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Cholesterol-loaded cyclodextrin is efficient in preserving sperm quality of cryopreserved ram semen with low freezability

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ABSTRACT

Semen freezability is positive correlated with the cholesterol content in the sperm cell. Freeze-thawing mainly cause temperature chock and change on media osmolarity, which can modify plasma membrane lipids content and sperm conformation, resulting in decreased fertility. Therefore, the aim of this study is to investigate the effect of adding cholesterol-loaded cyclodextrin (CLC) to the cryopreservation process of ram semen with low freezability. For that, two experiments were performed using 5 ejaculates of 6 rams, totalizing 30 samples. For experiment 1 the following treatments were tested: *in natura* (IN), Tris solution (CON), CLC + Tris solution (CLC), and pure methyl-β-cyclodextrin + Tris solution (MCD). For experiment 2 treatments CON and CLC were tested in samples subdivided into three freezability classes: high (n = 10), intermediate (n = 10) and low (n = 10). Freezability classes were based on the variation of sperm motility between IN and CON groups from the first experiment. Sample analyzes included sperm motility, sperm morphology, plasma and acrosome membrane integrity, mitochondrial membrane potential, reactive oxygen species content, lipid peroxidation, and fluidity of plasma membrane. Results showed that CLC treatment was more efficient in maintaining sperm motility, integrity of plasma membrane, integrity of acrosome, and mitochondria membrane potential. In addition, CLC treatment in the groups with low and intermediate freezability showed improvement on progressive motility and percentage of rapid cells. In contrast, no difference was noted between CLC and CON treatments in the high freezability group. Therefore, the addition of CLC to semen extender improved sperm cryopreservation, especially in rams with low freezability.

1. Introduction

Sperm viability post-thawing still faces major obstacles, despite the scientific and technological advances. The mainly cause of decreased viability is changes on media osmolarity and changes on sperm conformation during the freeze-thawing procedures, which lead to decreased fertility rates post-breeding [1,2].

Events during cryopreservation may cause changes in sperm plasma membrane lipids and cold chock-induced damage [3,4]. In addition, sperm is vulnerable to reactive oxygen species (ROS) due to low cytoplasmic antioxidant capacity and high content of polyunsaturated fatty acids in the cell membrane [5–7]. This characteristic lead to lipid peroxidation, which is a known factor in decrease of sperm quality, leading to a loss in function due to inhibition of ATP production, loss of membrane integrity and fluidity [8]. Thus, different freezing protocols may influence fluidity, permeability and distribution of phospholipid in the sperm membrane [9]. Therefore, several studies have been led to evaluate different extenders [10,11], lipids supply [12], lipids concentration [13], and other substitutes [14] to improve semen cryopreservation process and post-thaw viability.

Although advances on cryopreservation protocols were able improve post-thawed semen viability, the difference in semen freezability

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between animals [15], and even between ejaculates [16], has yet to be considered. The bottom line of semen freezability differences lies in the ability of spermatozoa to shift membrane hydraulic permeability [15] and cope with temperature-induced membrane phase changes and domain reorganizations [16]. Particularly, these differences in semen freezability potential have been reported in rams [17,18]. More explicitly in stallions, this freezability difference has been noted either among ejaculates and/or different cholesterol concentrations [19].

Sperm cells with lower cholesterol: phospholipid ratio are more susceptible to be affected by chock-induced damage [20]. The addition of cholesterol through cyclodextrin increased the proportion of cholesterol: phospholipid in the membranes, holding the sperm cells to a similar stage to those that did not suffer thermal shock caused by freezing [21]. Cyclodextrins are cyclic oligosaccharides with an external hydrophilic face and an internal hydrophobic core [4]. They have a strong affinity for sterols and, when pre-loaded with cholesterol, can insert this cholesterol in the cell membrane [22]. Cholesterol-loaded cyclodextrin (CLC) had been widely used in various animal species, such as boar [23], bull [24,25], stallion [26] rooster [27] and ram [21], to increase preservation of sperm membranes and motility. However, the addition of CLC has not been tested on ram ejaculates that exhibit low freezability. This raises the question whether cyclodextrin has a distinct effect on ram semen with different freezability, and if its effect is more significant in low freezability animals.

Based on this previous information, this study will evaluate the effects of the addition of CLC on ram semen cryopreservation. The following hypothesis will be tested: (1) CLC treatment preserves motility and cell integrity after cryopreservation; and (2) CLC preservation effect is greater in semen with low freezability. The goal is to develop a new cryopreservation protocol that would increase sperm cryopreservation, especially on semen with low freezability.

2. Material and methods

2.1. Animals and facilities

This study was performed in agreement with the "Ethics Committee in the Use of Animals" of the School of Veterinary Medicine and Animal Science of the University of São Paulo, protocol number 9949250515.

The present study was carried out in two sequential experiments. Six White Dorper rams with age of 10 ± 0.89 months and weight of 30.8 ± 7.57 kg were used. The animals were kept in a paddock and were previously evaluated through complete reproductive examination.

2.2. Cholesterol-loaded cyclodextrin preparation

The cyclodextrin used in this experiment was the methyl-β-cyclodextrin (MβCD, C4555 -Sigma-Aldrich Co, Saint Louis, Missouri, USA) with a concentration of 2 mg of cyclodextrin/120 × 10^6 spermatozoa, as described for ovine by Mocé et al. [21] and Purdy et al. [28]. MβCD was pre-loaded with cholesterol for the preparation of the CLC, as described by Purdy and Graham [24], and stored in an amber glass container at −20 °C until use.

To incorporate CLC to sperm, a solution was prepared by adding 10 mg of CLC to 200 µL Tris solution (200 mM Tris; 71 mM citric acid; 55 mM fructose; adjusted to pH 7.4 and 300 mOsm) without extender, at 37 °C, and homogenized with a vortex shaker immediately before adding semen. Subsequently, an aliquot of the Tris + CLC solution was added to the semen (calculated to a final concentration of 2 mg CLC/120 × 10^6 spermatozoa), and incubated for 15 min at 22 °C.

2.3. Experiment 1: the effect of the addition of cholesterol-loaded cyclodextrin on the cryopreservation of ram semen

2.3.1. Experimental design

In this experiment, the effect of the incorporation of cholesterol through cyclodextrin to improve ram sperm freezability was evaluated. The experimental design of this study is outlined in the Fig. 1. Briefly, five ejaculates from each ram were collected. Semen was evaluated *in natura* (IN), diluted in the three treatments: Control group, semen diluted in the extender (CON, n = 30); Treated group, semen diluted in extender + Cholesterol-loaded cyclodextrin (CLC, n = 30) and; Negative control group, semen diluted in extender + pure methyl-β-cyclodextrin (MCD, n = 30). After dilution, semen was packed in 0.5 mL straws, frozen by an automated method and stored for later analysis post-thawing. Details of each procedure were described in the next subitems.

