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Effect of Adding Cholesterol to Bull Sperm Membranes on Sperm Capacitation, the Acrosome Reaction, and Fertility[1]

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ABSTRACT

When cholesterol is added to sperm membranes before cryopreservation, higher percentages of motile and viable cells are recovered after thawing. However, because one of the first steps in sperm capacitation is cholesterol efflux from the sperm plasma membrane, adding cholesterol to enhance cryosurvival may retard sperm capacitation. These studies evaluated the ability of sperm treated with cholesterol-loaded cyclodextrins (CLC) to capacitate, acrosome react, and fertilize oocytes. Control (non-CLC-treated) and CLC-treated sperm were treated with heparin, dilauroylphosphatidylcholine (PC12), or calcium ionophore A23187 (A23187) to capacitate and induce the acrosome reaction. Sperm capacitation, assessed by an increase in intracellular calcium level, and acrosome-reacted sperm were measured using flow cytometry. Fresh CLC-treated sperm cells underwent capacitation and/or the acrosome reaction at rates different from control samples, and the differences detected were dependent on the method used to induce sperm capacitation and the acrosome reaction. After cryopreservation, however, CLC-treated and control sperm underwent capacitation and the acrosome reaction at similar rates regardless of the method used to induce capacitation and the acrosome reaction. The primary concern for CLC-treated sperm, however, is whether this treatment would affect in vitro or in vivo fertility. Adding either control or CLC-treated cryopreserved sperm to bovine oocytes in vitro resulted in similar oocyte cleavage rates and blastocyst formation rates. In addition, when inseminated into heifers, pregnancy rates for control and CLC-treated sperm were also similar. Therefore, treating bull sperm with CLC permits greater numbers of sperm to survive cryopreservation while preserving the fertilizing potential of each individual sperm.

acrosome reaction, gamete biology, sperm capacitation

INTRODUCTION

One of the initial steps in sperm capacitation is a loss of cholesterol from the plasma membrane [1, 2]. This cholesterol efflux induces plasma membrane lipid reorganization, ultimately increasing membrane permeability to Ca^{2+}, HCO_3^- and K^+ [3]. High intracellular concentrations of these ions are required for a spermatozoon to undergo the acrosome reaction [4] as well as fuse with the oocyte [5]. If concentrations of any of these ions in the medium are too low, sperm capacitation and the acrosome reaction can be retarded, and excluding Ca^{2+} from the medium can inhibit these processes completely [3]. The rise of intracellular Ca^{2+} is important to the early stages of capacitation and precedes rises in intracellular cAMP production, protein phosphorylation, and pH [3]. Therefore, although the rise in intracellular Ca^{2+} does not encompass all of capacitation, it can be used to indicate whether capacitation in a particular cell has been initiated [4, 6, 7].

The time at which a particular sperm initiates capacitation and the acrosome reaction as well the rate at which a cell undergoes these phenomena depend in large part on a cell's membrane status and in particular on the amount of cholesterol contained in the plasma membrane. During capacitation, cholesterol is lost from the sperm plasma membrane, and when sufficient cholesterol is removed, the membrane becomes unstable, enhancing its ability to fuse with the outer acrosomal membrane, resulting in the acrosome reaction [7]. Sperm capacitation can be inhibited or the sperm even decapacitated by adding seminal plasma or lipid vesicles composed of synthetic phospholipid liposomes containing cholesterol to the sperm [8], as these prevent the loss of cholesterol from the membrane. Indeed, when cholesterol was added to the incubation medium, sperm from several species were inhibited from undergoing the acrosome reaction when treated with progesterone to induce the acrosome reaction [9, 10]. Therefore, added cholesterol does not need to incorporate into the sperm membranes to affect capacitation; but the mere presence of cholesterol in the medium can inhibit capacitation and the acrosome reaction [9]. On the other hand, if compounds that enhance cholesterol removal from sperm membranes are added to the sperm medium, such as BSA or cyclodextrins containing no cholesterol, sperm capacitation is enhanced [10].

Altering the lipid composition of sperm plasma membranes not only affects the ability of sperm to capacitate and acrosome react, it also affects the way sperm respond to cryopreservation. When cyclodextrins, cyclic oligosaccharides of glucose that contain a hydrophobic center capable of incorporating lipids [11], are preloaded with cholesterol (CLC) and then incubated with bull sperm before cryopreservation, higher percentages of motile and viable cells are recovered after freezing and thawing, compared with control sperm [12, 13]. This added cholesterol most likely benefits cells by eliminating or at least lowering the temperature at which the sperm plasma membranes undergo the lipid phase transition from the fluid to the gel state as the cells are cooled [14]. Similarly, adding liposomes composed of lipids from the plasma membrane of sperm heads as well as liposomes composed of defined phospholipids improved the cryosurvival (percentages of motile and viable sperm) of boar sperm compared with untreated sperm [15]. Of equal importance is that the lipid-treated boar sperm were capable of undergoing an acrosome reaction when treated with calcium ionophore A23187 [15].

