6) were diluted with PBS containing 10% DMSO to a 1ml final volume in a 1.5ml centrifuge tube. The samples were then put into the freezing container, which was filled with 100% isopropyl alcohol, and placed into a -70°C mechanical freezer for 4 hours. The freezing container was then removed, and the samples were plunged into liquid nitrogen for long-term storage.

Staining and flow cytometry

Aliquots (1ml) of CLC treated samples (10×10^6 sperm cells/ml in Tris buffered diluent or 1×10^6 CHO cells/ml in DPBS) were stained with 5µl of PI (2.4mM solution in distilled/deionized water), and 10µl of N-((4-(6-phenyl-1,3,5-hexatrienyl) phenyl)propyl) trimethylammonium p-toluenesulfonate (TMAP-DPH) (Molecular Probes, Eugene OR; 200µM solution in DPBS). The samples were incubated at their respective temperatures (5°C, 23°C or 23°C following cryopreservation) for 15 minutes, filtered through a 20µm mesh, and the fluidity of the cell membranes was analyzed by flow cytometry as described by Fox and Delohery (1987). Briefly, an EPICS V flow cytometer (Coulter Electronics, Miami, FL) was equipped with a half wave retarder set to 355nm, a 50% beam splitter and vertical and horizontal polarizers to detect TMAP-DPH fluorescence (Fox and Delohery, 1987). A 418nm laser blocking filter, a 590nm dichroic mirror and a

610nm longpass filter were used to detect PI. TMAP-DPH and PI were excited using an argon laser tuned to ultraviolet (351nm) at a power of 200mW (Fox and Delohery, 1987). Histograms of the difference between $I_{\parallel}$ and $I_{\perp}$ and the sum of $I_{\parallel}$ and $I_{\perp}$ were collected; their polarization histograms were made by the ratio of the difference and sum histograms using the Cyclops software (Cytomation, Fort Collins, CO) ($P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$) (Fox and Delohery, 1987). The resulting mean channel was converted to a fluorescent polarization value (FPV) by dividing by 255, the largest value on the FPV scale. At least 50,000 viable cells were analyzed for each sample.

Statistics

Percentage data was transformed using arcsine, and treatment differences of fluorescence polarization values were determined using analysis of variance (Zar, 1996). Proportions of bull sperm cells in low fluidity populations (2) were normalized by dividing each datum by its corresponding 23°C control value. Differences were determined using analysis of variance (Zar, 1996). Treatment means were separated using Student Newman Keuls multiple range test (Zar, 1996).

RESULTS

Chinese hamster ovary cells

Addition of 1.5 and 5.0mg of CLC to CHO cells at 23°C resulted in significantly greater fluorescence polarization values (FPV) (.318* and .320*, respectively) compared to control cells (FPV=.300; $P<0.05$), indicating that cholesterol addition decreases membrane fluidity in a dose dependent manner (Table 4.1, Figure 4.1A,B,C). . Cooling CHO cells to 5°C following CLC treatment resulted in significantly greater FPVs

(decreased fluidity) for the 1.5 and 5.0mg doses (.309* and .325**, respectively) compared to the control (.286; $P<0.05$) (Table 4.1; Figure 4.1D,E,F). When CHO cells were cryopreserved, mean fluorescence polarization values for the 1.5 and 5.0mg CLC treatment (.297 and .300, respectively) were not significantly different compared to the control (.296; $P>0.05$) (Table 4.1; Figure 4.1G,H,I).

Analysis of CHO cells treated with PC-12 resulted in an FPV of .270 (Table 4.1; Figure 4.1J), which was significantly different from all other treatments ($P<0.05$).

The viability of the CHO cells, determined by PI exclusion, was similar (> 85%) for all treatments ($P>0.05$; Table 4.2).

