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—Original Article—

Egg Yolk and Glycerol Requirements for Freezing Boar Spermatozoa Treated with Methyl β-Cyclodextrin or Cholesterol-loaded Cyclodextrin

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Abstract. Egg yolk (EY) and glycerol are common constituents of extenders used for sperm cryopreservation. It has been demonstrated that using cholesterol-loaded cyclodextrins (CLC) improves sperm cryosurvival in several species. However, standard freezing extenders might not be the most appropriate for CLC-treated sperm. This study evaluated the EY and glycerol requirements for freezing CLC-treated boar spermatozoa. Semen samples from 34 ejaculates coming from 4 boars were used. Each ejaculate was split into three aliquots: one was used untreated (control), and the other two were treated with 1 mg of CLC or methyl-β-cyclodextrin/120 × 10^6 sperm for 15 min at 22 C prior to cryopreservation. Our results indicated that reducing the concentration of EY was detrimental for sperm viability after thawing (31.57 ± 2 *vs.* 19.89% ± 2 for 20 and 10% EY, respectively; $P < 0.05$), even in semen treated with CLC. On the other hand, it was observed that the traditional concentration of glycerol (3%) was not the appropriate for freezing CLC-treated sperm (61.10 ± 3 vs. 47.87% ± 3 viable sperm for control and CLC-treated sperm, respectively; $P < 0.05$). Thus, CLC-treated sperm showed a higher tolerance to high glycerol concentrations (5%) in terms of sperm viability (59.19% ± 3) than non-treated sperm (45.58% ± 3; $P<0.05$). Therefore, it could be necessary to modify the freezing extenders for CLC-treated sperm. Nevertheless, additional studies will be needed to evaluate alternative cryoprotectants and to determine the effect of high glycerol concentrations on sperm functionality.
Key words: Cholesterol, Cryopreservation, Pig, Semen.

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Egg yolk (EY) and glycerol are currently indispensable ingredients of freezing extenders for boar sperm cryopreservation. The protective effect of EY, minimizing cold stress, is related to its content in cholesterol and phospholipids, which are in part moved to sperm membranes [1, 2]. Glycerol, as a cryoprotectant, is essential, since it lowers salt concentration and raises the percentage of unfrozen water at any given temperature (for a review, see [3]). However, glycerol is also cytotoxic causing osmotic stress due to its low permeability through the membrane as a consequence of its high molecular weight [4].

Pre-freezing treatment with cholesterol-loaded cyclodextrins (CLC) adds cholesterol to sperm membranes, improving cryosurvival in spermatozoa of several mammalian species (for a review, see [5]). Cholesterol plays an important role in the thermotropic behavior of the membranes [6] by preventing the molecular packing of the phospholipids (which is required to form the gel phase) as the temperature decreases [7] and minimizing cooling shock. Spermatozoa treated with CLC prior to cryopreservation are currently frozen using standard freezing extenders characterized by a high EY concentration. Under this freezing condition, the effect of CLC treatment could be understated. Since CLC treatment increases the amount of cholesterol in sperm membranes [8–12], the EY in freezing extenders could be partially or even completely withdrawn, always considering that the cryoprotective effect of EY is partially due to its high cholesterol content.

In addition, CLC treatment could also modify the membrane permeability to penetrating cryoprotectants, since cholesterol is one of the most important regulators of membrane fluidity and permeability [13]. In this way, CLC-treated stallion sperm showed changes in the membrane permeability to water and cryoprotectants [14]. Besides, CLC treatment has widened the sperm osmotic tolerance limits in all species studied, including pigs [15]. Therefore, it is likely that the concentrations of glycerol usually included in boar sperm freezing extenders are not the most appropriate for CLC-treated sperm. In the light of this background, the aim of this study was to determine the most appropriate requirements of EY and glycerol for freezing CLC-treated boar spermatozoa.