He et al. [15] did not measure the lipid changes that occurred in boar sperm after adding liposomes to the cells.

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However, the level of CLCs added to bull sperm that optimized cryosurvival increased the cholesterol content of the sperm membranes 2–3 times that in native sperm [12]. Because the amount of cholesterol in sperm membranes is so important in maintaining membrane integrity and in the capacitation process, it is reasonable to expect this added cholesterol could affect sperm capacitation and the acrosome reaction. These studies were conducted to determine if increasing sperm membrane cholesterol levels, by adding CLCs to bull sperm, alter the ability of fresh and cryopreserved sperm to undergo capacitation and the acrosome reaction and the ability of cryopreserved sperm to fertilize oocytes in vitro and in vivo.

MATERIALS AND METHODS

Animals

Bulls were housed at the Animal Reproduction Laboratory, fed a diet providing 100% of their nutritional needs, and provided water ad libitum. All animal care and procedures used to collect semen were approved by the Animal Care and Use Committee of Colorado State University.

Cholesterol-Loaded Cyclodextrin Preparation

Methyl-β-cyclodextrin (Sigma Aldrich, St. Louis, MO) was loaded with cholesterol, as previously described by the authors [12]. Briefly, 200 mg of cholesterol was dissolved in 1 ml of chloroform. In a second tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol and 0.45 ml of the cholesterol solution added. The combined cyclodextrin/cholesterol solution was thoroughly mixed, and the solvents then removed using a stream of nitrogen gas. The resulting crystals were stored at 22°C until use. To add cholesterol to sperm, a solution of CLC was made by adding 50 mg of CLC to 1 ml TALP [7] at 37°C and mixing vigorously using a vortex mixer. The CLC working solution was mixed again, using a vortex mixer, before any aliquot was removed for addition to sperm.

Capacitating Agent Preparation

Liposomes composed of dilauroylphosphatidylcholine (PC12) were prepared as described by Nolan et al. [7]. Briefly, solvent was removed from PC12 (10 mg/ml in chloroform) using nitrogen gas and the lipids suspended in TALP medium. Liposomes were formed by extruding this solution through polycarbonate membranes possessing 0.1 μm pores (Lipex Biomembranes, Inc., Vancouver, BC, Canada). The liposomes were stored in 0.5-ml aliquots at −70°C until use. The phospholipid concentration of liposome preparation was determined using ammonium ferrothiocyanate [16].

A solution of calcium ionophore A23187 was prepared by diluting ionophore into dimethylsulfoxide (4.77 mM). This solution was stored at 5°C until use. Prior to adding to sperm, a working solution of A23187 (9.54 μM) was made by diluting the stock solution into TALP.

Solutions of heparin (2 mg/ml) and lysophosphatidylcholine (0.5 mg/ml) were prepared daily by dissolving these compounds in TALP.

Flow Cytometric Evaluation of Sperm

Intracellular calcium was determined using the fluorescent probe Indo1-AM (Molecular Probes, Eugene, OR) and measuring fluorescence intensities of calcium-free and calcium-bound Indo-1 AM using an Epics V flow cytometer (Coulter Electronics, Miami, FL) equipped with an ultraviolet laser tuned to 351 nm (100 mW) and a visible laser tuned to 488 nm (50 mW) with a 7-μsec delay between the beams. The flow cytometer set-up included a 408-nm laser blocking filter, a 440-nm short-pass filter to detect calcium-bound probe, and a 490-nm long-pass filter to detect calcium-free probe. The cells fluorescing positive for calcium-bound probe were considered the viable population because membrane-damaged cells do not maintain the enzymes necessary for Indo1-AM to bind calcium [17]. The ratios of the fluorescence signals (bound probe:free probe) were determined (Cyclops software; Cytomation, Fort Collins, CO), as described by Brewis et al. [17]. Spermatozoa having undergone the acrosome reaction were simultaneously determined using a 488-nm dichroic mirror, a 500-nm long-pass filter, and a 525-nm bandpass filter to detect cells staining positive for fluorescein-labeled lectin from the peanut plant, *Arachis hypogaea* (FITC-PNA), as described by Graham et al. [18].