Table 4.1. Fluorescence polarization values determined by flow cytometric assessment of the stain TMAP-DPH for Chinese hamster ovary cells (n=3 preparations) treated with 0, 1.5 or 5.0mg cholesterol loaded cyclodextrin (CLC) and analyzed at room temperature (23°C), cooled to 5°C or following cryopreservation. In addition, cells were analyzed following treatment with 30 μM dilauroylphosphatidylcholine (PC-12).

	Fluorescence polarization value			
CLC dosage	23°C	5°C	Cryopreserved	PC-12
0	.300^{cd}	.286^{d}	.296^{cd}	.270^{e}
1.5mg	.318^{ab}	.309^{cb}	.297^{cd}	--
5.0mg	.320^{ab}	.325^{a}	.300^{cd}	--
SEM	.004	.004	.004	.004

Superscripts indicate significant differences at $P<0.05$.

Table 4.2. Viability of Chinese hamster ovary (CHO) cells treated with 0, 1.5 or 5.0mg cholesterol loaded cyclodextrin and analyzed at room temperature (23°C), cooled to 5°C or analyzed following cryopreservation. Viability was also determined for CHO cells treated with 30μM dilauroylphosphatidylcholine (PC-12). Viability was determined by flow cytometric assessment of the stains TMAP-DPH and propidium iodide.

	Viability			
CLC dosage	23°C	5°C	Cryopreserved	PC-12
0	90	90	91	95
1.5mg	85	96	94	--
5.0mg	95	97	94	--

