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Effect of Cholesterol-loaded-cyclodextrin in Presence and Absence of Egg Yolk during Freezing Step on Quality of Markhoz Buck's Spermatozoa

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ABSTRACT : Cryopreservation protocols induce partially irreversible damage to mammalian sperm plasma membranes. Previous studies have indicated that adding cholesterol to the plasma membrane, as cholesterol-loaded-cyclodextrins, improves cryosurvival of sperm. Therefore, the purpose of this study was to determine if treating sperm of Markhoz bucks with cholesterol-loaded-cyclodextrins (CLC) (0, 0.75, 1.5, 2.25 and 3 mg/ml diluted 240×10^6 sperm/ml) in Tris-citric acid-glucose diluents with and without egg yolk (containing 5% glycerol) would improve the post-thaw sperm quality. The motion characteristics were evaluated with a Computer Assisted System Analyzer (CASA); acrosome integrity and vitality were measured with the triple-stain technique. Samples were recovered before and after freezing by means of putting straws into 37°C water for 30 sec and then parameters were assessed. The results showed that the treatments significantly affected motility, progressive motility, recovery rate, curvilinear velocity, beat cross frequency, live sperm with reacted acrosome, live sperm with unreacted acrosome, dead sperm with reacted acrosorne, and dead sperm with unreacted acrosome during freezing ($p<0.05$). However; no significant differences were found for average path velocity, straight line velocity, amplitude of lateral head displacement, straightness and linearity ($p>0.05$). The best results were observed for extender containing 2.25 mg/ml (240×10^6 sperm/ml) CLC supplemented with 2.6% egg yolk. In conclusion, the findings of this study indicate improved Markhoz sperm viability and motility following treatment in the presence of egg yolk. (**Key Words :** Markhoz Goat, Cholesterol, Cyclodexterin, Cryopreservation, Spermatozoa, Buck)

INTRODUCTION

Cryopreservation induces partially irreversible damage to sperm membranes, which decrease the quality of sperm after freezing and thawing (Moore et al., 2005). During this process, spermatozoa experience both cold shock and freezing damage that results in loss of motility, osmotic changes across the membrane and lipid-protein reorganization within the cell membranes (Drobnis et al., 1993; Bailey et al., 2000; Purdy and Graham, 2005). It has been suggested that the sperm plasma membrane is the primary site of damage induced by cryopreservation and is one of the main reasons for reduced motility and fertility of sperm during cryopreservation (Chakrabarty et al., 2007), and that the damage occurs when cell membranes undergo the transition from liquid crystalline to a gel phase (Darin-Bennett and White, 1977). Drobnis et al. (1993) reported that cold shock resulted in the plasma membrane undergoing a lipid phase transition during the cooling process and was inversely correlated with the proportion of cholesterol within the plasma membrane. Also, cryopreservation induces cholesterol depletion from the membrane, which in turn causes membrane destabilization (Bailey et al., 2008). Based on these observations, it has been suggested that cholesterol plays an important role in regulating fluidity and stability (Langlais et al., 1988; Purdy and Graham, 2005) and can easily be incorporated into or extracted from the plasma membrane of cells using cyclodextrin (Moore et al., 2005). It was concluded, when plasma membrane exposed to low temperatures, that addition of sufficient levels of liposome to sperm plasma membrane increased its cryoresistance and it did not undergo a phase transition (Parks et al., 1981; Wilhelm et al., 1996).

Numerous studies indicated that treatment of

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spermatozoa with cholesterol-loaded-cyclodextrin (CLC) increased cholesterol content in the plasma membrane and consequently improved the resistant capacity of sperm to cryoinjury due to cold shock and freezing damage (Klein et al., 1995; Zahn et al., 2002; Purdy and Graham, 2004a; Moore et al., 2005; Purdy and Graham, 2005; Moca and Graham, 2006). Furthermore, cholesterol increased the membrane integrity (Klein et al., 1995; Zahn et al., 2002), caused more sperm binding to the zona pellucida of oocytes after cryopreservation (Da Silva, 2006), improved the rate of water permeability, mediated the freezing-induced loss of intracellular water or water transport (Li et al., 2006; Walter et al., 2008), and decreased the osmotic stress (Walter et al., 2008). However, the exact mechanism by which added cholesterol interacts with the cell membrane and protects spermatozoa remains to be elucidated. There are suggestions that cholesterol added to a biological membrane locates within the cell membrane (Purdy and Graham, 2004a) and increases membrane hydrophobicity, and therby spermatozoa acquire the potential to resist cryoinjury (Chakrabarty et al., 2007). Under cryopreservation conditions, it is possible that sperm cells have the capability to preferentially discard hydrophilic lipids from the cell membrane and also absorb specific lipid components from the surrounding medium, thereby yielding modulated cells with markedly higher membrane hydrophobicity that confers cryoresistance potential (Chakrabarty et al., 2007).