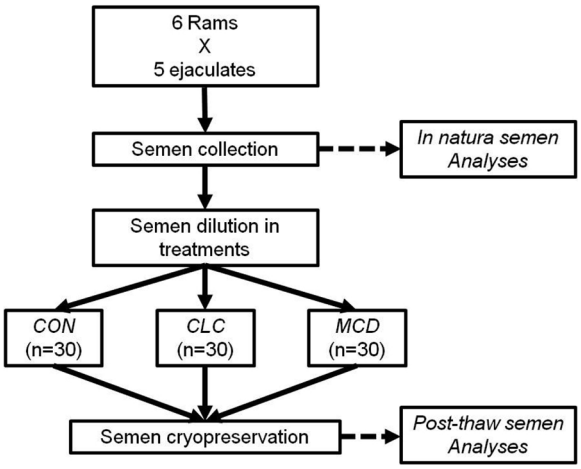

Fig. 1. Experimental design of the Experiment 1.
Legend: **CON**: control group, semen diluted in the extender. **CLC**: treated group, semen diluted in extender + Cholesterol-loaded cyclodextrin. **MCD**: negative control group, semen diluted in extender + pure methyl-β-cyclodextrin.

2.3.2. Semen collection

Semen from rams was collected in pre-warmed (37 °C) graduated conical tubes by artificial vagina. After collection, semen was transported to the laboratory and maintained at 37 °C temperature until the *in natura* (IN) semen analysis was performed.

2.3.3. Analysis of in natura semen

Semen was analyzed *in natura* (IN) for: sperm concentration, subjective and computerized motility, morphology, membranes integrity and reactive oxygen species (ROS). Only ejaculates with sperm motility over 70 % and a total abnormal sperm morphology below 30 % in IN analysis were selected for cryopreservation.

Sperm concentration was carried out by diluting semen in a 1:400 in 4 % formaldehyde Dulbecco's phosphate buffered saline (DPBS). Spermatozoa count was determined in Neubauer chamber under 400x magnification.

Sperm motility was evaluated both subjectively, through phase contrast microscopy, and objectively with the computer-assisted sperm analysis (CASA) system. For both motility analyses, semen was diluted in Tyrode's lactate pyruvate albumin medium (TALP sperm [29]) to 12.5 × 10^6 sperm/mL. For subjective motility (Mot, %) and vigor (Vig, 1–5) analysis, one drop of diluted semen was placed between a slide and cover slip, and analyzed at 100x magnification under contrast microscopy. CASA analysis was performed using the sperm class analyzer software (SCA - Microptics Barcelona, Spain), with prior setup for ovine semen. Diluted semen sample was deposited in a Makler™ chamber, and the image was obtained using a camera attached to the microscope equipped with phase contrast (Nikon, Model Eclipse Ni-U 80i). The following characteristics were analyzed: total motility (TM, %), progressive motility (PM, %), rapid cells (RAP, %), path velocity (VAP, µm/s), progressive velocity (VSL, µm/s), curvilinear velocity

(VCL, μm/s), lateral head displacement (ALH, μM), beat cross frequency (BCF, Hz), retilinearity (STR, %) and linearity (LIN, %).

For sperm morphology, semen samples were fixed in 4 %- DPBS and pre-warmed to 37 °C. Then, 200 cells were analyzed by differential interference contrast (DIC, Nikon 80i model) microscopy, using the wet chamber technique, at 1,000x magnification. Abnormal sperm cells were classified according to Blom [30] in percentage of major, minor and total defects.

Evaluation of plasma and acrosome membrane integrity and mitochondrial membrane potential was carried out by the association of fluorescent probes (propidium iodide, Hoescht 33342, FITC-PSA and JC-1), as described by Celeghini et al. [31], in epifluorescence microscopy (model 80i, Nikon, Tokyo, Japan). Sperm cells were classified according to structure integrity: cells presenting plasma and acrosome membrane integrity and high mitochondrial potential (PIAIH), cells with plasma membrane integrity (PI), cells with acrosome integrity (AI), and cells with high mitochondrial potential (HMP).

Reactive oxygen species were assessed by the fluorescent probe CellRox Deep Red Reagent® 2.5 mM (Invitrogen) as described by Alves et al. [32], and also analyzed by epifluorescence microscopy.

2.3.4. Semen cryopreservation

After *in natura* analysis, each ejaculate was divided into three aliquots and diluted into two steps. In the first step, aliquots were extended (120 × 10^6 sperm/mL) as the following treatments: Tris working solution (CON, n = 30); Cholesterol-loaded cyclodextrin resuspended in Tris working solution (CLC, n = 30); and pure methyl-β-cyclodextrin resuspended in Tris working solution (MCD, n = 30). This dilution resulted in 2 mg cyclodextrin/120 × 10^6 cells. After this first dilution, semen was homogenized and incubated for 15 min at 22 °C. In the second step, each sample was diluted in the commercial extender (Botubov®, BotuPharma) to a final concentration of 60 × 10^6 sperm/mL, homogenized and incubated for 10 min at 22 °C for interaction. After dilution, semen was packed in French straws (0.5 mL), labeled with name/number of animal, date and treatment.

Semen was cryopreserved in an automated device (TK3000 S Compact, Uberaba-SP, Brazil) with a cooling rate of −0.25 °C/min until temperature reached 5 °C, and a freezing rate of −20 °C/min until reaching −120 °C. A balance point was performed when semen reached 5 °C for 30 min. Finally, straws were plunged in liquid nitrogen at −196 °C. Afterward, straws were placed in racks and stored in a cryogenic tank for analysis.

2.3.5. Analysis of post-cryopreservation semen

For post-thawed semen analysis, two straws of semen from each sample of each treatment were thawed at 37 °C for 30 s, homogenized in a microtube and kept in a dry water bath at 37 °C. Subjective motility, vigor, CASA and morphology analyses were performed as described for the *in natura* semen. In addition to these evaluations, sperm membrane integrity and function, lipid peroxidation and sperm lipid bilayer membrane disorder were assessed using a flow cytometry, as well as the evaluation of reactive oxygen species and mitochondrial membrane potential by epifluorescence microscopy, as described below.

2.3.5.1. Analysis of integrity of plasma and acrosome membranes, lipid peroxidation and disorder of the sperm lipid bilayer. These analyses were performed using a flow cytometry (BD FACS Aria flow cytometer - Beckton-Dickinson, San Jose, CA, USA) controlled by BD FACS Diva 6.0 software (Beckton-Dickinson, San Jose, CA, USA). For the analyses, the samples were diluted (final concentration of 5 × 10^6 spermatozoa/mL) in modified TALP (TALPm: 114 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2.6H_2O$, 0.4 mM $NaH_2PO_4.H_2O$, 5 mM glucose, 10 mM sodium lactate, 0.1 mM sodium pyruvate, 10,000 IU/100 mL of sodium penicillin), and pH was adjusted to 7.4 using 5 N NaOH. Incubations were performed with different fluorescent probes described below. After the addition of fluorescent probes, samples were analyzed with an acquisition rate of approximately 600-1,000 events/s, acquiring 5000–10,000 cells per analysis. Cells were excited simultaneously by an argon laser at 488 nm by a Near UV laser at 375 nm range.

To analyze the integrity of plasma and acrosomal membranes, 150 μL of semen samples were diluted in TALPm, adding 2 μL of Hoechst 33342 (H342, 0.5 mg/mL, M-1399, Molecular Probes Inc., Eugene, Oregon, USA), with subsequent incubation for 10 min at 37 °C [33]. After incubation, 3 μL of propidium iodide (PI, 0.5 mg/mL, P4170, Sigma-Aldrich Co., Saint Louis, Missouri, USA) were added to the sample, with 10 μL of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA, 100 μg/mL, L-0770, Sigma-Aldrich Co, Saint Louis, Missouri, USA). The, samples were incubated for 10 min at 37 °C, followed by flow cytometer analysis.

