EFFECTS OF CHOLESTEROL ON BOVINE SPERM SURVIVAL DURING COOLING & FREEZING

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ABSTRACT

Damage caused to sperm due to cooling and freezing has long limited the potential of cryopreserved semen. Considering that much of this damage is attributed to injury to the plasma membrane of the sperm cell, methods that stabilize the membrane would greatly improve the prospects of cryopreservation. One such stabilizing factor is cholesterol, which decreases the phase transition of membrane lipids that occurs during the cooling and freezing process. One known method of membrane cholesterol enrichment employs cholesterol loaded cyclodextrin (CD/CHOL). The purpose of this study was to confirm current CD/CHOL results in the bull as well as to examine an alternative method of cholesterol enrichment that utilizes high cholesterol egg yolk. For the cyclodextrin studies, fresh bovine ejaculates were incubated with or without CD/CHOL and cooled and frozen in various concentrations and preparations of egg yolk. Percent motility of the sperm was examined prior to freezing, after cold shock treatment, 0 and 3 hrs after cooling, and post-thaw by phase contrast microscopy. Percent viability was evaluated post-thaw by propidium iodide (PI) and SYBR 14 staining using the LIVE/DEAD Sperm Viability Kit protocol.

For the study using high cholesterol yolk, domestic hens were fed one of three diets supplemented with 0.5%, 1.0%, or no cholesterol. Eggs from these hens were used to examine the effects of cooling and freezing with various preparations and levels of yolk. Just as in the CD/CHOL study, fresh ejaculates were extended in their respective treatment, cooled, frozen, and evaluated for percent motility and viability. No treatments yielded significant effects of CD/CHOL incubation on percent sperm motility or viability while studies using high cholesterol yolks demonstrated improved

motility with both the 0.5% and 1.0% diet. The results of this study indicate that treatment with CD/CHOL does not affect motility or viability, while treatment with high cholesterol yolk does.

BIOGRAPHICAL SKETCH

Kathryn Bach was born in Rockville, Connecticut on March 10, 1982. She graduated from the Loomis Chaffee School in Windsor, Connecticut in 2000 and obtained her B.S. in Animal Science cum laude from Cornell University in 2004. She began work on her Masters Degree in Animal Science at Cornell University in the fall of 2004.

for my parents

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AI	artificial insemination
CD/CHOL	cholesterol loaded cyclodextrin
CHOL	cholesterol
CPA	cryoprotective agent
EYT	egg yolk Tris
HEPES	N-2 hydroxyethylpiperazine-N-2-ethane acid
HDL	high density lipoproteins
LDL	low density lipoproteins
TALP	modified Tyrodes solution
Tris	hydroxylmethylaminomethane
MBCD	methyl-β-cyclodextrin
PI	propidium iodide

Chapter 1: Introduction

For decades there has been a major goal in the dairy industry for genetic improvement of dairy cattle. The potential for this advancement has been greatly enhanced by the development of artificial insemination (AI) and semen cryopreservation. With these two techniques combined, a genetically superior bull can inseminate exponentially more females on far more farms than would otherwise be possible during its reproductive lifetime, with greatly decreased risk of disease or injury. There are still some obstacles to overcome to bring these techniques to their full potential, especially with regard to sperm cryopreservation.

Successful semen cryopreservation has been achieved for many species over the years, though not without substantial reduction in the viability of the sperm [1]. Approximately 50% of the sperm do not survive the freezing process compared to sperm in fresh ejaculates and sperm that do survive often suffer some sort of impairment [2-4]. This decrease in the number of viable sperm is generally associated with biophysical effects to sperm membranes encountered during cooling and freezing semen. Improved methods for overcoming low temperature damage will help to optimize sperm cryosurvival.

Mammalian Sperm

Mammalian sperm are terminally differentiated haploid cells that consist of a head, a midpiece, and a tail (Figure 1.1) [5]. Within the head of the sperm lies the highly condensed nucleus which is surrounded, in the apical region, by the Golgi derived acrosome [6]. This acrosome is a vesicle that forms a cap-like structure over the sperm head. The inward facing membrane of the vesicle overlies the nuclear membrane, while the outer membrane lies directly beneath the plasma membrane [7].

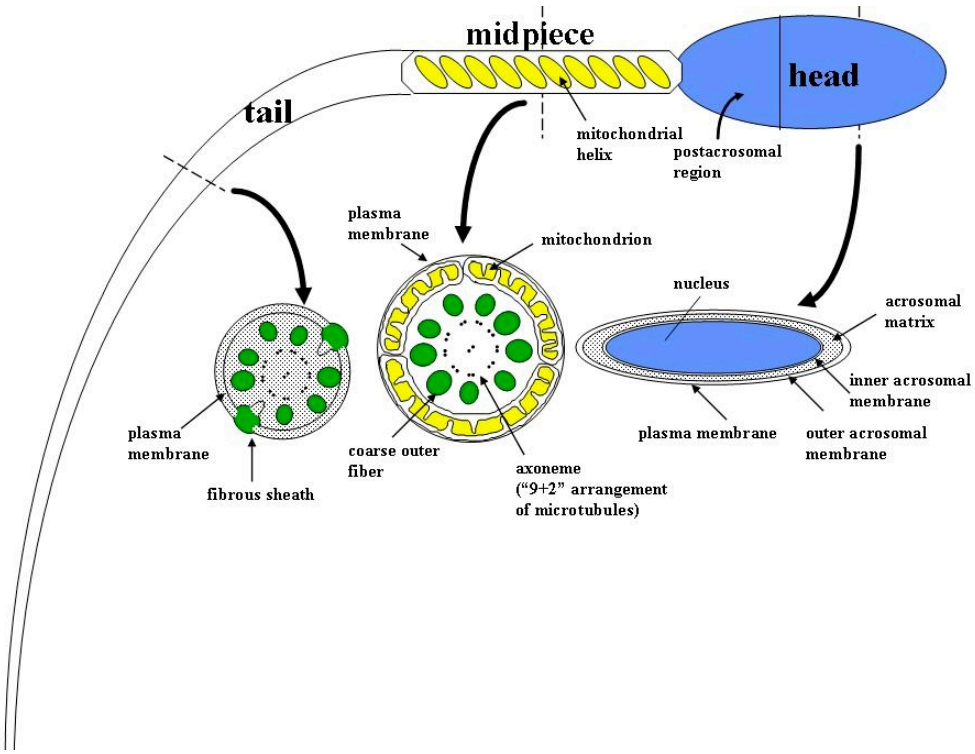

Figure 1.1. Structure of the bovine sperm (from Parks, 1997). Used with permission.

The acrosome contains hydrolytic enzymes that are released by exocytosis during binding to and penetration of the zona pellucida of an ovulated oocyte.

The midpiece of the sperm contains mitochondria that are organized end-to-end in a helical arrangement. The midpiece also contains the axoneme which consists of a 9+2 arrangement of microtubles surrounded by nine coarse outer fibers. The axoneme and the coarse outer fibers extend past the midpiece and also into the principal piece, where they are surrounded by a fibrous sheath [8]. These structures contribute to the characteristic "whiplash" motion of the flagellum [7]. Because sperm are extremely compact with a condensed nucleus, reduced organelles and cytoplasm, it is important to note that virtually no transcription or biosynthesis occurs within the ejaculated cell [9].

The function of the mammalian sperm is to fuse with an oocyte in order to form a zygote. To facilitate fusion, however, sperm must undergo a series of processes. The first of these is capacitation, which occurs after ejaculation within the female tract. Capacitation is thought of as a series of complex modifications that the sperm must undergo in order for it to fertilize an egg.

An apparent key early event of capacitation is cholesterol efflux. This event seems to be mediated by sterol-binding proteins such as high density lipoproteins (HDL) [10]. Cholesterol efflux is important because it affects the fluidity and ionic permeability of the sperm membrane. Consequences of these changes include an increase in intracellular pH, Ca^{2+}, and HCO_3^- [11, 12]. The increased Ca^{2+} and HCO_3^- have a marked effect on signal transduction within the sperm activating soluble adenylyl cyclase signaling pathways. These pathways in turn increase activity of tyrosine kinases and therefore tyrosine phophorylation [13-17] . It is believed that some of these consequences of cholesterol efflux predispose the sperm to zona binding and induction of the acrosome reaction [18, 19].

One result of these biochemical changes in the sperm is a mechanical change. During capacitation sperm acquire hyperactive motility. This is triggered by an increase in flagellar Ca^{2+} [20]. Hyperactivity is marked by a shift from movement in a linear path caused by symmetrical flagellar beating to movement in a non-linear path caused by asymmetrical flagellar beating. This change in motility aids movement of sperm through the female reproductive tract and penetration of the oocyte zona pellucida, thus allowing for fertilization to take place [21, 22].

The next step that occurs in the sequence of events leading to fertilization is the acrosome reaction. The main purpose of the acrosome reaction is to allow the sperm to penetrate the zona pellucida and to eventually fuse with the plasma membrane of the oocyte.

During the acrosome reaction, a continued increase in pH and Ca^{2+} can be observed as well as an orderly fusion of the spermatozoal plasma membrane and the outer acrosomal membrane. This fusion releases hydrolytic enzymes that allow the sperm to penetrate the zona pellucida through both chemical and mechanical means. With the completion of the acrosome reaction and penetration of the zona, the equatorial region on the sperm head fuses with the plasma membrane of the ooctye. Once the sperm and oocyte membranes have fused, egg activation, pronuclear formation, and syngamy occur resulting in embryo formation [18].

Sperm Membranes

The biological membranes of the sperm, as with most biological membranes, are believed to conform to the "fluid mosaic model" proposed by Singer and Nicholson in 1972 [23]. This model states that biological membranes are composed of an asymmetric bilayer of polar phospholipids and glycolipids containing integral proteins [23]. Phospholipids in the bilayer include ethanolamine and choline

phosphoglycerides, lysophosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylinositol [24].

Asymmetry of the phospholipid bilayer must be maintained for proper membrane function. Lateral diffusion of phospholipids is continuously occurring within the plane of the bilayer and various ATP dependent enzymes, including flippases and scramblases, are employed to ensure proper membrane organization. The main function of flippase is to keep phosphatidylserine sequestered from the cell surface. The action of scramblase is much less specific and functions more to randomly distribute newly synthesized lipids [25].

The majority of these membrane phospholipids create fluid bilayers spontaneously when placed into aqueous solution. This is, however, not true for all phospholipids. There are some lipids, known as hexagonal II preferring lipids that, when allowed to aggregate, will form arrays within the membrane. In normal physiological conditions this does not become a problem. This is mainly due to the fact that these hexagonal II preferring lipids are constrained to a bilayer formation by their association with other lipids or proteins [26, 27]. Specific distributions of phospholipids throughout the membrane is important because phospholipids membrane fusion events in the sperm [28].