Materials and Methods

Reagents and media

All chemicals used were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain) except flow cytometry fluorochromes, which were purchased from Invitrogen (Barcelona, Spain). Beltsville Thawing Solution (BTS, Minitub Ibérica S.L., Tarragona, Spain) was used as basic semen extender; it was supplemented with bovine serum albumin (BSA: 6 mg/ml) and used for post-thawing sperm dilution. The basic freezing extender (FE-1) was composed by lactose

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(321 mM) and EY [16]. The concentrations of EY ranged from 10 to 20% according to the experiment (see the section covering the experimental design). The FE-1 was centrifuged (12,000 *g*, 20 min, 5 C) and the supernatant was recovered and then filtered through 5-µm, 3-µm and 1.2-µm membrane filters (Millipore Ibérica, Madrid, Spain) in a three-step process. The FE-1 supplemented with 1.5% of Orvus Es Paste (Equex STM®, Nova Chemical Sales Inc., Scituate, MA, USA) and glycerol was used for a second semen dilution at 5 C before freezing (FE-2). The concentrations of glycerol also varied according to the experiment (see the section covering the experimental design). The final concentrations of EY and glycerol in the standard FE-2 were 20 and 9%, v/v, respectively. The pH values of FE-1 and FE-2 were both 6.2, and the osmolarity of the FE-1 ranged from 274 to 356 mOsmol/kg; that of the FE-2 ranged from 1200 to 3760 mOsmol/kg, and these values ranged according to the glycerol concentration used (from 6 to 21%, v/v). Phosphate buffered saline (PBS) was used as the basic medium for mitochondrial membrane potential evaluation and was composed by NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (0.86 mM) and $Na_2HPO_4 \cdot 7H_2O$ (6.4 mM) (pH 6.8 and 292 mOsmol/kg).

Preparation of methyl-β-cyclodextrin and CLC

Methyl-β-cyclodextrin (MBCD) was preloaded with cholesterol, and working solutions of CLC and MBCD were prepared by mixing 50 mg of CLC or MBCD in 1 ml of BTS using a vortex mixer, in accordance with the protocol developed by Purdy and Graham [11], as described elsewhere [17].

Semen collection and dilution

Mature Pietrain boars (n=4) were housed in the experimental farm of Centro de Tecnología Animal (CITA-IVIA, Segorbe, Castellón, Spain). Boars were fed a commercial diet (once/day) and provided water *ad libitum*. All boars were maintained under guidelines that were approved by the Ethics Committee of the Instituto Valenciano de Investigaciones Agrarias and that met the requirements of the European regulations for the Care and Use of Animals for Scientific Purposes (EC Directive 2010/63/EU).

Sperm-rich ejaculate fractions were collected weekly by the gloved-hand method and placed in pre-warmed thermo flasks covered with a gauze tissue to hold in the ejaculate gel fraction. Thereafter, sperm-rich ejaculate fractions were diluted (1:1, v/v) with BTS at 37 C and slowly cooled to 22 C over the course of 2 h.

Sperm cryopreservation

Only those ejaculates with >75% motile sperm were cryopreserved. The ejaculates were split into 3 fractions: one fraction was untreated, and the other two were treated with 1 mg/120 × 10^6 sperm of MBCD or CLC, respectively. The treated samples were incubated with MBCD or CLC for 15 min at 22 C [17]. Thereafter, the three aliquots were cooled to 16 C over the course of 2 h.

After cooling to 16 C, the 3 aliquots were processed using the straw-freezing procedure described by Westendorf *et al.* [18] with some modifications [17]. The straws were thawed in a water bath at 39 C for 30 sec. Post-thaw sperm quality was assessed in semen samples extended in BTS-BSA (25 × 10^6 sperm/ml) and incubated in a water bath at 39 C for 10 min.

Sperm quality evaluation

Sperm motility was objectively evaluated using a computer-aided sperm analysis system (ISAS® 1.0.17, Proiser R&D, Paterna, Spain) according to the protocol described by Blanch *et al.* [17]. The proportions of total motile sperm (%TMS, VAP>10 µm/sec) and rapid progressive motile sperm (%RPM, VAP > 45 µm/sec and STR ≥ 45%) were recorded.