Experiment 1: Effect of CLC Addition on Bull Sperm Capacitation and the Acrosome Reaction

Ejaculates were collected from 5 bulls using an artificial vagina, and 1-ml aliquots were diluted in separate 15-ml tubes 1:1 with TALP and centrifuged at 600 × *g* for 8 min. The supernatant was removed and the sperm pellet suspended with 1 ml TALP containing 10 mM calcium chloride [7]. The concentration of sperm in each sample was determined spectrophotometrically [19]. Spermatozoa to be analyzed without cryopreservation were diluted to 120×10^6 cells/ml in 1 ml of 10 mM Ca^{2+} TALP containing either 0 or 1.5 mg CLC and incubated at 23°C for 10 min before analysis.

Spermatozoa to be cryopreserved were resuspended to 120×10^6 cells/ml in 1 ml Tris buffered diluent (25 mM Tris, 8 mM citric acid, 7 mM glucose), treated with 0 (control) or 1.5 mg CLC and then frozen according to Purdy and Graham [12]. Briefly, control and CLC-treated samples were diluted 1:1 (v:v) with egg yolk Tris diluent (25 mM Tris, 8 mM citric acid, 7 mM glucose, 40% egg yolk) and cooled to 5°C over a 2-h period. After reaching 5°C, the samples were diluted 1:1 (v:v) with egg yolk Tris glycerol diluent (25 mM Tris, 8 mM citric acid, 7 mM glucose, 20% egg yolk, 14% glycerol) and allowed to equilibrate for 15 min. Following equilibration, the samples were packaged into 0.5-ml French straws and frozen in liquid nitrogen vapor (4.5 cm above the liquid nitrogen) for 12 min before being plunged into liquid nitrogen for storage. Straws (2 per treatment/bull) were thawed in a 37°C water bath for 30 sec and the samples diluted (1:1; v:v) with 1 ml of 10 mM Ca^{2+} TALP and centrifuged at 600 × *g* for 8 min. The supernatant was removed, and the sperm pellet was suspended at 120×10^6 cells/ml in 10 mM Ca^{2+} TALP.

Intracellular calcium levels were determined for both fresh and cryopreserved sperm as an indicator of sperm capacitation [17]. Sperm containing low intracellular calcium levels were considered uncapacitated, while sperm possessing high levels of intracellular calcium were considered to be either capacitated or starting to capacitate. To evaluate intracellular calcium, aliquots (100 μl) of sperm from each sample (fresh or frozen-thawed) were stained with Indo-1 AM (41.5 μl of 40 μM stock solution in water) and FITC-PNA (5 μl of 1 mg/ml in water) and incubated for 10 min. The sperm were then treated, in separate analyses, with PC12 liposomes, calcium ionophore A23187, or heparin in combination with lysophosphatidylcholine (heparin/LPC).

Spermatozoa to be treated with PC12 were diluted with 146 μl of the PC12 stock solution (60 μM PC12), resulting in a final PC12 concentration of 30 μM and final sperm concentration of 40×10^6 cells/ml. The samples were incubated at 39°C and subsamples removed at 5, 10, 15, 20, 25, and 30 min for analysis.

Spermatozoa capacitated with A23187 were treated with 30 μl of the A23187 working solution and an additional 824 μl of TALP, resulting in final A23187 and sperm concentrations of 286.2 nM and 12×10^6 cells/ml, respectively. The samples were incubated at 39°C and subsamples removed at 5, 10, 15, 20, 25, and 30 min for analysis.

Spermatozoa capacitated with heparin and LPC were first treated with heparin (5 μl of stock solution), resulting in final heparin and sperm concentrations of 60 μg/ml and 80×10^6 cells/ml, respectively. The samples were incubated at 39°C for 15, 45, 75, 105, 135, and 165 min, after which 24 μl of LPC was added (final LPC concentration of 70 μg/ml) and the sperm incubated an additional 15 min before analysis.

At the designated times, aliquots containing 1×10^6 cells were removed from each sample and diluted into 0.5 ml in TALP. The intracellular calcium level and acrosomal status for each of 50 000 sperm were then determined using flow cytometry, as described above.