Lipid peroxidation was analyzed using another 150 μL aliquot of semen samples diluted in TALPm and incubated with 0.5 μL of C11-BODIPY581/591 probe (1 mg/mL, D-3861, Molecular Probes Inc., Eugene, Oregon, USA) for 30 min at 37 °C [34]. To analyze disorder of sperm lipid bilayer, another semen aliquot was stained with 0.5 μL of Yo-Pro-1 (7.5 μM, Molecular Probes Inc., Eugene, Oregon, USA), incubated for 20 min, and then adding the fluorescent probe Merocyanine 540 (810 μM-Molecular Probes Inc., Eugene, Oregon, USA) for 70 s. Flow cytometry analysis followed to evaluate the spermatozoa with intact plasma membrane (Yo-Pro-1 negative) with an increase in the disorder of sperm lipid bilayer (positive merocyanine 540).

2.3.5.2. Analysis of mitochondrial membrane potential and reactive oxygen species production. Mitochondrial membrane potential was analyzed as previously described by adding 2 μL of H342 (0.5 mg/mL, Molecular Probes) and 2 μL 5,5',6,6' tetracloro 1,1',3,3' tetraetilbenzimidazolil carbocyanine iodide (JC-1, 153 μM, Sigma-Andrich). Samples were incubated at 37 °C for 8 min, and analyzed under epifluorescence microscopy (model 80i, Nikon, Tokyo, Japan) at 1,000x magnification [31]. Production of reactive oxygen species of sperm cells was evaluated by the probe Cell ROX Deep Red Reagent® 2.5 mM (Invitrogen) by epifluorescence microscopy at 1,000x magnification as described previously [32].

2.3.6. Statistical analysis

Data from the experiment were previously assessed for normality of residues and homogeneity of variances (Shapiro-Wilk test). Data that did not meet the requirements were transformed and reassessed. If it still didn't meet the premises, data were analyzed by non-parametric statistical order (Kruskal-Wallis test). Comparisons of sperm characteristics between groups (CON, CLC and MCD) were analyzed through combined procedure (PROC MIXED) of SAS (version 9.3; SAS Institute, Inc., Cary, NC, USA). Differences between groups were analyzed using the Tukey test. Differences were considered significant when $P \leq 0.05$; when probability ranged between 0.05 and 0.1, it was considered as difference approaching significant (statistical trend).

2.4. Experiment 2: the effects by adding cholesterol-loaded cyclodextrin on different classes of ram semen freezability

2.4.1. Experimental design

This experiment aimed to evaluate the different actions of CLC according to freezability of ram ejaculates. The experimental design of this study is outlined in the Fig. 2. Five ejaculates from six White Dorper Rams (n = 30) were used. After collection, semen was evaluated *in natura* (IN) and diluted into two treatments: Control group, semen diluted in the extender (CON) and Treated group, semen diluted in extender + Cholesterol-loaded cyclodextrin (CLC). After dilution, semen was packed in 0.5 mL straws, cryopreserved by an automated method, thawed (37 °C/30 s) and analyzed post-cryopreservation.

2.4.1.1. Experimental groups. To separate ejaculates according to the

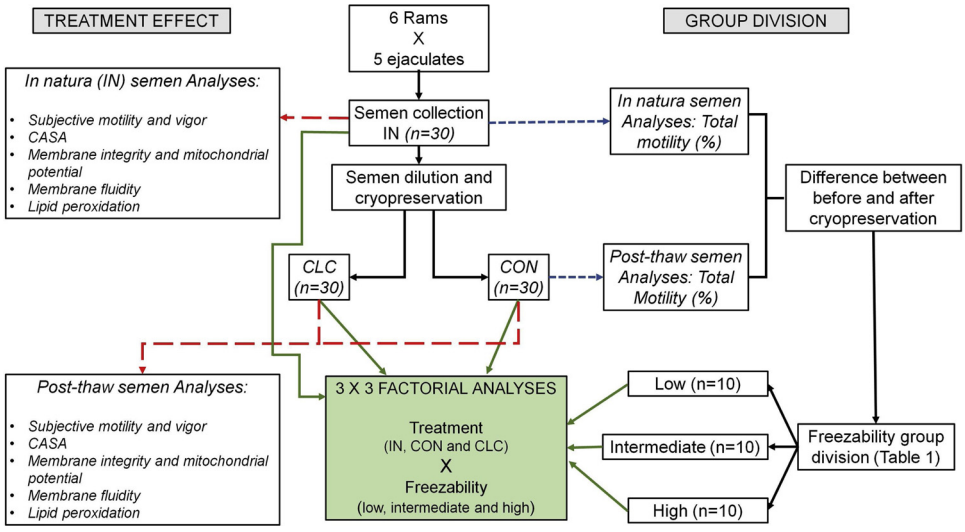

Fig. 2. Experimental design of the Experiment 2.
Legend: **IN**: in natura semen. **CON**: control group, semen diluted in the extender. **CLC**: treated group, semen diluted in extender + Cholesterol-loaded cyclodextrin. **MCD**: negative control group, semen diluted in extender + pure methyl-β-cyclodextrin.

Table 1
Variables and values used for the division of the ram ejaculates into three groups by freezeability: low, intermediate and high.

Variables	Freezeability		
	Low	Intermediate	High
PTTM[1]	≤ 40 %	> 40 % and < 60 %	≥ 60 %
%RTM[2]	≥ 60 %	> 40 % and < 60 %	≤ 40 %

[1]PTTM: post-thaw total motility; [2] %RTM: percentage of reduction in total motility.

susceptibility to cryopreservation process (freezability), the difference between total motility obtained of the previous analyses of *in natura* and post-thawed semen only of the control group (CON) was considered (Fig. 2). This division was performed using the total motility of each sample, taking into consideration: post-thaw total motility (PTTM) and the percentage of reduction in total motility (% RTM), $\%RTM = innatura\ \text{totalmotility} - PTTM$. The results obtained from

Table 3
Mean (± SEM) of abnormal sperm morphology of *in natura* and frozen-thawed semen in the treatment groups: only extender (CON), cholesterol-loaded-cyclodextrin (CLC) and extender plus methyl-β-cyclodextrin (MCD).

Sperm Defect	*In Natura* (n = 30)	Treatment			Mean	P value
		CON (n = 30)	CLC (n = 30)	MCD (n = 30)		
MaD[1] (%)	3.45 ± 0.82[c]	15.54 ± 1.75[a]	17.41 ± 3.21[a]	8.75 ± 1.96[b]	11.29 ± 1.30	< 0.0001
MiD[2] (%)	2.62 ± 0.64[c]	7.20 ± 1.02[b]	11.25 ± 1.24[a]	2.54 ± 0.59[c]	5.90 ± 0.68	< 0.0001
TD[3] (%)	6.08 ± 1.41[c]	22.75 ± 2.33[a]	28.66 ± 3.73[a]	11.29 ± 2.17[b]	17.19 ± 1.79	< 0.0001

[a,b,c] Lines with different lower case superscript letters are statistically different (P < 0.05) by Tukey test.
Legend: [1]MaD: major defects, [2]MiD: minor defects, [3]TD: total defects.