In addition to the various types of phospholipids, biological membranes also contain cholesterol, which is a sterol composed of four rigid sterol rings, a hydrophobic hydrocarbon tail and a polar beta-hydroxyl head group. Cholesterol has a unique effect on the membrane, because the sterol rings of cholesterol interact with the hydrocarbon tails of the phospholipids imparting order and constraining motion of the phospholipids in the membrane (Figure 1.2) [5]. This order reduces membrane permeability and fluidity [29]. It has been shown that reduction in membrane fluidity

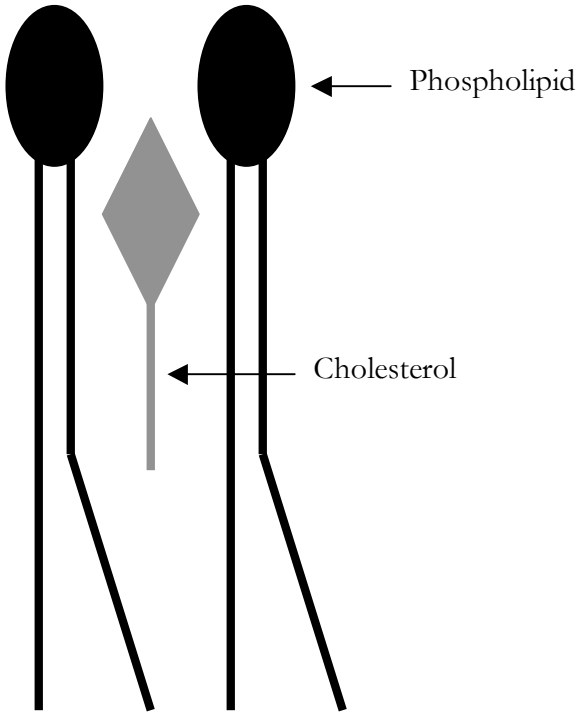

Figure 1.2. Interaction of cholesterol within the plasma membrane (from Parks, 1997). Used with permission.

caused by cholesterol actually reduces the transition temperature of membranes. This, in effect, allows the membrane to stay at a fluid state at lower temperatures [30].

Effects of Cooling and Freezing on Sperm

Cooling and freezing have many effects on sperm. One of these effects is caused by freezing of intracellular water. After cooling has been achieved, there are various rates at which sperm can be frozen. If sperm are frozen too slowly, the extracellular water freezes before the intracellular water creating an osmotic effect that results in dehydration of the cell. If sperm are frozen too rapidly, the intracellular water freezes rapidly, creating a lethal event known as intracellular ice formation. Fortunately, water damage can be reduced by finding an optimal cooling rate for the cell as well as by using various cryoprotective agents (CPAs) [31, 32]. The earliest of these CPAs discovered was glycerol in 1949 [33]. The permeating effects of glycerol reduce cell damage by reducing the amount of intracellular ice formed [34].

Another effect of cooling and freezing on the sperm cell is known as cold shock. Cold shock was originally discovered by Milovanov in 1934 when he noted sperm's sensitivity to hypothermia [35]. Later work by Watson showed more specific reasons for this sensitivity. Extensive damage to the plasma membrane was noted, leading to disruption of the acrosome and premature loss of the acrosomal contents as well as effects on mitochondrial arrangement in the midpiece [36, 37]. Cold shock has also been shown to alter membrane permeability leading to the loss of numerous intracellular and membrane components and decreased ability of the sperm to generate ATP [36, 38, 39].

Many of the effects of cold shock can be attributed to lateral phase separation (Figure 1.3) [5]. This phenomenon involves the non-bilayer preferring lipids that are normally dispersed throughout the lipid bilayer. During cooling, these lipids undergo

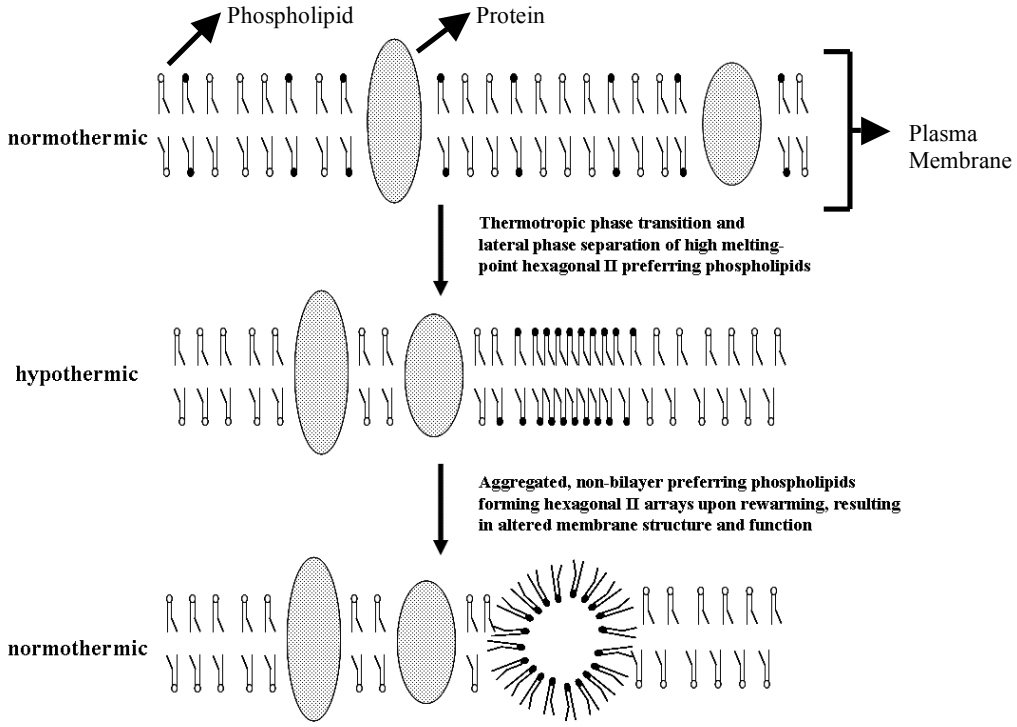

Figure 1.3. Phase separation model for biological membranes. White phospholipid head groups indicate bilayer preferring phospholipids and black phospholipids head groups indicated hexagonal II (non-bilayer preferring) phospholipids (from Parks, 1997). Used with permission.

thermotropic phase transitions (phase changes from normothermic liquid-crystalline state to a hypothermic gel state) at higher transition temperatures than bilayer preferring phospholipids. During this process the bilayer preferring lipids are still in the liquid-crystalline state allowing the non-bilayer preferring lipids to become sequestered in domains within the membrane. Because the non-bilayer preferring lipids have diffused laterally to create these domains, there are altered lipid-lipid and lipid-protein interactions as a result of their movement. In addition to interrupting interaction, these domains are free to form non-bilayer configurations within the membrane when cells are rewarmed which in turn alters membrane permeability and stability [26, 27].

Sperm from difference species vary in their susceptibility to cold shock. The boar is one of the most sensitive species, while the bull, ram, and stallion are slightly less sensitive. The least susceptible species are man and the rooster [36, 38]. This susceptibility is related to membrane lipid composition, especially the cholesterol to phospholipid molar ratio. The greater this ratio is, the less likely the sperm will be vulnerable to cold shock [2]. Considering the increased stability and constrained lateral movement effects cholesterol has on the membrane, the effect of the cholesterol to phospholipid ratio is consistent with protection during cooling.

Media for Freezing Sperm

In order to minimize the detrimental effects of cooling and freezing, much research has been done to develop media to improve the post thaw viability of sperm. In 1940, Phillips and Lardy discovered that egg yolk has a protective effect [40]. Since that time, the use of egg yolk has been widespread for many species and various improvements have been made on Phillips and Lardy's original phosphate buffered egg yolk media [41-44]. Because there was still room for improvement, further

research was done to examine which components of egg yolk actually have the protective effect. It was discovered that the low density lipoproteins contained in egg yolk, especially the phospholipid phosphatidylcholine, were responsible for beneficial effects [45-47].

Another component of egg yolk that seems to have beneficial effects on sperm during cooling and freezing is cholesterol. As stated earlier, cholesterol constrains phospholipid motion and reduces thermotropic phase transition temperature and membrane permeability [29, 30]. Reduction in membrane transition temperature is one of cholesterol's most important properties with respect to membrane resistance to cold shock. Studies with liposomes showed that when cholesterol was added to the system, phase transition temperature decreased, and was even eliminated if sufficient amounts of cholesterol were incorporated [48, 49]. Thus, if sperm membranes could be enriched with cholesterol, the effects of phase transition might be almost, if not totally, eliminated.

Modification of cholesterol content in various cell membranes using methyl-β-cyclodextrin has been reported, including in sperm [50]. This cyclic oligosaccharide consists of seven β glucopyranose units that form a ring-shaped structure (Figure 1.4) [51]. The external portion of the ring is hydrophilic and the internal core is hydrophobic [52]. This unique conformation allows cyclodextrin to be water-soluble and also to solubilize non-polar, hydrophobic chemicals like cholesterol, by allowing them to reside within its hydrophobic core [53].

In order to see if this compound could be used to not only remove, but also to enrich sperm membranes with cholesterol, cyclodextrin has been pre-loaded with cholesterol and used in extenders for both equine and bovine sperm [50, 54]. In the research done by Purdy and Graham on bovine sperm, increased cholesterol content

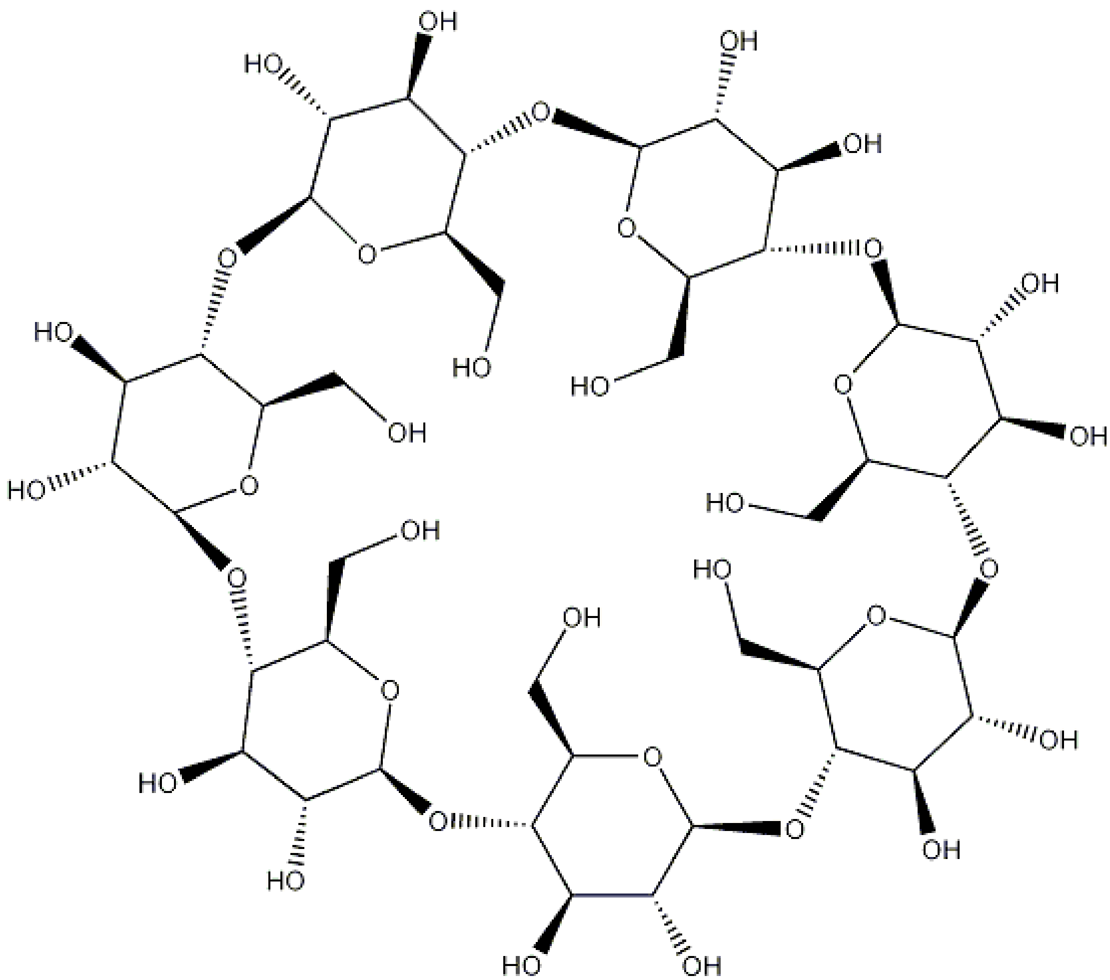

Figure 1.4. Molecular structure of β cyclodextrin (from Holden, 2006).

and improved cryosurvival were reported. This improved cryosurvival was also shown in more recent work from Graham's lab on equine sperm [55].

