THE INFLUENCE OF CHOLESTEROL LOADING AND SUBSEQUENT UNLOADING IN PRESERVATION OF STALLION SPERMATOZOA

by

Crystal R. Anderson

A Thesis Submitted to the Faculty of the

DEPARTMENT OF ANIMAL SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

The University of Arizona

2 0 0 5

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Crystal R. Anderson

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Mark J. Arns
Professor of Animal Science

May 11, 2005
Date

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES - Continued

ABSTRACT

The purpose of this study was to determine the influence of loading cholesterol into the stallion spermatozoa membrane prior to cold storage or cryopreservation, followed by the subsequent unloading of cholesterol from the membrane after preservation. Cholesterol loaded cyclodextrin (CLC) was added to spermatozoa samples before preservation to incorporate cholesterol into the membrane. Methyl β cyclodextrin (MβCD) was added following preservation to remove excess cholesterol that may inhibit the potential for fertilization. Experiment I consisted of dose response trials to determine the optimal amount of CLC and MβCD to include based on percentages of progressively motile spermatozoa (PMS) following cold storage or cryopreservation. In experiment II, the influences of CLC and MβCD on PMS, the percentages of live intact (LI) and live non-intact (LNI) spermatozoa were determined following 24 and 48 h of cold storage of spermatozoa cooled in the presence or absence of seminal plasma. Experiment III consisted of cholesterol loading prior to cryopreservation and subsequent removal of cholesterol post-thaw. The addition of CLC resulted in an increase in PMS when compared to the control ($P < 0.05$). Treatment with MβCD did not negatively influence PMS when compared to CLC treated spermatozoa that underwent cold storage or cryopreservation ($P > 0.05$). Addition of CLC improved ($P < 0.05$) PMS and LI following both cold storage and cryopreservation when compared to the control; however, CLC incorporation does not influence LNI when compared to non-CLC treated spermatozoa. The subsequent unloading of cholesterol using MβCD does not alter PMS, LI nor LNI when compared to CLC treated spermatozoa. Therefore, it was concluded

that addition of CLC was beneficial to the survival of spermatozoa following either cold storage or cryopreservation and the addition of a MβCD solution in small amounts does not negatively influence PMS, LI or LNI.

INTRODUCTION

Preservation of semen has proven to be valuable for advancement of artificial insemination in the domestic animal. Advancements in technology, such as development of semen extenders by Ivanoff in 1922 and the discovery of glycerol as a cryoprotectant by Polge et al. in 1949, have led to improvements in survival of spermatozoa following preservation. Evolution of these techniques has enabled the transportation of semen across the United States as well as between countries. With the ability to transport semen has come improvements in herd genetics, as a greater variety of quality animals are available to the breeder (Graham, 1996b). Despite the benefits of transporting semen, there are drawbacks to the preservation process, including reduced fertility in some species, such as the stallion.

In the equine industry, cold storage is the predominant method for semen transport. However, cooling the stallion ejaculate can cause cold shock, which can result in cell death (Watson, 2000). In addition, the percentages of progressively motile and viable spermatozoa decline over time (Foote, 2002), resulting in a narrow window in which producers may collect an ejaculate and inseminate a mare. The use of transported cooled semen can become costly when delays in shipping occur or there are errors in handling, resulting in loss of spermatozoa viability.

Cryopreservation of the stallion ejaculate enables long-term preservation of spermatozoa; however, there are problems associated with the cryopreservation process. In some stallions, membrane damage, such as that caused by irregular membrane fluidity associated with decreasing temperatures, results in a significant decrease in percentages

of viable spermatozoa (Watson, 2000). There is a correlation between decreased per cycle pregnancy rates and impaired spermatozoa parameters, such as PMS and membrane integrity (Wilhelm et al., 1996).

Preservation of stallion spermatozoa is an inefficient process that causes damage to the spermatozoa membrane primarily due to the cooling process. Altering phase transition temperature of the membrane may enable spermatozoa to withstand temperature changes and cold shock, therefore improving survival rates (Darin-Bennett and White, 1977). One method to alter membrane fluidity is through introduction of cholesterol in the form of cholesterol-loaded cyclodextrin (CLC). Cyclodextrins consist of a hydrophilic exterior and hydrophobic interior face that enable cyclodextrin to internalize hydrophobic substances through inclusion complexes, thus causing the insoluble substance to become water-soluble (Klein et al., 1995). When preloaded with cholesterol, CLC can deliver cholesterol to the spermatozoa membrane. Addition of CLC to stallion spermatozoa has led to improved spermatozoal motility following cold storage (Kirk et al., 2001) and cryopreservation (Combes et al., 2000; Vidament et al., 2001). Moreover, post-thaw membrane integrity is improved when spermatozoa are frozen in the presence of CLC (Combes et al., 2000). Adding cholesterol to the membrane improves survival after preservation.

Although cholesterol loading has been shown to improve spermatozoal motility and membrane integrity following preservation, Zahn et al. (2002) demonstrated that cholesterol addition could over-stabilize the membrane. Over stabilization prevents normal spermatozoal maturation and decreases per cycle pregnancy rates (Zahn et al.,

2002). When cyclodextrin is added to stallion spermatozoa without prior loading of cholesterol, it enhances capacitation through removal of cholesterol (Pommer et al., 2003). This mechanism may be beneficial for the unloading of cholesterol from those samples treated with CLC prior to insemination. Therefore, the objective of the present study was to determine the influence of cholesterol loading and subsequent unloading on spermatozoal motility and viability following cold storage and cryopreservation under conditions that are the similar to those currently being used in the equine industry.

LITERATURE REVIEW

Anatomy of Spermatozoa

The basic structure of a spermatozoon consists of a head and tailpiece. The head contains an oval nucleus with an overlying acrosome that is essential for the fertilization process (Flesch and Gadella, 2000). If the acrosome is damaged or prematurely reacted, fertilization of the oocyte will not occur (Graham, 1996a). Similarly, if the plasma membrane that encases and protects spermatozoa is not intact, spermatozoa will not be capable of fertilization (Wilhelm et al., 1996). Parameters that correlate with fertility include whether the spermatozoal membrane and the acrosome are intact. Those that remain intact are considered viable and more likely to have retained their competency to undergo fertilization.

The tailpiece is where motility is generated. Mitochondria, located in the midpeice, produce energy to move the axoneme, which results in forward motion (Garner and Hafez, 2000). If spermatozoa lack the ability to move progressively forward, there will be poor per cycle pregnancy rates (Graham, 1996a). There are currently no infallible predictors for fertility; however, parameters, including membrane integrity and percent progressively motile spermatozoa, may offer a means to analyze the potential for fertility (Wilhelm et al., 1996).

Plasma Membrane

Spermatozoa are completely encased in a phospholipid bilayer, or plasma membrane. A plasma membrane is an amphipathic molecule, consisting of both

hydrophilic and hydrophobic components (Berg et al., 2002). The stallion spermatozoa membrane consists of 57% phospholipid, 37% cholesterol and 6% glycolipid (Gadella et al., 2001). The primary phospholipids of the plasma membrane include phosphatidyl choline, phosphatidyl ethanoline, and sphingomyelin (Ladha, 1998). The glycerol backbone of a phospholipid is esterified with polyunsaturated long chain fatty acids, such as docosahexaenoic acid; saturated aliphatic chains, such as plasmenylcholine; and saturated alkyl groups, such as plosphatidylcholine (Flesch and Gadella, 2000). Phospholipids are arranged so that the hydrophobic tails interact to create a hydrophobic interior and permeable barrier (Berg et al., 2002). The phospholipid content of the spermatozoa membrane is an important factor in determining membrane fluidity.

A second set of components of the spermatozoa plasma membrane consists of neutral lipids, where cholesterol is the primary sterol component (Flesch and Gadella, 2000). The structure of cholesterol is based on four linked hydrocarbon rings, a hydrocarbon tail at one end, and a hydroxyl group at the other (Berg et al., 2002). The cholesterol molecule is situated so that it is parallel to the phospholipids, enabling the hydroxyl group to interact with the phospholipid head groups (Berg et al., 2002). Depending on the species, a majority of cholesterol is found in the acrosomal region (Ladha, 1998). Cholesterol is an important component in the regulation of membrane fluidity, since it aids in the stabilization of the membrane and the efflux of cholesterol leads to capacitation.

The third component of the plasma membrane consists of glycolipids, such as seminolipid, which is involved in the acrosome reaction and fusion of the equatorial segment during fertilization (Flesch and Gadella, 2000).

Plasma membrane constituents are asymmetrically distributed and are rapidly diffused throughout the surface of the membrane (Berg et al., 2002). Randomization of lipid distribution is prevented due to region specific membrane compositions that perform a specialized function (Ladha, 1998). Barriers exist within the spermatozoa to separate the membrane into domains. Pronounced structural barriers are not present in the head of the spermatozoa; however, freeze fracture and lectin-binding patterns have aided in the detection of distinct domains (Suzuki and Yanagimachi, 1989). These include acrosomal, equatorial, and postacrosomal domains that operate as functional barriers (Flesch and Gadella, 2000). An example of the formation of barriers and the varying lipid content of each barrier can be seen in how there is approximately four times as much cholesterol located in the acrosomal region than in other regions along the spermatozoal structure (De Leeuw et al., 1990). The posterior ring forms a separation in the plasma membrane between the head and the tail where the midpiece and principal piece are located (Flesch and Gadella, 2000). The formation of barriers and domains may dictate the location and quantity of cholesterol incorporated into the membrane.

