Research Report

Cholesterol protects stallion sperm from cold shock but egg yolk is necessary for optimal cryopreservation

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Abstract

Membrane cholesterol affects sperm membrane fluidity and stability at low temperatures. Sperm from species with high cholesterol:phospholipid ratios (human and rabbit) are resistant to cooling damage whereas equine sperm with low cholesterol:phospholipid ratios (~ 0.3) are sensitive to cooling and freezing damage. Cholesterol-loaded cyclodextrins (CLC) improve sperm cryosurvival; however, cholesterol-treated sperm have always been diluted with egg yolk or milk-based cryopreservation media that also protect sperm from cooling damage. Therefore, cholesterol's effect on mitigating cooling or freezing damage remains unclear. We examined how CLC affect stallion sperm membrane cholesterol composition and whether cholesterol alone can mitigate cold shock and cryopreservation damage. Stallion sperm treated with CLC (0-1.8 mg) were subjected to cold shock (0 °C) or cryopreservation, with or without egg yolk, using 1 or 2 step cryopreservation protocols; CLC increased sperm cholesterol:phospholipid ratios 2.7 fold. After cold shock, sperm motility was lower ($p < 0.05$) in control (8%) than CLC-treated sperm (39-65%), and CLC maintained high motility in cold shocked sperm, similar to fresh sperm. Sperm motility was similar for sperm cryopreserved in medium with egg yolk in either 1 or 2 step cryopreservation protocols. Although CLC alone provided modest cryopreservation protection, the combination of CLC and egg yolk resulted in greater motility and viability ($p < 0.05$) than either CLC or egg yolk alone. Furthermore, the cholesterol:phospholipid ratio that protected sperm from cold shock damage varied widely among stallions (0.33-0.79). In conclusion, adding cholesterol to sperm membrane eliminated cold shock damage but egg yolk provided additional cryoprotection, particularly at low CLC concentrations.

Keywords: Equine semen, membrane composition, cold shock, cholesterol-loaded cyclodextrins, membrane cholesterol, cholesterol:phospholipid ratio, cryosurvival

Introduction

Cryopreserved sperm use has expanded globally in a wide variety of species, enabling the distribution of unique genetics across distant regions. However, cryopreservation induces structural and functional damage to sperm, resulting in reduced cell viability, longevity, and fertility, compared to fresh sperm.[1] At least part of this damage occurs when sperm are cooled from room temperature (~ 22 °C) to 5 °C; when stallion sperm membranes undergo a phase transition from the fluid to the gel phase.[2,3] Membrane changes, including lipid loss[2,4] and lipid/protein rearrangements[3] that occur during this phase transition are at least partly responsible for the loss of sperm viability, longevity, and fertility, after cooling and/or cryopreservation.[1] However, adding egg yolk or milk low-density lipoproteins to sperm and cooling them slowly to 5 °C largely mitigates these membrane changes.[2,3]

Cholesterol has a key role in regulating membrane fluidity, particularly at low temperatures. In model membranes, addition of cholesterol lowers the temperature at which the gel-phase transition occurs, and with sufficient cholesterol, this transition can be eliminated.[5] Cholesterol has been added to the plasma membrane of several cell types,[6,7] including sperm (reviewed[8]) to improve cell function at low temperatures. In addition, sperm that naturally possess high cholesterol:phospholipid ratios (human and rabbit sperm) do not exhibit the cooling damage that occurs in sperm with lower cholesterol concentrations (most other mammalian species).[9]

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Cholesterol-loaded cyclodextrins (CLC) have been used extensively to increase cholesterol content and improve cryosurvival rates of sperm in many domestic[8] and nondomestic[10,11] species. However, how cholesterol protects sperm from cold shock or cryopreservation damage is not completely understood. This is partially due to the fact that after cholesterol addition, the treated sperm have always been diluted into cryopreservation media containing egg yolk or milk, before cooling and freezing.[10-19] Therefore, even though cholesterol-treated sperm survive cryopreservation better than non-treated sperm, the protective effects of the low-density lipoproteins in egg yolk and milk-based cryopreservation media likely mask at least some effects of cholesterol on sperm survival during cooling and freezing.