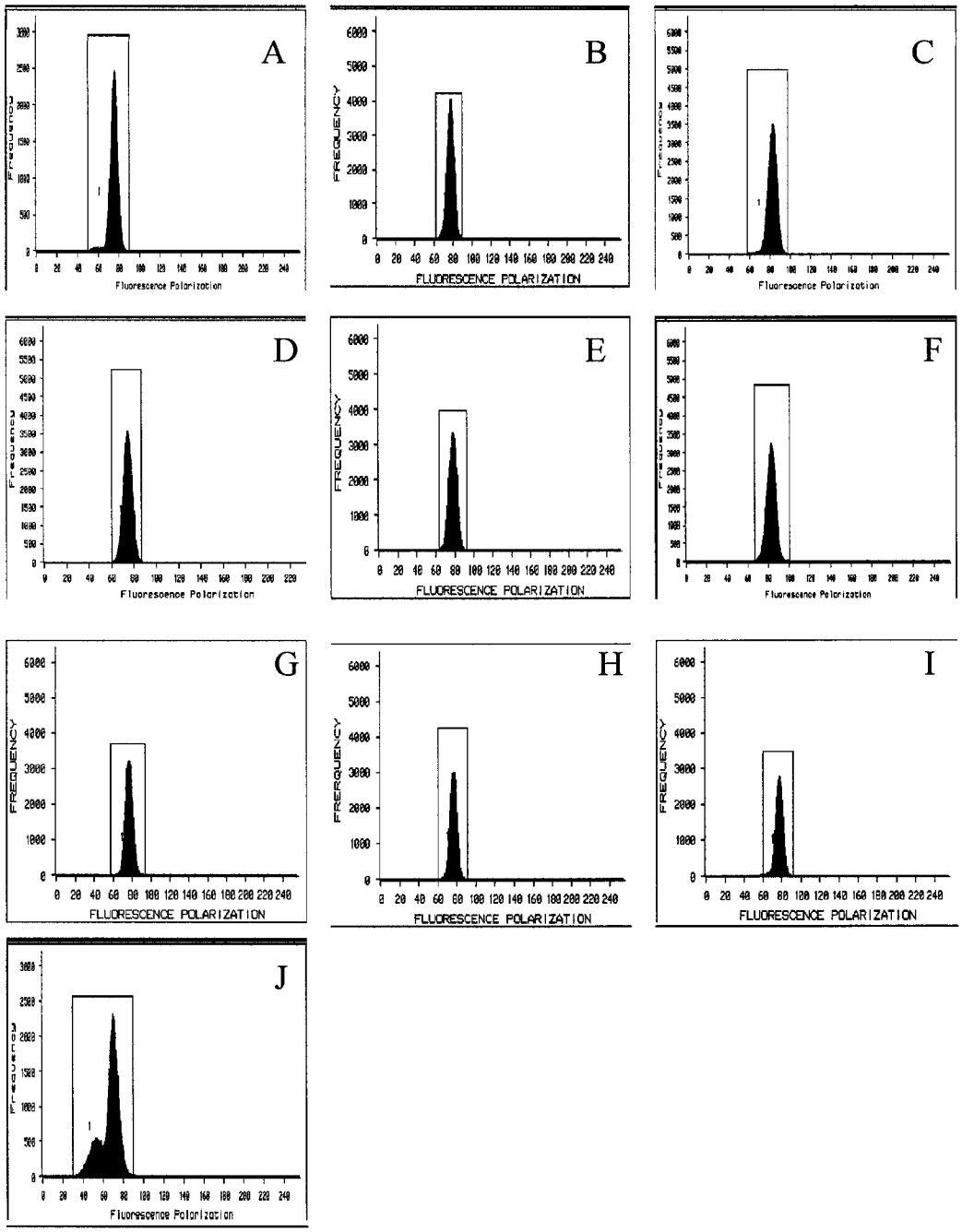

Figure 4.1. Histograms of CHO cells analyzed using an EPICS V flow cytometer and the fluorescent stains TMAP-DPH and propidium iodide to measure membrane fluidity and viability, respectively. Histograms represent A-C: Samples analyzed at 23°C following treatment with 0, 1.5 or 5mg cholesterol-loaded cyclodextrin (CLC), respectively; D-F: Samples treated with 0, 1.5 or 5mg CLC, respectively, and cooled to 5°C for analysis; G-I: Samples that were treated with 0, 1.5 or 5mg CLC, respectively, and cryopreserved. Analysis was post-thaw at 23°C; J: A sample treated with 30μM dilauroylphosphatidylcholine.

Bull spermatozoa

Flow cytometric analysis of the membrane fluidity of bull sperm cells resulted in 2 distinct populations of cells (Figure 4.2A) compared to the single population of cells seen with the CHO cell analyses (Figure 4.1). At 23°C bull sperm treated with 0, 1.5 and 5.0mg of CLC resulted in decreasing FPVs (.172, .152 and .148, respectively) for sperm population 1 (cells with high membrane fluidity) and increasing FPVs (.469, .472 and .479, respectively) for sperm population 2 (cells with low membrane fluidity) (Table 4.3; Figure 4.2 A,B,C). No differences in the percentages of viable sperm at 23°C were detected ($P>0.05$; Table 4.4). Proportions of cells in population 2 were significantly different across CLC dosages at 23°C (Table 4.5). The 0, 1.5 and 5.0mg CLC treatments had 34^{a}, $35^{ab}$and $42\%^{b}$ ($P<0.05$), respectively, of cells in the population 2, indicating that as the cholesterol dose increased, cells were converting from population 1 to population 2, a less fluid membrane state.

At 5°C the high fluidity population (1) becomes more fluid (.185, .173 and .161) with increasing levels of CLC (0, 1.5 and 5.0mg, respectively) although no significant differences were detected, while population 2 remains similar (.485, .486 and .488, respectively) (Table 4.3). After cooling to 5°C, control samples had significantly fewer viable cells (59%) than sperm samples treated with 1.5 or 5.0mg CLC (89 and 95%, respectively; $P<0.05$; Table 4.4). Results of the 5°C population distribution analysis were very different from results obtained at 23°C. While the cells shifted to the less fluid population at 23°C, the cells at 5°C shifted to the more fluid population (1) (Figure 4.2 D,E,F) as indicated by the decrease in proportion of viable cells in population 2 (47^{a}, 38^{b} and $38^{b}\%$) as CLCs were added (0, 1.5 and 5.0mg, respectively; $P<0.05$; Table 4.5).