Oddly, though, other research done on boar sperm by Zeng and Terada reported that exposure to cyclodextrin, without pre-loading it with cholesterol, improved cryosurvival. These studies also showed that when cholesterol was added along with the cyclodextrin, the beneficial effects of cyclodextrin were abolished, thus indicating that cyclodextrin alone was creating the improved viability [56, 57]. However, these findings have been recently contradicted by another study done with boar sperm by Galantino-Homer *et al.* whose results were similar to the initial work done in the equine and bovine. Galantino-Homer *et al.* hypothesized that in the previous studies by Zeng, the cyclodextrin may have been acting as a shuttle to move the cholesterol from the egg yolk extender to the sperm membrane, therefore indirectly enriching the membrane [58].

Conclusion

Even though it appears that cholesterol enrichment via cyclodextrin improves the viability of post-thaw semen, some problems remain with the concept. A recent publication by Graham (2006) reports mixed reviews regarding the fertility of sperm frozen with CD/CHOL [59]. He hypothesizes that this is due to the fact that CD/CHOL treated sperm need more time to capacitate due to the increase levels of cholesterol that must efflux from the membranes. There also may be other cytotoxic effects of the cyclodextrin causing low fertility rates. Regardless, increasing cholesterol in the membrane does appear to be beneficial to sperm during cooling and freezing. Finding another method to increase membrane cholesterol would be beneficial.

Because there is already a cholesterol source in conventional sperm extenders made with egg yolk, one way to enrich the membrane that might be successful is by altering the cholesterol content of the yolk itself. According to a study by Naber *et al.* in 1985, if supplemental cholesterol was added to conventional hen diets, the cholesterol content of the egg yolk was significantly increased [60]. This yolk could then be used at the same levels as conventional cryopreservation protocols but yield higher percentages of cholesterol. This higher percentage would help create a positive concentration gradient that would allow for cholesterol to leave the media and enter the sperm plasma membrane. If yolk enrichment could be found effective this would open up an easy and effective means of enriching sperm membranes.

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Chapter 2: Effects of Cholesterol Loaded Cyclodextrin on Cooling and Freezing of Bovine Sperm

Abstract

Very little research has been reported on the effects of cholesterol loaded cyclodextrin (CD/CHOL) on bull sperm, though published research indicates that CD/CHOL has a positive effect on sperm cryosurival. The main purpose of this study was to confirm the positive effects and determine whether or not CD/CHOL could result in increase post-thaw motility and viability.

Semen was diluted to a concentration of 120 x 10^6 sperm/ mL in 10% egg yolk Tris (EYT) with (control) or without pre-incubation in Tris buffer containing 2.0 mg/mL CD/CHOL for treated samples. Samples were incubated for 15 min before further dilution 1:1 (v/v) with either 10% EYT (control) or 20% EYT for treated samples (CD/CHOL) pre-incubated without yolk. Prior to cooling, 200 µl aliquots of the diluted semen were removed for a 30 min cold shock treatment in a 0°C water bath. The remainder was cooled, frozen, and stored until evaluation. Motility was assessed prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability was assessed after freezing.

No effect of CD/CHOL was observed for pre-freeze or post-thaw motility or post-thaw viability. However, samples treated with CD/CHOL did not differ from control samples when subjected to cold shock treatment prior to freezing, indicating some protective effect. These results indicate that CD/CHOL was not effective in providing protection during cooling and freezing and therefore do not confirm previous reports in the bull.

Introduction

Great genetic advancements have been achieved in the dairy industry from the extensive use of AI in combination with cryopreserved semen from superior sires. However, the substantial damage that occurs to sperm cells as a result of the processing required for cryopreservation has limited the potential of these technologies. Approximate 50% of sperm cells do not survive the freezing process and among those that do survive, damage to the sperm is often detected, resulting in reduced fertility [1].

One of the major sites of this damage is centered on the sperm plasma membrane. During cooling and freezing, the phospholipids of the sperm membrane have been shown to undergo lipid phase transition, in which non-bilayer preferring (hexagonal II) lipids begin to gel and aggregate at higher temperatures than the bilayer preferring lipids. Upon thawing, this results in regions of non-bilayer conformations within the membrane that interrupt lipid-lipid and lipid-protein interactions [2].

Sperm from all species are not affected by this phenomenon to the same extent. Boar sperm appear to be highly susceptible, while rooster and human sperm are much less vulnerable [3]. This lack of vulnerability has been correlated to the extremely high ratio of cholesterol to phospholipid within the sperm plasma membranes of these species. The high level of cholesterol is believed to increase stability of the membrane and to limit the lateral phase separation of phospholipids within the bilayer during cooling [4].

Considering that endogenous cholesterol levels in sperm membranes seem to have a significant influence on low temperature survival, research has been done to determine how the sperm plasma membrane could be enriched in cholesterol in order to decrease damage to the cell membrane. Studies with liposomes showed that by enriching sperm membranes with cholesterol, lipid phase transition could not only be

reduced, but also eliminated [5]. In order for sperm membranes to be effectively enriched with cholesterol, a technique was needed to allow water insoluble cholesterol to be solubilized in media for processing and freezing sperm.

In capacitation studies it was shown that cholesterol could be removed from cell membranes using the cyclic oligosaccharide methyl-β-cyclodextrin [6]. Cyclodextrin's unique structure allows cholesterol to insert itself into the hydrophobic core of the molecule, while the hydrophilic outer ring solubilizes anything contained within. If an excess of cyclodextrin molecules are introduced to semen, the cholesterol molecules contained within the sperm membrane leave the membrane and insert themselves into the cyclodextrin. Combes *et al.* discovered that stallion sperm treated with cyclodextrin preloaded with cholesterol caused the opposite effect and the cholesterol transferred from the cyclodextrin into the sperm plasma membrane [7].

Further research using cholesterol loaded cyclodextrin (CD/CHOL) for freezing bull [8] and ram [9] semen showed improved cryosurvival for both of these species. Because information on CD/CHOL usage with bull semen cryopreservation is limited, the main purpose of this study was to confirm whether CD/CHOL has a beneficial effect on sperm motility and viability post-thaw as compared to standard freezing protocols using 10% egg yolk extender.

Materials and Methods

Materials

Methyl-β-cyclodextrin (MBCD) (C4555), cholesterol (C8667), and all reagents used for media preparation were purchased from Sigma Chemical Company (St. Louis, MO). LIVE/DEAD Sperm Viability Kits were purchased from Molecular Probes Inc (Eugene, OR). VWR Special Select microscope slides (3 in x 1 in) and Corning No. 1 coverslips (18 mm x 18 mm) were purchased from VWR (Rochester,

NY). Corning 15 and 50 mL capped plastic conical tubes were purchased from Krackeler Scientific (Albany, NY). Capped, polypropylene microfuge tubes (1.5 mL) were purchased from LPS (Rochester, NY). French straws (0.5 mL) were purchased from IMV Technologies (L'Aigle, France).

Preparation of Cyclodextrin/Cholesterol (CD/CHOL) Complexes and Stock Solution

CD/CHOL complexes were prepared as described by Purdy and Graham [8] with the following modifications. Cholesterol (200 mg) was dissolved in 1.0 mL of redistilled chloroform in a 7 mL screw cap glass vial. In a separate glass vial, 1.0 g MBCD was dissolved in 2.0 mL of redistilled methanol. A 0.45 mL aliquot (90 mg) of the cholesterol solution was added to the methanol solution, mixed and allowed to warm for approximately 30 min at 37°C. Aliquots of 120 µL were placed in 2 mL screw cap glass vials and dried under a stream of nitrogen gas for approximately 1 h to apparent dryness. Vials were allowed to air dry for an additional 24 h before 0.8 mL of modified Tyrodes solution (TALP) [10] (92.9 mM NaCl, 3.1 mM KCl, 0.3 mM dibasic sodium phosphate, 0.4 mM magnesium chloride, 10 mM calcium chloride, 0.2 mM sodium pyruvate, 5.0 mM glucose, 25.6 mM lactic acid, 10 mM N-2 hydroxyethylpiperazine-N-2-ethane acid (HEPES), 24.8 mM sodium bicarbonate, 3 mg/mL BSA (fraction V, ≥ 96% essentially fatty acid free), 200 units/mL penicillin G, pH 7.4, 300 mOsm) was added. The vials were then placed on a 37°C slide warmer for 90 min, vortexed for 30 s and frozen at -20 °C until used.

Media Preparation

Semen was diluted in Tris (hydroxylmethylaminomethane) [11] (25.0 mmol Tris base, 8.90 mmol citric acid, 7.00 mmol glucose, 50000 IU penicillin G, and 50

mg streptomycin per 100 mL volume) containing 10% egg yolk (v/v) for control samples and diluted with Tris containing 2.0 mg/mL CD/CHOL for the treated samples. Treated samples were adjusted to 10% yolk prior to cooling and semen was frozen in 10% egg yolk Tris (EYT) with 14% glycerol added.

Semen Collection and Preparation

Fresh bovine semen was provided by Genex Cooperative, Inc. (Ithaca, NY) after collection by artificial vagina. All initial semen dilutions were performed at the Genex laboratory in 15 mL capped plastic conical tubes to a concentration of 120 x 10^6 sperm/mL in appropriate treatment media and performed within 1 h of collection. Each experiment was replicated on 14 ejaculates.

Semen Cooling and Freezing

Semen was cooled from 21°C to 4 °C at a constant rate over a period of 90 min during transport from the Genex facility to our laboratory. After cooling, samples were diluted with an equal volume (1:1, v/v) of either 2.5% or 10% egg yolk Tris (EYT) plus 14% glycerol added in 4 equal aliquots at 15 min intervals for 1 h and then allowed to equilibrate for an additional 1 h. Semen was then loaded into 0.5 mL french straws, placed 4 cm above liquid nitrogen in a 20 in x 10 in x 9 in Styrofoam box, and frozen for 15 min. Straws were then stored in liquid nitrogen until evaluated.

Evaluation of Sperm Motility

Sperm were evaluated subjectively using an Olympus CH-2 phase contrast microscope at 100x magnification with a heated stage (37°C). Each sample was warmed for 5 min in a 1.5 mL capped, polypropylene microfuge tube on a 37°C slide warmer prior to analysis in 6 μL aliquots on a slide with a coverslip. Percent

progressive motility was visually estimated in five fields per sample prior to cooling, after cold shock treatment, and following cooling (0 and 3 h). The five fields were then averaged to determine the progressive motility for the sample. For post-thaw samples, subjective motility was determined on 2 straws per sample and in 5 fields per sample after thawing in a 37°C water bath for 1 min. Estimates from the 5 fields were averaged to determine the progressive motility of each straw and an average of both straws was used to determine the progressive motility of the sample.