Capacitation and Acrosome Reaction

Capacitation is the process spermatozoa must undergo before the zona pellucida induced acrosome reaction can occur. Capacitation involves the destabilization of the

spermatozoa membrane leading to fusion of the plasma membrane and the acrosome membrane (Gadella et al., 2001). After ejaculation, spermatozoa plasma membranes have an elevated cholesterol to phospholipid ratio that will decrease as cholesterol is depleted from the membrane (Cross, 1998). Cholesterol acceptors, such as albumin or liposomes, act as the rate-limiting step of cholesterol efflux (Parks and Ehrenwald, 1989). During the process of capacitation, Gadella et al. (2001) proposed that activation of scramblase by a bicarbonate-mediated signaling pathway causes the alteration of spermatozoa membrane phospholipid asymmetry, which results in cholesterol loss by the presence of cholesterol acceptors, lateral removal of seminolipid, and phoshorylation of tyrosine residues of transmembrane proteins.

Once spermatozoa have undergone capacitation, the acrosome reaction can occur upon binding to the zona pellucida of the oocyte. Progesterone receptors are uncovered through the capacitation process and bind to progesterone that is saturated in the zona pellucida (Flesch and Gadella, 2000). Binding of progesterone to its receptor initiates an influx of calcium through calcium channels and phosphorylated tyrosine transmembrane proteins bind to the zona pellucida, thus inducing exocytosis or the acrosome reaction (Gadella et al., 2001). Once the equatorial segment binds the zona pelucida of the oocyte, stored enzymes, which are necessary to dissolve the protein coat surrounding the oocyte (Gadella et al., 2001), are released from the acrosome (Garner and Hafez, 2000). Therefore, capacitated but acrosome intact spermatozoa bind to the zona pellucida, where a receptor-ligand mediated event results in a zona pellucida induced acrosome reaction (Pommer et al., 2003).

Membrane Fluidity

The fluidity of a membrane is dependent on the temperature at which cell components alter their state of fluidity or phase transition temperature (Buhr et al., 1994). When external temperature is above the phase transition temperature, the spermatozoa membrane will be in a more fluid or liquid state; however, when external temperature is below the phase transition temperature, the spermatozoa membrane will be in a more rigid or gel-like state (Parks et al., 1981). Increasing temperature results in a more random order of the phospholipid membrane, whereas decreasing the temperature will result in a more structured order of the membrane (Berg et al., 2002).

Fluidity of the plasma membrane varies with the proportion of lipid components and is partially determined by the ratios of cholesterol to phospholipid and polyunsaturated to saturated fatty acids (Darin-Bennett and White, 1977). Cholesterol disrupts the normal phospholipid chain interactions, and when present in large amounts causes the membrane to be less fluid (Berg et al., 2002). Cholesterol aids in control of phospholipid hydrocarbon chains; therefore, the membrane will be more stable over a wider temperature range (Darin-Bennett and White, 1977). Darin-Bennett and White (1977) demonstrated that species with greater cholesterol to phospholipid ratios, such as rabbits (0.88) and humans (0.99), have greater survival of spermatozoa following cold shock when compared to animals with lower cholesterol to phospholipid ratios, such as the ram (0.38) and bull (0.45).

An additional factor that influences membrane fluidity is the ratio of polyunsaturated and saturated fatty acids. The melting point of unsaturated fatty acids is lower than saturated fatty acids of the same length; therefore, the ratio of unsaturated to saturated fatty acids in phospholipids will reduce the transition temperature (Berg et al., 2002). According to Darin-Bennett and White (1977), spermatozoa that contain high amounts of polyunsaturated fatty acids have lower levels of cholesterol that results in the membrane lacking cohesiveness and therefore resulting in a greater susceptibility to thermal stress, such as cold shock.

Damage to the Spermatozoa Membrane

To successfully preserve the ejaculate, damage to the spermatozoa membrane must be minimized. Damage can occur from many factors including thermal stress, such as cold shock associated with cold storage and cryopreservation, or the presence of factors that can be detrimental to membrane integrity, such as those present in seminal plasma.

During cold storage, membrane damage is closely associated with the rate of cooling when temperatures are rapidly reduced greater than 8-10°C at a time (Magistrini, 2000). Cold shock, induced by rapid cooling, is associated with temperature related phase transition of lipids in the spermatozoa membrane. Park and Lynch (1992) further examined the relationship of cold shock and membrane components between species and determined that similar to Darin-Bennett and White (1977), species that were more resistant to cold shock had a high degree of saturation of phospholipids and high

cholesterol to phospholipid ratio. It was determined that cold shock damage was caused by membrane disruption after re-warming due to the occurrence of multiple gel phases that were aggravated by proteins in the membrane causing faulty packing, which resulted in membrane structural defects (Parks and Lynch, 1992). Park and Lynch (1992) detected multiple phase transition temperatures at approximately 26˚C at which the transition temperature of phospholipids was associated and 35-38˚C at which mammalian glycolipids were associated. This final transition temperature, believed to be due to glycolipid, was absent in the rooster and is thought to be a possible reason for the small amount of cold damage in this particular species (Park and Lynch, 1992). Reducing the number of phase transition temperatures may decrease the amount of damage caused by preservation.

Cryopreservation alters the membrane fluidity such that post thaw spermatozoa lose fluidity faster than freshly ejaculated spermatozoa; therefore reducing the survival time of spermatozoa post thaw (Buhr et al., 1994). Cholesterol appears to be beneficial in the resistance of cold shock due to the alteration of membrane fluidity (Darin-Bennett and White, 1977). Purdy et al. (2002) demonstrated, using transbilayer transport of a propyltrimethylammonium derivative of dipheylhexatriene (TMAP-DPH) and flow cytometric analysis of spermatozoa, that regions consisting of high and low fluidity in the spermatozoa membrane were dependent on the temperature and amount of cholesterol present; however, regardless of the amount of cholesterol in the membrane, fluidity of the post thaw spermatozoa will be reduced.

Along with a dramatic change in temperature, additional stressors associated with cryopreservation include osmotic stress on the plasma membrane, formation of intracellular ice crystals (Watson, 2000), or dehydration from diffusion of unfrozen intracellular water (Graham, 1996b). Damage to the plasma membrane related to cryopreservation, such as the damage seen in the acrosomal region, can result in low fertility rates due to a premature release of acrosomal enzymes (De Leeuw et al., 1990). Increasing membrane fluidity as the spermatozoa are thawed can cause capacitation like changes in the plasma membrane (Neild et al., 2003). Therefore, increasing the amount of cholesterol to the acrosomal region should result in the membrane at this location to remain in a more fluid state throughout the freeze-thaw cycle (De Leeuw et al., 1990), thus preventing some of the negative influences associated with cryopreservation.

Exposure to small amounts of seminal plasma is beneficial to survival and function of spermatozoa; however, excessive exposure to seminal plasma for an extended period of time, such as that associated with cold storage, can damage the spermatozoa membrane (Magistrini, 2000). Seminal plasma consists of secretions from the epididymis, ampulla, prostate, seminal vesicles, and bulbourethral glands (Garner and Hafez, 2001). Some beneficial constituents of seminal plasma include proteins, which attach to the plasma membrane surface and are necessary for fertilization (Magistrini, 2000). Prolonged exposure to seminal plasma may cause decreased percentages of motile and viable spermatozoa and may increase susceptibility to cold shock (Magistrini, 2000). When seminal plasma of stallions with poor post warming PMS were recombined with stallions who had high PMS following warming, PMS was diminished in the

stallions exposed to seminal plasma from poor quality stallions and motility was improved in stallions exposed to seminal plasma of high quality stallions (Aurich et al., 1996). Addition of semen extenders containing skim milk or egg yolk will dilute the seminal plasma and reduce some of the negative influences associated with storing semen in the presence of seminal plasma (Rigby et al., 2001). Removal of seminal plasma by centrifugation and the replacement with an electrolyte-supplemented extender may be beneficial for some stallions. Dilution of semen using a modified Tyrode's media was beneficial to the improvement of motility following cold storage (Webb and Arns, 1995). Dilution of semen using a modified PBS extender was beneficial to improving post-warming percentages of progressively motile spermatozoa and acrosome intact spermatozoa (Dawson et al., 2000). Therefore, the replacement of seminal plasma with an electrolyte-supplemented extender may be advantageous for those stallions susceptible to toxic influences present in seminal plasma.

Characteristics of Cyclodextrin

A method to modify cholesterol content of the spermatozoa membrane is through the use of cyclodextrin. Cyclodextrin is a cyclic oligosaccharide that contains six (α-cyclodextrin), seven (β-cyclodextrin), or eight (γ-cyclodextrin) glucopyranose units (Ohvo and Slotte, 1996). The external face of the molecule is hydrophilic whereas the cavity of cyclodextrin is hydrophobic; therefore, cyclodextrin can encapsulate non-polarized materials thus causing non-soluble substances to become soluble (Yancey et al., 1996). Current research in the pharmaceutical industry is utilizing the carrier mechanism

of cyclodextrin as a way to deliver and release hydrophobic medicines (Ohvo and Slotte, 1996). Cyclodextrin is also being used in cellular research to study cholesterol loading and efflux mechanisms (Klein et al., 1996; Hartel et al., 1998).

A determining factor, as to the guest molecule that is taken up into the cyclodextrin, is the size of the cyclodextrin cavity (Hartel et al., 1998). The β-cyclodextrin family has a high affinity for sterols, especially cholesterol (Yancey et al., 1996). Cholesterol found in phospholipid membranes will also be internalized by cyclodextrin (Ohvo and Slotte, 1996). The hydroxyl group of β-cyclodextrin can be modified to improve solubility in water and the ability to form an inclusion complex with cholesterol (Yancey et al., 1996). Yancey et al. (1996) determined that when the efficiency of methyl-β-cyclodextrin (MβCD), 2-hydroxypropyl-β-cyclodextrin (2OHpβCD), and β-cyclodextrin were compared, MβCD was the most efficient at encapsulating cholesterol. In addition, Christian et al. (1997) determined that MβCD is less vulnerable to precipitation of cholesterol after dilution, and is more effective at loading cholesterol into cells at lower concentrations than 2OHpβCD. Therefore, MβCD may prove to be more beneficial when it comes to research of the spermatozoal membrane.