Table 2
Mean (± SEM) of subjective motility, vigor and kinetics by CASA of *in natura* and frozen-thawed semen in the treatment groups: only extender (CON), cholesterol-loaded-cyclodextrin (CLC) and extender plus methyl-β-cyclodextrin (MCD).

Sperm Characteristics	*In Natura* (n = 30)	Treatments			Mean	P value
		CON (n = 30)	CLC (n = 30)	MCD (n = 30)		
Mot[1] (%)*	79.83 ± 1.387[a]	49.81 ± 4.25[c]	65.00 ± 3.44[b]	1.62 ± 1.38[d]	49.18 ± 3.12	< 0.0001
Vigor (1–5)	4.26 ± 0.10[a]	2.68 ± 0.10[b]	2.82 ± 0.11[b]	0.20 ± 0.11[c]	2.50 ± 0.14	< 0.0001
CASA						
TM[2] (%)	75.47 ± 2.04[a]	49.02 ± 4.22[c]	65.49 ± 3.40[b]	1.59 ± 1.23[d]	47.88 ± 2.99	< 0.0001
PM[3] (%)	56.49 ± 2.03[a]	26.19 ± 2.76[c]	37.39 ± 2.53[b]	0.69 ± 0.60[d]	30.19 ± 2.13	< 0.0001
RAP[4] (%)	66.06 ± 2.34[a]	31.35 ± 3.67[c]	43.95 ± 3.69[b]	0.70 ± 0.63[d]	35.52 ± 2.59	< 0.0001
VAP[5] (μm/s)	135.56 ± 3.88[a]	83.16 ± 4.78[b]	88.18 ± 4.39[b]	8.88 ± 3.90[c]	78.94 ± 4.65	< 0.0001
VSL[6] (μm/s)	118.35 ± 3.24[a]	70.94 ± 4.22[b]	74.89 ± 3.85[b]	7.59 ± 3.34[c]	67.95 ± 4.05	< 0.0001
VCL[7] (μm/s)	146.35 ± 4.09[a]	96.00 ± 5.22[b]	101.22 ± 4.70[b]	11.14 ± 4.65[c]	88.68 ± 5.04	< 0.0001
ALH[8] (μm)	2.47 ± 0.08[b]	2.65 ± 0.04[a]	2.53 ± 0.06[ab]	0.34 ± 0.16[c]	0.99 ± 0.10	0.0597
BCF[9] (Hz)	9.07 ± 0.22[a]	8.76 ± 0.15[a]	9.01 ± 0.15[a]	1.10 ± 0.47[b]	6.97 ± 0.34	< 0.0001
STR[10] (%)	87.50 ± 0.81[a]	85.12 ± 0.64[b]	84.56 ± 0.83[b]	19.72 ± 6.81[c]	69.09 ± 3.15	0.0109
LIN[11] (%)	81.05 ± 0.98[a]	73.43 ± 1.04[b]	73.09 ± 1.37[b]	13.19 ± 5.15[c]	60.08 ± 2.87	< 0.0001

[a,b,c,d] Lines with different superscript lower case letters differ statistically (P < 0.05) Tukey test.
* Indicate statistical difference in line (P < 0.05) by Kruskal-Wallis test.
Legend: [1]Mot: subjective motility, [2]TM: total motility, [3]PM: progressive motility, [4]RAP: fast cells, [5]VAP: path velocity, [6]VSL: progressive velocity, [7]VCL: curvilinear velocity, [8]ALH: lateral head displacement, [9]BCF: beat cross frequency, [10]STR: straightness, [11]LIN: linearity.

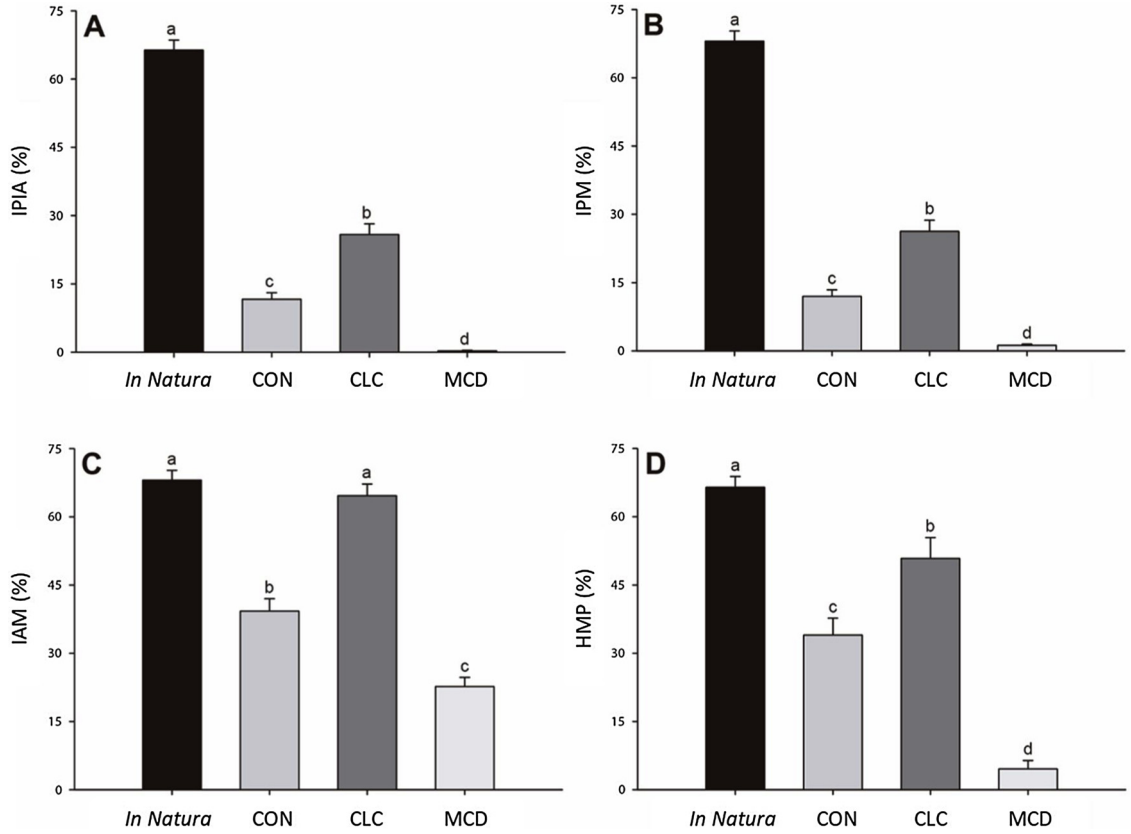

Fig. 3. Mean (± SEM) of integrity of plasma and acrosome membrane and mitochondrial potential of *in natura* and treatment groups: CON (only extender), CLC (cholesterol-loaded-cyclodextrin) and MCD (extender plus methyl-β-cyclodextrin).
[a,b,c,d] Bars with different letters are statistically different (P < 0.05) by Kruskal-Wallis test. (A and B) or by Tukey test (C and D).
Legend: A: IPIA, sperm presenting intact plasma and acrosome membranes and high mitochondrial potential; B: IPM, sperm presenting intact plasma membrane; C: IAM: sperm presenting intact acrosome membrane; D: HMP: sperm presenting high mitochondrial potential.