On the other hand, differences among species in the sensitivity of their sperm to cooling are largely attributable to compositional variations of the spermatozoa plasma membrane (Bailey et al., 2000). Therefore, sperm cells have different inherent resistance toward cryopreservation (Chakrabarty et al., 2007). For sperm of species known for higher resistance against cold susceptibility, the cholesterol content is high (rabbit and human) whereas plasma membranes of the bull, ram and boar possess lower cholesterol levels, which are considered to be sensitive (Bailey et al., 2000). However, there is little available information about the interaction of cholesterol with plasma membrane on the motility and viability of goat sperm during the freezing and thawing process. Therefore, the goal of this study was to determine if supplementing goat sperm with cholesterol using cyclodextrin as a carrier molecule in association with egg yolk, was an important membrane stabilization factor during the freezing process that would improve cryosurvival and post-thaw sperm quality.

MATERIALS AND METHODS

Chemical reagents

The following chemicals were used: methyl-β-cyclodextrin, cholesterol, Rose Bengal, and Trypan Blue from Sigma-Aldrich Company (St. Louis, MO) and tris (hydroxymethyl) aminomethane, glucose, citric acid, glycerol, Bismarck Brown (Vesuvine), glutaraldehyde, chloroform and methanol were obtained from Merck (Darmstadt, Germany).

Preparation of cholesterol loading methyl-β-cyclodextrin

Cholesterol loading methyl-β-cyclodextrin (CLC) was prepared as described by Purdy and Graham (2004a). Briefly, to load cholesterol into the methyl-β-cyclodextrin, 500 mg of methyl-β-cyclodextrin was dissolved in 1 ml of methanol in a glass test tube. In a second glass test tube, 200 mg of cholesterol was dissolved in 1 ml of chloroform. An aliquot of 0.225 ml of cholesterol solution was combined with the methyl-β-cyclodextrin solution, stirred until the combined solution became clear, after which the mixture was poured into a glass petri dish and the solvents removed using a stream of nitrogen gas. The resulting crystals were stored in a glass vial at 22°C until used. To add cholesterol to sperm, a working solution of the cholesterol-loaded methyl-β-cyclodextrin was prepared by adding 50 mg of CLC to 1 ml TALP at 37°C and the solution mixed briefly using a vortex mixer.

Semen collection, evaluation, freezing and thawing

This study was conducted at the animal husbandry station of the Agricultural College of the University of Kurdistan, located in Sanandaj, Iran at 35° 20' N latitude and 47°E longitude, Semen from mature Iranian Markhoz (Angora) bucks (*Capra hircus*) was collected twice weekly from four fertile males using an artificial vagina. Immediately after collection, the fresh semen was transported to the laboratory and kept in a water bath at 37°C. Ejaculates were evaluated for their initial quality before every experiment. The qualified ejaculates from all four bucks were pooled to obtain sufficient semen volume, re-examined for quality, then divided into 10 equal volumes (2×5 groups) and diluted (1:10) with extender in four steps. In the first step, all treatments were diluted with Tris (3.786%)-citric acid (2.172%)-fructose (1%) without egg yolk. Then different CLC solutions (0, 0.75, 1.5, 2.25 and 3 mg/ml for 240×10^6 spermatozoa) were added to the ten treatments and incubated for 15 min. In the third step, 2.6% egg yolk (final concentration) was added to five treatments. In the last step, 5% glycerol (final concentration) was added to all ten treatments. The diluted semen was packaged into 0.5 ml French straws, the open end of the filled straws was sealed with polyvinyl chloride powder, and then cooled to 5°C within 3.5 h. Before freezing, one straw from each sample was recovered in a water bath at 37°C for 30 s for analysis of motion characteristics at time 0. The sperm were then frozen in static liquid nitrogen vapor (4.5 cm above the

liquid nitrogen) for 10 min, before being plunged into liquid nitrogen for storage. Prior to analysis, straws were thawed by placing them into a 37°C water bath for 30 s.