When cyclodextrin is compared to other cholesterol acceptors, such as high density lipoproteins (HDL), cyclodextrin is more effective at modifying cholesterol due to the size of cyclodextrin when compared to HDL. The diameter of cyclodextrin is about 15 Å and the diameter of HDL particles ranges from 70 to 120 Å (Yancey et al., 1996). Therefore, Yancey et al. (1996) hypothesized that cyclodextrin is capable of

accessing the cholesterol domains on the plasma membrane more efficiently due to its small size and, unlike HDL particles, cyclodextrin can diffuse readily through the media and filter through barriers, such as the spermatozoa glycocalyx.

The mechanism for cyclodextrin-induced cholesterol efflux pertains to the difference in activation energy (Christian et al., 1997). Activation energy for cholesterol efflux by a phospholipid acceptor is approximately 20 kcal/mol; whereas, during the cholesterol efflux mediated by cyclodextrin, the activation energy is approximately 7 kcal/mol (Yancey et al., 1996). The difference in activation energy is due to the cholesterol molecules being ejected through an aqueous phase prior to reabsorption by the liposome (Yancey et al., 1996). However, with cyclodextrin, cholesterol molecules incorporate directly into the cavity bypassing an aqueous phase (Christian et al., 1997). Therefore, the incorporation of cholesterol into cyclodextrin is more efficient than other cholesterol acceptors.

The amount of cholesterol mobilized is dependent on the length of time cyclodextrin has access to the cellular membrane and the amount of surface area exposed. The rate of cholesterol diffusion is dependent on duration of incubation, whether the sample is being stirred, and whether the sample is in a suspension or single layer (Yancey et al., 1996). When there is a gradient in cholesterol concentration, cholesterol from loaded cyclodextrin molecules appears to be incorporated through diffusion to areas in the membrane that are undersaturated (Christian et al., 1997; Hartel et al., 1998).

When CLC is compared to cholesterol-loaded liposomes, Hartel et al. (1998) demonstrated that cyclodextrin was more efficient at increasing cholesterol content. In a

study using liver microsomes, it was demonstrated by Hartel et al. (1998) that cholesterol loaded cyclodextrin can increase the cholesterol 4 times its original content compared to liposomes, which increase the cholesterol content by approximately 140%. It was hypothesized that cholesterol enrichment by liposomes utilized a receptor-mediated endocytosis of the LDL receptor (Brown and Goldstein, 1986) that will be limited due to negative feedback, resulting in fewer LDL receptors in response to increasing levels of cholesterol (Hartel et al., 1998). The incorporation of cholesterol by cyclodextrin was not limited by these restrictions.

Cholesterol-Loaded Cyclodextrin and Spermatozoa

The amount of cholesterol loaded into the spermatozoa membrane is dependent on the dose of CLC. There is a linear relationship between cholesterol incorporation in the membrane and amount of CLC added to the media (Purdy and Graham, 2004b). Spermatozoa cholesterol content can be increased two to three times the original amount due to the addition of CLC (Purdy and Graham, 2004b). Vidament et al. (2001) demonstrated that the cholesterol to phospholipid ratio of CLC-treated spermatozoa is higher (1.0) when compared to non-treated spermatozoa (0.62). When the cholesterol to phospholipid ratio is approximately 1.0, it is more similar to the cholesterol to phospholipid ratio of rabbits (0.88) and humans (0.99), which are unaffected by cold shock damage (Darin-Bennett and White, 1977). This suggests that loading cholesterol into the spermatozoa membrane will decrease the negative influences due to cold shock.

The percent of viable spermatozoa is improved due to the protective qualities of CLC. The percentages of spermatozoa with intact membranes following cryopreservation were improved in the bull (Purdy and Graham, 2004b) and stallion (Combes et al., 2000; Zahn et al., 2002). Total and progressively motile spermatozoa were improved after cold storage (Kirk et al., 2001) and post thaw in the stallion (Combes et al., 2000) and bull (Awad et al., 2002; Purdy and Graham, 2004b). Similarly, curvilinear and straight-line velocities were unaffected in CLC treated spermatozoa in the stallion (Combes et al., 2000) and bull (Awad et al., 2002) after cryopreservation. The improvement of these parameters would suggest that spermatozoa treated with CLC will be of a higher quality following preservation and therefore have greater potential for increased per cycle pregnancy rates.

Purdy and Graham (2004b) utilized the fluorescent molecule 22-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminl-23,24-bisnor-5-cholen-3B-ol (NBD) to label cholesterol loaded into cyclodextrin to determine the location of cholesterol loaded into the spermatozoa membrane. It was determined that cholesterol was incorporated throughout the length of the plasma membrane with greater amounts localized in the acrosomal and mitochondrial regions (Purdy and Graham, 2004b). During cold storage or cryopreservation, damage to the plasma membrane will occur along the length of the spermatozoa (Blottner et al., 2001). The protective qualities of CLC are evident since cholesterol is incorporated throughout the length of the membrane; therefore, the increased percentages of intact membranes after preservation may be due to the altered membrane fluidity (Combes et al., 2000; Purdy and Graham 2004b). Purdy and Graham

(2004b) suggested that the incorporation of cholesterol increases PMS of spermatozoa following preservation due to protection of the mitochondria. During the cryopreservation process, membrane damage occurs to the spermatozoa acrosomal region (Blottner et al., 2001); therefore, addition of cholesterol to the acrosomal region will aid in maintaining an intact membrane. However, addition of cholesterol to the acrosomal region may decapacitate or have a negative impact on the capacitation process and acrosome reaction since these processes require cholesterol to be lost from the acrosomal region to enable fertilization (Davis, 1978). Loading of cholesterol into the spermatozoa membrane aids in its stabilization; however, fertilizing capacity of the spermatozoa is thought to be inhibited.

An example of the over-stabilization of the spermatozoa membrane is evident when CLC is added to freshly ejaculated bull sperm. Purdy and Graham (2004a) evaluated induction of the acrosome reaction in fresh, CLC-loaded semen with dilauroylphosphatidylcholine (PC12), calcium ionophore A23187, and heparin followed by lysophosphatidylcholine (LPC), and determined that CLC retarded the capacitation process and/or acrosome reaction of fresh spermatozoa. However, when Purdy and Graham (2004a) used the same methods to evaluate induction of capacitation and acrosome reaction in thawed-cryopreserved spermatozoa; CLC-treated and control samples had similar percentages of capacitated and acrosome-reacted spermatozoa. Procedures for *in vitro* fertilization did not need to be altered when using CLC treated cryopreserved spermatozoa since additional capacitation time was not required to fertilize the oocyte (Purdy and Graham, 2004a). There were no differences in the percentages of

oocytes cleaved and embryos that developed to blastocyst when CLC treated and non-treated post-thaw spermatozoa were used (Purdy and Graham, 2004a). When CLC treated spermatozoa were tested *in vivo*, there was a 59% fertility rate with CLC treatment versus a 50% fertility rate when non-treated spermatozoa were utilized, thus leading Purdy and Graham (2004a) to conclude that, although not statistically significant, more spermatozoa were surviving cryopreservation in CLC-treated spermatozoa to influence the fertility rate. Purdy and Graham (2004a) theorized that the difference between fresh and post thaw cryopreserved spermatozoa is due to the loss of membrane fluidity due to the cryopreservation process, thus producing spermatozoa whose membranes were destabilized, or capacitated, following cryopreservation.

The concept of the cryopreservation process resulting in larger percentages of precapacitated spermatozoa was not evident in the stallion. Zahn et al. (2002) reported a post-thaw reduction in the percentage of CLC-treated spermatozoa to undergo an acrosome reaction when compared to non-treated spermatozoa; these authors concluded that CLC resulted in the stabilization of the membrane, which improved membrane integrity after thawing, but resulted in a reduction of fertility due to the inhibition of the acrosome reaction.

Unloading of Cholesterol by Methyl β-Cyclodextrin

The cholesterol efflux mechanism has been applied to studies to determine the role of cholesterol in capacitation and the fertilization process. The cholesterol efflux mechanism caused by MβCD has been demonstrated to stimulate capacitation *in vitro*

(Choi and Toyoda, 1998) and thought to increase the degree of acrosome response during the acrosome reaction (Cross, 1999). Choi and Toyoda (1998) established that the cholesterol efflux mechanism of cyclodextrin could result in the capacitation of spermatozoa in a protein free environment, leading to the conclusion that capacitation as well as fertilization can occur *in vitro* with MβCD as a BSA substitute. These researchers also concluded that the influence of cyclodextrin on percentages of viable spermatozoa was dependent on the concentration of MβCD and the incubation time (Choi and Toyoda, 1998). Fertilization rates declined when MβCD was present in both the capacitation and fertilization media and all spermatozoa were dead when incubated for over six hours (Choi and Toyoda, 1998). These results suggest that prolonged exposure and excess amounts of MβCD can be detrimental to spermatozoa survival.

Cyclodextrin has been demonstrated to enhance the binding of spermatozoa to zonae pellucidae. Parinaud et al. (2000) demonstrated how greater numbers of spermatozoa were capable of binding to the zona pellucida due to greater availability of ZP3 receptors within the spermatozoa membrane, therefore concluding that ZP3 receptors were more accessible as a result of cholesterol depletion (Parinaud et al., 2000). The correlation between cholesterol depletion and acrosomal response was also examined by Cross (1999), who demonstrated that MβCD increased the spermatozoal responsiveness to progesterone, which is known to induce acrosome reactions in some species (Flesch and Gadella, 2000), due to a rapid decrease in cholesterol.