We tested 2 primary hypotheses: 1. there is an optimal cholesterol:phospholipid ratio at which stallion sperm maintain membrane integrity when they are cold shocked or cryopreserved and 2. stallion sperm that contain sufficient cholesterol survive cooling in ice water or cryopreservation without egg yolk. Experiments were conducted to determine: 1. cholesterol:phospholipid ratios of stallion sperm at various concentrations of CLC addition; 2. sperm viability after exposure to 0°C water to induce cold shock; 3. if cholesterol alone protects stallion sperm during cryopreservation without low-density lipoproteins; and 4. whether the cryopreservation medium should be added to cholesterol-treated sperm using either 1 or 2 step protocols.

Materials and methods

Semen collection and initial preparation

Stallion sperm were chosen because, unlike other species, stallions have not undergone intensive selection for sperm cryosurvival; therefore, stallions have a wide range of sperm cryosurvival rates.[20] Ejaculates were collected from 11 stallions (5-28 years), representing various breeds (Quarter Horse, Irish Draft, and grade breeds), following a routine semen collection schedule (3 times per week for at least 2 weeks prior to the collection used). Semen was collected using a Colorado model artificial vagina with an in-line gel filter (Animal Reproduction Systems, Chino, CA, USA). Each ejaculate was diluted 1:1 (volume/volume) with a modified Whitten's medium (MWM) that contained NaCl 100 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 5.5 mM, Na-pyruvate 1 mM, Na-lactate 4.8 mM, HEPES 22 mM, and BSA 0.3% with a pH 7.2. Diluted samples were transported (at room temperature) to the laboratory, where processing occurred within 1 hour. All procedures were conducted in accordance with the guidelines approved by the Colorado State University Animal Care and Use Committee (Protocol number 5747).

On arrival at the laboratory, ejaculates were centrifuged at 600 x g for 7.5 minutes at room temperature, supernatant was removed, sperm were resuspended in MWM to a final volume of ~ 7 ml; and sperm concentration was determined using a densimeter (Model 590a Densimeter, Animal Reproduction Systems).

Cyclodextrin preparation

Cholesterol loaded-cyclodextrins (CLC) were prepared as described.[21] Briefly, 200 mg cholesterol was dissolved in 1 ml chloroform in a glass tube. In a second glass tube, 1 mg methyl-β-cyclodextrin was dissolved in 2 ml methanol. Then 0.45 ml of the cholesterol solution was added to the cyclodextrin solution and the mixture stirred until the solution became clear. This solution was then poured into a glass petri dish and the solvent was removed using a stream of nitrogen gas; resulting crystals were allowed to dry for 24 hours and were scraped from the dish and stored in a glass container at 22°C until use.

To add cholesterol to sperm, a working solution of CLC was prepared by adding 50 mg of CLC in 1 ml MWM and mixing using a vortex mixer. The solution was maintained at 37°C until needed. Prior to removing aliquots for sperm addition, the CLC working solution was mixed using a vortex mixer.[12]

Preparation of Percoll gradient

A 90% Percoll solution was prepared by diluting 1 volume of 3 M phosphate buffer saline with 9 volumes of Percoll and a 45% Percoll solution was then prepared by diluting 90% Percoll 1:1 (volume:volume) with MWM.

Sperm analysis

Motility assessment

Sperm motion characteristics were assessed using a Computer-Assisted Sperm Analysis (CASA) system (Sperm Vision Therio, Minitube, Tiefenbach, Germany). Seven fields per sample were evaluated, with 30 frames collected per field at a rate of 60 frames per second. Sperm were identified based on a head length of 3-6 µm and width of 2-4 µm. Percentage of both total motile and progressively motile sperm were recorded, with progressive motility defined as sperm moving at velocities > 5 µm/second. For analysis, a 6 µl aliquot from each sample was placed on a preheated (37°C) Cell-Vu microscope slide (Millennium Sciences, New York, USA), and a minimum of 400 sperm per sample were evaluated.

Subjective motility assessments (percentage of total motile sperm) were performed by a single observer using a phase contrast microscope equipped with a slide warmer set at 37°C and an attached television monitor (final magnification, x 200).