Evaluation of Sperm Viability

Sperm were evaluated by propidium iodide (PI) and SYBR 14 staining using the LIVE/DEAD Sperm Viability Kit protocol. Both stains detect DNA; PI permeates only dead sperm while SYBR 14 is also permeable to live sperm. Each sample was evaluated in duplicate following a 1 min thaw in a 37°C water bath. Stain was incubated with each sample for 5 min at 37°C prior to analysis. Measurements were done using a BD FACSAria (Bectin Dickenson) and analyzed using BD FACSDiVa software (San Jose, Ca). SYBR 14 fluorescence detector E (530/30nm BP filter) while PI was detected with detector B (695/40nm BP filter) with an LP filter of 502 nm and 635 nm before the BP for detectors E and B, respectively. Particles were gated so that only those positive for DNA fluorescence were counted.

Experimental Procedure

Semen was diluted in 10% EYT with (control) or without pre-incubation with 2.0 mg/mL CD/CHOL in Tris-buffered medium (treated). All samples were incubated for 15 min before further dilution 1:1 (v/v) with either the respective EYT for control samples or with 20% EYT for treated samples to achieve 10% egg yolk concentrations for both samples. Prior to cooling, 200 µl aliquots of the diluted semen were removed

for a 30 min cold shock in a 0°C water bath. The remainder of the diluted semen was cooled, frozen and stored until evaluation. Motility data were collected prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability data were collected after freezing.

Experimental Design

Percent motility and viability data were collected for both treatments and subjected to a one-way analysis of variance using Minitab Release 15 Statistical Software for Windows. Factors and interactions demonstrating significant differences were tested using Tukey's test for multiple comparisons.

Results

Table 2.1 shows a summary of the effect of CD/CHOL and cooling on mean bull sperm motility prior to freezing. Factors demonstrating significant differences are presented in table 2.2. For treatments prior to freezing, addition of CD/CHOL had no effect on sperm motility. There was a significant decrease in motility of cold shock treated aliquots for both control and CD/CHOL (48.8 ± 2.94%; $P < 0.05$) compared to the other three pre-freeze treatments (Table 2.2). Addition of CD/CHOL to extended semen had no effect on motility on samples cooled to 4°C upon rewarming to 37°C or following 3 hr of incubation at 37°C. Neither percent motility nor viability was statistically different between control and CD/CHOL treated samples post-thaw in this experiment ($P > 0.05$). Tables 2.3 and 2.4 summarize post-thaw results for motility and viability, respectively.

Table 2.1. Summary of effects of CD/CHOL and cooling on bull sperm motility prior to freezing (mean ± SEM). n = 14

Treatment	Motility
Control	
Precool	67.9 ± 2.9
Cold Shock	46.3 ± 4.0
Postcool (t=0 hr)	67.5 ± 2.8
Postcool (t=3 hr)	65.6 ± 4.0
CD/CHOL	
Precool	67.9 ± 4.4
Cold Shock	51.3 ± 4.4
Postcool (t=0 hr)	67.1 ± 2.8
Postcool (t=3 hr)	63.8 ± 3.2

Table 2.2. Effect of cooling on bull sperm motility prior to freezing (mean ± SEM). n = 14

Treatment	Motility
Precool	67.9 ± 2.0
Cold Shock	48.8 ± 2.9*
Postcool (t=0 hr)	67.3 ± 1.9
Postcool (t=3 hr)	64.9 ± 2.5

*P < 0.001

Table 2.3. Summary of effects of CD/CHOL on bull sperm motility post-thaw (mean ± SEM). n = 14

Treatment	Motility
Control	54.4 ± 3.0
CD/CHOL	52.9 ± 3.2

Table 2.4. Summary of effects of CD/CHOL on mean bull sperm viability post-thaw (mean ± SEM). n = 14

Treatment	Viability
Control	48.4 ± 3.2
CD/CHOL	50.4 ± 3.5

Discussion

Cooling and freezing has serious detrimental effects on sperm. Some of these effects are due to the consequences of thermotropic phase transitions of various membrane phospholipids between liquid crystalline and gel phases particularly on plasma membrane organization and stability [2]. High levels of cholesterol decrease these detrimental effects by creating stability and reducing lateral diffusion within the plasma membrane of the sperm [4]. Species whose membranes contain high cholesterol to phospholipids ratios, such as the rooster and human, appear to be extremely resistant to the effects of lipid phase transition, while species, such as the boar, which have low ratios, are extremely susceptible [3].

Research using liposomes as artificial membranes has shown that enriching membranes with cholesterol can decrease and even eliminate the effects of lipid phase transition [5]. Research on stallion, bull, and ram sperm membranes enriched with cholesterol using CD/CHOL also showed an increase in viability and motility, indicating that the added cholesterol improves the freezing tolerance of the cells [7-9, 12-14].

In this study, there was no significant effect of CD/CHOL on the motility or viability of bull sperm either pre-freeze or post-thaw. There are a few possibilities for why this might be the case. In previous research, Purdy (2004) mentions the importance of incubating sperm with CD/CHOL in a lipid free environment in order for proper membrane enrichment [8]. This is crucial because because presence of yolk droplets provides an excess of cholesterol accepting particles that will limit the amount of cholesterol available to sperm membranes. However, all treated samples in this study were pre-incubated in a virtually lipid free environment prior to addition of EYT, so there should not have been any cholesterol loss to egg yolk lipid droplets during incubation.

Another possibility might have to do with insufficient cholesterol and cyclodextrin coupling. This would become a problem because there needs to be a concentration gradient in order for the cholesterol to leave the cyclodextrin and enter the sperm membrane [15]. If there is an excess of uncoupled cyclodextrin molecules, there is a risk of removing cholesterol from the sperm membrane, therefore affecting sperm plasma membrane stability and potential for fertilizing the egg.

It appears from the cold shock motility data that lack of cholesterol saturation is not an issue as Tris alone will not protect against cold shock [16] and there was no significant difference in motility after cold shock between control samples that have the protection of egg yolk and for CD/CHOL treated samples. These data suggest that samples treated with CD/CHOL were enriched and not depleted of cholesterol, allowing for protection against the drastic temperature changes during cold shock.

An interesting aspect of this study compared to the current research using CD/CHOL treatment on bovine semen that might explain the lack of statistical significance is that motility and viability percentages for control samples were much higher for this study compared to the previous research, even with half the concentration of egg yolk used. This study reports control motility post-thaw as 54.4% while Purdy and Graham reported control motility at 42% [8]. The viability difference was not as drastic, but this study still found control viability post-thaw to be 48.4% compared to 46% reported by Purdy and Graham [8]. Even with motility and viability percentages the same as Purdy found, these higher control values reduce the treatment differences observed.

The difference between mobility and viability percentages found post-thaw can be explained by two methods used to evaluate the samples. All motility evaluations were based on subjective estimates as compared to the very objective measurements

for viability using flow cytometry. It is also possible that sperm may be permeable to vital stains and still remain motile.

Regardless of the lack of significance seen between control and CD/CHOL treated samples in this study, it does appear that there was some effect of CD/CHOL as percent motility after cold shock treatments was not different between the two treatment groups. The lack of statistical significance everywhere else may be due to loss of cholesterol to yolk lipid droplets or due to the higher control values seen in this study compared to the previous research, though further studies would have to be done to examine these possibilities.

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Chapter 3: Effects of Cholesterol Loaded Cyclodextrin and Various Yolk Levels and Yolk Preparations on Cooling and Freezing of Bovine Sperm

Abstract

This study was designed to examine the effects of yolk precipitation (removal of lipid droplets by dilution and centrifugation, leaving most of the protective LDL in the supernatant) and levels of egg yolk below the conventional 20% (v/v) in combination with CD/CHOL addition.

Eight treatments were evaluated in this experiment, 4 without CD/CHOL treatment and 4 with. For samples without CD/CHOL treatment, semen was diluted to a concentration of 120 x 10^6 sperm/ mL in egg yolk Tris (EYT) containing either 2.5% or 10% whole yolk (v/v) or 2.5% or 10% precipitated yolk. For CD/CHOL samples, both whole yolk and precipitated yolk were incubated for 15 min with 5 mM CD/CHOL. The CD/CHOL yolk was then added to semen as above. After initial semen dilution, all samples were incubated for 15 min before further dilution 1:1 (v/v) with their initial diluent, but lacking CD/CHOL. Prior to cooling, 200 µl aliquots of the diluted semen were removed for a 30 min cold shock in a 0°C water bath. The remainder of the diluted semen was cooled, frozen, and stored until evaluation. Motility was assessed prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability was assessed after freezing.

No effect of CD/CHOL was observed other than during cold shock treatment. In fact, this study found that CD/CHOL treatment was actually significantly less effective at protection during cooling than control treatments (50.8% ± 1.9; 52.7% ± 1.9; $P < 0.05$). In addition, atypical effects of 2.5% EYT as well as precipitated yolk were found. These results indicate that, regardless of yolk preparation or level,

CD/CHOL was ineffective at increasing pre-freeze and post-thaw motility as well as post-thaw viability.

Introduction

As presented in chapter 2, there is a great need for advancement in the basic science of semen cryopreservation. Currently, several investigators are studying the use of CD/CHOL to improve sperm survival during the cooling and freezing process. Because our previous study did not support findings of improved post-thaw survival after treatment with CD/CHOL by other authors, further research was needed to determine if an effect could be found.

Current research protocols for bull sperm usually include a 10 to 15 min incubation with CD/CHOL followed by a dilution with egg yolk tris (EYT) made from whole yolk [1-4]. The incubation without yolk appears to be quite important as Purdy states that the cholesterol is very likely to enter the egg yolk lipid droplets instead of the sperm membranes if the complexes are added directly to semen diluted in EYT [2]. In order to prevent this problem, precipitated yolk was examined in the current study. Precipitated yolk refers to yolk that has been diluted to facilitate sedimentation of lipid droplets and HDL during high speed centrifugation while retaining over 90% of the protective LDL in the supernatant, so dilution of cholesterol within droplets should be minimized [5].

The previous study also demonstrated higher control motility and viability values than the previous research, so a lower yolk percentage was examined to determine if these effects were from CD/CHOL or from the EYT. Sperm treated with yolk concentrations below 15% should demonstrate poor cryosurvival [6]. If CD/CHOL has a protective effect during freezing, as with cold shock in the previous study, this would be more apparent at the lower concentrations of yolk. Therefore, the current study examined the effects of CD/CHOL treatment with two different

concentrations of egg yolk, 2.5% and 10%, in EYT were prepared with both whole and precipitated yolk.

Materials and Methods

Materials

Methyl-β-cyclodextrin (MBCD) (C4555), cholesterol (C8667), and all reagents used for media preparation were purchased from Sigma Chemical Company (St. Louis, MO). LIVE/DEAD Sperm Viability Kits were purchased from Molecular Probes Inc (Eugene, OR). VWR Special Select microscope slides (3 in x 1 in) and Corning No. 1 coverslips (18 mm x 18 mm) were purchased from VWR (Rochester, NY). Corning 15 and 50 mL capped plastic conical tubes were purchased from Krackeler Scientific (Albany, NY). Capped, polypropylene microfuge tubes (1.5 mL) were purchased from LPS (Rochester, NY). French straws (0.5 mL) were purchased from IMV Technologies (L'Aigle, France).