Phophorylation of tyrosine residues was enhanced by MβCD. Pommer et al. (2003) demonstrated that the addition of MβCD to stallion spermatozoa to cause

capacitation resulted in the enhancement of phosphorylation of tyrosine residues with increasing MβCD concentration. The correlation between intensity and MβCD indicates that cholesterol efflux is associated with the membrane events that cause capacitation and lead to the acrosome reaction (Pommer et al., 2003). Cholesterol efflux leading to the phosphorylation of protein tyrosines was also demonstrated in the human, mouse, and bull (Visconti et al., 1999).

MATERIALS AND METHODS

Loading of Cholesterol into Methyl β-Cyclodextrin

All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich Company (St. Louis, MO). Procedures for preparing CLC were adapted from those developed by Purdy and Graham (2004) and Klein and associates (1995). Briefly, 200 mg of cholesterol was dissolved into 1 ml chloroform. In a separate glass container, 500 mg MβCD was dissolved into 1 ml methanol. An aliquot of 0.225 ml of cholesterol solution was combined with the MβCD solution, which was then placed in a 60 ˚C waterbath and mixed until clear. The solution was placed in a lyophilizer until contents were dry and the CLC was stored in a desiccator at 22 ˚C until use.

Working Solution of Cholesterol Loaded Cyclodextrin and Methyl β-Cyclodextrin

The working solution of CLC was prepared immediately prior to collection of stallions by combining 50 mg CLC to 1 ml semen extender with ticarcillin (E-Z Mixin – "BFT", Animal Reproduction Systems, Chino, CA) at 37 ˚C. The solution was mixed with a vortex until dissolved, then centrifuged for 5 min at 100 rpm to remove undissolved particles. The solution of MβCD was prepared by adding 50 mg MβCD to 1 ml Tyrode's salt solution at 37 ˚C.

Semen Collection and Processing

Mature stallions of known fertility were stabled at the University of Arizona Equine Center. All procedures were approved by the Institutional Animal Care and Use

Committee of The University of Arizona. The ejaculate was collected from stallions through the use of a Missouri style artificial vagina (NASCO, Fort Atkinson, WI). Stallions were collected for ten days prior to the initiation of experimental trials to deplete the extragonadal reserves and were maintained on a schedule of every other day collections. The raw ejaculate was filtered to remove the gel fraction and debris. Spermatozoa concentration was determined using a SpermaCue Photometer (MiniTube, Verona, WI). Bright field microscopy was used to determine percent progressively motile spermatozoa (PMS) from semen that had been diluted with skim milk extender containing ticarcillin.

Experiment I: Dose Response Trials

CLC Dose Response of Cold Stored Spermatozoa. A dose response of CLC was conducted on three ejaculates from each of three stallions (N = 9) to determine the amount of CLC needed to optimize PMS following 24 h of cold storage. After each ejaculate was collected, it was subdivided and extended into five, 5 ml aliquots containing 50 x 10^6 total spermatozoa per ml. The aliquots received 0, 6.0, 9.0, 12, or 25 µg CLC per 10^6 spermatozoa. Samples were equilibrated for 15 min after the addition of CLC before determination of PMS, and then subsequently brought to 5 ˚C using a static cooling device (Equine Express, Exodus Breeders Supply, York, PA). Approximately 24 h later, samples were warmed to 37 ˚C to determine PMS in a double blind procedure.

MβCD Dose Response of Cold Stored Spermatozoa. A dose response of MβCD was performed on treatments that had been loaded with cholesterol prior to cold storage

to determine the ideal amount of MβCD to unload cholesterol while maintaining the motility of the sample. Three ejaculates from three stallions were collected. Each ejaculate was subdivided and extended into two 5 ml aliquots containing 50 x 10^6 spermatozoa per ml. To each sample, either 0 or 9.0 μg per 10^6 spermatozoa was added. After 24 h, the sample containing CLC was subdivided into 1 ml aliquots where 0, 0.5, 0.75, 1.0 mM of MβCD was added and allowed to incubate for 15 min after which time PMS was determined.

MβCD Dose Response of Cryopreserved Spermatozoa. Cryopreserved spermatozoa from three ejaculates of three stallions (N = 9) were subjected to increasing concentrations of MβCD to determine any adverse effects on PMS. One straw per treatment containing either 0 or 12 μg CLC per 10^6 spermatozoa was thawed by submersion in a 37 ˚C water bath for 1 min. Samples were then diluted in 5 ml of skim milk extender. Subsamples containing 1 ml of cholesterol treatment were placed in separate containers where 0, 0.5, 1.0, or 2.0 mM of MβCD was added. After 15 and 60 min of incubation, PMS was determined.

Experiment II: Cold Stored Spermatozoa

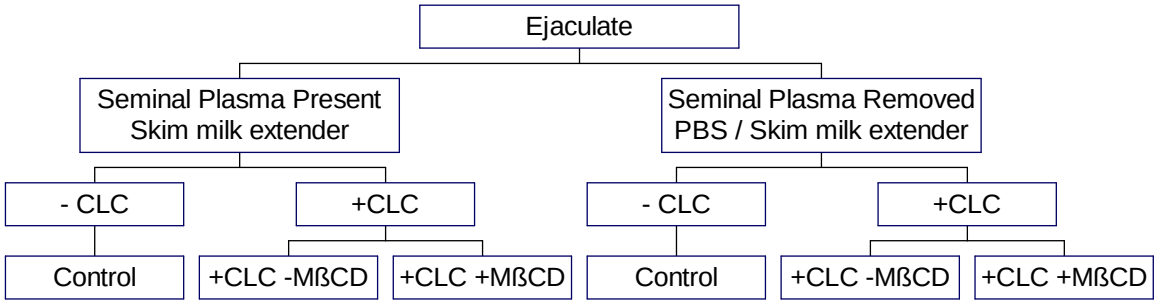
Addition of CLC Prior to Cold Storage. The influence of CLC on cold stored spermatozoa in the presence of seminal plasma was determined through the addition of CLC to each of three ejaculates from five stallions (N = 15). After each ejaculate was collected, it was subdivided and extended into two, 12 ml aliquots containing 50 x 10^6 spermatozoa per ml (600x10^6). To each sample was added 0 or 12 μg CLC per 10^6

spermatozoa. Each 12 ml aliquot was subdivided into two 5 ml samples and one 2 ml sample. The PMS was determined after a 15 min equilibration period following addition of CLC. The 5 ml aliquots were brought to 5 ˚C using a static cooling device. Samples were analyzed following 24 and 48 h of cold storage.

To determine the effects of CLC in the absence of seminal plasma on cold stored spermatozoa, CLC was added to three ejaculates from five stallions. The ejaculate was subdivided and extended 1:1 with extender prior to being placed in a centrifuge for 15 min at 300 g. Concentration of spermatozoa was adjusted by 10% to accommodate loss due to centrifugation. The supernatant was discarded and the pellet re-suspended with 1:3 ratio (vol:vol) of phosphate buffered saline (PBS) to skim milk extender (Webb and Arns, 1997). Either 0 or 12 μg CLC per 10^6 spermatozoa was added to each treatment group, which was then subdivided into two 5 ml samples and one 2 ml sample. From the 2 ml sample, PMS was determined 15 min after the initial addition of CLC. The 5 ml subsamples were brought to 5 ˚C using a static cooling device and samples were analyzed following 24 and 48 h of cold storage.

Evaluation of Cooled Samples at 24 & 48 hours. After 24 and 48 h of cold storage, treatments were warmed to 37 °C, and PMS and percent viable spermatozoa analyzed. Prior to sample warming, 1.0 mM MβCD was added to 1 ml subsamples of CLC positive treatments. Following 15 min of incubation, PMS was determined.

Figure 1. Organizational chart demonstrating the treatments of cold stored spermatozoa



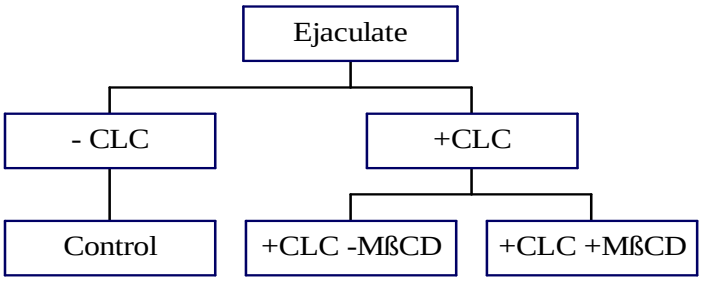
Flow cytometric analysis was performed by the Biotechnology Cytometry Facility of the University of Arizona to determine the integrity of the spermatozoa membrane by analyzing the percent live intact (LI) and the percent live non-intact (LNI) spermatozoa. A 1 ml subsample per treatment was transferred to be analyzed by a Becton-Dickinson FACStar PLUS (San Jose, CA) flow cytometer equipped with an Innova 90-5 (Coherent, Inc. Santa Clara, CA) 5 W water cooled laser tuned to 488 nm. Acquisition and analysis software was performed on Cellquest (Becton-Dickinson San Jose, CA) using a Macintosh G3 computer running OS 8.6 operating system.

Flow cytometric procedures were based on those presented by Graham in 2001. Peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA) (Molecular Probes, Eugene, OR), consisting of 0.01 mg FITC-PNA per ml PBS that had been frozen in micro-centrifuge tubes, thawed and added approximately 30 min prior to flow cytometric analysis. Propidium iodide (PI) (Component B of LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR) consisted of 0.1 mg PI per ml distilled water and was added approximately 5 min prior to flow cytometric analysis.