Sperm motility assessment

The sperm motion characteristics (Table 1) were determined using a computer-assisted sperm motility analysis (CASA) system (VideoTesT, ltd: version Sperm 2.1© 1990, 2004, Russia). For measurement of total motile sperm in a minimum of 200 cells, at least five areas of the slide were used with the following parameters: frames acquired, 50; minimum contrast, 20; minimum cell size, 6; threshold straightness, 60; medium VAP cut-off, 60; low VAP cut-off, 25; low VSL cut-off, 10; non-motile head size, 17; non-motile head intensity, 70; magnification, 1.95; static size limits, 0.46-2.47; static intensity, 1.28; and static elongation limits, 5-60. Moreover, recovery rate was computed by the following formula;

Recovery rate
= (Motility after freezing/Motility before freezing)×100

Assessment of acrosome integrity and vitality

Acrosome reaction and viability were measured by a modified triple-stain technique described by Didion and Graves (1986). Briefly, a 200 ml aliquot of the sperm suspension was mixed 1:1 with 2% trypan blue and incubated in a centrifuge tube for 13 min at 37°C. The sperm suspension was diluted to 400 ml with physiological serum (removal of excess stain) and centrifuged at 800×g for 6.5 min. This washing step was repeated (usually two times) until the supernatant was clear or pale blue. Sperm were subsequently fixed for 15 min in 3% glutaraldehye in phosphate buffer (pH 7.4). Then, the sample was washed in physiological serum similarly to the first washing step, placed on slides and air-dried. Finally, the smears were stained for 1 min in 0.8% Bismark brown (pH 2.0) in water at 40°C and rinsed again in water. The smears were then stained for 12 min in 0.8% Rose Bengal in 0.1 M Tris buffer (pH 5.3 in 25°C), rinsed in water, and placed under a coverslip. After staining, slides were microscopically examined (1,000×) and spermatozoa were classified into the following four categories; 1- live sperm with unreacted acrosome (LUR), 2- live sperm with reacted acrosome (LAR), 3- dead sperm with unreacted acrosome (DUR) and 4- dead sperm with reacted acrosome (DAR). Two-hundred cells were counted on a slide of each replication in a treatment. To validate the triple-stain technique, Iborra and et al. (2000) reported that there were no differences in the percentages of acrosome reaction as measured by the triple-stain technique, transmission electron microscopy and lectin pisum sativum-fluorescein isothiocyanate technique.

Table 1. Sperm motion characteristics according to user guide VideoTesT, ltd: version Sperm 2.1© 1990 .2004, Russia

Parameter	Abbreviation	Unit
Motility	MOT	%
Progressive motility	PMT	%
Recovery rate*	REC	%
Average path velocity	VAP	micron/s
Straight line velocity	VSL	micron/s
Curvilinear velocity	VCL	micron/s
Amplitude of lateral head displacement	ALH	micron
Beat cross frequency	BCF	Hertz
Straightness	STR	%
Linearity	LIN	%

Statistical analysis

We designed this study with 6 replications of the treatments and the data were analyzed by a factorial arrangement of 2×4 treatments using the mixed procedure of SAS (Statistical Analysis System, Institute Inc., 2001) to determine the effects of CLC and egg yolk. Comparison of treatment means for main effects was performed by Duncan's multiple range test. All data are given as means with pooled SEM.

RESULTS

Sperm motion parameters

The effect of 0 and 2.6% egg yolk concentrations on motion parameters (motility, progressive motility and recovery rate) of Markhoz goat sperm after freezing and thawing are shown in Figure 1 (n = 6). The results showed that diluents containing 2.6% egg yolk were, in regard to sperm motion parameters, significantly better than extender without egg yolk ($p<0.05$). In addition, the results of different CLC concentrations (0, 075, 1.5, 2.25 and 3 mg/ml for 240×10^6 spermatozoa) showed that an increasing concentration of CLC significantly increased ($p<0.05$) motility, progressive motility and recovery rate of frozen-thawed sperm (Figure 2). Furthermore, as shown in Figure 2, the concentration of 2.25 mg/ml CLC for 240×10^6 spermatozoa was significantly better ($p<0.05$) than other levels of CLC. Results for the interaction between CLC and egg yolk are presented in Table 2. With regard to motility, progressive motility and recovery rate, extenders containing different concentrations of CLC and 2.6% egg yolk were significantly better ($p<0.05$) than diluents without egg yolk. Furthermore, the motion parameters for diluents containing 1.5 and 2.25 mg/ml CLC for 240×10^6 spermatozoa and 2.6% egg yolk were significantly ($p<0.05$) higher than at other CLC levels, but there were no significant differences