Preparation of Cyclodextrin/Cholesterol (CD/CHOL) Complexes and Stock Solution

CD/CHOL complexes were prepared as described by Purdy and Graham [2] with the following modifications. Cholesterol (200 mg) was dissolved in 1.0 mL of redistilled chloroform in a 7 mL screw cap glass vial. In a separate glass vial, 1.0 g MBCD was dissolved in 2.0 mL of redistilled methanol. A 0.45 mL aliquot (90 mg) of the cholesterol solution was added to the methanol solution, mixed and allowed to warm for approximately 30 min at 37°C. Aliquots of 120 µL were placed in 2 mL screw cap glass vials and dried under a stream of nitrogen gas for approximately 1 h to apparent dryness. Vials were allowed to air dry for an additional 24 h before 0.8 mL of modified Tyrodes solution (TALP) [7] (92.9 mM NaCl, 3.1 mM KCl, 0.3 mM

dibasic sodium phosphate, 0.4 mM magnesium chloride, 10 mM calcium chloride, 0.2 mM sodium pyruvate, 5.0 mM glucose, 25.6 mM lactic acid, 10 mM N-2 hydroxyethylpiperazine-N-2-ethane acid (HEPES), 24.8 mM sodium bicarbonate, 3 mg/mL BSA (fraction V, ≥ 96% essentially fatty acid free), 200 units/mL penicillin G, pH 7.4, 300 mOsm) was added. The vials were then placed on a 37°C slide warmer for 90 min, vortexed for 30 s and frozen at -20 °C until used.

Media Preparation

Tris (hydroxylmethylaminomethane) [8] (25.0 mmol Tris base, 8.90 mmol citric acid, 7.00 mmol glucose, 50000 IU penicillin G, and 50 mg streptomycin per 100 mL volume) with 50% egg yolk (v/v) was made using both whole yolk and precipitated yolk. To produce precipitated yolk, whole yolk was diliuted 1:1 (v/v) with water and centrifuged at 4300 x g for 20 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge to remove lipid droplets and high density lipoproteins (HDL), leaving approximately 90% of the low density lipoproteins (LDL) in the resulting supernatant. This LDL supernatant was then recovered and used as the precipitated yolk for this study. Five mM CD/CHOL was added to treated samples and incubated for 15 min prior to further dilution. All samples were adjusted to either 2.5% or 10% egg yolk (v/v) with Tris buffer prior to semen dilution.

Semen Collection and Preparation

Fresh bovine semen was provided by Genex Cooperative, Inc. (Ithaca, NY) after collection by artificial vagina. All initial semen dilutions were performed at the Genex laboratory in 15 mL capped plastic conical tubes to a concentration of 120 x 10^6 sperm/ mL in appropriate treatment media within 1 h of collection. Each experiment was replicated on 6 ejaculates.

Semen Cooling and Freezing

Semen was cooled from 21°C to 4 °C at a constant rate over a period of 90 min during transport from the Genex facility to our laboratory. After cooling, samples were diluted with an equal volume (1:1, v/v) of either 2.5% or 10% egg yolk Tris (EYT) plus 14% glycerol added in 4 equal aliquots at 15 min intervals for 1 h and then allowed to equilibrate for an additional 1 h Semen was then loaded into 0.5 mL french straws, placed 4 cm above liquid nitrogen in a 20 in x 10 in x 9 in Styrofoam box, and frozen for 15 min. Straws were then stored in liquid nitrogen until evaluated.

Evaluation of Sperm Motility

Sperm were evaluated subjectively using an Olympus CH-2 phase contrast microscope at 100x magnification with a heated stage (37°C). Each sample was warmed for 5 min in a 1.5 mL capped, polypropylene microfuge tube on a 37°C slide warmer prior to analysis in 6 μL aliquots on a slide with a coverslip. Percent progressive motility was visually estimated in five fields per sample prior to cooling, after cold shock treatment, and following cooling (0 and 3 h). The five fields were then averaged to determine the progressive motility for the sample. For post-thaw samples, subjective motility was determined on 2 straws per sample and in 5 fields per sample after thawing in a 37°C water bath for 1 min. Estimates from the 5 fields were averaged to determine the progressive motility of each straw and an average of both straws was used to determine the progressive motility of the sample.

Evaluation of Sperm Viability

Sperm were evaluated by propidium iodide (PI) and SYBR 14 staining using the LIVE/DEAD Sperm Viability Kit protocol. Both stains detect DNA; PI permeates only dead sperm while SYBR 14 is also permeable to live sperm. Each sample was

evaluated in duplicate following a 1 min thaw in a 37°C water bath. Stain was incubated with each sample for 5 min at 37°C prior to analysis. Measurements were done using a BD FACSAria (Bectin Dickenson) and analyzed using BD FACSDiVa software (San Jose, Ca). SYBR 14 fluorescence detector E (530/30nm BP filter) while PI was detected with detector B (695/40nm BP filter) with an LP filter of 502 nm and 635 nm before the BP for detectors E and B, respectively. Particles were gated so that only those positive for DNA fluorescence were counted.

Experimental Procedure

Eight treatments were evaluated in this experiment, 4 without CD/CHOL treatment and 4 with. For samples without CD/CHOL treatment, semen was diluted in EYT containing either 2.5% or 10% whole yolk (v/v) or 2.5% or 10% precipitated yolk. For CD/CHOL samples, both whole yolk and precipitated yolk were incubated for 15 min with 5 mM CD/CHOL. The CD/CHOL yolk was then used as above. After initial semen dilution, all samples were incubated for 15 min before further dilution 1:1 (v/v) with their respective preparation and percentage of yolk. Yolk used for these dilutions were not treated with CD/CHOL. Prior to cooling, 200 µl aliquots of the diluted semen were removed for a 30 min cold shock in a 0°C water bath. The remainder to the diluted semen was cooled, frozen and stored until evaluation. Motility data were collected prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability data were collected after freezing.

Experimental Design

Percent motility and viability data were collected using a factorial arrangement of treatments. Treatment effects were analyzed by means of a one-way analysis of variance using Minitab Release 15 Statistical Software for Windows. Main effects in

the statistical model included ejaculates, CD/CHOL treatment, level of yolk, preparation of yolk, and cooling. Factors and interactions demonstrating significant differences were tested using Tukey's test for multiple comparisons.

Results

Pre-freeze Motility

Table 3.1 shows a summary of the effect of CD/CHOL, level and preparation of egg yolk, and cooling on mean bull sperm motility prior to freezing. Factors demonstrating significant differences are presented in tables 3.2 - 3.4. For motility prior to freezing, control samples were significantly higher than samples treated with CD/CHOL (Table 3.2). Yolk level had a significant effect on the motility prior to freezing when examined by treatment. Motility following cold shock treatment was significantly higher for samples containing 10% yolk than those with 2.5% yolk (30.4 ± 1.59%; 20.2 ± 2.0%; $P < 0.05$). For both levels of yolk, treatments rewarmed to 37°C and incubated for 0 hr or 3 hr were significantly lower than precool treatments and significantly higher than cold shock treatments (Table 3.3). Yolk level also had a significant effect on prefreeze motility when compared with yolk preparation. Samples that contained 2.5% whole yolk had significantly lower motility rates (47.6 ± 2.90%; $P < 0.05$) than any of the other samples.

Post-thaw Motility

Table 3.5 summarizes the effects of CD/CHOL and level and preparation of yolk on mean bull sperm motility post-thaw. Table 3.6 displays the only significant effect on post-thaw motility, which was attributed to yolk preparation. Samples containing precipitated yolk showed significantly higher percent motility than those with whole yolk (46.5 ± 2.03%; 44.0 ± 1.88%; $P < 0.05$). Percent motility for 10%

whole yolk samples treated with CD/CHOL yolk did appear to have a lower percent motility than the remaining treatments, though this was not found to be significant ($P > 0.05$).

Post-thaw Viability

Table 3.7 shows a summary of the effects of CD/CHOL and level and preparation of egg yolk on mean bull sperm viability post-thaw. Factors demonstrating significant difference are presented in tables 3.8 and 3.9. Significant differences in post-thaw sperm viability were seen for both yolk preparation and yolk level. Samples containing precipitated yolk had significantly higher viability values than those containing whole yolk (Table 3.8). Viability was also higher for samples containing 2.5% yolk compared to those containing 10% yolk ($45.2 \pm 2.46\%$; $40.0 \pm 1.83\%$; $P < 0.05$).

Table 3.1. Summary of effects of CD/CHOL, level and preparation of egg yolk, and cooling on bull sperm motility prior to freezing (mean ± SEM). n = 6

Treatment	Yolk			
	Whole		Precipitated	
	2.5%	10%	2.5%	10%
Control				
Precool	65.8 ± 2.7	65.8 ± 2.7	69.2 ± 3.0	68.3 ± 3.6
Cold Shock	20.8 ± 4.7	35.0 ± 2.9	21.7 ± 4.0	31.7 ± 4.0
Postcool (t=0 hr)	57.5 ± 3.8	60.0 ± 3.7	60.0 ± 4.5	63.3 ± 4.0
Postcool (t=3 hr)	55.0 ± 4.3	56.7 ± 4.4	58.3 ± 4.8	59.2 ± 4.4
CD/CHOL				
Precool	64.2 ± 3.0	65.8 ± 2.7	68.3 ± 2.8	68.3 ± 2.1
Cold Shock	17.5 ± 4.2	28.3 ± 2.8	20.8 ± 4.0	26.7 ± 2.5
Postcool (t=0 hr)	55.0 ± 4.8	59.2 ± 4.4	60.8 ± 4.0	61.7 ± 5.1
Postcool (t=3 hr)	50.0 ± 5.2	53.3 ± 4.8	57.5 ± 4.2	55.0 ± 4.3

Table 3.2. Effects of CD/CHOL on bull sperm motility prior to freezing (mean ± SEM). n = 6

Treatment	Motility
Control	52.7 ± 1.9*
CD/CHOL	50.8 ± 1.9

*P < 0.05

Table 3.3. Effect of level of egg yolk and cooling on bull sperm motility prior to freezing (mean ± SEM). n = 6

Treatment	Yolk Level	
	2.5%	10%
Precool	66.9 ± 1.4[c]	67.1 ± 1.3[c]
Cold Shock	20.2 ± 2.0[a]	30.4 ±1.6[a]*
Postcool (t=0 hr)	58.3 ± 2.1[b]	61.0 ± 2.0[b]
Postcool (t=3 hr)	54.0 ± 2.5[b]	56.0 ± 2.1[b]

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

*Indicates treatment differences within rows ($P < 0.001$)

Table 3.4. Effects of level and preparation of egg yolk on bull sperm motility prior to freezing (mean ± SEM). n = 6

Yolk Preparation	Yolk Level	
	2.5%	10%
Whole	47.6 ± 2.9*	53.0 ± 1.8
Precipitated	52.1 ± 3.0	54.3 ± 2.5

*Indicates difference in type by yolk ($P < 0.001$)

Table 3.5. Summary of effects of CD/CHOL and level and preparation of egg yolk on bull sperm motility post-thaw (mean ± SEM). n = 6

Treatment	Yolk			
	Whole		Precipitated	
	2.5%	10%	2.5%	10%
Control	44.2 ± 4.9	45.8 ± 3.8	45.8 ± 4.9	45.8 ± 3.5
CD/CHOL	44.2 ± 3.8	41.7 ± 3.3	46.7 ± 4.2	47.5 ± 4.6

Table 3.6. Effect of preparation of egg yolk on bull sperm motility post-thaw (mean ± SEM). n = 6

Yolk Preparation	Motility
Whole	44.0 ± 1.9
Precipitated	46.5 ± 2.0*

*P < 0.05

Table 3.7. Summary of effects of CD/CHOL and level and preparation of egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Treatment	Yolk			
	Whole		Precipitated	
	2.5%	10%	2.5%	10%
Control	44.7 ± 5.7	39.5 ± 4.3	46.5 ±5.2	41.6 3.7
CD/CHOL	43.8 ± 4.8	37.9 ± 3.5	45.9 ± 5.3	40.5 ± 4.0

Table 3.8. Effect of preparation of egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Yolk Preparation	Viability
Whole	41.5 ± 2.2
Precipitated	43.6 ± 2.2*

*P < 0.05

Table 3.9. Effect of level of egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Yolk Level	Viability
2.5%	45.2 ± 2.5*
10%	40.0 ± 1.8

*P < 0.001

Discussion

The importance of discovering an effective method of enriching sperm membranes with cholesterol was discussed in chapter 2. Previous studies using CD/CHOL have been successful in producing more motile and viable post-thaw sperm, while studies described in the preceding chapter were unable to duplicate those effects. In the current study, additional yolk preparations and yolk concentrations were observed to determine if CD/CHOL addition to extenders could improve post-thaw sperm survival.