Experiment III: Cryopreservation of Spermatozoa

Addition of CLC prior to Cryopreservation. To determine the effects of CLC on cryopreservation of spermatozoa, three ejaculates were collected from five stallions. The ejaculate was diluted 1:1 with extender and allowed to equilibrate for 5 min. For the CLC treatment, 12 μg per 10^6 spermatozoa was added to the extender and the treatment allowed to incubate an additional 15 min. Both treatments were placed in a centrifuge for 15 min at 300 g. After the supernatant was discarded, egg yolk freezing extender (E-Z Freezin - "MFR5", Animal Reproduction Systems, Chino, CA) at room temperature was utilized to resuspend the pellet of spermatozoa to a final concentration of 330 x 10^6 spermatozoa per ml. French straws (0.5 ml) were filled with extended semen and subsequently heat-sealed. Straws were placed approximately 3 cm above liquid nitrogen for approximately 15 min before submersion. Samples were then transferred to a storage tank where they remained in liquid nitrogen until evaluation.

Figure 2. Organizational chart demonstrating the treatments of cryopreserved spermatozoa



Evaluation of Cryopreserved Samples. Cryopreserved samples were thawed by submersion in a 37 ˚C water bath for 1 min. Contents were then diluted in 5 ml skim milk extender and placed in an incubator. Subsamples (1 ml) CLC treated spermatozoa

were incubated for 15 min with 1.0 mM MβCD prior to analysis (Figure 2). At 15 and 60 min post thaw, PMS was determined.

Flow cytometric analysis was employed to determine the integrity of the spermatozoa membrane through the assessment of LI and LNI. A 1 ml sample per treatment was transferred to be analyzed on a Becton-Dickinson FACScan (San Jose, CA) flow cytometer equipped with a 488 nm 200 mW argon laser. Acquisition and analysis was performed on Cell Quest Pro software on a Macintosh G3 (Santa Clara, CA) computer running OS 9.2. Single color controls were used to electronically compensate for spectral overlap. All parameters were read on a log scale and 10,000 events were collected in a gate defined by forward versus side scatter.

Flow cytometric procedures were based on those presented by Nagy et al. (2003). Phycoprobe-Peanut Agglutinin (PE-PNA), consisting of 1 mg PE-PNA per ml, was added 30 min prior to flow cytometric analysis. Approximately 15 min prior to flow cytometry, SYBR® 14 dye reagent (Component A of LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR) consisting of 1 mM solution in DMSO was added. Addition of PI was added to the sample 5 min prior to flow cytometric analysis.

Statistical Analysis

Statistical analysis was performed using the Linear Mixed Effects Model Package of R (R Development Core Team, 2004). A split plot design was employed such that the individual stallion was considered the random variable with variable, ejaculate, nested within stallion. The response variable included PMS prior to preservation, PMS, LI, and LNI for spermatozoa following 24 and 48 h of cold storage, and post thaw. Data is presented as least squares means $\pm$ standard error of the mean. The p-value was determined through the use of ANOVA tables while least squares means function of R was used to reveal relationships within treatments. Each time interval was examined separately. When determining the significance of cold storage treatments, the significance of seminal plasma was determined prior to statistical analysis of the individual treatments. Significance was determined at the $\alpha = 0.05$ level.

The model for the analyses was:

$$y_{ijk} = \mu + \alpha_i + \beta_{ij} + \gamma_k + \alpha\gamma_{ik} + \beta\gamma_{ijk} + \varepsilon_{ijk}$$

μ = Mean of the response variable (PMS, LI, or LNI)

i = Individual stallion

j = Ejaculate

k = Cholesterol treatment (control, cholesterol-loaded, cholesterol-unloaded)

α_i = Mean of the response variable by stallion

β_{ij} = Mean of the response variable by ejaculate nested within stallion

γ_k = Mean of the response variable by cholesterol treatment

$\alpha\gamma_{ik}$ = Interaction between stallion and cholesterol treatment

$\beta\gamma_{ijk}$ = Interaction between ejaculate within stallion and cholesterol treatment

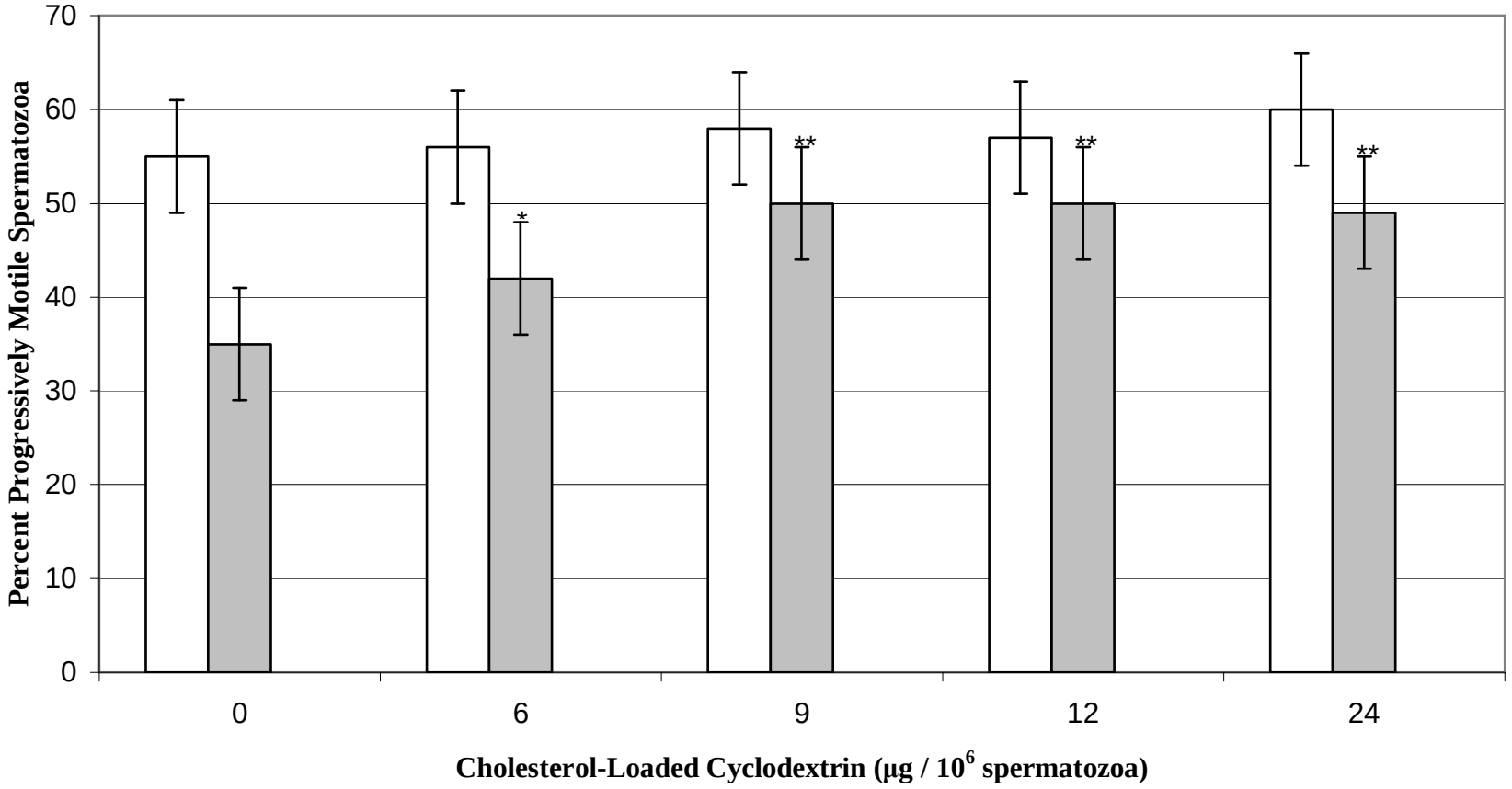
ε_{ijk} = Error between individual stallion, ejaculate and cholesterol treatment

RESULTS AND DISCUSSION

Dose Response Trials

Dose Response of Cholesterol-Loaded Cyclodextrin. Prior to cold storage, incorporation of increasing concentrations of CLC did not increase PMS. However, following 24 h of cold storage, all samples containing CLC had a higher ($P < 0.05$) PMS when compared to non-treated spermatozoa (figure 3). Moreover, the response occurred in a dose dependent fashion and reached a response plateau at 9 μg CLC per 10^6 spermatozoa. This indicates that the ideal amount of CLC ranges between 9.0 and 25 μg per 10^6 spermatozoa. The amount of CLC in this range is similar to what has been reported to be optimal when freezing equine (Combes et al., 2000) and bovine (Purdy and Graham, 2004b) spermatozoa. Although both parties utilized different procedures for loading cholesterol into cyclodextrin, the final cholesterol concentrations were similar. When the amount of cholesterol in a CLC dose is calculated, Combes et al. (2000) added approximately 0.78 μg cholesterol per 10^6 spermatozoa for the stallion whereas the ideal range for the bull, as determined by Purdy and Graham (2004b), was 1.125 to 2.25 μg cholesterol per 10^6 spermatozoa. In the present study, the calculated amount of cholesterol in a CLC dose was determined to range between 0.81 and 2.25 μg cholesterol per 10^6 spermatozoa. The stallion has less cholesterol present in the membrane than the bull and may therefore benefit more from incorporation of greater amounts of cholesterol when spermatozoa are subject to stressors; such as cold shock or cryopreservation. Both Combes et al. (2000) and Purdy and Graham (2004a; 2004b) added CLC to spermatozoa

Figure 3. Dose Response of Cholesterol-Loaded Cyclodextrin (CLC) and its Influence on Progressively Motile Spermatozoa Prior to and Following Cold Storage. Percent progressively motile spermatozoa was determined approximately 15 minutes after the addition of various dosages of CLC (white shading) and after 24 hours of cold storage (light grey shading). * Means are significantly different ($P < 0.05$). ** Means are significantly different ($P < 0.001$) from control (0 µg CLC) .



that underwent cryopreservation unlike the CLC dose response of the present paper, where CLC was incorporated into samples prior to cold storage.