Flow cytometry analyses were performed under dimmed light using either an EPICS XL flow cytometer (Coulter Corporation, Miami, FL, USA) equipped with an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 nm, or a Coulter FC500 flow cytometer (Coulter Corporation, Miami, FL, USA) equipped with an argon ion and an helium-neon laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 and 630 nm, respectively. Data were analyzed using the EXPO 2000 software (Coulter Corporation). A total of 10,000 events with forward and side scatter properties similar to sperm cells were initially gated. From these events, those without reasonable DNA content after SYBR-14/propidium iodide (PI) staining were gated out (double-gated sperm analysis). In subsequently carried out cytometry test, the percentages of these non-DNA events with scatter characteristics similar to sperm cells were taken into account to calculate the true sperm counts [19].

Sperm viability (% SV) was evaluated by assessment of plasma membrane integrity using a LIVE/DEAD Sperm Viability Kit (L-7011; Molecular Probes Europe BV, Leiden, the Netherlands) and following the procedure described by Purdy and Graham [11] and modified by Blanch *et al.* [17]. The samples were stained and incubated at 39 °C in the dark for 10 min. The fluorescence spectra of SYBR-14 and PI were detected using 505–545 and 605–635-nm band-pass filters, respectively. The analyzed sperm were categorized as follows: intact plasma membrane (SYBR-14+/PI−) and damaged plasma membrane (PI+). Only the sperm population that exhibited an intact plasma membrane is shown in the results.

Mitochondrial membrane potential (MMP) was analyzed using a triple fluorescent procedure for flow cytometric analysis (SYBR 14/PI/Mitotracker Deep Red 633, MTDR) described by Januskauskas *et al.* [20] and Martínez-Pastor *et al.* [21] with modifications, as indicated below. Fifty microliter aliquots of thawed sperm (25 × 10^6 sperm/ml) were diluted in 450 µl of PBS containing 25 µl of SYBR-14 (100 nm in DMSO), 2.5 µl of PI (1.5 mM in purified water) and 3.75 µl of MTDR (6.6 µM in DMSO) and incubated at 37 C in the dark for 20 min. The fluorescence spectra of SYBR-14, PI and MTDR were detected using 505–545-, 605–635- and 600–650-nm band-pass filters, respectively. The analyzed sperm were categorized as follows: viable sperm with high MMP (SYBR-14+ and high MTDR fluorescence), viable sperm with low MMP (SYBR-14+ and low MTDR fluorescence), and dead cells (PI+). Only viable sperm with low MMP are shown in the results.

Experimental design

Experiment 1: Requirement of EY for freezing CLC- or MBCD-treated boar spermatozoa

Each of the semen samples from 8 ejaculates was split into three aliquots and treated as described previously (control, MBCD treatment or CLC treatment). After seminal plasma removal, each aliquot was

split into two subsamples. Subsample one was diluted with FE-1 containing 20% of EY, whereas subsample two was diluted with FE-1 containing 10% of EY. Thereafter, both subsamples were frozen (final glycerol concentration of 3%) and thawed following the protocol described above. In a preliminary experiment, EY concentrations lower than 10% (5 and 2.5%) were used, but sperm samples frozen with these freezing extenders exhibited extremely low viability and motility after thawing; therefore, these percentages were discarded for subsequent experiments (results not shown).

Experiment 2: Requirement of glycerol for freezing CLC- or MBCD-treatment boar spermatozoa

Each of the semen samples from 13 ejaculates was split into three aliquots and treated as described previously (control, MBCD treatment or CLC treatment). After this, the aliquots were cooled to 16 C, centrifuged, resuspended with FE-1 containing 20% of EY and cooled to 5 C. Once at 5 C, each aliquot was split into four subsamples and diluted with FE-2 containing 6, 9, 12 or 15% of glycerol (final concentrations of 2, 3, 4 and 5%, respectively). After, semen aliquots were frozen and thawed as described above.

Experiment 3: Effect of increasing glycerol concentration on cryosurvival of CLC-treated boar sperm

Consequent to the results obtained in the previous experiment, in this third experiment, we evaluated if the highest glycerol concentrations further improved the quality of cryopreserved CLC-treated sperm. Each of the semen samples from 13 ejaculates was split into three aliquots and treated as described previously (control, MBCD treatment or CLC treatment). Thereafter, aliquots were cooled to 16 C, centrifuged, resuspended with FE-1 with 20% EY and cooled to 5 C. Then, CLC aliquots were split into two subsamples and diluted with FE-2 containing 9 or 21% of glycerol (final concentrations of 3 and 7%, respectively). The MBCD and control aliquots were diluted with FE-2 containing 9% of glycerol (final concentration of 3%). Samples were frozen and thawed as described above.