Assessment of sperm plasma membrane integrity by flow cytometry

Percentages of sperm with intact plasma membrane were determined by flow cytometry, using a modified procedure.[22] Briefly, after thawing, sperm in 0.5 ml were stained with SYTO-17 (2 µl of a 0.2 mM solution in water) and PI (2.0 µl of a 2.4 mM solution in water) for 10 minutes prior to analysis by flow cytometry. Samples were then diluted to 3 x 10^6 sperm/ml with MWM and 40,000 sperm were analyzed to determine plasma membrane integrity, using a BD Accuri C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Propidium iodide fluorescence was detected with a 610 nm long pass filter and SYTO-17 fluorescence detected with a 675 nm band pass filter. This protocol resulted in all sperm staining with SYTO-17 and allowed sperm to be distinguished from egg yolk particles that do not stain with SYTO-17.[23] Each sperm could then be categorized as having an intact plasma membrane (without PI) or having a damaged plasma membrane (staining with PI) as described.[21]

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Sperm lipid analyses

To remove unincorporated cholesterol in the medium from sperm, samples (control and CLC-treated) were layered on top of 45% Percoll solution and centrifuged at 600 x g for 25 minutes. The supernatant was discarded, the sperm pellet was resuspended in 1 ml MWM and stored at –20°C until sperm lipids were extracted. In addition to removing unincorporated cholesterol, this step also removed any remaining seminal plasma proteins and lipids, and many nonviable and abnormal sperm. This resulted in a subpopulation of the original ejaculate that was assayed and utilized in subsequent steps.

Lipids were extracted from sperm using chloroform-methanol (2:1; volume:volume) as described.[24] Briefly, sperm samples were thawed and mixed with 10 ml chloroform-methanol (2:1; volume:volume) and stored at 5°C for a minimum of 24 hours. Each sample was then filtered through a fat-free ashless filter disk (Whatman 1541-125), and 5 ml water was added to each sample. Samples were then mixed using a vortex mixer and each sample held at 5°C for 24 hours to allow the solution to separate into 2 phases. The upper phase, containing nonlipid contaminants, was removed and discarded. The remaining chloroform phase, containing lipids, was then evaporated under a stream of nitrogen gas.

Lipids in each sample were resuspended in 3 ml of 1% acetic acid in chloroform. Subsamples were removed to spectrophotometrically quantify the phospholipid content as described[25] and the cholesterol content as described.[26] The cholesterol:phospholipid (C:PL) ratio for each sample was then determined.

Experimental design

Experiment 1. Determine the amount of cholesterol incorporated into stallion sperm

Ejaculates, from 11 stallions, having 65-80% total motile sperm (determined by CASA) were centrifuged as described above, and split into 7 subsamples. Subsamples were treated with 0-1.8 mg CLC/120 x 10^6 sperm (8 ejaculates were treated with 0, 0.6, 0.8, 1.0, 1.2, 1.4, or 1.8 mg, and the last 3 ejaculates were treated with 0, 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 mg CLC, to focus on lower CLC concentrations) and sperm were incubated at 22°C for 15 minutes. During incubation, the percentage of motile sperm for the control sample (0 mg CLC) was evaluated visually. Following incubation, 4 aliquots were removed from each subsample to evaluate the ability of sperm to withstand damage from cold shock and cryopreservation, and the remainder of each subsample was processed for lipid analysis.

Experiment 2. Determine the cholesterol content that maximizes sperm survival after cold shock

To evaluate the effect of cholesterol to protect stallion sperm from cold shock damage, 100 µl aliquots from each subsample, described above, were plunged into ice water (0°C) for 30 minutes to induce cold shock damage. Each aliquot was then diluted to 30 x 10^6 sperm/ml with MWM at 37°C and percentage of motile sperm was assessed visually and by CASA.

Experiment 3. Determine the effect of CLC treatment on sperm cryopreservation

Sufficient sperm from each CLC-treated subsample (generally ~ 100 µl, for each), of 6 stallions made in Experiment 1, were removed and diluted in cryopreservation medium. Three freezing methods were tested. Sperm were cryopreserved using a 1 step freezing protocol in lactose EDTA containing 20% egg yolk (LEDTA) cryopreservation medium with 5% glycerol[12] to create 2 ml samples with 30 x 10^6 sperm/ml. Sperm were also frozen using a 2 step protocol, in which sperm were initially diluted to 1 ml at 60 x 10^6 sperm/ml in LEDTA with no glycerol; and then diluted 1:1 (volume:volume) with LEDTA with 10% glycerol. Finally, sperm were frozen using a 1 step protocol by diluting the sperm in MWM containing 5% glycerol to 2 ml at 30 x 10^6 sperm/ml.