Table 1. Influence of increasing concentrations of methyl beta-cyclodextrin (MβCD) on the percentage of progressively motile spermatozoa in cholesterol loaded spermatozoa stored at 5˚C for 24 hours.

	Treatment (mM MβCD)				
	Control*	0.00	0.50	0.75	1.00
PMS (%)	35±10[a]	50±10[b]	55±10[b]	52±10[b]	51±10[b]

[ab] Means in a row with different superscripts are different ($P < 0.05$).

* Control refers to non-cholesterol loaded spermatozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

Dose Response of Methyl ß-Cyclodextrin. Following 24 h of cold storage, the addition of varying amounts of MβCD to CLC-treated spermatozoa to unload cholesterol did not alter PMS (table 1). Similarly, when varying amounts of MβCD were added post thaw to cryopreserved CLC-treated spermatozoa, PMS was not altered after 15 and 60 min post thaw (table 2). These results indicate that the unloading of cholesterol through the use of small amounts of MβCD did not have negative influences on PMS. When MβCD is used in increasing amounts, capacitation can be enhanced in stallion spermatozoa (Pommer et al., 2003) that have not been previously loaded with cholesterol. In large dosages, we found (unpublished observations) that MβCD was detrimental to spermatozoa regardless of CLC treatment. Death in the presence of large amounts of MβCD was theorized by Cross (1999) to be due to the acceleration of maturation caused by a rapid loss of cholesterol from the spermatozoa membrane. It may be beneficial to use small amounts of MβCD to unload excess cholesterol that may inhibit fertilization;

however, addition of MβCD in larger amounts may induce premature capacitation or cell death.

Table 2. Influence of increasing concentrations of methyl beta-cyclodextrin (MβCD) on percentage of progressively motile spermatozoa in cholesterol loaded cryopreserved spermatozoa 15 and 60 minutes post-thaw.

		Treatment (mM MβCD)				
Post Thaw (min)	PMS (%)	Control*	0.00	0.50	1.00	2.00
15		24±4[a]	37±4[b]	34±4[b]	38±4[b]	38±4[b]
60		24±4[a]	41±4[b]	37±4[b]	38±4[b]	35±4[b]

[ab] Means in a row with different superscripts are different ($P < 0.05$).

* Control refers to non-cholesterol loaded spermatozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

Treatment with MβCD in small amounts did not express negative influences on spermatozoa PMS, suggesting that CLC-treated spermatozoa are unaffected by MβCD. The lack of an affect, especially at the highest dose, may be due to competitive interference between MβCD and cholesterol found in the skim milk and egg yolk media utilized. There are approximately 0.174 μg cholesterol per mg of skim milk (Nutrient Data Laboratory, 2005) and skim milk-based extenders contain approximately 24 mg nonfat milk per ml extender (Magistrini, 2000); therefore, there is approximately 4.17 μg cholesterol per ml skim milk extender. Egg yolk based freezing extender contains 2% egg yolk (Wilhelm et al., 1996) with approximately 12.4 mg cholesterol per mg of egg yolk (Nutrient Data Laboratory, 2005); therefore, egg yolk based extenders contain 270 μg cholesterol per ml. After dilution with a skim milk extender (1:10), the estimated cholesterol content of the egg yolk plus skim milk extender is approximately 31.2 μg cholesterol per ml (table 3). Exogenous cholesterol in the media may bind to the

cyclodextrin, inhibiting its influence or ability to remove cholesterol from the spermatozoa membrane.

Table 3. Estimated cholesterol content of skim milk and egg yolk based extenders following treatment with cholesterol-loaded cyclodextrin (CLC).

Cholesterol Content (mg/ml)	**Skim Milk Base**	**Egg Yolk Base**
Extender	4.20	27.0
Additional Extender	NA	4.20
CLC treatment	54.0	54.0
Total Cholesterol	**58.2**	**85.2**

The quantity of cholesterol unloaded using MβCD can be estimated based on the amount of cholesterol saturation utilized to load cholesterol into MβCD. If the initial concentration of cholesterol loaded into the cyclodextrin represents the point of saturation, the addition of 0.00, 0.50, 0.75, 1.00 and 2.00 mM MβCD removes 0.0, 60, 90, 120, 240 μg cholesterol per ml. Therefore, the addition of 0.50 mM or larger amounts of MβCD in skim milk extender should be sufficient to remove excess cholesterol present in the media and from CLC incorporation (table 4). The addition of 1.00 mM or larger amounts of MβCD in egg yolk based extender diluted in skim milk extender should be sufficient to remove excess cholesterol present in the media due to CLC incorporation

Table 4. Estimated cholesterol content of cholesterol loaded cyclodextrin (CLC) and skim milk extender following treatment with varying dosages of methyl beta-cyclodextrin (MβCD).

	Treatment (mM MβCD)			
	0.00	0.50	0.75	1.00
Cholesterol Removed by MβCD (μg/ml)	0.00	60.0	90.0	120
Calculated Amount of Cholesterol Removing or Removed (μg/ml)	**45.0**	**-15.0**	**-45.0**	**-75.0**

and lipids found in the skim milk and egg yolk (table 5). To determine the influence of lipids in the media, further tests to quantify the amount of cholesterol present in the membrane after CLC or MβCD treatment and the type of extender used should be conducted.

Table 5. Estimated cholesterol content of cholesterol loaded cyclodextrin (CLC) and egg yolk extender following treatment with varying dosages of methyl beta-cyclodextrin (MβCD).

	Treatment (mM MβCD)			
	0.00	0.50	1.00	2.00
Cholesterol Removed by MβCD (mg/ml)	0.00	60.0	120	240
Calculated Amount of Cholesterol Removing or Removed (μg/ml)	**85.0**	**25.0**	**-35.0**	**-155**

Progressively Motile Spermatozoa

After 1 h of incubation, there were no differences in PMS between CLC-treated spermatozoa and non-treated samples (table 6). Although CLC has been reported to load cholesterol into the spermatozoa membrane in approximately 15 min (Combes et al., 2000; Purdy and Graham, 2004b), it does not alter initial PMS when compared to the control, which demonstrates that incorporation of cholesterol into the membrane has no immediate benefits on PMS.

Loading cholesterol into the spermatozoa membrane was beneficial to PMS following cold storage at 5˚C. After 24 and 48 h of cold storage, PMS of CLC-treated spermatozoa was improved ($P < 0.05$) when compared to non-treated spermatozoa. The PMS was maintained after addition of MβCD to the CLC treated spermatozoa (table 6). Following a period of cold storage, PMS of CLC treated spermatozoa was improved. Addition of a MβCD solution to the CLC treated spermatozoa does not negatively

influence PMS. These results correspond to Kirk et al. (2001) who observed improved PMS and total motile spermatozoa after 24 and 48 h of cold storage.

Table 6. Influence of cholesterol-loaded cyclodextrin (CLC) and cyclodextrin (MβCD) on percent progressively motile spermatozoa prior to and following 24 and 48 h of cold storage at 5˚C

Period (Hours)	Treatment		
	Control*	+CLC -MβCD	+CLC +MβCD
1	56±2[a]	57±2[a]	
24	40±4[a]	49±4[b]	52±4[b]
48	28±5[a]	36±5[b]	39±5[b]

[ab] Means in a row with different superscripts are significantly different ($P < 0.05$).
* Control refers to non-cholesterol loaded spematozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

There were no differences in PMS between treatments where seminal plasma was removed and where seminal plasma was present. It was hypothesized that the removal of seminal plasma through centrifugation in combination with the use of CLC would be beneficial towards the survival of spermatozoa. Instead, there were no differences between PMS of those treatments where seminal plasma was removed and where seminal plasma was present. This contradicts the findings of Dawson et al. (2000), where PMS was improved when seminal plasma was removed and replaced with a PBS-skim milk extender solution. The differences between Dawson et al. (2000) and the present study may be due to variation between stallions.

Samples treated with CLC had improved PMS at 15 ($P = 0.025$) and 60 min ($P = 0.006$) post thaw when compared to non-treated cryopreserved spermatozoa. Addition of a MβCD solution to unload cholesterol did not affect PMS after 15 and 60 min post thaw (table 7). These results indicate that loading cholesterol into the spermatozoa membrane

is beneficial in maintaining PMS of post thaw cryopreserved samples. These results correspond with those of Combes et al. (2000). Since post thaw PMS was maintained after 60 min of incubation, spermatozoa treated with CLC are capable of remaining motile for a period of time, therefore demonstrating longevity. The ability of spermatozoa to remain motile over a length of time may indicate that the addition of MβCD does not have negative influences on PMS post thaw. In order to determine whether the modification of cholesterol in the membrane affects the longevity of the post thaw spermatozoa, longer periods of incubation should be examined.

Table 7. Influence of cholesterol-loaded cyclodextrin (CLC) and cyclodextrin (MβCD) on percent progressively motile cryopreserved spermatozoa 15 and 60 minutes post thaw.

Incubation (Min)	**Treatment**		
	Control*	**+CLC -MβCD**	**+CLC +MβCD**
15	25±4[a]	33±4[b]	35±4[b]
60	25±4[a]	34±4[b]	36±4[b]

[ab] Means in a row with different superscripts are significantly different ($P < 0.05$).
* Control refers to non-cholesterol loaded spermatozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

Membrane Integrity

Following 24 and 48 h of cold storage, the percent of LI was enhanced in CLC treated spermatozoa in the presence of seminal plasma ($P < 0.05$) upon comparison to non-treated samples. The percent LNI was not different when comparing CLC treated and non-treated spermatozoa in the presence of seminal plasma (table 8). These results indicate improved spermatozoal membrane integrity and the absence of negative influences on acrosomal integrity after cold storage since the membrane was stabilized through the loading of cholesterol in the presence of seminal plasma.