In the current study, the only positive effects seen with CD/CHOL were during cold shock experiments where motility with CD/CHOL treatment (prior to further dilution with EYT) was equivalent to control values (10% EYT). However, when examined over all treatment groups, yolk levels and yolk preparations, CD/CHOL actually had significantly lower pre-freeze motility when compared to controls. This indicated that while CD/CHOL might be as effective at preventing cold shock damage as conventional EYT treatments, it does not appear to be as beneficial during controlled cooling. Considering that current published data on the bull is only on post-thaw motility [1-4], comparison of these results with other studies cannot be performed and therefore further examination of this phenomenon remains to be completed.

Additional pre-freeze differences were seen between samples treated with 2.5% EYT and those treated with 10% EYT. Previous research has indicated that effective concentrations of egg yolk for cooling and freezing bull sperm must be within a range of 15% to 30% [6]. Samples treated with 10% EYT are not far enough below that range for the yolk to no longer provide enough low density lipoproteins and cholesterol to be protective [9-11], while 2.5% EYT is. Both yolk levels also resulted

in a decrease in sperm motility after cooling, which is to be expected as cooling has damaging effects on sperm [12-14].

Differences among the preparation of yolk in combination with level of yolk were also seen. Motility percentages prior to freezing were significantly lower for samples treated with 2.5% whole yolk. As indicated above, the lower percentages of yolk are predicted to result in lower motility values due to the low concentrations of lipoproteins and cholesterol. The difference seen between whole and precipitated yolk, however, is very interesting. Precipitating yolk decreases the amount of high density lipoproteins and lipid droplets within the yolk therefore clarifying the yolk [5]. Due to the process, which includes a dilution and centrifugation, a certain amount of low density lipoproteins and cholesterol loss would be expected [5]. Perhaps the reason for the higher percentage of observed motility is due to the more clarified media. Whole yolk still contains lipid droplets which may be difficult for sperm to navigate through while being observed under a coverslip on a slide. Sperm can be observed moving, though not progressively, in contact with lipid droplets. Precipitated yolk does not have this opacity, so more progressively motile sperm could be more readily observed.

After freezing, the only treatment difference seen for motility was between precipitated and whole yolk. As with cooling, the precipitated yolk appeared to be more effective at protecting the sperm. This was also apparent in the viability of the sperm post-thaw. Because viability measurements would not be affected by the presence of lipid droplets like motility, another reason for this improvement must be determined. Perhaps, with the removal of high density lipoproteins, there is less competition for cholesterol in the low density lipoproteins to enter the sperm membrane. More studies would have to be done on these precipitated yolks to determine what it actually is causing this treatment difference.

Post-thaw viability data also showed an interesting effect of yolk level. Samples treated with 2.5% EYT had significantly higher viability than those treated with 10% EYT regardless of CD/CHOL treatment or yolk preparation. This also is an unexpected result. As mentioned earlier, lower yolk levels should be less effective at protecting sperm during freezing than higher yolk levels and the only way to better understand these effects would be to further study the underlying mechanisms of how the CD/CHOL behaves in various concentrations of egg yolk.

In general, this study found control post-thaw motility and viability values closer to those reported by Purdy and Graham in their previous reports [1, 2] and no positive effects of CD/CHOL on either sperm motility or viability post-thaw, regardless of reduction of yolk level or removal of lipid droplets using precipitation. Many of the results of this study were also unexpected in regard to yolk level and use of precipitated yolks, which makes drawing results from this study difficult. Considering the lack of encouraging results from the use of CD/CHOL to enrich sperm membranes with cholesterol, alternative methods must be examined. One method that might be found helpful could be utilizing high cholesterol egg yolk rather than attempting to enrich the sperm prior to yolk addition.

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Chapter 4: Effects of Cholesterol Enriched Egg Yolk on Bovine Sperm during Cooling and Freezing

Abstract

The objective of this study was to examine a method of enriching sperm membranes with cholesterol utilizing cholesterol enriched egg yolk produced by feeding hens a high cholesterol diet.

Control, 0.5% cholesterol supplemented, and 1.0% cholesterol supplemented diets were fed to 5 Babcock B-300 hens for 21 days. Eggs were collected daily starting on day 3 and ending on day 24. Semen was diluted in one of nine treatments consisting of 2.5% and 10% whole yolk EYT and 10% precipitated yolk EYT (v/v) using eggs from each of the three diet groups. After initial dilution, all samples were incubated for 15 min before further dilution 1:1 (v/v) with their respective preparation and percentage of yolk. Prior to cooling, 200 µl aliquots of the diluted semen were removed for a 30 min cold shock in a 0°C water bath. The remainder of the diluted semen was cooled, frozen and store until evaluation. Motility was evaluated prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability was evaluated after freezing. Yolk cholesterol content was also examined.

Though no significant effects of supplemented yolks were seen for pre-freeze or post-thaw motility, there was an observed effect on post-thaw viability. Samples treated with 2.5% whole yolk from hens fed the 1.0% cholesterol supplemented diet had a significantly higher percentage of viable sperm cells post-thaw than almost all other treatments. Samples treated with 10% EYT from hens fed the 0.5% cholesterol supplemented diet also showed significantly higher percentages of viable cells post-thaw regardless of yolk preparation. In addition, yolks from the 1.0% supplemented diet were found to have a significantly higher cholesterol concentration than the eggs from the control diet. These results indicate that increasing cholesterol of egg yolk

through diet is possible and that there are some positive effects of treatment with these enriched yolks.

Introduction

During the cooling and freezing procedures required for semen cryopreservation, to the sperm plasma membrane incurs irreversible damage. A generally accepted phenomenon that contributes to this damage is lipid phase transition that occurs during cooling. During lipid phase transition from liquid crystalline to gel phase, non-bilayer preferring lipids, particularly hexagonal II lipids, aggregate during cooling due to their high melting points. This disrupts normal lipid-lipid and lipid-protein interactions within the membrane and also creates non-bilayer arrangements upon warming [1].

The higher the ratio of cholesterol to phospholipids found in membranes, the less likely phase transition and lateral phase separation is to occur. For example, with liposomes, the more cholesterol inserted into the phospholipids bilayer, the less lateral movement of lipids occurred [2]. This has also been shown in intrinsic resistance to lipid phase transition found with human and rooster sperm. These species have very high cholesterol to phospholipid ratios compared to other species, such as the bull, stallion and boar, and are more resistant to the effects of freezing on the membrane [3].

Research done on enriching equine and bovine sperm membranes with cholesterol using cholesterol loaded cyclodextrin (CD/CHOL) have also shown improvement in sperm survival as compared to control samples [4-6]. However, in a current review by Graham *et al.* (2006), fertility results have been extremely varied using CD/CHOL treated semen. In some studies, the fertility rates were not any better than control samples, and in some, fertility was actually reduced to only half that of

control samples. Graham *et al.* (2006) hypothesized that this might be because CD/CHOL treated sperm may require more time for capacitation (membrane and associated changes in the female tract required for the acquisition of sperm fertility) due to the extra cholesterol that must transfer out of the sperm membrane [7]. There also may be other cytotoxic effects of the cyclodextrin that affect fertility, which leaves room for research on alternative ways to enrich membranes with cholesterol without using cyclodextrin.

Current extenders used in cryopreservation protocols already contain a cholesterol source provided by egg yolk. By increasing the cholesterol concentration of the yolk itself, extenders could be made with the same yolk content while having greater concentrations of cholesterol. This would allow concentration differences between sperm and extender that would favor net transfer of cholesterol to sperm similar to the CD/CHOL enrichment. This method would also eliminate the need for the addition of a chemical, such as cyclodextrin, to solubilize the cholesterol in order to enrich an existing extender.

Naber *et al.* (1985) showed that feeding domestic hens a diet high in cholesterol could significantly increase the free cholesterol content of yolk [8]. The main objective of this study was to generate high cholesterol yolks to determine whether or not they provided added protection to bull sperm during cooling and freezing. Yolks were both used in their whole yolk and precipitated yolk form at two different concentrations to determine an optimal extender for processing bull sperm for cryopreservation.

Materials and Methods

Materials

Methyl-β-cyclodextrin (MBCD) (C4555), cholesterol (C8667), and all reagents used for media preparation were purchased from Sigma Chemical Company (St. Louis, MO). LIVE/DEAD Sperm Viability Kits and Amplex Red Cholesterol Assay Kits were purchased from Molecular Probes Inc (Eugene, OR). VWR Special Select microscope slides (3 in x 1 in) and Corning No. 1 coverslips (18 mm x 18 mm) were purchased from VWR (Rochester, NY). Corning 15 and 50 mL capped plastic conical tubes were purchased from Krackeler Scientific (Albany, NY). Capped, polypropylene microfuge tubes (1.5 mL) were purchased from LPS (Rochester, NY). French straws (0.5 mL) were purchased from IMV Technologies (L'Aigle, France).

Production of High Cholesterol Eggs

Control and cholesterol supplemented diets were made following the basic recipe by Naber *et al.* [8] with modification listed (fig. 4.1). Each diet was fed to 5 Babcock B-300 hens for 21 days. Eggs were collected daily starting on day 3 and ending on day 24. Eggs were labeled with the diet fed, the hen number, and the date laid.

Table 4.1. Composition of experimental diets for production of high cholesterol egg yolks.