Statistical analyses

Statistical analyses were performed using the SAS software package (version 9.0, 2002, SAS Institute, Cary, NC, USA). Data from the three experiments were analyzed using a mixed model analysis of variance (ANOVA). In the first experiment, semen treatment (three levels: control, MBCD, CLC), EY concentration (two levels: 20 and 10%) and their interaction were included as fixed effects. In the second experiment, semen treatment (three levels: control, MBCD, CLC), glycerol concentration (four levels: 2, 3, 4 and 5%) and their interaction were included as fixed effects. In the third experiment, treatment (four levels: control, MBCD, CLC-glycerol 3%, CLC-glycerol 7%) was included as a fixed effect. In all the experiments, the ejaculate within the boar was included in the model as a random effect. When this analysis showed a significance effect, the means were compared using the Tukey test. A P-value of <0.05 was considered to be statistically significant. All of the data are shown as least squares means ± standard error. The percentage of progressively motile sperm in the first experiment and the percentage of viable sperm with low mitochondrial membrane potential in the third experiment were normalized using Freeman-Tukey arcsine transformation [22] before the analysis. Data are shown in tables in original form.

Results

Experiment 1: Requirement of EY for freezing CLC- or MBCD-treated boar spermatozoa

While motility parameters were not affected by CLC and MBCD treatment or EY concentration, the percentage of SV was negatively influenced ($P<0.05$) by CLC or MBCD treatment and by the lowest EY concentration (Table 1). The interaction between semen treatment and EY concentration was not significant.

Experiment 2: Requirements of glycerol for freezing CLC- or MBCD-treated boar spermatozoa

The interaction between glycerol concentration and semen treatments was significant ($P<0.05$) for all the quality parameters (Table 2). On the one hand, increasing glycerol above 3% resulted in decreased SV in the control and samples treated with MBCD, but SV gradually improved with increased concentrations of glycerol in the sperm samples treated with CLC prior to cryopreservation. On the other hand, when the glycerol concentration exceeded 3 or 4%, a decrease in the percentages of TMS and RPM was observed in MBCD-treated and control samples, respectively. By contrast, increasing the glycerol concentration from 3 to 4% exerted a positive effect on the percentages of TMS and RPM in CLC-treated samples ($P<0.05$; Table 2).

Experiment 3: Effect of increasing the glycerol concentration on cryosurvival of CLC-treated boar sperm

The increment in the glycerol concentration in the CLC-treated sperm samples had contradictory effects on SV and sperm motility (Table 3). Similar to the previous experiments, the concentration of 3% significantly decreased the percentage of SV in comparison with control samples. However, this difference in SV between CLC-treated and control samples disappeared when the glycerol concentration was increased to 7% in the CLC-treated samples. An opposite trend was observed in sperm motility parameters CLC-treated sperm samples frozen with 3% of glycerol exhibited similar percentages of TMS and RPM compared with control samples, while those frozen with 7% showed lower percentages of TMS and RPM ($P<0.05$; Table 3) than control samples.

With respect to the mitochondrial membrane potential detected by Mitotracker Deep Red 633, when sperm were treated with CLC and frozen with 3 or 7% of glycerol, the percentages of viable spermatozoa showing low MMP were higher than for control and MBCD-treated sperm samples.