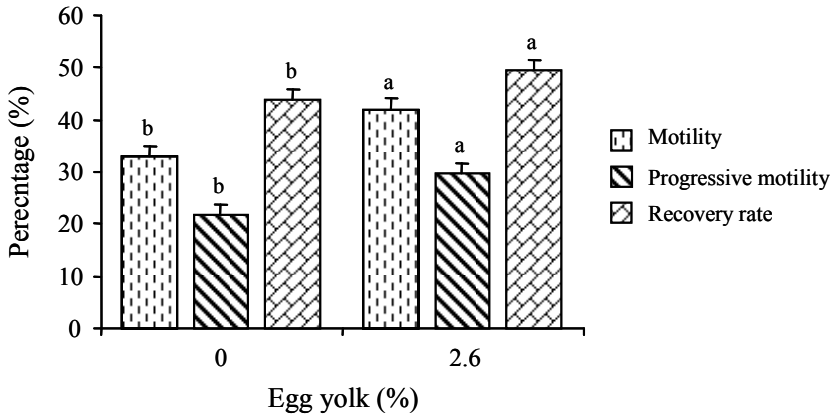

Figure 1. Effect of egg yolk on the mean percentages of motion parameters in pooled buck's semen after freezing-thawing (n = 6). Different letters indicate significant differences between means of the two treatments at $p<0.05$.

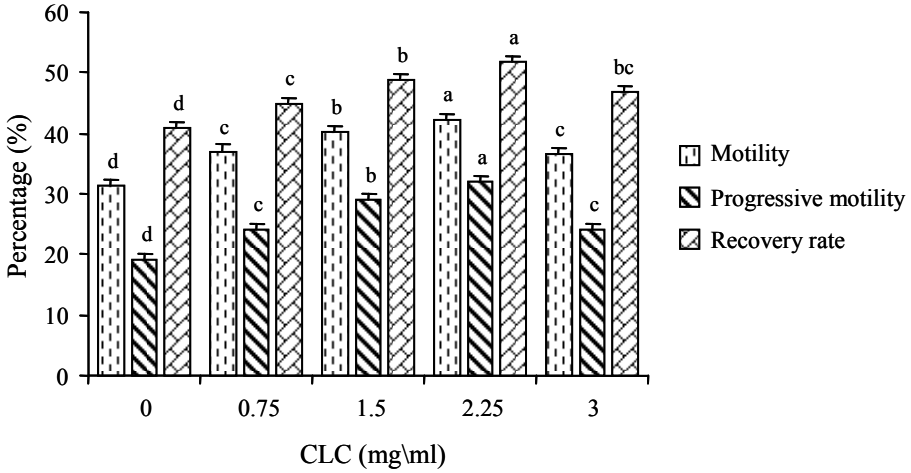

Figure 2. Effect of CLC concentration on the mean percentages of motion parameters in pooled buck's semen after freezing and thawing (n = 6). Different letters indicate significant differences between means of the treatments at $p<0.05$.

Table 2. The effect of egg yolk on the mean percentages of kinematical parameters in buck's semen after freezing-thawing (n = 6)

Egg yolk (%)	0	2.6	SEM
VAP (micron/s)	36.14	36.14	0.35
VSL (micron/s)	31.93	32.43	0.34
VCL (micron/s)	59.71	60.21	0.11
ALH (micron)	2.15	2.25	0.05
BCF (Herz)	8.52^{b}	8.73^{a}	0.06
STR (%)	88.52	89.75	0.58
LIN (%)	53.75	54.12	0.65

Different superscript letters indicate significant differences between means of the two treatments at $p<0.05$. SEM indicates standard error of the mean.

Table 3. Effects of CLC concentration with egg yolk on the mean percentages of motion parameters in buck's semen after freezing-thawing (n = 6)

Egg yolk (%)	0					2.6					SEM
CLC (mg/ml)	0	0.75	1.5	2.25	3	0	0.75	1.5	2.25	3	
Motility (%)	25.3^{g}	33.7^{ef}	35.8^{ed}	37.9^{cd}	31.6^{f}	37.2^{de}	40.4^{bc}	44.8^{a}	46.5^{a}	41.4^{b}	1.17
Progressive motility (%)	14.2^{fe}	21.4^{de}	25.1^{c}	28.3^{b}	20.5^{e}	24.5^{cd}	27.2^{bc}	33.0^{a}	36.1^{a}	28.1^{b}	0.87
Recovery rate (%)	35.2^{d}	43.7^{c}	45.9^{bc}	50.9^{a}	44.3^{c}	46.4^{b}	46.5^{b}	51.4^{a}	53.4^{a}	49.6^{ab}	0.73

Different superscript letters indicate significant differences between means of the treatments at p<0.05. SEM indicates standard error of the mean.