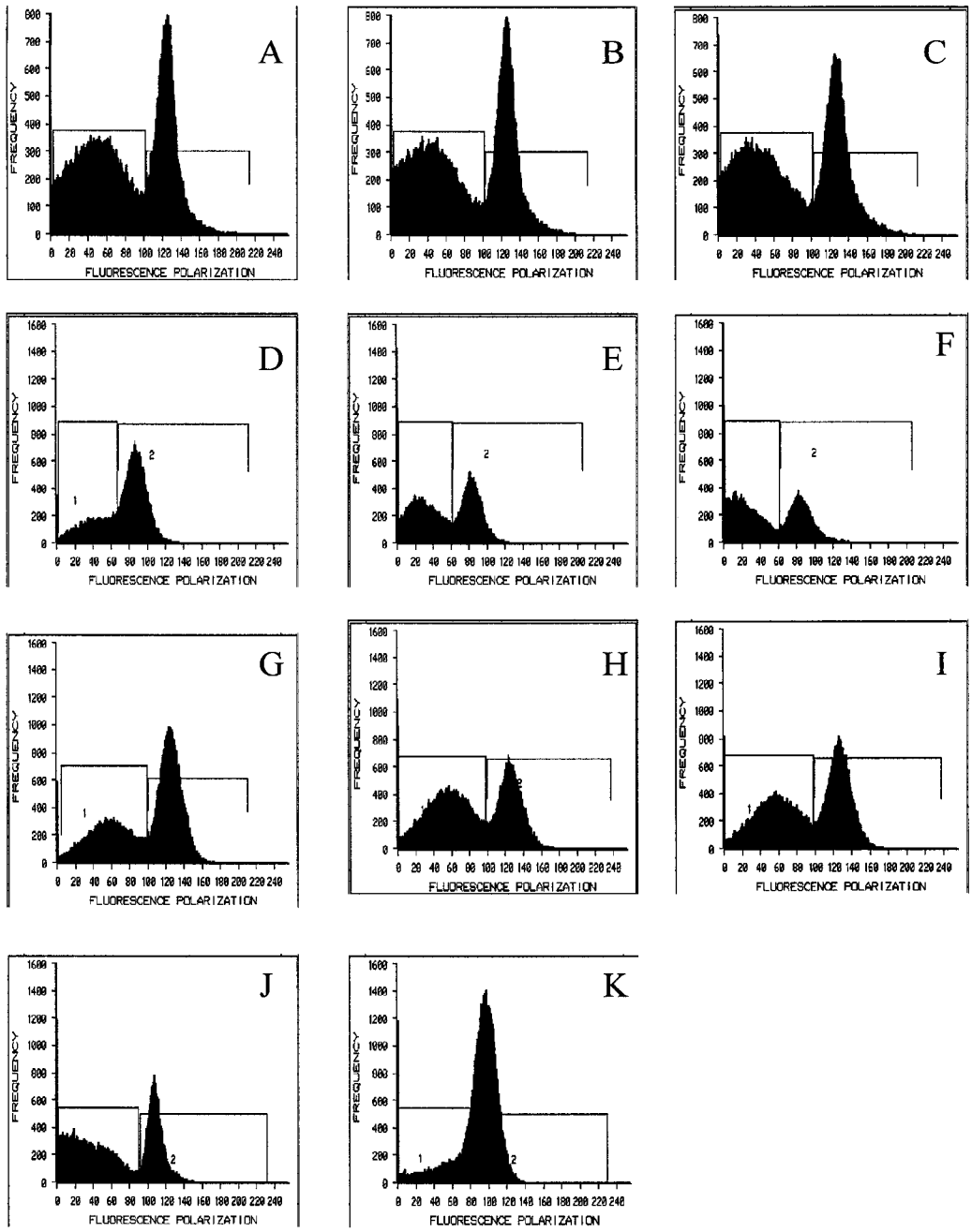

Figure 4.2. Histograms of bull sperm cells analyzed using an EPICS V flow cytometer and the fluorescent stains TMAP-DPH and propidium iodide to measure membrane fluidity and viability, respectively. Histograms represent A-C: Samples analyzed at 23°C following treatment with 0, 1.5 or 5mg cholesterol-loaded cyclodextrin (CLC), respectively; D-F: Samples treated with 0, 1.5 or 5mg CLC, respectively, and cooled to 5°C for analysis; G-I: Samples that were treated with 0, 1.5 or 5mg CLC, respectively, and cryopreserved. Analysis was post-thaw at 23°C; J-K: Samples that were treated with 0 (control) or 30μM dilauroylphosphatidylcholine, respectively.