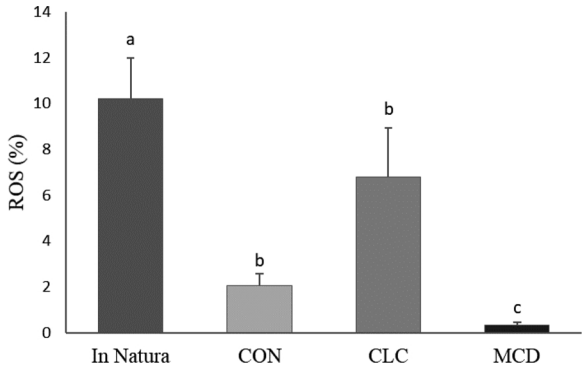

Fig. 4. Mean (± SEM) of ROS production of *in natura* and treatment groups: CON (only extender), CLC (cholesterol-loaded-cyclodextrin) and MCD (extender plus methyl-β-cyclodextrin).
[a,b,c] Lines with different lower case superscript letters are statistically different (P < 0.05) by Kruskal − Wallis test.

both were used to divide ejaculates retrospectively into three classes of freezability as high (n = 10), intermediate (n = 10) and low (n = 10) (Table 1).

2.4.2. Semen collection, analysis, dilution and cryopreservation

Semen from rams was collected by artificial vagina in pre-warmed (37 °C) graduated conical tubes. Afterward, it was maintained at 37 °C temperature until *in natura* semen analysis.

In natura semen was analyzed for: sperm concentration, subjective and computerized motility, morphology, membranes integrity and reactive oxygen species (ROS), as described in Experiment 1.

After *in natura* analyzes, the semen was divided into two aliquots and diluted in the CON and CLC treatments and cryopreserved using an automated system in the same manner as described in Experiment 1.

To compare semen freezability classes (high, intermediate and low) according to each treatment (IN, CON and CLC) the following sperm characteristics were considered: subjective motility and vigor, CASA, morphology, acrosome and plasma membrane integrity and mitochondrial potential, as described for *in natura* analisys in Experiment 1.

In addition, post-cryopreservation semen of CON and CLC groups according to semen freezability classes (high, intermediate and low) were evaluated for sperm plasma membrane integrity and mitochondrial potential, lipid peroxidation and sperm membrane permeability, through flow cytometry, as also described in Experiment 1.

2.4.3. Statistical analysis

Data from the experiment were previously assessed for normality of residues and homogeneity of variances (Shapiro-Wilk test). Data that did not meet the requirements were transformed and reassessed. Data that still failed to meet the premises were analyzed by non-parametric statistical order (Kruskal-Wallis test). Comparisons were made by analysis of variance (ANOVA) in 3 × 3 factorial: three treatments (IN, CON and CLC) X three freezability groups (high, intermediate and low). Analyses were performed by the combined procedure (PROC MIXED) SAS (version 9.3; SAS Institute, Inc., Cary, NC, USA). When only the main effects were statistically significant (no significant interaction), comparisons were performed using the Tukey test. When significant interaction was observed, means were then separated by the SAS

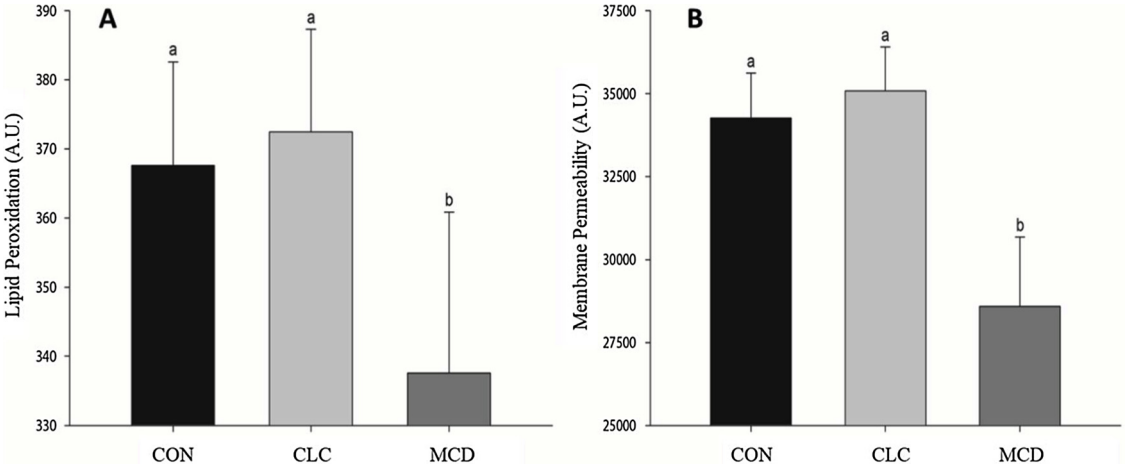

Fig. 5. Mean (± SEM) of lipid peroxidation (panel A) and membrane permeability (panel B) of *in natura* and treatment groups: CON (only extender), CLC (cholesterol-loaded-cyclodextrin) and MCD (extender plus methyl-β-cyclodextrin).
[a,b] Bars with different letters are statistically different (P < 0.05).

Table 4
Mean (± SEM) of subjective vigor and kinetics by CASA from ram ejaculates in different treatments: *in natura* (IN), control group (CON) and cholesterol-loaded cyclodextrin group (CLC); within different freezability groups: high, intermediate and low.

Groups	Treatments	Sperm characteristics					
		Vigor (1–5)	VSL[1] (μm/s)	LIN[2] (%)	STR[3] (%)	ALH[4] (μm)	BCF[5] (Hz)
High	*in natura*	4.25 ± 0.14	120.37 ± 4.00	80.67 ± 1.63	87.25 ± 1.34	2.59 ± 0.15	9.08 ± 0.36
	CON	2.95 ± 0.10	80.98 ± 2.92	71.66 ± 1.14	83.04 ± 0.64	2.75 ± 0.05	8.62 ± 0.16
	CLC	2.95 ± 0.10	82.44 ± 3.30	73.24 ± 1.49	83.92 ± 1.17	2.73 ± 0.06	8.91 ± 0.18
Intermediate	*in natura*	4.35 ± 0.19	118.5 ± 7.29	80.74 ± 1.93	87.13 ± 1.42	2.40 ± 0.12	8.77 ± 0.36
	CON	2.80 ± 0.08	77.15 ± 6.72	75.15 ± 1.76	86.37 ± 1.11	2.62 ± 0.09	8.67 ± 0.27
	CLC	3.00 ± 0.14	77.51 ± 6.24	73.47 ± 2.05	84.71 ± 1.36	2.59 ± 0.08	9.26 ± 0.30
Low	*in natura*	4.18 ± 0.23	115.03 ± 6.10	82.00 ± 1.63	88.34 ± 1.60	2.39 ± 0.12	9.41 ± 0.46
	CON	2.00 ± 0.22	48.14 ± 8.99	74.03 ± 2.92	86.91 ± 1.40	2.50 ± 0.07	9.14 ± 0.44
	CLC	2.35 ± 0.32	60.30 ± 9.95	75.90 ± 2.58	85.33 ± 2.07	2.14 ± 0.13	8.85 ± 0.38
Main effects							
Treatments							
in natura (n = 30)		4.26 ± 0.10[a]	118.35 ± 3.24[a]	81.05 ± 0.98[a]	87.50 ± 0.81[a]	2.47 ± 0.08	9.07 ± 0.22
CON (n = 30)		2.68 ± 0.10[b]	70.94 ± 4.22[b]	73.43 ± 1.04[b]	85.12 ± 0.64[ab]	2.65 ± 0.04	8.76 ± 0.15
CLC (n = 30)		2.82 ± 0.11[b]	74.89 ± 3.85[b]	73.96 ± 1.10[b]	84.56 ± 0.83[b]	2.53 ± 0.06	9.01 ± 0.15
Groups							
High (n = 10)		3.41 ± 0.12[A]	94.60 ± 3.63[A]	75.19 ± 1.04	84.74 ± 0.68	2.69 ± 0.05[A]	8.87 ± 0.14
Intermediate (n = 10)		3.38 ± 0.15[A]	91.08 ± 5.22[A]	76.45 ± 1.21	86.07 ± 0.75	2.54 ± 0.06[B]	8.90 ± 0.18
Low (n = 10)		2.95 ± 0.26[B]	74.49 ± 7.67[B]	77.52 ± 1.51	86.85 ± 0.99	2.33 ± 0.07[B]	9.13 ± 0.24
Total mean		3.28 ± 0.10	88.06 ± 3.14	76.20 ± 0.704	85.74 ± 0.46	2.55 ± 0.039	8.95 ± 0.10
Probability	Treatment	< 0.0001	< 0.0001	< 0.0001	0.0181	0.2048	0.6061
	Group	0.0026	0.0002	0.325	0.1115	0.0001	0.6464
	Interaction	0.2688	0.149	0.7069	0.6048	0.3174	0.618