After dilution in cryopreservation medium, sperm were packaged in 0.5 ml straws and placed in a 13 x 11 cm rectangular plastic box containing 300 ml water at 20°C; box was placed in a 5°C cold-room, and sperm were cooled to 5°C for 2 hours. On reaching 5°C, straws were frozen in liquid nitrogen vapor, 4.5 cm above liquid nitrogen, for 15 minutes. Straws were then plunged into liquid nitrogen and stored at –196°C for a minimum of 24 hours before thawing and assessment. Straws were thawed in 37°C water for 30 seconds and samples were assessed for percentage of motile sperm (visually and CASA) and for percentage of sperm with intact plasma membranes (flow cytometry).

Data analyses

For Experiment 1, differences among the C:PL ratios of sperm samples treated with various concentrations of CLC, were compared with analysis of variance.[27] Regression analysis was used to determine the relationship between CLC addition and the C:PL ratio of the sperm.

For Experiments 2 and 3, percentage data were transformed by arcsine and treatment (amount of CLC added) differences for the percentage of motile sperm (assessed visually and CASA) and sperm with intact plasma membranes were determined using analysis of variance and treatment means separated by Student-Newman-Keuls test.[27] Differences between cryopreservation techniques (cold shocked sperm versus sperm cryopreserved in MWM; sperm cryopreserved without egg yolk versus sperm cryopreserved with egg yolk) were determined by paired comparisons after blocking by CLC added. Values presented in the figures represent the means and standard errors for each treatment. Treatments were considered different at $p < 0.05$.

For each stallion, polynomial regression analysis was performed to determine how CLC treatment affected sperm C:PL ratio determined in Experiment 1.[27] In addition, regression analysis was used to determine the relationship between CLC concentration and percentage of motile sperm (total motility determined by CASA) or sperm with intact plasma membrane. First derivatives of these equations were solved to determine the CLC concentration that maximized the percentage of motile or viable sperm, after cold shock and after cryopreservation. The C:PL ratio for sperm associated with maximum cell survival for cold shock and cryopreservation survival, for each stallion, was then determined using each stallion's regression analysis. In addition, differences among sperm C:PL ratios for each stallion that provided the maximum percentage of motile sperm after cold shock and after cryopreservation with and without egg yolk were determined by analysis of variance and the means separated by Student-Newman-Keuls test.[27]

Finally, paired t-tests were used to compare the initial percentage of motile sperm (assessed visually) to the percentage of motile sperm after treatment with 1.2 mg CLC/120 x 10^6 sperm, followed by either cold shock or cryopreservation.[27]

Results

Experiment 1

Untreated stallion sperm evaluated in this experiment had a C:PL ratio of 0.3 ± 0.1. When sperm were treated with increasing amounts of CLC, the C:PL ratio increased, reaching a maximum C:PL ratio of ~ 0.8 ± 0.2 with > 1.2 mg CLC/120 x 10^6 sperm (Figure 1). However, individual C:PL ratios for control sperm from these stallions ranged from 0.112-0.585 and sperm maintained wide variations in C:PL ratios when CLC was added (Figure 1). Therefore, C:PL ratios were only an upward trend with increasing CLC.

Experiment 2

After sperm were cold shocked, percentage of motile sperm was lower ($p < 0.05$) for untreated sperm than for sperm treated with any CLC concentration, regardless of whether motility was evaluated as the percentage of total motile sperm (evaluated subjectively or with CASA) or progressively motile sperm (Figure 2). For all concentrations of CLC, percentages of motile sperm were similar regardless of the evaluation method used.

Experiment 3

Percentage of motile sperm was similar for stallion sperm cryopreserved in LEDTA using 1 or 2 step cryopreservation protocols, when no cholesterol was added (mean total motilities and SEMs were 45 ± 7 and 43 ± 7, respectively) and at all concentrations of CLC addition ($p > 0.05$; data available on request). Stallion sperm frozen in LEDTA without CLC had 45 ± 7% total motility (measured by CASA) and although not different, sperm treated with 1.4 mg CLC had 62 ± 8% total motility (Figure 3). Sperm frozen without egg yolk or CLC had only 18 ± 5% motile sperm ($p < 0.05$) and unlike egg yolk, adding 0.2 mg CLC did not benefit sperm motility. However, adding > 0.4 mg CLC resulted in a modest benefit to sperm, for sperm frozen without egg yolk.