Table 4.3. Fluorescence polarization values determined by flow cytometric assessment of the stain TMAP-DPH for bull sperm cells treated with 0, 1.5 or 5.0mg cholesterol loaded cyclodextrin (CLC) and analyzed at room temperature (23°C), cooled to 5°C or post thaw (Cryo). In addition, cells were also analyzed following treatment with 30μM dilauroylphosphatidylcholine (PC-12).

	Fluorescence polarization value						
	High fluidity (1)			Low fluidity (2)			
CLC Dose	23°C	5°C	Cryo	23°C	5°C	Cryo	PC-12
0mg	.172[a]	.185[a]	.239[b]	.469[a]	.485[ab]	.524[b]	.414[c]
1.5mg	.152[a]	.173[a]	.237[b]	.472[a]	.486[ab]	.529[b]	
5.0mg	.148[a]	.161[a]	.235[b]	.479[ab]	.488[ab]	.527[b]	
SEM	.009	.009	.009	.012	.012	.012	.003

[ab] Indicates treatment is significantly different within high or low fluidity region ($P<0.05$).
[c] Indicates treatment is significantly different within a row, across high and low fluidity regions ($P<0.05$).

Table 4.4. Viability of bull sperm treated with 0, 1.5 or 5.0mg cholesterol loaded cyclodextrin and analyzed at room temperature (23°C), cooled to 5°C or analyzed post thaw (Cryo). Viability was also determined for bull sperm treated with 30μM dilauroylphosphatidylcholine (PC-12). Viability was determined by flow cytometric assessment of propidium iodide exclusion.

	Viability			
CLC Dose	23°C	5°C	Cryo	PC-12
0mg	96	59[a]	60[a]	61
1.5mg	96	89[b]	76[b]	
5.0mg	96	95[b]	78[b]	
SEM	.78	2.9	2.4	

[a,b]Indicate significant differences within a column ($P<0.05$).

Table 4.5. Proportion of bull sperm in the low fluidity population following treatment with cholesterol-loaded cyclodextrin (0, 1.5 or 5.0mg) and analyzed flow cytometrically with the fluorescent probe TMAP-DPH at room temperature (23°C), cooled to 5°C or post thaw (Cryo).

	Population of low fluidity (2)		
CLC Dose	23°C	5°C	Cryo
0mg	34[a]	47[a]	54[a]
1.5mg	35[a,b]	38[b]	48[b]
5.0mg	42[b]	38[b]	46[b]
SEM	2.7	3.1	3.7

[a,b]Indicate significant differences within a column ($P<0.05$).

FPVs for control and CLC treated sperm were similar ($P>0.05$) after sperm were frozen and thawed (Table 4.3). However, differences were detected in FPVs within populations 1 and 2 across the temperatures (23°C vs. 5°C vs. frozen-thawed; Table 4.3). In the high fluidity population (1), the frozen thawed cells had FPVs that were significantly higher than unfrozen cells ($P<0.05$; Figure 4.2). Similarly, after cryopreservation the low fluidity population (2) had higher FPVs than unfrozen cells ($P<0.05$; Figure 4.2). This indicates that the bull sperm that survive cryopreservation have less fluid membranes than unfrozen cells. Addition of cholesterol also resulted in higher percentages of cells surviving cryopreservation (>76%) compared to control sperm (60%; $P<0.05$; Table 4.4). Therefore, cholesterol supplementation enhances cell survival through cryopreservation although FPVs are not affected.