Table 4. Effect of CLC concentration on the mean percentages of kinematical parameters in buck's semen after freezing-thawing (n = 6)

CLC (mg/ml)	0	0.75	1.5	2.25	3	SEM
VAP (micron/s)	35.42^{ab}	36.31^{ab}	37.26^{a}	37.17^{a}	34.46^{b}	0.33
VSL (micron/s)	32.12	32.13	33.03	32.75	30.97	0.35
VCL (micron/s)	60.63^{a}	60.35^{a}	60.13^{ab}	59.23^{b}	59.5^{ab}	0.12
ALH (micron)	2.15	2.16	2.14	2.17	2.44	0.06
BCF (Herz)	8.51	8.53	8.56	8.86	8.63	0.05
STR (%)	90.73	88.43	88.72	87.81	89.72	0.59
LIN (%)	53.32	53.42	55.36	55.32	52.01	0.67

Different superscript letters indicate significant differences between means of the treatments at p<0.05. SEM indicates standard error of the mean.

(p>0.05) between 1.5 and 2.25 mg/ml CLC. However, the results for diluent containing 1.5 mg/ml CLC were slightly lower than extender with 2.25 mg/ml CLC.

Sperm kinetic parameters

According to Table 3, the results showed that extenders containing 2.6% egg yolk were slightly better than than those without egg yolk. In addition, extenders containing different CLC concentrations showed (Table 4) no significant differences (p>0.05) for sperm kinetic parameters obtained after freezing and thawing, except VAP and VCL (p<0.05). However, the highest values for VAP were observed in extender with 1.5 mg/ml CLC concentration (37.2 micron/sec) and VCL in diluents with 0, 0.75 and 1.5 mg/ml CLC concentrations (60.6, 60.3 and 60.1 micron/sec, respectively). Concerning CLC and egg yolk concentrations, the results presented in Table 5 showed no significant differences (p>0.05) between the effects of CLC and egg yolk concentrations on kinetic parameters after freezing and thawing of sperm. A significant difference was obtained for VCL (p<0.05) but, apart from this result, the results of CLC extenders containing 2.6% egg yolk were slightly better than other groups.

Acrosome integrity and vitality

The effect of 0 and 2.6% egg yolk concentrations on acrosome integrity and vitality of Markhoz goat sperm after freezing and thawing are shown in Figure 3. In regard to acrosome integrity and vitality, the results showed that diluents containing 2.6% egg yolk were significantly better than extender without egg yolk (p<0.05). Furthermore, Figure 4 shows that concentrations of 1.5 and 2.25 mg/ml CLC for 240×10^{6} spermatozoa were significantly better (p<0.05) than other levels of CLC. In addition, the results

Table 5. Effects of CLC concentration with egg yolk on the mean percentages of kinematical parameters in buck's semen after freezing-thawing (n = 6)

Egg yolk (%)	0					2.6					SEM
CLC (mg/ml)	0	0.75	1.5	2.25	3	0	0.75	1.5	2.25	3	
VAP (micron/s)	35.22	36.53	37.26	36.75	35.05	35.65	36.22	37.24	37.74	33.73	0.35
VSL (micron/s)	31.83	32.26	32.64	31.65	31.42	32.42	31.91	33.43	33.75	30.34	0.34
VCL (micron/s)	59.65^{a}	59.57^{a}	61.05^{a}	59.02^{b}	59.63^{a}	61.62^{a}	61.13^{a}	59.21^{a}	59.55^{a}	59.46^{a}	0.12
ALH (micron)	2.02	2.05	2.26	2.23	2.35	2.23	2.25	2.04	2.02	2.47	0.07
BCF (Herz)	8.44	8.44	8.57	8.73	8.43	8.74	8.63	8.52	8.83	8.81	0.06
STR (%)	90.42	88.43	87.61	86.11	89.71	91.05	88.42	89.81	89.54	89.63	0.58
LIN (%)	53.61	54.45	53.83	53.74	52.82	52.94	52.51	56.83	56.91	51.17	0.65

Different superscript letters indicate significant differences between means of the treatments at p<0.05. SEM indicates standard error of the mean.