[a,b,c] columns with different superscript lower case letters within treatments are statistically different (P < 0.05), by Tukey test.
[A,B,C] columns with different superscript case letters within groups are statistically different (P < 0.05), by Tukey test.
[1]VSL: progressive velocity, [2]LIN: linearity, [3]STR: retilinearity, [4]ALH: lateral displacement head, [5]BCF: beat cross frequency.

procedure PDIFF. Differences were considered significant when P ≤ 0.05; when probability ranged between 0.05 and 0.1, it was considered as difference approaching significant (statistical trend).

3. Results

3.1. Experiment 1: the effect of cholesterol-loaded cyclodextrin addition on the cryopreservation semen of ram

The hypothesis that CLC treatment preserves motility and cell integrity after cryopreservation was supported. Regardless of treatment, sperm motility decreased after cryopreservation. Cholesterol treatment showed greater protective effect, as observed in post-thaw sperm motility, in which the CLC group was better (P < 0.05) in preserving subjective motility, total motility, progressive motility and percentage of rapid cells than the CON group (Table 2). Furthermore, cholesterol removal in the MCD group presented greater sperm deterioration for the same features when compared with both the CON and CLC groups.

Frozen-thawed sperm had higher percentage of abnormal cells, as seen when *in natura* and treated groups (CON, CLC, MCD), were compared (Table 3). Furthermore, cholesterol treatment did not prevented cell abnormalities, presenting similar percentage of major and total defects to CON. However, both were higher than MCD group (CLC = CON > MCD) (Table 3).

When sperm membrane preservation was evaluated, cholesterol treatment was greater than the other groups in preserving membrane

Table 5
Mean (± SEM) of sperm membranes characteristics from ram ejaculates in different treatments: *in natura* (IN), control group (CON) and cholesterol-loaded cyclodextrin group (CLC); within different freezability groups: high, intermediate and low.

Groups	Treatments	Sperm characteristics			
		PIAIH[1] (%)	MI[2] (%)	AI[3] (%)	HMP[4] (%)
High	*in natura*	70.50 ± 3.07	72.62 ± 3.04	71.75 ± 2.94	71.08 ± 3.22
	CON	15.41 ± 1.78	15.95 ± 1.76	47.41 ± 2.68	41.96 ± 6.36
	CLC	32.65 ± 3.08	33.05 ± 3.13	72.93 ± 1.96	58.83 ± 7.00
Intermediate	*in natura*	65.30 ± 3.55	66.65 ± 3.63	66.45 ± 3.30	64.75 ± 4.15
	CON	12.88 ± 2.33	13.07 ± 2.33	40.69 ± 4.45	34.05 ± 4.83
	CLC	26.91 ± 3.62	27.15 ± 3.64	65.81 ± 4.73	57.30 ± 5.69
Low	*in natura*	61.31 ± 4.97	62.87 ± 4.97	64.50 ± 5.37	61.68 ± 5.19
	CON	5.90 ± 2.75	6.21 ± 2.78	29.23 ± 6.89	21.87 ± 6.71
	CLC	14.10 ± 3.99	14.92 ± 4.26	50.47 ± 4.16	30.73 ± 8.58
Main effects					
Treatments					
in natura (n = 30)		66.31 ± 2.19^{a}	68.03 ± 2.21^{a}	68.05 ± 2.15^{a}	66.46 ± 2.37^{a}
CON (n = 30)		12.03 ± 1.42^{c}	12.39 ± 1.43^{c}	40.32 ± 2.83^{b}	33.97 ± 3.70^{c}
CLC (n = 30)		25.79 ± 2.39^{b}	26.25 ± 2.42^{b}	64.57 ± 2.61^{a}	50.83 ± 4.54^{b}
Groups					
High (n = 10)		39.52 ± 4.17^{A}	40.54 ± 4.29^{A}	64.03 ± 2.45^{A}	57.29 ± 3.81^{A}
Intermediate (n = 10)		35.03 ± 4.49^{A}	35.62 ± 4.58^{A}	57.65 ± 3.23^{A}	52.03 ± 3.66^{A}
Low (n = 10)		27.10 ± 5.55^{B}	28.00 ± 5.66^{B}	48.07 ± 4.31^{B}	38.10 ± 5.23^{B}
Total mean (± SEM)		34.71 ± 2.70	35.55 ± 2.77	57.64 ± 1.95	50.42 ± 2.51
Probability	Treatment	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	Group	< 0.0001	< 0.0001	< 0.0001	0.001
	Interaction	0.463	0.5431	0.374	0.4071

^{a,b,c} columns with different superscript lower case letters within treatments are statistically different (P < 0.05), by Tukey test.
^{A,B,C} columns with different superscript case letters within groups are statistically different (P < 0.05), by Tukey test.
[1]PIAIH: spermatozoa presenting intact plasma and acrosome membranes and High mitochondrial potential, [2]MI: spermatozoa presenting integrity of plasma membrane, [3]AI: spermatozoa cells presenting intact acrosome, [4]HMP: spermatozoa presenting high mitochondrial potential.

integrity, as seen in the higher percentage of cells with intact plasma and acrosome membranes and high mitochondrial potential concomitantly (IPIAH), intact plasma membrane (IPM), intact acrosome membrane (IAM) and high mitochondrial potential (HMP) (Fig. 3). The MCD group presented the lowest values compared to the other groups. Cholesterol treatment presented better acrosome integrity preservation, with percentage of cells similar to *in natura* semen (Fig. 3, panel C).

The three treatments showed less ROS production in relation to the *in natura* semen (Fig. 4). The CON and CLC groups presented similar response P > 0.05) (Fig. 4). Cholesterol treatment did not differ in lipid peroxidation (Fig. 5A) and disorder of the sperm lipid bilayer (Fig. 5B) when compared to CON group. However, the MCD group presented the lowest rates of these characteristics (Figs. 4 and 5).