Treating bull sperm with 30μM PC-12 resulted in a single population of cells instead of the two populations of cells seen in untreated samples (Figure 4.2, J and K). The FPV (.414; Table 4.4) was significantly different from all other values ($P<0.05$) and resulted in 61% cell viability for the samples.

DISCUSSION

Analysis of membrane fluidity has been assessed for a variety of cell types using TMAP-DPH or similar probes such as TMA-DPH or DPH (Holt and North, 1986; Fox and Delohery, 1987; Tahara et al., 1996; Giraud et al., 2000). However, only Fox and Delohery used flow cytometry to assess cell membrane fluidity. The advantage of using flow cytometry for these types of experiments is that flow cytometry allows a rapid assessment of the membrane fluidity of large numbers of individual cells. Furthermore, these measurements may be conducted on only the viable cells by use of the vital stain PI, while spectrophotometric analyses determine the average membrane fluidity on total cell populations (Giraud et al., 2000) or membrane extracts (Holt and North, 1986; Rana and Majumder, 1990). By analyzing individual sperm using flow cytometry two distinct populations of bull sperm, which differ in membrane fluidity, were detected (Figure 4.2A), whereas spectrofluorometric analysis of sperm results in only a single numerical value for the population mean (Giraud et al., 2000). We are confident that the two populations of bull sperm cells are genuine and not an artifact of sperm shape due to the presentation of the populations. The flow cytometer was equipped with a beveled needle to orient the sperm, which should minimize effects of shape on the emitted fluorescence, but more importantly, data that is artifactual tends to be displayed as a "smear" and not in the distinct regions presented in our findings (Figure 4.2).

Research by Rottem et al. (1973) documented the role of cholesterol in determining membrane fluidity. Cholesterol at room temperature decreases membrane fluidity whereas at decreased temperatures, cholesterol has the opposite effect, namely increasing the membrane fluidity (Rottem et al., 1973). When CHO cells were treated

with either cholesterol dose at 23°C membrane fluidity decreased. Likewise, the membrane fluidity of CHO cells also decreased when the cells were cooled or cryopreserved. The decrease in fluidity for cryopreserved human sperm samples was reported by Giraud et al. (2000) and occurred because of membrane reorganization. Contrary to our predictions, the CHO cells treated with CLCs and cooled to 5°C did not experience an increase in membrane fluidity. Based on the work by Rottem et al. (1973) we anticipated that the CLC treated samples would become more fluid at 5°C but the opposite effect occurred. We hypothesize that incorporating cholesterol into the plasma membrane of CHO cells may result in a reorganization of the phospholipids and reorder their interactions with integral proteins consequently causing the decreases in membrane fluidity.

The bull sperm analyses illustrate increases and decreases in membrane fluidity for both the high fluidity (population 1) and low fluidity (population 2) regions similar to the CHO cells at 23°C but with distinctly different results at 5°C and at 23°C following cryopreservation (Table 4.3). These increases and decreases in membrane fluidity are likely due to membrane reorganization at reduced temperatures as well as loss of viable cells. Treating cells with CLCs causes sperm to respond to the environmental temperature changes and cryopreservation as Rottem et al. (1973) predicted. CLC treatment, and the uptake of cholesterol into cells at 23°C, causes these cells to change their membrane fluidity and move from population 1 to population 2. At 5°C, as predicted and documented by Rottem et al. (1973), CLC treatment induces the cells to become more fluid and change from population 2 to population 1 but, following cryopreservation, there is a decrease in the fluidity of population 2 (Table 4.5). Giraud et

al. (2000) also reported a decrease in membrane fluidity for human sperm similar to the population 2 shift, but our research also shows that we have a CLC dose-dependent decrease in population 2 following cryopreservation (Table 4.5). The FPVs recorded for population 2 are quite high and not something normally seen with somatic cells but we are confident that these values are genuine as Giraud et al (2000) reported similar results. This means that following cryopreservation the samples treated with CLCs will have more cells with increased fluidity (population 1) than the control. Furthermore, analysis of the viability of the treatments demonstrates that there is a direct benefit to using CLCs in the cooling and cryopreservation processes (Table 4.4). These results demonstrate that the membrane fluidity of the bull sperm cell population shifts in response to temperature and cholesterol incorporation. Cholesterol supplementation is able to modify the plasma membranes of bull sperm so that they become more fluid at lower temperatures and consequently are better able to survive cryopreservation (Table 4.4).