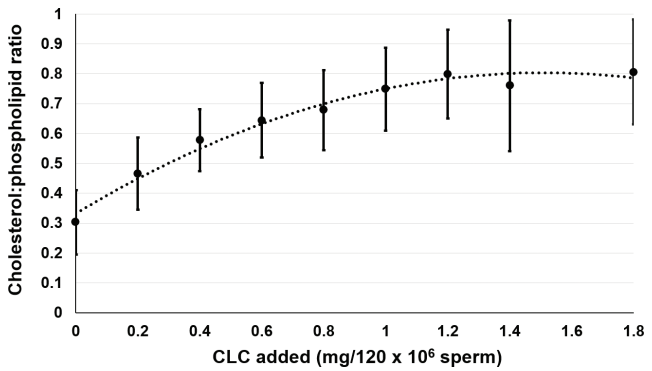

Figure 1. Cholesterol:phospholipid ratios of stallion sperm after treatment with various concentrations of cholesterol-loaded cyclodextrins (CLC)

Sperm viability (Figure 4) was similar to sperm motility (Figure 3) when stallion sperm were cryopreserved with egg yolk, but was very low for sperm cryopreserved with only CLC. In addition, percentages of viable sperm frozen without egg yolk were lower ($p < 0.05$) than those for sperm frozen with egg yolk at every CLC concentration (Figure 4).

Higher C:PL ratios were required to maximize sperm motility ($p < 0.05$) when sperm were cryopreserved without egg yolk than when sperm were frozen with egg yolk (Figure 5).

A C:PL ratio of ~ 0.8 provided maximal survival for stallion sperm diluted in only MWM when cold shocked or cryopreserved (Figure 4). A C:PL of 0.8 was achieved when stallion sperm were treated with 1.2 mg CLC/120 x 10^6 sperm (Figure 1). Comparisons were made among the initial motility of samples and the motility of samples treated with 1.2 mg CLC/120 x 10^6 sperm, and after they were cold shocked and cryopreserved. Percentage of motile sperm, determined visually was similar for sperm prior to cooling (70 ± 3%) and for sperm treated with 1.2 mg CLC/120 x 10^6 sperm and then cold shocked (64 ± 4%). However, percentage of motile sperm initially (70 ± 3%) was higher ($p < 0.05$) than the percentage of motile sperm after sperm had been treated with 1.2 mg CLC/120 x 10^6 sperm and cryopreserved without egg yolk (35 ± 15%).

Discussion

Using cholesterol-loaded cyclodextrins we increased the C:PL ratio ~ 2.7 fold, similar to stallion[12] or bull sperm[21] after CLC treatment. Similarly, methyl-β-cyclodextrins delivered comparable concentrations of cholesterol enrichment to various cell types.[28] Although the overall increase in membrane cholesterol was similar in these studies, we used smaller increments in the CLC treatment concentrations to determine more precisely the cholesterol concentration that protects stallion sperm from cold-shock and freezing damage.

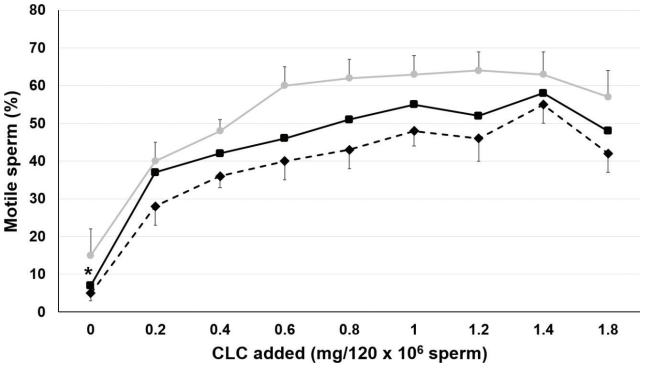

Figure 2. Percentage of motile sperm (total motile assessed visually ●—●, total motile assessed by CASA ■—■, progressively motile assessed by CASA ♦- - -♦) after sperm had been treated with 0-1.8 mg CLC, then plunged into 0 °C water for 30 minutes and diluted in 37 °C MWM prior to assessment. Sperm treated without CLC exhibited lower ($p < 0.05$) sperm motility for all measurements than sperm treated with CLC

*difference between the percentage of total motility determined subjectively versus CASA ($p < 0.05$)