Experiment 2: Effect of Cholesterol Addition to Bull Sperm on In Vitro Fertilizing Capacity

Semen from 5 bulls was diluted to 120×10^6 cells/ml in TALP and 1-ml samples were treated with either 0 or 1.5 mg of CLC and incubated at 23°C for 10 min. After incubation, samples were diluted with 1 ml of egg-yolk-TALP extender (5% egg yolk) and the samples cooled to 5°C over 2 h. Upon reaching 5°C, the samples were further diluted with 2 ml of egg-yolk-TALP diluent (2.5% egg yolk), supplemented with glycerol (14%), and allowed to equilibrate for 15 min. The samples were then packaged into 0.5-ml straws and frozen as described above.

Oocytes from slaughterhouse ovaries were aspirated from 2- to 6-mm follicles. Acceptable oocytes possessing a homogeneous cytoplasm and an intact, unexpanded complement of cumulus cells were washed twice in H-199 medium [20], placed into wells containing 0.5 ml Hepes-buffered TCM-199 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-treated estrus cow serum, 1 μg/ml of estradiol 17-β, 1 μg/ml of LH

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(NIH-oLH-26), and 0.05 μg/ml of FSH (NIH-FSH-S-17), and incubated at 39°C (5% CO_2) for 21–24 h to mature the oocytes [20]. Prior to fertilization, one straw containing control sperm and one straw containing CLC-treated sperm were thawed in a 37°C water bath for 30 sec, and the sperm washed by layering the cells on top of a 90/45% Percoll column and centrifuging for 20 min at 700 × *g*. The supernatant was removed, the sperm pellet suspended in 100 μl of CDM-1 [20], and the sperm concentration of each sample determined using an hemacytometer. The sperm were then diluted in CDM-1 to a final concentration of 200 × 10^6 motile cells/ml. Oocytes (20 per treatment) were removed from maturation medium, washed twice in H-CDM [20], and placed into 45-μl drops of CDM-1 under paraffin oil. Oocytes were inseminated with 5 μl of sperm sample, containing 1 × 10^6 motile sperm, either immediately after sperm centrifugation and dilution or after sperm had been incubated at 39°C for 60 min, to permit the sperm to capacitate before insemination. Sperm and oocytes were incubated together at 39°C in an atmosphere of 5% CO_2 for 18 h, after which the oocytes were removed from the drops, placed into 0.5-ml microcentrifuge tubes containing 50 μl H-CDM, and shaken using a vortex mixer for 60 sec to remove the cumulus cells. The contents of the tube were placed into a culture dish and each tube rinsed with H-CDM multiple times, until all oocytes were observed in the dish. The oocytes were washed three times in H-CDM before being placed in fresh 50-μl drops of CDM-2 [20] under paraffin oil for additional culture at 39°C (5% CO_2, 5% O_2, and 90% N_2). Resulting embryos were placed into fresh drops of CDM-2 every 48 h for 8 days [20]. Oocytes were observed for cleavage 72 h postfertilization and the number of embryos that developed to the blastocyst stage evaluated 8 days postfertilization.

Experiment 3: Effect of Cholesterol Addition to Bull Sperm on In Vivo Fertilizing Capacity

Sperm from three bulls were cryopreserved as described in experiment 1 except that, in this experiment, after sperm were treated with either 0 or 1.5 mg CLC and cooled to 5°C, they were diluted to a concentration of 3.0 × 10^6 cells/ml in egg yolk-Tris-glycerol diluent and packaged into 0.25-ml straws before freezing.

A total of 77 Holstein heifers had their reproductive cycles synchronized using two injections of prostaglandin $F_{2\alpha}$ (5 ml per treatment) 12 days apart. Animals were observed for estrus, starting 36 h after the second prostaglandin treatment and then again every 12 h later for the next 4 days. Heifers in estrus were inseminated using a single 0.25-ml straw (750 000 total sperm), 12–24 h after estrus detection, until 64 heifers (32 animals per treatment) were inseminated. Pregnancy was determined for each animal using ultrasound through the rectal wall 50 days postinsemination. A single technician performed all inseminations and palpations.

Statistical Analysis

Percentage data for sperm possessing high intracellular calcium, for sperm having undergone an acrosome reaction, and for viable cells were transformed using arcsine before analysis. Regression analysis determined the rate at which cells possessing high intracellular calcium, having undergone an acrosome reaction, and lost viability changed over time [21]. Paired *t*-tests were used to determine if the slopes of the control (0 mg) and CLC-treated cells were different ($P < 0.05$) for each of the capacitating agents (PC12, A23187, and heparin/LPC) [21]. Differences between samples at time zero and samples at other time periods, within CLC-treatment, were determined by ANOVA [21].

Treatment differences between control and CLC-treated sperm, for cleavage and blastocyst rates, were determined by ANOVA [21].