Treatment of either CHO or bull sperm cells with PC-12 resulted in greater membrane fluidity. In addition, bull sperm treated with PC-12 resulted in a single cell population (Figure 4.2K) instead of the 2 populations seen prior to PC-12 addition. PC-12 increases membrane permeability and fluidity, and results in membrane lipid loss similar to the acrosome reaction (Graham and Foote, 1987; Cross, 1998; Visconti and Kopf, 1998). Because the population of higher fluidity (population 1) in the bull sperm analyses already has less membrane cholesterol (Flesch et al., 2001) we interpret the single population as a shift of the cells of lower fluidity (population 2) and a removal of population 1 due to cell death. As sperm cells progress through the processes of capacitation and the acrosome reaction, membrane reorganization occurs due to loss of

plasma membrane cholesterol that causes increased membrane fluidity. Therefore, we speculate that during capacitation and the acrosome reaction, the cells naturally travel from population 2 to population 1. Flesch et al. (2001) also observed the difference between the populations of cells and noted that the sperm cells in the population of higher fluidity (1) could be capacitated with bicarbonate, but the cells in the lower membrane fluidity population (2) were incapable of undergoing this process.

In conclusion, adding cholesterol to cells changes membrane fluidity and these fluidity differences can be detected at room temperature or at cold temperatures. Addition of cholesterol enhances the number of bull sperm that survive cryopreservation and this increased survival is a result of increasing the fluidity of the cells' plasma membranes at low temperatures. This research increases the present understanding of the importance of membrane fluidity in cryopreservation.

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CHAPTER V

CONCLUSIONS

During cryopreservation, cells are exposed to a variety of stresses that result in destabilization of the plasma membrane (Mazur, 1977; Steponkus et al., 1983). Particularly, membrane destabilization occurs as temperature decreases and the membrane undergoes a phase transition from the fluid phase to the gel phase. These effects may be overcome by supplementing cell membranes with lipids, particularly cholesterol. Rottem et al. (1973) demonstrated that after treating yeast cells with cholesterol, the plasma membrane phase transition was eliminated at all of the low temperatures tested.

Cryopreservation: In the present study, bull sperm were treated with cholesterol loaded cyclodextrin (CLC) to increase the plasma membrane cholesterol content. This treatment resulted in greater percentages of motile and viable sperm following cryopreservation due to a decrease in the phase transition temperature. These experiments also demonstrated that CLC treatment results in cholesterol incorporation into sperm membranes in a dose dependent manner to the point that the cholesterol becomes deleterious to sperm function.

Intracellular calcium, capacitation and the acrosome reaction: Intracellular calcium levels and the ability of sperm to undergo the acrosome reaction were monitored in sperm cells following treatment with CLCs. The rationale for these experiments was

that cholesterol supplementation could inhibit capacitation and the acrosome reaction, because cholesterol efflux is necessary to initiate an increase in intracellular calcium and the events of these processes (Visconti and Kopf, 1998). Our results demonstrated that cholesterol treatment does modulate calcium activity, capacitation and the acrosome reaction, but the outcome depends on the acrosome reaction inducer used. Regardless, cholesterol supplementation did not inhibit capacitation or the acrosome reaction, regardless of the compound used to induce the acrosome reaction.