Diet	1	2	3
Ingredient		(%)	
Corn, ground yellow	49.51	49.51	49.51
Soybean meal (48% crude protein)	19.61	19.61	19.61
Meat & bone meal (53% crude protein)	4.70	4.70	4.70
Alfalfa meal, dehydrated (17% crude protein)	2.84	2.84	2.84
Cellulose	1.96	1.96	1.96
Limestone, ground	8.33	8.33	8.33
Dicalcium phosphate	0.98	0.98	0.98
Vitamin-mineral premix*	2.25	2.25	2.25
Soybean oil	9.80	9.30	8.80
Cholesterol	0.00	0.50	1.00
Total	100.00	100.00	100.00

*Vitamin-mineral premix contained the following, expressed per kilogram of diet: 2200/1100 IU vitamin AD3, 10 IU vitamin E, 2.0 mg menadione sodium bisulfite, 7.0 mg riboflavin, 3.8 mg pantothenate (Ca salt), 0.2 mg biotin, 5.8 ug vitamin B12, 0.78 g DL-methionine, 0.25 g ethoxyquin, 0.5 g iodized salt, 100 mg manganese oxide, 125 mg zinc oxide, 0.24 mg sodium selenite, 16.697 g corn meal

Media Preparation

Tris (hydroxylmethylaminomethane) [8] (25.0 mmol Tris base, 8.90 mmol citric acid, 7.00 mmol glucose, 50000 IU penicillin G, and 50 mg streptomycin per 100 mL volume) with 50% egg yolk (v/v) was made using both whole yolk and precipitated yolk. To produce precipitated yolk, whole yolk was diliuted 1:1 (v/v) with water and centrifuged at 4300 x g for 20 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge to remove lipid droplets and high density lipoproteins (HDL), leaving approximately 90% of the low density lipoproteins (LDL) in the resulting supernatant. This LDL supernatant was then recovered and used as the precipitated yolk for this study. All samples were adjusted to either 2.5% or 10% egg yolk (v/v) with Tris buffer prior to semen dilution.

Semen Collection and Preparation

Fresh bovine semen was provided by Genex Cooperative, Inc. (Ithaca, NY) after collection by artificial vagina. All initial semen dilutions were performed at the Genex laboratory in 15 mL capped plastic conical tubes to a concentration of 120 x 10^6 sperm/ mL in appropriate treatment media within 1 h of collection. Each experiment was replicated on 6 ejaculates.

Semen Cooling and Freezing

Semen was cooled from 21°C to 4 °C at a constant rate over a period of 90 min during transport from the Genex facility to our laboratory. After cooling, samples were diluted with an equal volume (1:1, v/v) of either 2.5% or 10% egg yolk Tris (EYT) plus 14% glycerol added in 4 equal aliquots at 15 min intervals for 1 h and then allowed to equilibrate for an additional 1 h. Semen was then loaded into 0.5 mL

french straws, placed 4 cm above liquid nitrogen in a 20 in x 10 in x 9 in Styrofoam box, and frozen for 15 min. Straws were then stored in liquid nitrogen until evaluated.

Evaluation of Sperm Motility

Sperm were evaluated subjectively using an Olympus CH-2 phase contrast microscope at 100x magnification with a heated stage (37°C). Each sample was warmed for 5 min in a 1.5 mL capped, polypropylene microfuge tube on a 37°C slide warmer prior to analysis in 6 μL aliquots on a slide with a coverslip. Percent progressive motility was visually estimated in five fields per sample prior to cooling, after cold shock treatment, and following cooling (0 and 3 h). The five fields were then averaged to determine the progressive motility for the sample. For post-thaw samples, subjective motility was determined on 2 straws per sample and in 5 fields per sample after thawing in a 37°C water bath for 1 min. Estimates from the 5 fields were averaged to determine the progressive motility of each straw and an average of both straws was used to determine the progressive motility of the sample.

Evaluation of Sperm Viability

Sperm were evaluated by propidium iodide (PI) and SYBR 14 staining using the LIVE/DEAD Sperm Viability Kit protocol. Both stains detect DNA; PI permeates only dead sperm while SYBR 14 is also permeable to live sperm. Each sample was evaluated in duplicate following a 1 min thaw in a 37°C water bath. Stain was incubated with each sample for 5 min at 37°C prior to analysis. Measurements were done using a BD FACSAria (Bectin Dickenson) and analyzed using BD FACSDiVa software (San Jose, Ca). SYBR 14 fluorescence detector E (530/30nm BP filter) while PI was detected with detector B (695/40nm BP filter) with an LP filter of 502 nm and

635 nm before the BP for detectors E and B, respectively. Particles were gated so that only those positive for DNA fluorescence were counted.

Cholesterol Assay

Yolk cholesterol content was measured using the Amplex Red Cholesterol assay kit. In the assay, recombinant cholesterol oxidase from *Streptomyces* oxidizes cholesterol to produce a ketone and H_2O_2. The hydrogen peroxide is then able to be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent). In the presence of horseradish peroxidase, Amlex Red reagent reacts with H_2O_2 to produce highly fluorescent resorufin. For all samples, fluorescence was measured with a Tecan SpectraFluor microplate reader (Männedorf, Switzerland) with an excitation wavelength of 540 nm and an emission wavelength of 595 nm. A standard curve was prepared using purified cholesterol provided by the kit. Both the standard curve and the yolk samples were incubated with reagent for 30 min at 37°C, while protected from light. Data were analyzed using Magellan software (Männedorf, Switzerland). Each yolk sample was evaluated in duplicate.

Experimental Procedure

Semen was diluted in one of nine treatments consisting of 2.5% or 10% whole yolk EYT or 10% precipitated yolk EYT (v/v) using eggs from each of the three diet groups. All eggs came from hens that had been on their respective diet for no less than 18 days and no more than 22 days. After initial dilution, all samples were incubated for 15 min before further dilution 1:1 (v/v) with their respective preparation and percentage of yolk. Prior to cooling, 200 µl aliquots of the diluted semen were removed for a 30 min cold shock in a 0°C water bath. The remainder of the diluted semen was cooled, frozen and stored until evaluation. Motility data were collected

prior to cooling, after cooling (0 h and 3 h), after cold shock, and after freezing. Viability data were collected after freezing.

Experimental Design

Percent motility and viability data were collected using a factorial arrangement of treatments. Treatment effects were analyzed by analysis of variance using Minitab Release 15 Statistical Software for Windows. Main effects in the statistical model included ejaculates, dietary cholesterol treatment, level of yolk, preparation of yolk, and cooling. Factors and interactions demonstrating significant differences were tested using Tukey's test for multiple comparisons.

Results

Yolk Cholesterol Content over Time

All diets increased the cholesterol content of the eggs over time though the relative changes over time for the different diets were not statistically significant (Chart 4.1). However, the absolute value of cholesterol for each diet was shown to be statistically different.

Pre-freeze Motility

Effects of dietary cholesterol enrichment, level and preparation of egg yolk, and cooling on mean bull sperm motility prior to freezing are summarized in table 4.2. Factors that differed significantly are presented in table 4.3. Motility was highest prior to cooling (Table 4.3). A small but significant decrease in motility was observed after controlled cooling immediately upon rewarming and after 3 hr at 37°C. Samples containing 2.5% whole yolk exhibited the lowest motility following cold shock (42.8 ± 3.06%; $P < 0.05$) compared to any other yolk level or yolk preparation.

Post-thaw Motility

Table 4.4 summarizes the effects of dietary cholesterol enrichment, and level and preparation of egg yolk on mean bull sperm motility post-thaw. Factors demonstrating significant differences are displayed in table 4.5. The only significant effect seen for post-thaw motility was due to yolk level of the samples treated with whole yolk. Overall, samples extended with 10% whole yolk exhibited significantly higher sperm motility than those containing 2.5% yolk (Table 4.5).

Post-thaw Viability

Table 4.6 summarizes the effects of dietary cholesterol enrichment, level and preparation of egg yolk on mean bull sperm viability post-thaw. Factors demonstrating significant differences are presented in tables 4.7 - 4.9. Sperm viability was significantly higher in post-thaw samples containing 2.5% whole yolk made from the control and 1.0% CHOL enriched diet as compared to control samples with 10% whole yolk. All other viability values were intermediate to these treatments. A trend toward higher viability was observed using 2.5% whole versus 10% yolk, though not statistically significant (Table 4.7). Samples extended with 10% precipitated yolk from the 0.5% CHOL diet retained significantly higher viability (49.4 ± 2.4%; $P < 0.05$) than samples with yolk from the control diet (46.4 ± 2.6%; $P < 0.05$). Precipitated yolk, in general, also showed significantly higher post-thaw viability than whole yolk (Table 4.9).

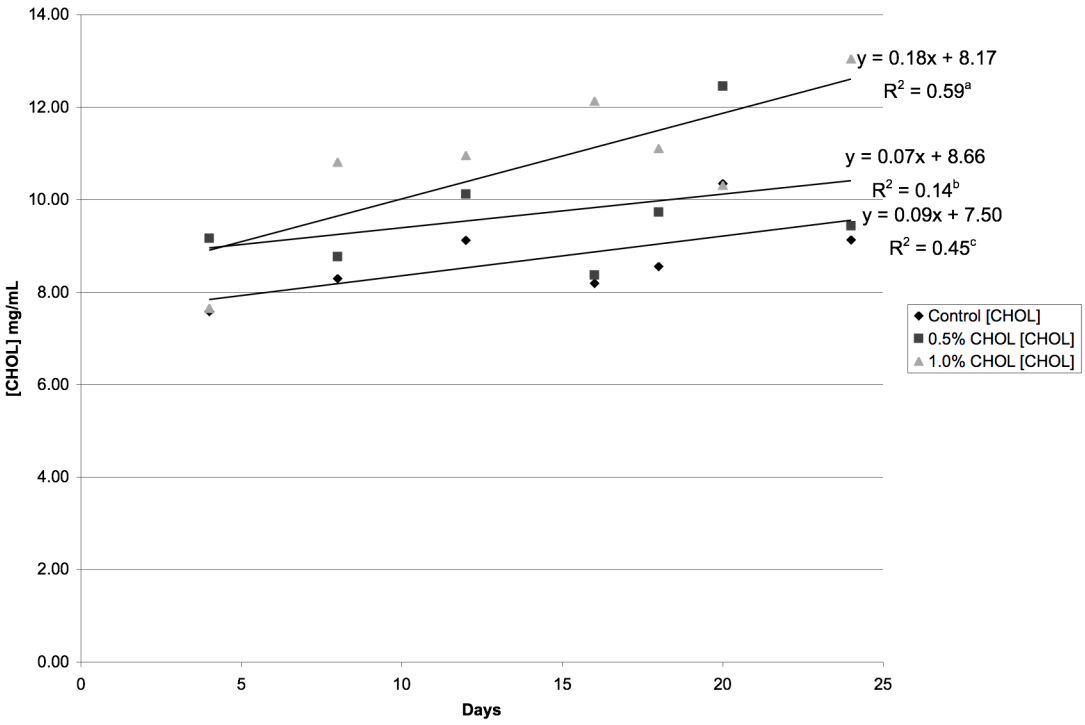

Figure 4.1. Regression equations for relationship between egg yolk cholesterol concentration and days of feeding different levels of cholesterol. Each diet was fed to 5 Babcock B-300 hens for 21 days. Eggs were collected daily starting on day 3 and ending on day 24. [a,b,c] Different superscripts indicate treatment differences ($P < 0.05$).