Chi-square was used to determine differences in pregnancy rate for control and CLC-treated sperm [21].

RESULTS

Experiment 1

Addition of CLC to sperm resulted in fewer fresh sperm accumulating high levels of intracellular calcium after challenge with PC12 or A23187 compared with control sperm ($P < 0.05$; Fig. 1). Both control and CLC-treated sperm responded similarly; however, when the sperm were treated with heparin followed by LPC ($P > 0.05$; Fig. 1a). Addition of CLC to sperm reduced the rate at which fresh CLC-treated sperm underwent an acrosome reaction compared with control sperm, when cells were treated with heparin

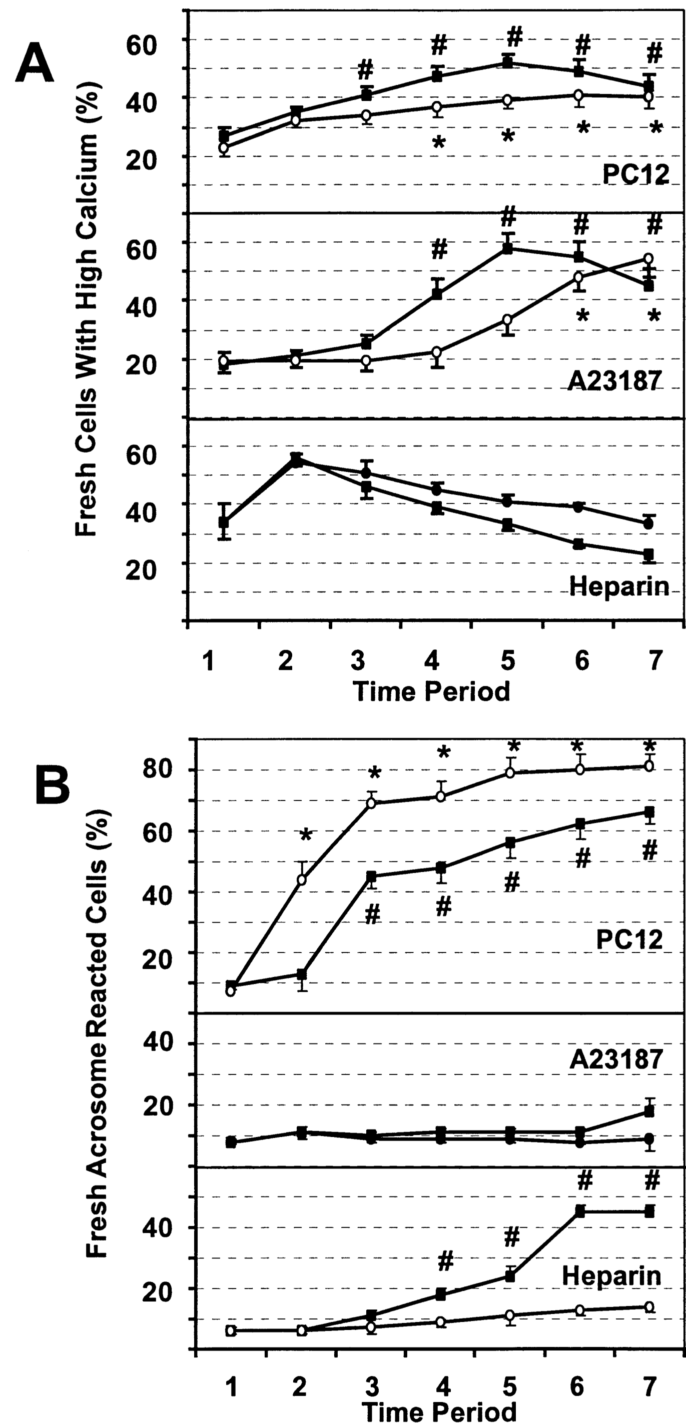

FIG. 1. Percentages of bull sperm, from five bulls, with high intracellular calcium levels (**A**) and live sperm having undergone an acrosome reaction (**B**), when fresh control sperm (black squares) and CLC-treated sperm (black circles) were subsequently treated with PC12 (PC12), ionophore A23187 (A23187), or the combination of heparin followed by lysophosphatidylcholine (Heparin) and incubated at 39°C to capacitate the sperm and induce the acrosome reaction. Sperm were assayed seven different times after capacitation treatments were initiated. Sperm treated with PC12 and A23187 were evaluated at 0, 5, 10, 15, 20, 25, and 30 min; sperm treated with Heparin were evaluated at 0, 30, 60, 90, 120, 150, and 180 min. Open circles indicate that CLC-treated sperm reacted differently than control sperm within a specific capacitating treatment ($P <$ 0.05). Superscripts or subscripts indicate that control (#) or CLC-treated (★) samples are different from similarly treated samples at Time 0 ($P <$ 0.05).