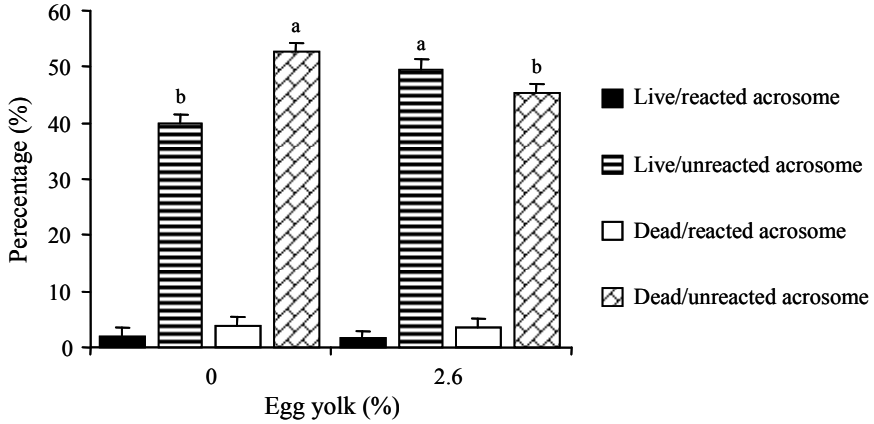

Figure 3. Effect of egg yolk on the mean percentages of acrosome integrity and vitality parameters in buck's semen after freezing-thawing (n = 6). Different letters indicate significant differences between means of the two treatments at $p<0.05$.

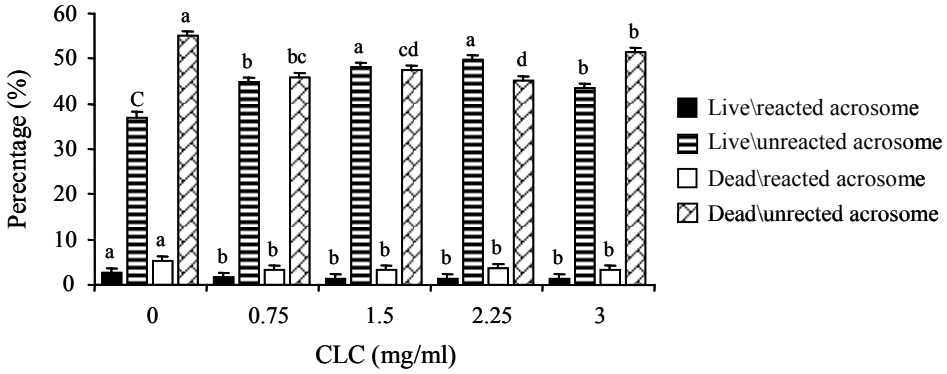

Figure 4. Effect of CLC concentration on the mean percentages of acrosome integrity and vitality parameters in buck's semen after freezing-thawing (n = 6). Different letters indicate significant differences between means of the treatments at $p<0.05$.

for both CLC levels (1.5 mg/ml and 2.25 mg/ml) showed lowest acrosome reaction and highest vitality rate. Concerning interaction between CLC and egg yolk, the results are presented in Table 6 and Figure 5. With regard to acrosome integrity and vitality after freezing and thawing of goat sperm, there were significantly higher results ($p<0.05$) for CLC concentrations containing 2.6% egg yolk in comparison with those without egg yolk. The best results ($p<0.05$) for acrosome integrity and vitality of frozen-thawed sperm were obtained in extenders containing 1.5 mg/ml and 2.25 mg/ml CLC concentrations containing 2.6% egg yolk.