Table 8. Influence of seminal plasma, cholesterol-loaded cyclodextrin (CLC) and cyclodextrin (MβCD) on membrane integrity determined by flow cytometry following 24 and 48 hours of cold storage.

		Treatment					
		Seminal Plasma Present			Seminal Plasma Removed		
Cold Storage		**Control***	**+CLC -MβCD**	**+CLC +MβCD**	**Control***	**+CLC -MβCD**	**+CLC +MβCD**
24 Hours	LI	63±4[a]	80±4[c]	80±4[c]	63±4[a]	70±4[a]	64±4[a]
	LNI	3±1[a]	3±1[a]	1±1[a]	5±1[a]	2±1[a]	2±1[a]
48 Hours	LI	59±4[b]	75±4[c]	74±4[c]	65±4[a]	68±4[a]	63±4[ab]
	LNI	2±1[a]	3±1[a]	4±1[a]	3±1[a]	4±1[a]	3±1[a]

[abc] Means in a row with different superscripts are different ($P < 0.05$).
* Control refers to non-cholesterol loaded spermatozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

Upon addition of a MβCD solution to CLC treated spermatozoa following 24 and 48 h of cold storage in the presence of seminal plasma, the percentages of LI and LNI were maintained when compared to non-treated samples (table 8). This indicates that addition of a MβCD solution does not have negative influences on the spermatozoa membrane integrity, nor does it induce capacitation in the cholesterol-loaded samples that may correspond to an absence of negative effects on fertility.

The removal of seminal plasma prior to preservation had an unexpected influence on spermatozoal membrane integrity following cold storage. After 24 h of cold storage, CLC treated spermatozoa, where seminal plasma had been removed, had a tendency to improve the percent LI ($P = 0.059$). There were no differences in LNI of CLC treated spermatozoa upon comparison to non-treated samples when seminal plasma was removed. Following 48 h of cold storage in the absence of seminal plasma, percentages

of LI and LNI of CLC treated spermatozoa were not different from the control (table 8). Therefore, CLC addition and the removal of seminal plasma from the ejaculate did not improve recovery rates of viable spermatozoa.

The differences in those treatments that contain seminal plasma and those where seminal plasma was removed may be due to processing of the ejaculate. In those treatments where seminal plasma was removed, CLC was added after centrifugation. The addition of CLC after centrifugation may not be as effective as loading cholesterol prior to centrifugation, like the procedures described for cryopreserved samples. Other researchers also used a variety of procedures to incorporate CLC. Therefore, future research for optimizing spermatozoa recovery rates due to CLC incorporation and the influences of seminal plasma, centrifugation, and extender may be beneficial to developing new procedures for preservation of semen.

Addition of MβCD did not alter the percentages of LI or LNI when compared to non-treated spermatozoa after 24 and 48 h of cold storage in the absence of seminal plasma (table 8). These results indicate that addition of MβCD to spermatozoa treated with CLC does not improve or damage the membrane integrity from the unloading of cholesterol.

It can be concluded from the results that stabilizing the spermatozoa membrane with cholesterol through the use of CLC is beneficial to the membrane integrity of cold stored spermatozoa. According to Purdy and Graham (2004b), the improvement in membrane integrity is due to cholesterol being evenly distributed throughout the population and loaded throughout the individual spermatozoa.

Table 9. Influence of cholesterol-loaded cyclodextrin (CLC) and cyclodextrin (MβCD) on membrane integrity of spermatozoa determined by flow cytometry following cryopreservation.

Method of Preservation		Treatment		
		Control*	**[+ CLC - MβCD]**	**[+ CLC + MβCD]**
Cryopreservation	LI	20±4[a]	30±4[b]	29±4[b]
	LNI	26±3[a]	26±3[a]	31±3[a]

[ab] Means in a row with different superscripts are significantly different ($P < 0.05$).
* Control refers to non-cholesterol loaded spermatozoa diluted in extender. All others had cholesterol loaded prior to MβCD treatment.

When the influences of CLC and MβCD are analyzed for spermatozoa that have undergone the freeze thaw cycle, percent LI were elevated ($P < 0.05$) in CLC treated samples when compared to non-treated cryopreserved spermatozoa. Removal of cholesterol, through the use of MβCD, did not affect the percent LI; however, there were no differences between treatments in percent LNI post thaw (table 9). These results indicate that loading cholesterol into the spermatozoa membrane is beneficial to preservation of membrane integrity in post thaw spermatozoa, which correspond with results presented by Combes et al. (2000). However, Zahn et al. (2002) indicated that the acrosomal region of CLC treated post thaw cryopreserved spermatozoa was inhibited due to decapacitation by cholesterol. In the present paper, there were no differences in the integrity of the acrosomal membrane due to there being no difference in the percent LNI.

Purdy and Graham (2004b) determined that the acrosome reaction absorbed large amounts of cholesterol that resulted in a greater percent of spermatozoa with intact membranes. The addition of cholesterol at the acrosome region is of concern due to the possibility of inhibiting capacitation since the degree of membrane order becomes

increasingly more stable thus preventing the outer acrosomal and plasma membrane from fusing (Nimmo and Cross, 2003). Inability of the membranes to fuse would inhibit the acrosome reaction (Davis, 1978), which would support the observations from Zahn et al. (2002). Therefore, we attempted to overcome this disability by the addition of MβCD to unload the excess cholesterol.

Capacitation was inhibited in CLC treated fresh bovine spermatozoa upon challenge by PC12, when compared to the control (Purdy and Graham, 2004a). However, after thawing of the cryopreserved spermatozoa, post thaw maturation and fertility were similar when compared to non-treated spermatozoa (Purdy and Graham, 2004a). Purdy and Graham (2004a) attributed the difference between CLC loaded fresh spermatozoa, where the acrosome reaction was inhibited, and CLC loaded post thaw cryopreserved spermatozoa, where the acrosome reaction was not inhibited, to the loss of cholesterol during the freeze-thaw procedure. When cryopreserved spermatozoa are thawed, cholesterol is lost in the process resulting in higher numbers of capacitated spermatozoa, which is one of the possibilities as to why cryopreserved spermatozoa do not survive as long as fresh or cooled spermatozoa (Pommer et al., 2003). Therefore, addition of CLC inhibits the acrosome reaction of freshly ejaculated spermatozoa but not those that have undergone stressors such as cryopreservation.

However, in the stallion, Zahn et al. (2002) indicated that cholesterol incorporation resulted in lower percentages of acrosome reactions and reduced fertility when compared to non-treated cryopreserved spermatozoa after 90 min. In contrast to Zahn et al. (2002), the results of the present study did not detect a difference in percent

LNI when CLC-treated spermatozoa were compared with non-treated cold-stored and cryopreserved spermatozoa after 60 min. However, this would indicate that the acrosomal region was not inhibited by cholesterol loading into the membrane. The difference between these studies may be due to variation in stallion response. In the present study, PMS of post thaw CLC loaded spermatozoa was improved whereas Zahn et al. (2002) did detect a difference.

The discrepancy between acrosomal status of Zahn et al. (2002) and the present paper, may be due to procedural differences; such as the technique of loading cholesterol into cyclodextrin, or variation in the procedures to add CLC to spermatozoa. For example, in the present study, CLC was added to a skim milk extender that was immediately incorporated into the spermatozoa samples and incubated for 15 min at 37 ˚C whereas Zahn et al. (2002) first diluted the ejaculate followed by centrifugation and then resuspended the pellet with a CLC solution in 50% Kenney and 50% Ringer with sodium lactate at 24 °C and centrifuged the sample a second time before adding a specially formulated freezing extender. This difference in procedures may be significant since Combes et al. (2000) indicated a difference in spermatozoa parameters when different procedures were used to load cholesterol in the membrane. Although the percent motile spermatozoa was improved when incubated in a skim milk extender containing CLC, more cholesterol was incorporated into the spermatozoa membrane when incubated with TALP containing CLC (Combes et al., 2000). Combes et al. (2000) theorized that the difference between CLC treatment procedures was due to competitive interference with lipids in the skim milk extender. Therefore, the procedure and

conditions utilized to incorporate CLC may affect the amount of cholesterol incorporated into the spermatozoa membrane.

The initial theory was that cholesterol loading inhibits capacitation, thus preventing the acrosome reaction. Consequently, it was hypothesized that the excess cholesterol could be subsequently unloaded through the use of a MβCD solution. Capacitation can be enhanced when MβCD is added to stallion spermatozoa, based on the concentration of MβCD (Pommer et al., 2003) that had not been previously loaded with cholesterol. The purpose of MβCD addition to CLC treated spermatozoa was not to induce capacitation but to remove the excess cholesterol that could inhibit fertilization. Addition of MβCD solution did not alter PMS or membrane integrity.

Overall, loading cholesterol into the membrane using CLC is beneficial in maintaining the quality of spermatozoa following preservation. Percentages of both progressively motile and membrane intact spermatozoa were improved following cold storage and cryopreservation when compared to non-treated spermatozoa. This corresponds to observations from Kirk et al. (2001), who observed improved total and progressively motile spermatozoa when preserved by cold storage, and Combes et al. (2000), who observed improved percentages of progressively motile and membrane intact spermatozoa following the freeze thaw cycle. Status of the acrosomal region does not appear to be affected by CLC incorporation; however, in those stallions where decapacitation from the addition of CLC influences the potential of per cycle pregnancy rates, such as those observed by Zahn et al. (2002), unloading of excess cholesterol through the use of MβCD may be beneficial.