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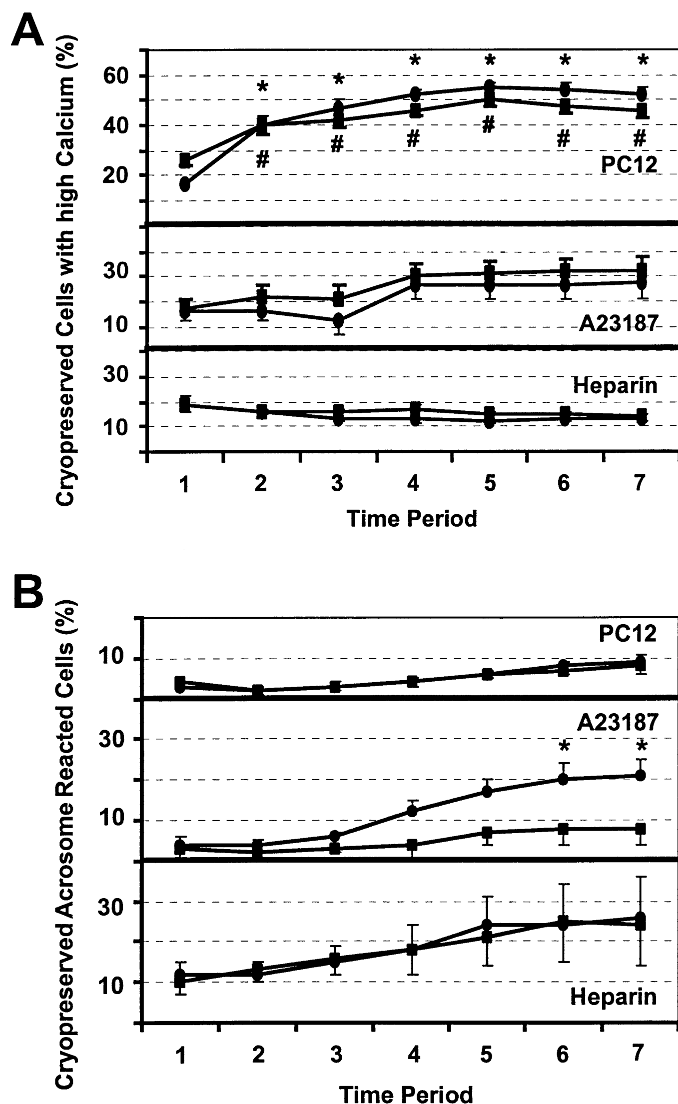

FIG. 2. Percentages of bull sperm, from five bulls, with high intracellular calcium levels (**A**) and live sperm having undergone an acrosome reaction (**B**), when cryopreserved control sperm (black squares) and CLC-treated sperm (black circles) were subsequently treated with PC12 (PC12), ionophore A23187 (A23187), or the combination of heparin followed by lysophosphatidylcholine (Heparin) and incubated at 39°C, to capacitate the sperm and induce the acrosome reaction. Sperm were assayed seven different times after capacitation treatments were initiated. Sperm treated with PC12 and A23187 were evaluated at 0, 5, 10, 15, 20, 25, and 30 min; sperm treated with Heparin were evaluated at 0, 30, 60, 90, 120, 150, and 180 min. Superscripts or subscripts indicate that control (#) or CLC-treated (★) samples are different from similarly treated samples at Time 0 ($P < 0.05$).

($P < 0.05$; Fig. 1b). However, CLC-treated sperm exhibited higher percentages of acrosome-reacted sperm and the cells acrosome reacted earlier than did control sperm, when treated with PC12 ($P < 0.05$; Fig. 1b). The percentages of viable sperm ranged from 89% to 96% across treatments and time periods ($P > 0.05$).

After cryopreservation, CLC-treated sperm and control spermatozoa exhibited similar percentages of cells with high intracellular calcium levels ($P > 0.05$; Fig. 2a) and similar percentages of acrosome-reacted cells ($P > 0.05$; Fig. 2b) within each capacitating treatment. The percentages of viable sperm ranged from 63% to 78% across treatments and time periods ($P > 0.05$).