Table 4.2. Summary of effects of dietary cholesterol enrichment, level and preparation of egg yolk, and cooling on bull sperm motility prior to freezing (mean ± SEM). n = 6

Treatment	Yolk		
	Whole		Precipitated
	2.5%	10%	10%
Control			
Precool	65.0 ± 2.6	64.2 ± 1.5	66.7 ± 2.5
Cold Shock	47.5 ± 7.0	47.5 ± 3.8	46.7 ± 4.0
Postcool (t=0 hr)	61.7 ± 1.7	60.8 ± 1.5	60.0 ± 1.3
Postcool (t=3 hr)	59.2 ± 2.4	59.2 ± 1.5	55.8 ± 1.5
0.5% CHOL			
Precool	64.2 ± 2.4	62.5 ± 2.5	65.8 ± 2.4
Cold Shock	40.0 ± 4.5	50.0 ± 3.7	45.8 ± 5.1
Postcool (t=0 hr)	60.8 ± 1.5	60.0 ± 1.8	62.5 ± 1.7
Postcool (t=3 hr)	58.3 ± 2.1	59.2 ± 1.5	58.3 ± 1.1
1.0% CHOL			
Precool	65.0 ± 1.3	65.0 ± 1.8	65.0 ± 2.2
Cold Shock	40.8 ± 4.4	50.8 ± 4.6	46.7 ± 4.2
Postcool (t=0 hr)	60.8 ± 0.83	60.0 ± 1.8	62.5 ± 1.1
Postcool (t=3 hr)	58.3 ± 1.1	58.3 ± 1.7	59.2 ± 0.83

Table 4.3. Effect of level of egg yolk and cooling on bull sperm motility prior to freezing (mean ± SEM). n = 6

Treatment	Yolk Level	
	2.5%	10%
Precool	64.7 ± 1.2[c]	63.9 ± 1.1[c]
Cold Shock	42.8 ± 3.1[a]	49.4 ± 2.2[a]*
Postcool (t=0 hr)	61.1 ± 0.76[b]	60.3 ± 0.95[b]
Postcool (t=3 hr)	58.6 ± 1.1[b]	59.0 ± 0.68[b]

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

*Indicates treatment differences within rows ($P < 0.001$)

Table 4.4. Summary of effects of dietary cholesterol enrichment and level and preparation of egg yolk on bull sperm motility post-thaw (mean ± SEM). n = 6

Diet	Yolk		
	Whole		Precipitated
	2.5%	10%	10%
Control	49.2 ± 2.4	52.5 ± 2.1	50.8 ± 1.5
0.5% CHOL	48.8 ± 1.9	50.0 ± 1.8	53.3 ± 2.5
1.0% CHOL	47.9 ± 1.4	50.8 ± 2.0	52.5 ± 1.7

Table 4.5. Effect of level of egg yolk on bull sperm motility post-thaw (mean ± SEM). n = 6

Yolk Level	Motility
2.5%	48.6 ± 1.05
10%	51.1 ± 1.01*

*P < 0.05

Table 4.6. Summary of effects of dietary cholesterol enrichment, level and preparation of egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Diet	Yolk		
	Whole		Precipitated
	2.5%	10%	10%
Control	49.8 ± 2.8	43.1 ± 2.4	46.4 ± 2.6
0.5% CHOL	48.1 ± 2.2	47.8 ± 2.0	49.4 ± 2.4
1.0% CHOL	49.5 ± 3.1	46.0 ± 2.2	47.4 ± 1.7

Table 4.7. Effect of dietary cholesterol enrichment and level of egg yolk on bull sperm viability post-thaw (mean ± SEM). = 6

Diet	Whole	
	2.5%	10%
Control	49.8 ± 2.8[b]	43.1 ± 2.4[a]
0.5% CHOL	48.1 ± 2.2[ab]	47.8 ± 2.0[ab]
1.0% CHOL	49.5 ± 3.1[b]	46.0 ± 2.2[ab]

[a,b] Different superscripts indicate treatment differences (P < 0.05)

Table 4.8. Effect of dietary cholesterol and 10% egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Diet	10%
Control	44.7 ± 1.8^{a}
0.5% CHOL	48.6 ± 1.5^{b}
1.0% CHOL	46.7 ± 1.4^{ab}

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

Table 4.9. Effect of preparation of egg yolk on bull sperm viability post-thaw (mean ± SEM). n = 6

Yolk Preparation	Viability
Whole	45.6 ± 1.3
Precipitated	47.7 ± 1.3*

*P < 0.05

Discussion

As discussed in the previous two chapters, a method to enrich sperm membranes with cholesterol for protective purposes during the cooling and freezing process would be extremely beneficial to the artificial insemination industry. However, the previous studies did not duplicate results published in studies using CD/CHOL to achieve improved post-thaw motility reflective of membrane cholesterol enrichment. The objective of this study was to examine whether or not effects of cholesterol enrichment could be achieved using high cholesterol egg yolk produced by feeding hens two different diets supplemented with cholesterol, one at 0.5% added cholesterol and the other at 1.0% added cholesterol.

The diets produced similar results to those previously reported [8]. When comparing regression equations for the three diet groups, the regression coefficients for the three diet groups were significantly different indicating that an increase in dietary cholesterol did result in an increase in yolk cholesterol. Some other measurement of cholesterol such as GLC or HPLC might be useful for reducing variability in cholesterol in future studies. The assay used for the current study required a large dilution of the yolk for sample cholesterol to fall on the standard curve which may have affected the results.

Prior to freezing, diluted sperm samples in extenders made with yolk from all preparations behaved the same. This is interesting considering that the cholesterol content of the eggs does increase respective to diet (Chart 4.1). One might assume that sperm incubated with the yolk from the highest cholesterol diet, which after 21 days would have a concentration of about 12.0 mg/mL cholesterol, would be able to incorporate a greater amount of cholesterol as compared to the sperm incubated with the control yolk which had only 9.4 mg/mL cholesterol.

It is unlikely the sperm are unable to incorporate the added cholesterol given that Purdy (2004) demonstrated increased cholesterol incorporation with his CD/CHOL treated sperm [5]. Perhaps, even though the increase of cholesterol in the 1.0% diet was significantly higher than the control diet, this increase was not enough to make a significant impact on the enrichment of the membrane. This also is hard to confirm because with the CD/CHOL work, an addition of only 1.5 mg/mL of CD/CHOL showed an increase on post-thaw motility [5]. Again, just as with previous results (see chapters 2 and 3), it is hard to compare these pre-freeze values due to the lack of published data prior to freezing. A further examination of this phenomenon might help explain the lack of treatment differences observed in this study.

Similar to chapter 3, no overall significant protective effect of cold shock or controlled cooling prior to freezing was observed regardless of yolk used. Cold shock resulted in the lowest motility, which is to be expected due to the detrimental effects of rapid cooling [10]. Samples treated with 2.5% EYT exhibited lower resistance to cold shock and than those treated with 10% EYT which is consistent with previous studies that showed that the optimal yolk concentrations for cooling and freezing fall within the range of 15% to 30% [11].

Sperm that were examined either 0 or 3 hr after a controlled cooling had significantly better motility than the cold shock treatments, though they had slightly lower, yet signicantly different motility as compared to the non-cooled sperm. This is to be expected because even if the sperm are cooled in a controlled way, cold shock damage still occurs [3].

Just as with motility prior to freezing, yolk from the various diets did not have different effects on the post-thaw motility. Regardless of diet, samples treated with 10% EYT demonstrated higher motility percentages than those treated with 2.5% EYT. As stated above, this is an expected outcome due to the reduced concentration of yolk provided in the extender. Presumably, lower yolk concentrations do not provide enough low density lipoproteins or cholesterol to provide the protective effects of egg yolk [11].

Interestingly, the various levels of yolk cholesterol did affect viability. When examining samples treated with whole yolk extender and diluted to 2.5% or 10%, those treated with 2.5% EYT made with the 1.0% cholesterol enriched yolks exhibited significantly higher percentages of viable sperm than other treatments. Samples treated with 2.5% EYT made with the control yolk also exhibited this significantly higher viability, though this effect was not also seen with 10% control yolks. The 10% control samples actually had the lowest viability in the study, which might indicate that the 2.5% EYT treatments are not a typical response. Further research would be needed to confirm this.

When examining samples treated with 10% EYT, regardless of yolk preparation, a significant difference was seen between the three diet groups. With the different diets, the 0.5% diet group showed the highest viability regardless of the preparation of yolk. The 1.0% diet was not significantly lower than the 0.5% diet, though it was also not significantly higher than the control diet indicating that this diet may have some improvement on the viability, though cholesterol concentrations may not have been high enough to create significance. As in chapter 3, the precipitated yolk showed increased viability, which is not to be expected and would need to be determined with further research.

The viability findings in this study are similar to those demonstrated with CD/CHOL [5-7, 12-14] which support that additional cholesterol can be introduced to the sperm membrane resulting in improved post-thaw motility and viability. This study also confirms that enriching yolks with cholesterol is possible through dietary means. Considering the lack of effect on motility with the yolks used in this study, there is a need for further examination of these yolks to determine if a great effect could be achieved.

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Chapter 5: Conclusions

In the search for a method of sperm membrane cholesterol enrichment, the results of this study were inconclusive in regards to CD/CHOL. In initial experiments examining CD/CHOL the only promise of treatment was in cold shock experiments where CD/CHOL treated samples and control samples were statistically similar. In further experiments, which included precipitated yolk and lower yolk levels in order to try and eliminate cholesterol loss to lipid droplets as well abnormally high control values from the first study, inconclusive results were also observed. No positive effects of CD/CHOL were observed other than cold shock protection. This experiment also found atypical effects of precipitated yolk for both post-thaw motility and viability, as well as for 2.5% yolk for post-thaw viability.

Experiments looking at high cholesterol egg yolks, however, showed much more promise in combating cooling and freezing effects. Enrichment of the yolks was quite successful as eggs from hens fed a diet supplemented with 1.0% cholesterol contained significantly more cholesterol in their yolks as compared to eggs from hens on the conventional diet. As for the effects of these yolks, not much significance was seen for either pre-freeze or post-thaw motility between diet treatment groups, though post-thaw viability did show effects of the high cholesterol yolks. Samples treated with 2.5% whole yolk enriched with 1.0% cholesterol showed significant improvement over almost all treatments as did samples treated with 10% precipitated yolk enriched with 0.5% cholesterol. Additionally, all samples made with enriched yolks were not significantly different from these above-mentioned treatments even if their viability values were not high enough to be deemed significant.

As with the CD/CHOL work, there were a few abnormal results observed with 2.5% yolk as well as with precipitated yolk. Samples treated with 2.5% control yolks showed significantly higher viability when looking at whole yolk and

10% precipitated yolk had significantly higher viability than 10% whole yolk. These findings, in combination with the unusual results seen in the CD/CHOL studies, bring up a need for further research using both of these methods of membrane cholesterol enrichment. This study examined yolk levels and types that have not typically been examined [1-6] in cholesterol enriched studies, and therefore, these abnormal results cannot be confirmed or refuted. Further research would have to be done on how both CD/CHOL and cholesterol enriched yolks behave when used in a more broad array of extender preparations in order to better understand the findings in this study.

In addition, considering the promise of cholesterol enriched yolks, further research needs to done not only on the usage of these yolks, but also on the dietary supplementation. Previous research showed a much greater increase in cholesterol using only 0.5% added cholesterol [7], which indicates that this study was unable to optimize the enrichment. Addition feeding trials may be able to fine-tune the production of these yolks.

Even though this study was unable to duplicate results from previous reports utilizing CD/CHOL [1-6], dietary supplementation appears to have promise, and with some continued research, may be found an effective alternative for enriching sperm membranes with cholesterol.

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