IMPLICATIONS

The addition of cholesterol loaded cyclodextrin was beneficial in improving the percentages of progressively motile and live intact spermatozoa following 24 and 48 h cold storage and post thaw. In addition, the use of a MβCD solution to unload cholesterol did not alter the semen evaluation parameters, which indicates that unloading of the cholesterol incorporated through the use of cyclodextrin is not detrimental to the spermatozoa. Future research is needed to fully investigate the influence of cholesterol removal following short and long-term preservation. This includes substantiation of cholesterol removal and the influence of different preservation media on MβCD efficacy. The influences of cholesterol loading of the spermatozoa membrane and the subsequent unloading of cholesterol on fertility are unknown. Cholesterol loaded cyclodextrin is a beneficial constituent for a semen extender intended for those stallions who are considered poor coolers or have poor post preservation quality inseminates.

WORKS CITED

Aurich, J. E., A. Kuhne, H. Hoppe, and C. Aurich. 1996. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. Theriogenology. 46:791-797.

Awad, M. M., and J. K. Graham. 2002. Effect of adding cholesterol to bovine spermatozoa on motility parameters and cell viability after cryopreservation. Cryobiology. 45: 256-257.

Berg, J. M., J. L. Tymoczko, and L. Stryer. 2002. Lipids and cell membranes. Page 319-342 in Biochemistry 2001. 5th edition. W. H. Freeman and Company. New York.

Blottner, S., C. Warnke, A. Tuchscherer, V. Heinen, and H. Torner. 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim. Reprod. Sci. 65:75-88.

Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. Science (WashDC). 232:34-47.

Buhr, M. M., E. F. Curtis, and N. Somnapan Kakuda. 1994. Composition and behavior of head membrane lipids of fresh and cryopreserved boar sperm. Cryobiology. 31:224-238.

Choi, Y. H., and Y. Toyoda. 1998. Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. Biol. Reprod. 59:1328-1333.

Christian, A. E., M. P. Haynes, M. C. Phillips, and G. H. Rothblat. 1997. Use of cyclodextrins for manipulating cellular cholesterol content. J. Lipid Res. 38:2264-2272.

Combes, G. B., D. D. Varner, F. Schroeder, R. C. Burghardt, and T. L. Blanchard. 2000. Effect of cholesterol on the motility and plasma membrane integrity of frozen equine spermatozoa after thawing. J. Reprod. Fertil. 56(Suppl.):127-132.

Cross, N. L. 1999. Effect of methyl β-cyclodextrin on the acrosomal responsiveness of human sperm. Mol. Reprod. Dev. 53:92-98.

Cross, N. L. 1998. Role of cholesterol in sperm capacitation. Biol. Reprod. 59:7-11.

Darin-Bennett, A. B., and I. G. White. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology. 14:466-470.

Davis, B. K. 1978. Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. Symposium on the pharmacological effect of lipids. Champaign, IL. The American Oil Chemists Society. 145-157.

Dawson, G. R., G. W. Webb, J. A. Pruitt, T. M. Loughin, and M. J. Arns. 2000. Effect of different processing techniques on motility and acrosomal integrity of cold-stored stallion spermatozoa. J. Equine Vet. Sci. 20:191-194.

De Leeuw, F. E., H. C. Chen, B. Colenbrander, and A. J. Verdleij. 1990. Cold-induced ultrastuctureal changes in bull and boar sperm plasma membranes. Cryobiology. 27:171-183.

Flesch, F. M., and Gadella, B. M. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim. Biophys. Acta. 1469:197-235.

Foote, R. H. 2002. The history of artificial insemination: selected notes and notables. J. Anim. Sci. 80(E Suppl 2): E1-E10.

Gadella, B. M., R. Rathi, J. F. H. M. Brouwers, T. A. E. Stout, and B. Colenbrander, B. 2001. Capacitation and the acrosome reaction in equine sperm. Anim. Reprod. Sci. 68:249-265.

Garner, D. L., and E. S. E. Hafez. 2000. Spermatozoa and seminal plasma. Page 96-109 in Reproduction in Farm Animals. 7th edition. Lippincott Williams and William, Philadelphia, PA.

Graham, J. K. 1996a. Analysis of stallion semen and its relation to fertility. Vet. Clin. North Am., Equine Pract. 12:119-130.

Graham, J. K. 1996b Cryopreservation of stallion spermatozoa. Vet. Clin. North Am., Equine Pract. 12:131-147.

Hartel, S., H. A. Diehl, and F. Ojeda. 1998. Methyl- β-cyclodextrins and liposomes as water-soluble carriers for cholesterol incorporation into membranes and its evaluation by a microenzymatic fluorescence assay and membrane fluidity-sensitive dyes. Anal. Biochem. 258:277-284.

Ivanoff, E. I. 1922. On the use of artificial insemination for zootechnical purposes in Russia. J. Agric. Sci. 12:244-256.

Kirk, E. S., J. K. Graham, and E. L. Squires. 2001. Increasing membrane cholesterol content benefits the motility of cooled equine semen. Anim. Reprod. Sci. 68:317-318.

Klein, U., G. Gimpl, F. Fahrenholz. 1995. Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry. 34:13784-13793.

Ladha, S. 1998. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian Spermatozoon. J. Membr. Bio. 165:1-10.

Magistrini, M. 2000. Semen Evaluation. Pages 91-108 in Equine Breeding Management and Artificial Insemination. W.B. Saunders Company. Philadelphia, PA. Samper, J. C. ed.

Nimmo, M. R., and N. L. Cross. 2003. Structural features of sterols required to inhibit human sperm capacitation. Biol. Reprod. 68:1308-1317.

Neild, D. M., B. M. Gadella, M. G. Chaves, M. H. Miragaya, B. Colenbrander, A. Aguero. 2003. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. Theriogenology. 59:1693-1705.

Nutrient Data Laboratory. 2005. USDA National Nutrient Database for Standard Reference, Release 17. Available: www.ndl.usda.gov/fnic/foodcomp. Accessed March 10, 2005.

Ohvo, H., and J. P. Slotte. 1996. Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. Biochemistry. 35:8018-8024.

Parinaud, J., and G. Vietez, C. Vieu, X. Collet, and B. Perret. 2000. Enhancement of zona binding using 2-hydroxypropyl-β-cyclodextrin. Hum. Reprod (Oxf.) 15:1117-1120.

Parks, J. E., and E. Ehrenwald. 1989. Cholesterol efflux from mammalian sperm and its potential role in capacitation. Proceedings of the international symposia on fertilization in mammals sponsored by Serono Symposia, Newton, MA. 155-167.

Parks, J. E., and D. V. Lynch. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 29:255-266.

Parks, J. E., T. N. Meacham, and R. G. Saacke. 1981. Cholesterol and phospholipids of bovine spermatozoa. I. Selection of a PIPES-buffered diluent for evaluating the effect of egg yolk lipoproteins on sperm cholesterol and phospholipids. Biol. Reprod. 24:393-398.

Polge, C., A. U. Simth, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature (Lond.) 164:666.

Pommer, A. C., J. Rutllant, and S. A. Meyers. 2003. Phosphorylation of protein tyrosine residues in fresh and cryopreserved stallion spermatozoa under capacitating conditions. Biol. Reprod. 68:1208-1214.

Purdy, P. H., M. H. Fox, C. U. Lim, and J. K. Graham. 2002. Flow cytometric assessment of fluctuations in the membrane fluidity and viability of Chinese hamster ovary (CHO) and bull sperm cells using the fluorescent stains TMAP-DHP and propidium iodide. Cryobiology. 45:240-241.

Purdy, P. H., and J. K. Graham. 2004a. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. Biol. Reprod. 71:522-527.

Purdy, P. H., and J. K. Graham. 2004b. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology. 48:36-45.

R Development Core Team. 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.

Rigby, S. L., S. P. Brinsko, M. Cochran, T. L. Blanchard, C. C. Love, and D. D. Varner. 2001. Advances in cooled semen technologies: seminal plasma and semen extender. Anim. Reprod. Sci. 68:171-180.

Suzuki, F., and R. Yanagimachi. 1989. Changes in the distribution of intramembranous particles and filipin-reactive membrane sterols during *In Vitro* capacitation of golden hamster spermatozoa. Gamete Research. 23:335-347.

Vidament, M., V. Prigent, F. Queva, V. Douard, and E. Blesbois. 2001. Cyclodextrin alone or in complex with cholesterol modulates cholesterol content and freezability of equine spermatozoa. Anim. Reprod. Sci. 68:325-326.

Visconti, P. E., H. Galantino-Homer, X. P. Ning, G. D. Moore, J. P. Valenzuela, C. J. Jorgez, J. G. Alvarez, and G. S. Kopf. 1999. Cholesterol efflux-mediated signal transduction in mammalian sperm. J. Biol. Chem. 274:3235-3242.

Watson, P. F. The causes of reduced fertility with cryopreserved semen. 2000. Anim. Reprod. Sci. 60-61:481-492.

Webb, G. W., and M. J. Arns. 1995. Influence of modified tyrode's media on motility of cold-stored stallion spermatozoa. J. Equine Vet. Sci. 15:441-444.

Wilhelm, K. M., J. K. Graham, and E. L. Squires. 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analysis, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology. 46:559-578.

Yancey, P. G., W. V. Rodriguez, P. C. Kilsdonk, G. W. Stoudt, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1996. Cellular cholesterol efflux mediated by cyclodextrins. J. Biol. Chem. 271:16026-16034.

Zahn, F. S., F. O. Papa, and J. A. Dell'Aqua. 2002. Cholesterol incorporation on equine sperm membrane: effects on post-thaw sperm parameters and fertility. Theriogenology. 58:237-240.