TABLE 1. The percentages of in vitro-matured bovine oocytes that cleaved and developed to the blastocyst stage when inseminated with 1 million motile sperm, after sperm from three bulls were treated with CLC (cholesterol-loaded cyclodextrins) and cryopreserved. Sperm were inseminated immediately after thawing or after 60 min of incubation at 39°C to permit sperm capacitation.

Treatment	Insemination time (min)	Cleaved (%)	Number of oocytes	% Blastocyst/cleaved oocyte
Control	0	48	127	25
CLC	0	46	140	26
Control	60	48	93	13
CLC	60	34	82	18

Experiment 2

When equal numbers of motile cryopreserved CLC-treated or control sperm were added to in vitro-matured bovine oocytes, similar percentages of oocytes cleaved and similar percentages of embryos developed to the blastocyst stage regardless of whether the sperm were inseminated immediately after preparation or whether they were incubated for 60 min before insemination ($P > 0.05$; Table 1).

Experiment 3

When an equal number of total cryopreserved sperm were artificially inseminated into synchronized heifers, 59% of the heifers inseminated with CLC-treated sperm became pregnant and 50% of the heifers inseminated with control sperm became pregnant ($P > 0.05$). Fertility rates of the three bulls used were not different ($P > 0.05$).

DISCUSSION

Mammalian sperm capacitation is a complex process that is characterized by cholesterol efflux from the plasma membrane, increased intracellular calcium, and an activation of protein phosphorylation cascades, all ultimately leading to the acrosome reaction [3, 22]. Many methods to capacitate sperm in vitro have been reported; however, for these experiments, we focused on only three. These three methods were chosen because each affects the sperm cell very differently, which we hoped would provide insight as to how additional cholesterol in the sperm membrane would affect sperm capacitation in vivo. Furthermore, there are many ways of measuring capacitation, but we chose to use the previously-documented rise in intracellular calcium [4, 17, 23], as it is one of the very early steps in capacitation and this rise can be easily measured using flow cytometry [17, 24].

The calcium ionophore A23187 induces the acrosome reaction by incorporating into the sperm cell plasma membrane and transporting calcium across the plasma membrane, resulting in high intracellular calcium concentrations necessary for sperm capacitation and the acrosome reaction [23]. Addition of PC12 liposomes to sperm results in PC12 monomers rapidly incorporating into the outer leaflet of the plasma membrane [7]. The short fatty acyl chains of PC12 remodel this leaflet, inducing membrane fusion (acrosome reaction) in the absence of true capacitation [7]. Initial sperm contact with heparin, followed by treatment with lysophosphatidylcholine, induces capacitation and the acrosome reaction in sperm by displacing sperm surface proteins, reorganizing membrane lipid domains, and increasing intracellular calcium levels in a way probably very similar to in vivo methods [25, 26].

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The PC12 treatment led to higher percentages of cells with high intracellular calcium levels and higher percentages of acrosome-reacted sperm 10 min after treatment in fresh control sperm compared with CLC-treated sperm. These results are similar to data reported previously [7, 27, 28]. It was anticipated that increasing the cholesterol content of the plasma membrane would retard sperm capacitation and the acrosome reaction. As expected then, CLC-treated sperm samples treated with PC12 exhibited fewer cells possessing high intracellular calcium levels than control sperm samples. However, PC12 induced more CLC-treated sperm to undergo an acrosome reaction than control sperm. Although capacitation, measured by increased intracellular calcium, was suppressed by cholesterol addition in this system, this did not delay the ability of PC12 to induce the membrane fusion required for the acrosome reaction. Yanagimachi [29] and Wooding [30] reported that various detergents can induce sperm to undergo an acrosome reaction in uncapacitated sperm. It is likely that PC12, possessing fatty acyl chains the same length (12 carbons) as sodium dodecyl sulfate, emulsifies the sperm plasma membranes sufficiently to permit fusion of the plasma membrane with the outer acrosomal membrane regardless of the capacitation status of the cell. Why added cholesterol enhances the effect of PC12 is not understood. Perhaps the cholesterol reorganizes the membrane lipids in such a manner that permits greater access or incorporation of the PC12 into the membrane, but further research is required to determine the interactions between membrane cholesterol and PC12. Similarly, the capacitation of fresh CLC-treated sperm was initially retarded, compared with control sperm, when sperm were challenged with the ionophore A23187 (Fig. 1); however, few sperm underwent an acrosome reaction, regardless of CLC-treatment.