An in vitro fertilization experiment and a breeding trial were also used to better understand the effects that cholesterol supplementation has on bull sperm capacitation and the acrosome reaction. These experiments also demonstrated that conception was possible with bull sperm treated with cholesterol. More importantly, these experiments combined with the results of the calcium/acrosome reaction experiments demonstrate that cholesterol supplementation is not contraceptive.

Membrane fluidity: The membrane fluidity of Chinese hamster ovary (CHO) cells and bull sperm was measured using flow cytometry. Overall, CHO cells and bull sperm responded as predicted depending on the cholesterol and temperature treatments used. Of significance in these experiments was that the flow cytometric analysis enabled observation of samples that were increasing and decreasing in plasma membrane fluidity. In addition, this methodology allowed observation of cells that were converting between low and high fluidity populations depending on the cholesterol and temperature treatment used. More importantly, these analyses demonstrated that increasing plasma membrane fluidity of bull sperm by cholesterol supplementation results in greater percentages of viable cells following cryopreservation.

The research presented in this report has provided a better understanding of the actions and interactions of cholesterol in bull sperm plasma membranes during cryopreservation. Hopefully, this research will allow improvements in cryopreservation methodologies for species with sperm that are traditionally poor freezers.

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APPENDIX

ANOVA Tables to accompany information in:

Table 2.2. Sodium citrate diluent motility

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	3	1214.33	404.78	1.71	0.185
Error	32	7580.89	236.90		
Corrected total	35	8795.22			

Table 2.2. Tris diluent motility

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	6	2111.49	351.92	2.28	0.049
Error	56	8654.45	154.54		
Corrected total	62	10765.94			

Table 2.2. Tris diluent viability

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	6	3298.71	549.785	3.37	0.0173
Error	21	3424.25	163.06		
Corrected total	27	6722.96			

Figure 2.1. Spectrofluorometry

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	5	3.93×10^{14}	7.86×10^{13}	4.79	0.0004
Error	174	2.86×10^{15}	1.64×10^{13}		
Corrected total	179	3.25×10^{15}			

Figure 2.1. HPLC

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	4	13.83	3.46	3.87	0.0146
Bull	6	40.15	6.69	7.49	0.0001
Error	24	21.45	0.89		
Corrected total	34	75.42			

Table 4.1. CHO fluorescent polarization values

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC dosage	8	262.13	32.77	12.65	<.0001
Error	18	46.63	2.59		
Corrected total	26	308.77			

Table 4.3. Population 1 fluorescent polarization values

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
Temperature treatment (Trt)	2	6990.96	3495.48	67.03	<.0001
CLC dosage	2	296.83	148.42	2.85	0.0639
Trt*CLC	4	106.99	26.75	0.51	0.7264
Error	81	4223.68	52.14		
Corrected total	89	11618.46			

Table 4.3. Population 2 fluorescent polarization values

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
Temperature treatment (Trt)	2	3068.11	1534.05	15.13	<.0001
CLC dosage	2	30.74	15.37	0.15	0.8595
Trt*CLC	4	14.9	3.725	0.04	0.9974
Error	81	8211.16			
Corrected total	89	11324.92			

Table 4.3. PC-12 fluorescent polarization values

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
CLC/PC-12 dosage	9	487.91	54.21	19.1	<.0001
Error	20	56.76	2.84		
Corrected total	29	544.67			

Table 4.4. Bull sperm viability

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
Temperature treatment (Trt)	2	9252.83	4626.41	94.47	<.0001
CLC dosage	2	5409.08	2704.54	55.22	<.0001
Trt*CLC	4	3584.35	896.09	18.3	<.0001
Error	81	3966.96	48.97		
Corrected total	89	22213.22			

Table 4.5. Proportion of sperm cells in population 2

Source	Deg. of freedom	Sum of squares	Mean square	F	Pr > F
Temperature treatment (Trt)	2	1365.53	682.76	6.68	0.0021
CLC dosage	2	373.6	186.8	1.83	0.1675
Trt*CLC	4	665.14	166.28	1.63	0.1756
Error	81	8281.8	102.24		
Corrected total	89	10686.07			