DISCUSSION

The cryopreservation process decreases the quality of sperm after freezing and thawing (Moore et al., 2005). During this process, spermatozoa experience irreversible loss of motility and osmotic changes across the membrane and lipid-protein reorganizations within the cell membranes (Drobnis et al., 1993; Purdy and Graham, 2005; Bailey et al., 2008). Therefore, in the first part of this study we investigated the effects of egg yolk. Based on results of the first part, the effects on Markhoz buck sperm of addition of CLC to extenders prior to cryopreservation was investigated

Table 6. Effects of CLC concentration with egg yolk on the mean percentages of acrosome integrity and vitality parameters in buck's semen after freezing-thawing (n = 6)

Egg yolk (%)	0					2.6					SEM
CLC (mg/ml)	0	0.75	1.5	2.25	3	0	0.75	1.5	2.25	3	
Live/reacted acrosome	3.0^{a}	1.9^{ab}	1.4^{b}	1.8^{ab}	1.8^{ab}	2.2^{ab}	1.5^{b}	1.4^{b}	1.5^{b}	1.6^{b}	0.13
Live/unreacted acrosome	30.5^{ef}	41.6^{de}	43.5^{cd}	45.4^{c}	38.4^{e}	43.8^{c}	48.6^{b}	53.3^{a}	54.3^{a}	49.2^{b}	0.97
Dead/reacted acrosome	5.6^{a}	3.9^{b}	3.6^{b}	3.6^{b}	3.3^{b}	5.5^{a}	3.3^{b}	3.4^{b}	3.5^{b}	3.6^{b}	0.15
Dead/unreacted acrosome	61.3^{a}	44.7^{c}	52.5^{c}	49.5^{c}	56.3^{b}	49.6^{cd}	48.1^{d}	44.2^{f}	41.1^{f}	46.8^{d}	0.86

Different superscript letters indicate significant differences between means of the treatments at $p<0.05$. SEM indicates standard error of the mean.

in the presence and absence of egg yolk. The effect of addition of 0 and 2.6% (v/v) egg yolk prior to freezing showed that the presence of 2.6% egg yolk in extenders significantly improved motion parameters and membrane integrity of sperm after freezing and thawing (Figures 1 and 3). The reason for this is a membrane protective capacity of still unknown egg yolk components (Witte et al., 2009), but the low-density lipoprotein fraction (LDF) of egg yolk (Awad and Graham, 2002) may interact with the plasma membrane of sperm and also decrease the efflux of cholesterol and phospholipids from the sperm membrane and thus prevent premature capacitation and subsequent acrosome reaction (reviewed by Purdy, 2006). Concerning combination effects of egg yolk and CLC, Purdy and Graham (2004a) suggested that cholesterol and sperm need to co-incubate in a medium which is lipid-free. Otherwise, most of the cholesterol added to sperm in the presence of egg yolk will be transferred to egg yolk lipid droplets rather than the sperm, and thereby cannot benefit sperm viability after freezing and thawing. Based on this suggestion, we studied the effect of incubation of cholesterol with egg yolk prior to cryopreservation. Concerning motion, acrosome integrity and vitality of frozen-thawed sperm, the results of the present study showed that samples incubated in extender containing egg yolk were significantly better than diluents without egg yolk (Tables 3 and 5). However, there were no significant differences for kinematical parameters (Table 2). These findings are in agreement with the results of Purdy and Graham (2004a) and Bailey et al. (2008). Concerning the effects of CLCs, the results with regard to motility, progressive motility and recovery rate showed significant improvement, which were in agreement with the findings of Purdy and Graham (2004a), Anderson (2005), Barrera-Compean et al. (2005), Moore et al. (2005), Purdy et al. (2005), Alvarez et al. (2006), Mocé and Graham (2006), Amorim et al. (2008), Bailey et al. (2008) and Walters et al. (2008). In addition, our results indicated that CLC treatment not only improved recovery of viable and motile sperm, but also increased the percentages of progressive motile sperm. Also, these results agree with the findings of Awad and Graham (2002) who reported that CLC treatment improved percentages of progressive motility. Concerning kinematical parameters, the present results showed no significant differences for CLC levels, except in VAP and VSL (Table 4). These results demonstrated that the CLC levels used could not significantly affect kinematical parameters, which is in agreement with the findings of Alvareze et al. (2006) who reported no significant differences in kinematical parameters using different CLC levels. Contrary to our results and those of Purdy and Graham (2004a, 2005), Moce and Graham (2006), Cormier et al. (1997), Bailey et al. (2008), Anderson (2005), Amorim et al. (2008), Alvarez et al. (2006), and Barrera-Compean et al. (2005), Zahn et al. (2002) found that cholesterol significantly decreased motion characteristics, but they suggested that cholesterol increased membrane integrity when added to sperm membranes. However, the comparison of these studies with our results shows that adding cholesterol to sperm membranes by CLC technology has been proven to be very useful in enhancing the cryosurvival of sperm of different species. However, the exact mechanism by which added cholesterol interacts with cell membranes and protects spermatozoa still remains to be elucidated. Purdy and Graham (2004a) suggested that, when a sufficient level of cholesterol was added to biological membrane, it locates within the cell membrane. Furthermore, the added cholesterol incorporates into all plasma membrane components and should affect each compartment of the membrane. This may inhibit these cells from undergoing premature capacitation at low temperatures, thereby eliminating the transition phase and also increasing the number of sperm that survive cryopreservation (Purdy and Graham, 2004a; Moore et al., 2005). In addition, the present study showed that increasing CLC concentration to 2.25 mg/ml CLC/240×10^6 sperm significantly and positively affected the motion characteristics. In agreement with our observation, Barrera-Compean et al. (2005) found the level of 2.5 mg/ml CLC /240×10^6 sperm was the best concentration. Alvarez et al. (2006) showed that the optimal CLC concentration was 2 mg/ml CLC/120×10^6 cells, which agrees with our results and the findings of Barrera-Compean et al. (2005). However, the level of 3 mg/ml CLC/240×10^6 used in our study decreased the quality of sperm after freezing and thawing. These observations were in agreement with other findings, which showed that freezing of sperm at a higher concentration of CLC decreased the rate of motility (Comier et al., 1997; Purdy and Graham, 2004a; Barrera-Compean et al., 2005; Alvarez et al., 2006). Purdy and Graham (2004a) suggested that when cholesterol concentration increased to 4-5 times that of the control it had a detrimental effect on sperm survival. Furthermore, it has been shown that higher CLC levels increased not only membrane rigidity, which decreased the motility rate of frozen-thawed sperm (Purdy et al., 2005), but the oxidation process of cholesterol also produced oxysterols which are toxic to sperm (Comier et al., 1997). Although the molecular events responsible for these membrane changes are presently unclear, there is increasing evidence that capacitation involves a reversible lowering of the cholesterol, and thereby cholesterol/phospholipid ratio, in sperm (Davies, 1981; Langlais et al., 1988; Gadella et al., 2001), and that when sufficient cholesterol is removed the membrane becomes unstable, enhancing its stability to fuse