Unlike PC12 or ionophore-treated sperm, the percentages of capacitated sperm were similar, regardless of CLC treatment, when sperm were challenged with heparin in combination with LPC. However, control samples exhibited higher percentages of acrosome-reacted sperm than CLC-treated samples (20% vs. 10%, respectively) 60 min following treatment with heparin and LPC, and this difference continued throughout the time sperm were evaluated. The percentages of acrosome-reacted cells achieved using heparin in combination with LPC in this study are similar in magnitude to, although occurred slightly earlier than, those reported by Parrish et al. [31, 32]. The reason for this is likely due to differences in the amount of heparin to which sperm were exposed. Parrish et al. [31] incubated 50×10^6 sperm with 10 μg of heparin, while in the current experiment, 80×10^6 sperm were incubated with 60 μg of heparin. Incubating the sperm with this higher amount of heparin likely reduced the time necessary for the acrosome reaction to occur. Regardless of the compound used to challenge fresh sperm, adding cholesterol to the sperm membranes using CLC technology retarded either sperm capacitation or the acrosome reaction or both processes.

After cryopreservation, CLC-treated and control sperm responded similarly to each other when treated with each capacitating agent. Although there are few reports of investigations into the capacitation of frozen-thawed bull sperm, our results are similar to those reported previously [33]. What is more interesting, however, are the reasons that cryopreserved CLC-treated sperm and control sperm act similarly when treated with capacitating agents, when fresh sperm do not. We believe that the CLC treatment altered the capacitation and acrosome-reaction rates of fresh samples by decapacitating the sperm; specifically, by increasing the cholesterol content in the plasma membranes of the treated sperm cells [8]. A possible explanation for the results observed with the cryopreserved cells is that cryopreservation alters the sperm membranes in ways that enhance sperm capacitation and the acrosome reaction that added cholesterol does not affect. Some of these membrane changes result from lipid-protein rearrangements within the membrane as cells are cooled [34], and some are due to the loss of lipids from the membrane during cryopreservation [35], all of which result in a rather homogeneous population of precapacitated sperm after thawing [36]. Therefore, whereas in vitro fertilization techniques using fresh sperm require strategies to capacitate the sperm, cryopreserved sperm require little or no capacitation before in vitro fertilization [25]. Additional support for such a hypothesis comes from in vitro-fertilization data collected in experiment 2, in which both control and CLC-treated sperm fertilized similar percentages of oocytes and no additional capacitation time was required for CLC-treated sperm.

The cleavage and development rates for in vitro-fertilized oocytes obtained in these studies are similar to those previously reported [20]. What is perhaps more interesting, however, is that, when equal numbers of motile sperm were added to oocytes, equal numbers of oocytes were fertilized and developed to the blastocyst stage. In addition, as mentioned above, the time required for sperm capacitation was the same for CLC-treated and control sperm. Therefore, on an individual motile sperm basis, CLC-treated and control sperm are equivalent, both in their fertilizing capability and time required for capacitation. This is important, as it indicates that CLC-treated sperm can be used for in vitro and potentially in vivo inseminations without having to modify current protocols.

Indeed, when CLC-treated sperm were used for artificial insemination using standard breeding procedures, pregnancy rates were similar to control sperm. The intent of the fertility trial conducted was not to differentiate between the fertility of control and CLC-treated sperm, but to determine if CLC-treated sperm could fertilize oocytes in vivo. With this objective in mind, we chose an insemination dose of 750 000 total sperm. This was an extrapolation from fertility rates achieved when inseminating heifers with low sperm numbers [37, 38] that was anticipated to result in a 50% pregnancy rate (G.E. Seidel Jr., personal communication), and this was exactly the pregnancy rate achieved. Although the number of heifers inseminated in each treatment group was too small to detect a difference between the control (50%) and CLC-treated sperm (59%), the trend is at least in the desired direction, suggesting that the increased number of sperm that survive freezing after CLC treatment may improve fertility rates or permit lower numbers of sperm to be used in an insemination dose without reducing fertility. However, a large fertility trial is required to determine if this is true.

In conclusion, increasing the cholesterol content of sperm cells to enhance cryosurvival retards the rates at which fresh sperm undergo capacitation and the acrosome reaction, but not the rates that cryopreserved sperm undergo capacitation or the acrosome reaction. After freezing and thawing, CLC-treated sperm undergo capacitation and the acrosome reaction at rates similar to control sperm and fertilize oocytes in vitro and in vivo similarly well. Therefore, adding cholesterol to sperm membranes using CLC technology may prove very useful in enhancing the cryosur-

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vival of bull sperm and perhaps sperm from other species as well.

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