3.2. Experiment 2: the effects by addingcholesterol-loaded cyclodextrin on different classes of ram semen freezability

The hypothesis that CLC preservation effect is greater in semen with low freezability was also supported. The results of sperm characteristics regarding the interaction between treatments (IN, CON and CLC) within the different freezability groups (high, intermediate and low), as well as the main effects of t isolated factors (treatments and freezability) are shown in Tables 4 and 5. The characteristics in these tables showed no interaction effects between treatment and freezability (P > 0.05); thus, the main effects are presented separately. However, the average of each treatment within each group were shown at the top of the table to allow inference of biological effects.

Vigor, VSL and linearity decreased in CLC treatment similar to CON group related to *in natura* semen, with the exception of STR, which had lower on CON group (Table 4). ALH was lower for Intermediate and low freezability, however no difference was observed for treatments. BCF showed no differences for freezability or treatment. Regarding the freezability groups, the low freezability presented the lowest values of vigor and VSL, with exception of ALH in which intermediate and low freezability groups showed lower values. LIN, STR and BCF were similar among freezability groups (Table 4).

Subjective and progressive motility (Fig. 6, panels A and C, respectively), as well as rapid cell (Fig. 6, panel D) presented reduction in all freezing groups of CON, with greater decline observed in the low freezability group (IN *vs* CON). Total motility decreased in the CON in intermediate and low freezability. However, CLC treatment, when used in the intermediate and low freezability semen, improved preservation of these characteristics, showing lower decrease than the CON, highlighting the total motility observed in the intermediate freezability group, where CLC was similar to *in natura* semen for this characteristic. No significant effect of cholesterol was found in high freezability semen. On the other hand, the VCL and VAP were lower after cryopreservation than the *in natura* semen for all freezability groups. Nevertheless, the CLC treatment did not improve VCL or VAP, and both were similar in the CON and CLC groups, as shown in Fig. 6 (panels E and F).

No interaction was found between treatments and freezability for membranes integrity and mitochondrial potential, only individual effects. The CLC treatment developed a greater protection of cell membranes, since was noted a smaller decline on post-thaw PIAIH, MI, AI and HMP cells for CLC group, mainly AI, in which the treatment maintained the integrity similar to that observed in the *in natura* semen (Table 5). There was also a clear difference within the freezability groups, in which the low freezability group had lower percentage of PIAIH, MI, AI and HMP cells (Table 5).

Lipid peroxidation and membrane permeability also showed no interaction between treatments and freezability. When analyzed the individual effects no differences were found between treatments for both lipid peroxidation and membrane permeability (Fig. 7A). As for

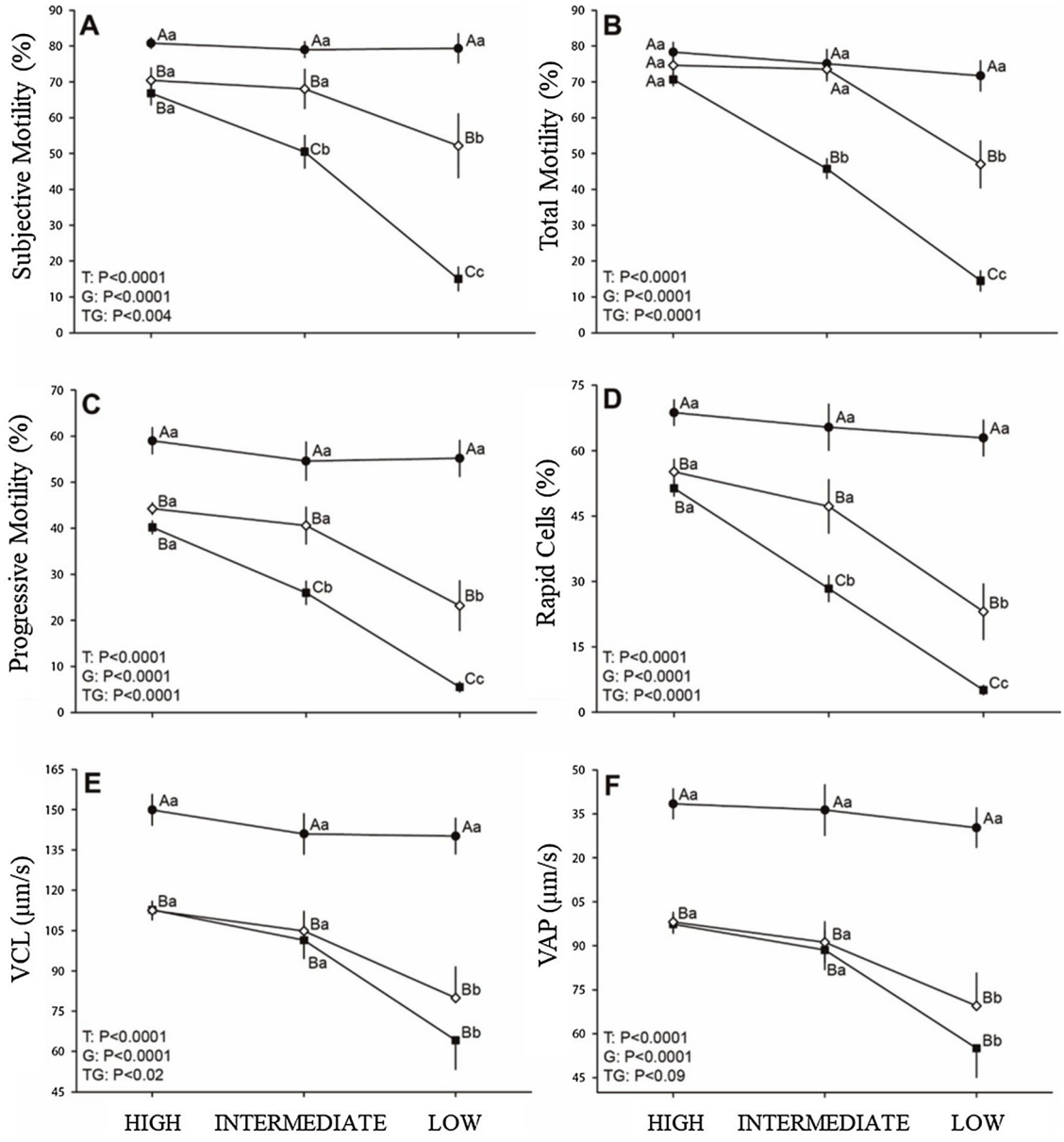

Fig. 6. Mean (± SEM) of motility characteristics (subjective and CASA) from ram ejaculates according freezability groups: high, intermediate and low; and different treatments: semen *in natura* (—●—), CON (—■—) and CLC (—◇—).
a, b, c, points with different lower case letters are statistically different ($P < 0.05$) in treatment.
A, B, C points with capital letters different are statistically different ($P < 0.05$) in the group.
Legend: VCL: curvilinear velocity; VAP: Path speed.
Probabilities for treatment (T), group (G) or interaction (treatment x group - TG) effect, which were statistically significant or showed statistical trend are shown in the left bottom.

freezeability, the intermediate freezability had higher number of cells with lipid peroxidation and a statistical trend for higher membrane permeability than the high freezability semen. Finally, the low freezability group presented the lowest values of lipid peroxidation as well as membrane permeability (Fig. 7B).