with other acrosomal membranes and resulting in the acrosome reaction (Langlais et al., 1988; Gadella et al., 2001; Purdy and Graham, 2004a). Similarly, Bailey et al. (2000, 2008), Chakrabary et al. (2007), Thomas et al. (2006) and Cromier et al. (1997) reported that sperm lose cholesterol from the plasma membranes after the cells have been cryopreserved. This may be one of the primary causes of a premature capacitation phenotype observed in cryopreserved spermatozoa (Thomas et al., 2006; Amorim et al., 2008; 2008). However, Gadella et al. (2001) suggested that the rate of capacitation depends on the rate of cholesterol depletion from the sperm plasma membrane, thus sperm with high cholesterol content undergo capacitation more slowly than those with lower cholesterol levels. In addition, the present results indicate (Figure 3) that incorporation of cholesterol into the membrane of spermatozoa significantly improved the vitality and acrosome integrity and prevented premature capacitation after freezing. Similarly, other studies suggested that added cholesterol decreased the acrosome reaction after freezing (Iborra et al., 2000; Khorasani et al., 2000; Shadan et al., 2004; Barrera-Compean et al., 2005).

With regard to effects of CLC levels on vitality and acrosome integrity, our results showed that addition of 1.5 and 2.25 mg/ml CLC were optimal, and direct comparison between the two levels showed no significant differences. However, Barrera-Compean et al. (2005) and Purdy and Graham (2004b) found no significant differences between sperm treated with cholesterol and the control. These results demonstrated, in contrast to our findings, that treating sperm with cholesterol prior to cryopreservation results in higher post-thaw motility without affecting plasma membrane and acrosomal membrane integrity. However, direct comparisons between these studies and our results are not appropriate, as different methods were utilized to determinate acrosome integrity and vitality and different media were used. Parks et al. (1981) also reported that loading of the sperm membrane with cholesterol may be deleterious. In conclusion, the present data indicated that adding cholesterol, using CLC technology, to goat sperm prior to freezing improved the rates of motility, viability and acrosome integrity of spermatozoa after freezing and thawing. However, additional investigations are required to determine the mechanism by which added cholesterol affects the sperm during freezing and thawing. In particular, the effects of loss of sperm/oocyte receptors, the role of membrane hydrophobicity, and interaction between cholesterol and the sperm membrane appear to be very important.

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