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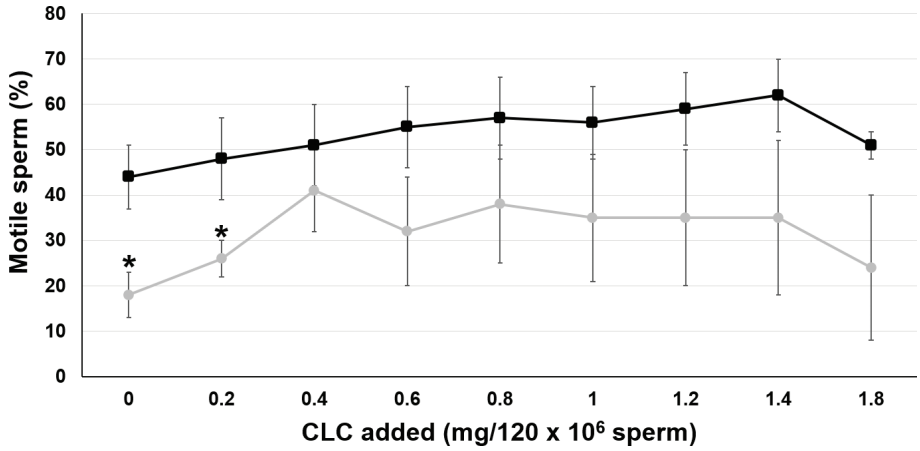

Figure 3. Percentage of total motile stallion sperm, determined by CASA, after sperm were treated with 0-1.8 mg of CLC/120 x 10^6 sperm, and cryopreserved in either the presence of egg yolk (■—■) or absence of egg yolk (●—●)
*indicate differences between egg yolk treatments

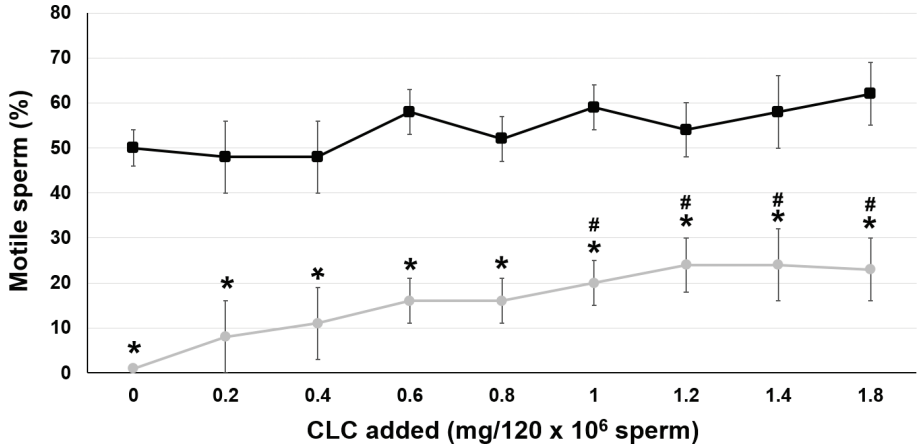

Figure 4. Percentage of viable stallion sperm after sperm were treated with 0-1.8mg of CLC/120 x 10^6 sperm and then cryopreserved in either with egg yolk (■—■) or without egg yolk (●—●)
*indicate differences between egg yolk treatments
#indicates mean of CLC treated sperm is different from control (no CLC) sperm

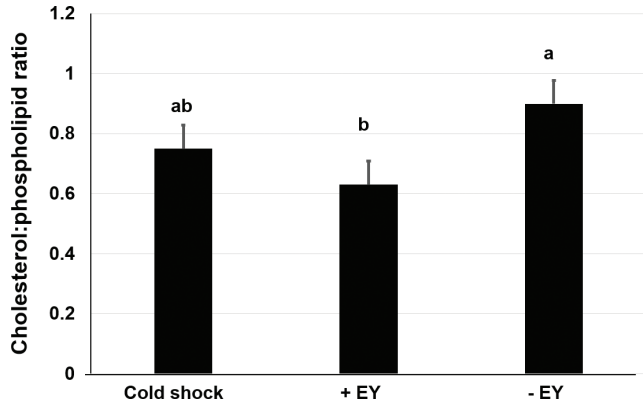

Figure 5. Cholesterol:phospholipid ratio that maximized the percentage of motile stallion sperm after cold shocking the sperm in ice water or after cryopreservation with egg yolk (+EY) or without egg yolk (-EY)
[a,b]different superscripts denote differences between treatments

Increasing cholesterol content of stallion sperm enabled sperm to survive cold shock (Figure 2). This would be expected, if cold shock damage occurs due to membrane damage caused by rearrangement of membrane components when the plasma membrane undergoes a phase change from a fluid membrane to a gel membrane;[1,3] and if by adding sufficient cholesterol that phase change is eliminated.[5]

Similarly, cryopreserved bull and goat sperm treated with CLC had similar survival rates as conventionally frozen untreated sperm, even when sperm were cryopreserved directly from room temperature, without first cooling slowly to 5 °C.[29] These data indicated that increasing cholesterol content of bull and goat sperm enabled sperm to avoid cold shock damage. These findings supported our observation that increasing membrane cholesterol helps stallion sperm resist cold shock damage.