Discussion

Since Phillips and Lardy [23] reported that EY is beneficial for sperm preservation, it has been routinely included in most freezing extenders for semen from domestic species [24]. However, using animal-derived additives such as EY in a semen extender implies sanitary risks and an extremely wide variability of their composition [25]. In that regard, there is increasing interest in finding a substitute for EY. Part of the positive effect of EY is likely due to the relatively high cholesterol content in the yolk [1]. Therefore, treating the sperm prior to cryopreservation with CLC could be a useful strategy to reduce

Table 1. Post-thaw percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM) and sperm with an intact plasma membrane (sperm viability; SV) in boar semen samples treated with 1 mg/120 × 10^6 sperm of methyl-β-cyclodextrin (MBCD) or methyl-β-cyclodextrin preloaded with cholesterol (CLC) or not treated (control) and subsequently frozen in a extender with 20 or 10% egg yolk

Semen treatments	Egg yolk	TMS (%)	RPM (%)	SV (%)
Control		20.06 ± 3	17.25 ± 3	34.35 ± 3 [a]
MBCD		15.41 ± 3	13.18 ± 3	20.66 ± 3 [b]
CLC		13.44 ± 3	11.19 ± 3	22.17 ± 3 [b]
	20%	18.12 ± 3	15.87 ± 2	31.57 ± 2 [x]
	10%	14.48 ± 3	11.87 ± 2	19.89 ± 2 [y]

[a,b] Differences (P<0.05) between semen treatments. [x,y] Differences (P<0.05) between egg yolk concentrations.

Table 2. Percentages of total motile sperm (TMS), rapid progressive motile sperm (RPM) and plasma membrane intact sperm (sperm viability; SV) after thawing when boar spermatozoa were treated with 1 mg/120 × 10^6 sperm of methyl-β-cyclodextrin (MBCD) or methyl-β-cyclodextrin preloaded with cholesterol (CLC) or were not treated (control) and then cryopreserved with different percentages of glycerol

Glycerol concentration	Semen treatment	TMS (%)	RPM (%)	SV (%)
2%	Control	50.46 [ab]	46.69 [ab]	56.15 [ab]
	MBCD	45.00 [abc]	40.23 [abc]	52.69 [bcd]
	CLC	32.46 [de]	28.61 [def]	47.31 [def]
3%	Control	53.18 [a]	49.29 [a]	61.10 [a]
	MBCD	36.58 [cde]	34.19 [cde]	46.59 [def]
	CLC	35.98 [cde]	33.19 [cde]	47.87 [cdef]
4%	Control	44.77 [abc]	41.46 [abc]	48.55 [cde]
	MBCD	26.61 [ef]	24.46 [ef]	41.43 [g]
	CLC	45.00 [abc]	41.08 [abc]	54.88 [abc]
5%	Control	37.23 [cd]	34.23 [cde]	45.58 [ef]
	MBCD	21.77 [f]	19.77 [f]	38.05 [fg]
	CLC	41.00 [bcd]	38.23 [bcd]	59.19 [ab]
s.e.		± 5	± 5	± 3

Data concerning the interaction between semen treatments and glycerol concentrations are shown. [a–g] Column means without common superscript letters are different (P<0.05).

the EY concentration in the freezing extenders, and this could be a first step towards the development of chemically defined extenders.

Our results indicated that reducing the concentration of EY from 20 to 10% in a lactose-based diluent was detrimental for SV after thawing in all the sperm samples, even those treated with CLC prior to freezing. However, similar to results achieved with ram [26] and rhesus monkey [27] spermatozoa, the post-thaw motility of sperm samples did not decrease when the EY concentration was lowered. This detrimental effect on SV could be related to either the largest proportion of lactose in the extender or the poor cryoprotective ability of the lowered EY concentration with respect to the lactose proportion. It is unlikely that the rise in lactose (from 8.8 to 9.9% in the diluents containing 20 and 10% EY, respectively) is responsible for the decrease observed in SV. In this regard, some authors did observe a negative effect of lactose on boar sperm motility but at concentrations of 10.8% [28] or 14% [29], both of which exceeded that used in our study. On the other hand, protection of the sperm by EY at these low concentrations could not have been fully achieved. Thus, low density lipoproteins (LDL), which are also constituents of EY, are also responsible for sperm protection (for a review, see [2]), and some authors have observed that the optimal LDL concentration for boar sperm cryosurvival was 9% (w/v) [30]. Considering that freezing extenders supplemented with 20% EY will contain approximately 6–7% (w/v) of LDL [31] at concentrations of 10% EY, the concentration of LDL (3–3.5%) would be one-third of that reported as optimal for boar sperm, which could explain the decrease in sperm quality at this EY concentration.

Cholesterol represents approximately 5% of the total lipid composition of EY [32], and it contributes to the sperm cryoprotective effects of EY. In addition, cholesterol plays an important role in the thermotropic behavior of the sperm membranes [6], and it is able to minimize the phase transition of the plasma membrane phospholipids [33] by reducing the transition temperatures of membranes and maintaining them in a fluid state at low temperatures [18]. Thus, the detrimental effect of the MBCD treatment on sperm freezing could be a priori expected, since this treatment has been proven to decrease the cholesterol content of the sperm membranes [34]. For the same reasons, a beneficial effect of CLC treatment on sperm freezing could be expected because it has been demonstrated that this treatment increases the cholesterol content of the sperm membranes [8–12]. The effectiveness should be even more relevant

Table 3. Percentages of total motile sperm (TMS), rapid progressive motile sperm (RPM), plasma membrane intact sperm (sperm viability; SV) and viable sperm with low mitochondrial membrane potential (MMP) after thawing when boar spermatozoa were treated with 1 mg/120 × 10^6 sperm of methyl-β-cyclodextrin (MBCD) or were not treated (control) and then cryopreserved with 3% of glycerol or treated with methyl-β-cyclodextrin preloaded with cholesterol (CLC) and then cryopreserved with different percentages of GLY

Semen treatment	TMS (%)	RPM (%)	SV (%)	Viable sperm with low MMP (%)
Control-glycerol 3%	51.40 ± 5.47 [a]	43.90 ± 4.73 [a]	57.94 ± 3.73 [a]	2.38 ± 1.26 [b]
MBCD-glycerol 3%	39.70 ± 5.47 [b]	34.00 ± 4.73 [b]	56.00 ± 3.73 [ab]	1.62 ± 1.26 [b]
CLC-glycerol 3%	49.20 ± 5.47 [a]	40.60 ± 4.73 [a]	51.15 ± 3.73 [b]	5.87 ± 1.26 [a]
CLC-glycerol 7%	39.30 ± 5.47 [b]	31.90 ± 4.73 [b]	59.80 ± 3.73 [a]	7.29 ± 1.26 [a]

[a–c] Column means without common superscript letters are different (P<0.05).

when spermatozoa are frozen using suboptimal EY concentrations. However, CLC treatment was not effective for freezing boar sperm, irrespective of the EY concentration of the freezing extender. The reason why boar sperm did not respond to CLC while spermatozoa of other cold-shock sensitive mammalian species did (for a review, see [5]) is not known. It could be related to the composition and the distribution of phospholipids in the sperm plasma membrane, which differ among mammalian species [36]. Alternatively, it could be also related to the specific mechanism by which sperm acquire resistance to cooling, which also differs among mammalian species [37]. Otherwise, the concentration of glycerol used traditionally might not be adequate for sperm treated with CLC, as seen in our results discussed below.