Previous studies with CLC treatment had beneficial effects for sperm cooled to 5 °C. However, in every study in which CLC-treated sperm have been cryopreserved, the treated sperm were subsequently diluted in cryopreservation diluents that contained either egg yolk[10-15] or milk[16-19] prior to cooling and cryopreserving. Since egg yolk and milk contain low-density lipoproteins (LDLs) that also ameliorate damage to sperm when they are cooled to 5 °C, it is difficult to separate the beneficial effects of cholesterol from the beneficial effects of the LDLs. In addition, cooling sperm slowly to 5 °C also helped sperm membrane to maintain normal structure, likely in combination with the effects of LDLs.[30] In the current study, if sufficient cholesterol was added to stallion sperm membrane, we could essentially eliminate the membrane damage that occurs due to cooling to 5 °C.

Addition of CLC to sperm prior to cryopreservation in an egg yolk freezing medium increased motile sperm percentage from 45% (0 CLC) to ~ 60% (0.8-1.2 mg CLC), representing a > 130% increase in the number of motile sperm surviving cryopreservation similar to what has been reported for stallion,[12,31] bull,[21,29,32] ram,[33] and goat[18,21] sperm. Similarly, CLC addition resulted in 2 fold increase in the percentage of motile sperm surviving cryopreservation (40 versus 18%; Figure 3), when sperm were frozen without egg yolk. In addition, when 0.2 mg CLC was added to sperm, samples frozen with cholesterol alone had fewer ($p < 0.05$) motile sperm than samples frozen with both cholesterol and egg yolk (Figure 3). However, at higher CLC concentrations, differences between cholesterol alone and cholesterol with egg yolk treatments were eliminated, although there was a trend for sperm frozen with only cholesterol to have lower motility than similarly treated sperm frozen with egg yolk. This indicated that when the cholesterol content of stallion sperm membrane increased, sperm more efficiently survived cryopreservation and cold shock. It should be noted, however, that the addition of egg yolk to sperm provided additional protection to sperm during cryopreservation than CLC addition alone (Figure 3).

This phenomenon was observed more dramatically when the effects of CLC and egg yolk on sperm viability (Figure 4) were examined. Although sperm viability and motility are generally highly correlated,[34] in this study, the samples used to evaluate motility and viability were treated very differently for these 2 assays. Sperm motility was evaluated directly from the frozen-thawed samples, as the sperm were frozen at a concentration (30 x 10^6 sperm/ml) suitable for visual assessment or CASA analysis. In contrast, flow cytometric analysis required diluting the samples to 2-5 x 10^6 sperm/ml. Therefore, sperm determined to be viable by flow cytometry not only needed to survive cryopreservation but also needed to withstand the osmotic stress associated with a 10-fold dilution in a medium without cryoprotectant. This dilution induced sperm swelling due to intracellular glycerol.[1,3] Comparing results from Figures 3 and 4, it is apparent that sperm treated with egg yolk were not severely affected by this dilution, as percentages of motile and viable sperm were similar. However, sperm cryopreserved with only CLC did not handle the osmotic swelling that occurs to sperm when they were diluted. This was particularly evident at the very low CLC concentrations. We inferred that cholesterol enrichment alone was not sufficient to protect sperm from osmotic damage during postthaw handling.

Stallion sperm are usually frozen using 1 step cryopreservation protocol, in which centrifuged sperm are simply diluted to the desired sperm concentration in the cryopreservation medium.[1] In contrast, sperm from many other species are frozen using 2 step protocol, in which the sperm are diluted twice to the desired sperm concentration, first in cryopreservation medium without cryoprotectant and then diluted 1:1 (volume:volume) with cryopreservation medium containing twice the desired concentration of cryoprotectant. Using a 2 step protocol, both the sperm concentration and the cryoprotectant concentration were exactly what was desired. Using the 1 step protocol, sperm concentration was exactly what was desired but the cryoprotectant concentration was slightly lower than what was added to cryopreservation medium. We tested both protocols with CLC-treated sperm but sperm survival rates were exactly similar. Canine sperm cryopreserved equally well using both methods[35,36] whereas bull sperm survived cryopreservation better using the 2 step protocol.[37] An issue arises when a 1 step protocol is used for samples with low initial sperm concentrations, as the sperm volume added to the cryopreservation medium dilutes the cryoprotectant to concentrations sufficiently low that optimum sperm cryosurvival is not achieved. However, for samples with high sperm concentrations, the sperm volume added to the cryopreservation medium is sufficiently small that changes in cryoprotectant concentration are very limited. Since we centrifuged the sperm to remove seminal plasma and unincorporated cholesterol, in this study, sperm concentration was sufficiently high that the small sperm volumes added to the 1 step cryopreservation medium did not alter the cryoprotectant concentration very much. Therefore, both protocols provided similar cryosurvival rates.

Adding cholesterol increased membrane fluidity at low temperatures.[5,38] In addition, adding cholesterol increased membrane permeability to cryoprotectants and water which increased membrane osmotic tolerance.[31,39,40] Therefore, the poor viability of frozen-thawed sperm treated with cholesterol alone was quite unexpected. Although cholesterol can have a major effect on plasma membrane fluidity, it also had a major impact on membrane organization and protein function,[41-43] including ion channels[42,44] that could affect ability of sperm to react to anisosmotic conditions after cryopreservation and subsequent dilution.

Amount of CLC that provided optimal cryosurvival for sperm varies among species and is at least partially correlated with the initial C:PL sperm ratio[33] and likely sperm membrane total lipid composition.[45] We hypothesized that the membrane composition of stallion sperm would be sufficiently

similar and that sperm from various stallions would survive cold shock at a similar C:PL ratio. However, this was not the case. Although the average C:PL ratio to maximize sperm survival from cold shock was ~ 0.75 (Figure 5), the individual C:PL ratios maximizing cold shock survival, for the stallions tested, ranged from 0.33-0.79. Therefore, even within a single species, sperm from various males differed in lipid and protein composition and had variable responses to a given treatment.

Although CLC were effective in protecting stallion sperm from cold shock damage (Figure 2) they were less effective in preserving sperm from cryodamage. Addition of egg yolk to CLC-treated sperm had an additive effect on sperm cryosurvival. It has been established that the main components in egg yolk or milk that benefited sperm during cryopreservation are low-density lipoproteins;[30] however, the mechanisms by which they protect sperm are still unknown. One hypothesis was that the LDLs adhere to the membrane during the cryopreservation process, preserving membrane structure.[46-48] If so, it is probable that the mechanisms by which CLC and LDLs protect sperm are different, and therefore their effects would be additive (Figures 3 and 4). It also appeared that during the cryopreservation process, maintaining the membrane structure, including protein-protein associations, was more important than simply maintaining a fluid membrane.

To achieve maximal cryosurvival using cholesterol alone, one would theoretically need a C:PL ratio of ~ 0.9 (Figure 5). This value was calculated using regression analysis for the total motility determined by CASA for individual stallions. However, even at the highest CLC concentrations, we only achieved a C:PL ratio of 0.8 in stallion sperm membrane. Interestingly, when egg yolk was added, the C:PL ratio that provided maximal cryopreservation was only ~ 0.6 ($p < 0.05$), again indicating that cholesterol and LDL have different mechanisms to preserve sperm plasma membrane from cryodamage.

Since increasing cholesterol content of stallion sperm membranes was very effective in preserving sperm membranes from cold shock but was less effective in preserving membranes from cryodamage, cooling membranes to < 0 °C must induce severe changes in lipid composition and/or protein structure that did not occur when the membranes are cooled to 0 °C. Additional studies are needed to determine what specific membrane changes occur when sperm membrane is cooled to < 0 °C.

In conclusion, our results demonstrated that increasing cholesterol content of stallion sperm to a C:PL ratio of ~ 0.75, ameliorated cold shock damage. However, elevated membrane cholesterol concentrations alone did not protect sperm from cryodamage unless egg yolk was added to the system. Therefore, it appeared that the LDL in egg yolk helped to maintain membrane structure that increased membrane fluidity at low temperatures.

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Authors' contribution statement and agreement

MC: conceptualization, methodology, investigation, validation, data curation, writing original draft; **PR:** conceptualization, methodology, investigation, data curation, writing, reviewing, editing; and **JG:** conceptualization funding acquisition, methodology, investigation, supervision, project administration, formal analysis, data curation, writing, reviewing, editing. Authors have read and approved the final submission.

Conflict of interest

None to declare.

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