Glycerol is the cryoprotectant most widely used for boar sperm cryopreservation. This cryoprotectant exerts both an extracellular effect by osmotic stimulation of cell dehydration and an intracellular effect, permeating the membrane and restricting the dehydration effect [38]. The general view is that relatively low concentrations of glycerol (1 to 3%) are appropriate for boar sperm cryopreservation [39]. The widening in the osmotic tolerance of CLC-treated boar sperm [15] indicates that their sperm permeability is altered, and for this reason, we studied if this type of sperm would have different requirements for glycerol concentrations than non-treated sperm. For this, we gradually increased the glycerol concentration of this cryoprotectant, starting from 2% (one of the concentrations commonly used in boar sperm protocols) and increasing it in increments of 1%. In our study, a glycerol concentration of 5% was detrimental for all the sperm quality parameters evaluated after thawing in the control samples, which is in agreement with previous reports [35, 40, 41]. However, the effects of the glycerol concentrations on sperm cryosurvival differ among the semen treatments evaluated. Control and MBCD-treated sperm showed the highest post-thaw sperm quality when cryopreserved with the lowest glycerol concentrations (2–3%), while the post-thaw quality of CLC-treated sperm was highest when frozen with the highest glycerol concentrations (4–5%) used in the second experiment. Consequently, a third experiment was carried out to evaluate the putative effectiveness of the highest glycerol concentrations to further improve the quality of cryopreserved CLC-treated sperm. Increasing the final glycerol concentration of freezing extender to 7% in CLC-treated sperm samples increased the percentage of sperm with an intact plasma membrane but decreased the percentage of motile spermatozoa after thawing. The dichotomy between the results observed for membrane integrity and motility corroborates previous results [35]. Thus, boar sperm tolerate differently concentrations of glycerol, depending on the parameter evaluated. When the percentage of cryoprotectant is increased in the freezing extender, more deleterious effects of osmotic stress induced by addition and removal of the cryoprotectant are expected [42]. Moreover, the osmotic effect is enhanced when the cryoprotectant used is glycerol, since it permeates and equilibrates across the plasma membrane slowly, inducing a sudden cell volume change [14]. This could explain the negative effects of high glycerol concentrations on post-thaw sperm quality in the MBCD-treated and control samples. However, the higher tolerance of CLC-treated sperm, in terms of sperm viability, to the highest glycerol concentrations could be due to the changes in permeability to glycerol and osmotic tolerance limits of the boar sperm membrane induced by the CLC treatment. It has been demonstrated that CLC treatment increases the osmotic tolerance limits of boar sperm [15], and it is then likely that cholesterol could increase the sperm membrane permeability to cryoprotectants lessening osmotic cell damage [14, 43]. However, the increase in the glycerol requirements in CLC-treated sperm required to achieve the quality (sperm viability) of non-treated boar sperm cryopreserved with lower glycerol concentrations (3%) could also indicate that the permeability of the membrane of CLC-treated sperm to this cryoprotectant is reduced and that higher glycerol concentrations are needed to counteract this effect. In a previous study, CLC treatment decreased the membrane permeability of bull sperm to water at subzero temperatures [44], although this was considered as a positive effect of this treatment (volume excursions due to osmotic stress would decrease also). Nevertheless, cholesterol enrichment of human red blood cells inhibits several carrier-mediated transport pathways [45] and the transport of small neutral molecules such as glycerol. Perhaps this phenomenon is predominating in boar sperm. Thus, the added cholesterol could be acting as a double-edged sword in boar sperm, having a positive (widening the osmotic tolerance limits and reducing the sensitivity to cold shock) and negative effect (reducing the permeability of the membrane to the cryoprotectant). Permeating cryoprotectants, depending on the concentrations used, can induce decreased motility [38]. In our study, CLC-treated sperm samples showed the highest percentages of viable spermatozoa presenting low mitochondrial membrane potential postthawing. However, these percentages were not high enough to fully explain the difference found between the data of viable and motile spermatozoa in the CLC-treated samples (20%). Perhaps this decrease in motility as the glycerol concentration increased could be also related to the higher degree of dehydration of the sperm when a high concentration of glycerol is used. In this regard, some authors suggested that the reduction in intracellular water content could be the cause of the inhibition of sperm motility, since the friction in the tail at a lower intracellular content would increase and this friction would in turn cause an inhibition of sliding of microtubules or other structural elements in the flagellum [29].

The improvement in sperm quality parameters after thawing observed in CLC-treated boar sperm samples as the glycerol concentration increased suggests that the permeability of the membrane of these sperm to the glycerol was altered. However, the post-thaw sperm quality of the CLC-treated samples frozen with a high glycerol concentration still did not exceed that shown by the control samples. Alternative cryoprotectants should be evaluated in future studies in order to optimize the cryopreservation protocol for boar spermatozoa.

In conclusion, the proportion of EY in the freezing extender cannot be reduced even if the sperm samples are treated with CLC prior to freezing. As regards the optimal glycerol concentration of the extender for freezing CLC-treated sperm samples, concentrations of 5–7% increased the percentages of viable sperm after thawing but decreased the percentage of motile sperm. Therefore, it seems clear that it would be necessary to adjust the freezing protocol for CLC-treated boar sperm and, studies will be needed to evaluate alternative cryoprotectants or different freezing and thawing rates for CLC-treated sperm.

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