4. Discussion

During the cryopreservation process, sperm cells undergo changes in their physical and chemical properties [35], which leads to a decrease in sperm quality and, consequently, a decline in fertility. Our results clearly show that cholesterol influx into cells improves the preservation of some sperm characteristics after cryopreservation. In addition, we also show the increased effect of CLC on low freezability groups. These results show great promise in the use of cryopreserved ram semen, allowing the use of semen samples that would not meet the minimum standards with the use of simple egg yolk extender, *i.e.* low freezability. To the best of our knowledge, the present study is the first to illustrate the effect of CLC in different classes of freezability in ram semen.

Sperm kinetics is an indicator of sperm quality [36]. Moreover, motility dynamics offered by the CASA system along with traditional semen assessments provide valuable information about semen quality before and after freezing [37,38]. Our results indicate an improvement in the preservation of motility and some of kinetic characteristics when CLC was added to the extender, corroborating the CLC addition in bovine [39,40], ovine [21,28] and caprine semen [39]. For all freezability groups, the CLC addition showed better preservation on characteristics

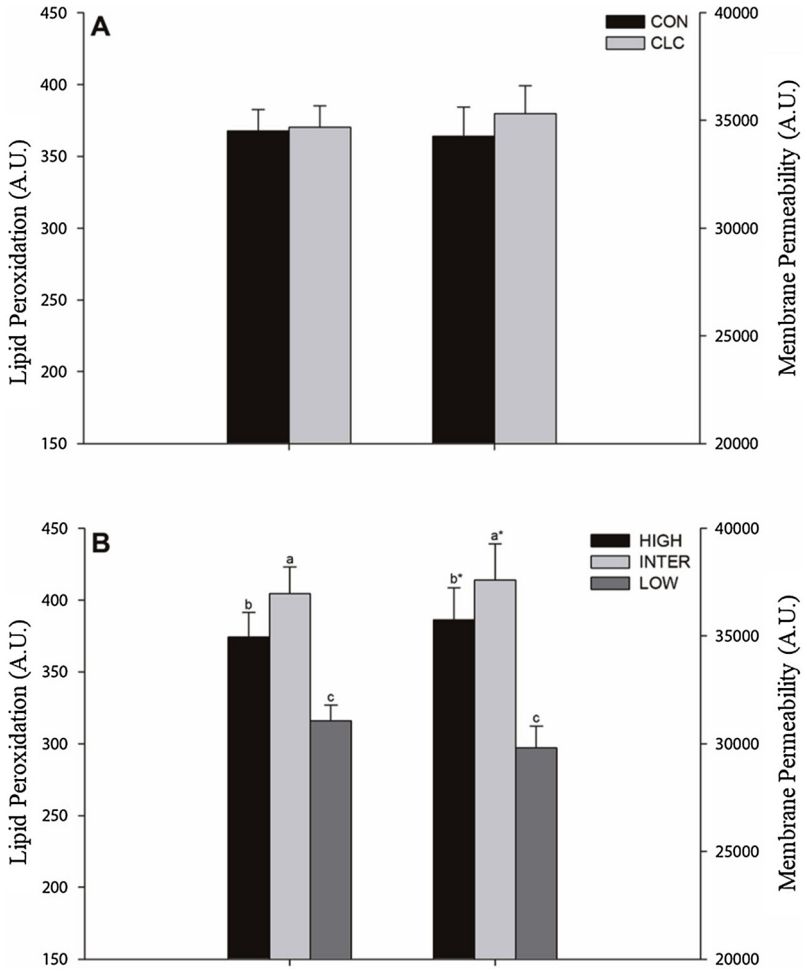

Fig. 7. Mean (± SEM) of the characteristics of lipid peroxidation and membrane permeability from ram ejaculates in different treatments: control group (CON) and cholesterol-loaded cyclodextrin treated group (CLC) (panel A) and in different freezability groups: high, intermediate and low (panel B).
[a,b,c]Bars with different lower case letters are statistically different ($P \leq 0.05$) in the evaluated characteristic.
* Bars with superscript asterisk indicate trend of statistical difference ($P > 0.05$ and < 0.1) between specified groups.

of sperm motility. However, the intermediate and low freezability groups stand out, due to the significantly increased sperm motility preservation when treated with CLC, with a total motility near that of *in natura* semen (Fig. 6). These differences in freezability groups may be due to the better preservation of sperm membrane stability caused by high cholesterol ratio [41], along with the possible instability of low freezability sperm membrane.

Sperm morphology is an important step of evaluation, due to the fact that it can dramatically affect fertility potential [42,43]. In our results, no difference was observed between the CLC and CON groups for sperm morphology. MCD group presented the lowest values of abnormal sperm, probably due to the death of sperm cells during the stabilization time of treatment; caused by cholesterol and phospholipids removal, which led to high permeability, and disruption of membrane architecture. Thus, the plasma membrane was no longer functional to respond to osmotic and structural changes caused by the extender and freezing process.

The integrity of the membranes is intimately linked to semen quality, as well as the structural integrity of sperm to maintain fertilizing capacity [44]. Interestingly treatment with CLC showed a great potential to protect plasma and acrosome membranes, as well as in maintaining high mitochondrial potential of cells. Our study found promising role of CLC in preserving the integrity of those membranes, and also maintain the integrity of the mitochondrial matrix, probably due to the increase in the intramembrane cholesterol ratio [41], decreasing the susceptibility of membranes to cryoinjury. This hypothesis could be supported by the negative control group with pure cyclodextrin, which not only presented a high affinity of cyclodextrin for the sperm membrane cholesterol, but due to high concentrations, also phospholipids removal [45], destabilizing the plasma membrane. However, there was no difference between the freezabilities when adding CLC for membrane integrity, despite the clear lower percentage of intact membrane cells in the low freezability ejaculates not treated with CLC.

CLC treatment showed a higher presence of superoxide and hydroxyl radicals evaluated by CellROX than CON group, despite no statistical difference, corroborating the higher mitochondrial potential found in the CLC group, since mitochondrial metabolism includes a buildup of ROS [46]. This higher presence may be due to the lower antioxidant activity in high cholesterol levels in the mitochondrial membrane [47] An increase in ROS was expected, since Maia et al. [48] showed that ROS in ram semen had a higher production from the time of adding extender until the freezing process. Any significant change in ROS would also be noticeable in the lipid peroxidation percentage, since sperm are highly vulnerable to ROS attack due to low cytoplasmic antioxidant capacity and high content of polyunsaturated fatty acids of the membrane [5,6]. However, we observed a clear difference between freezability groups regarding lipid peroxidation, in which the low freezability group showed the lowest level of peroxidation and the intermediate group presented the highest degree. This fact is probably

due to the minor mitochondrial activity found in the low freezability ejaculates, since mitochondria is the main site of ROS production [46], and, consequently, high ROS concentration leads to loss of membrane integrity and fluidity [8].

Cholesterol efflux in the membrane of sperm cells is an extremely important event for the start of training and activation process [49,50]. However, our results showed similar membrane disorder regarding addition or not of the CLC to extender. Nevertheless, low freezability ejaculates presented lower membrane permeability in relation to other groups. This lower rate of membrane permeability in the low freezability group is likely due to the small number of post-thaw viable cells, compared to the other groups. The use of CLC caused a decrease in the number of cells in cold chock-induced membrane alterations [19], due to the increase in membrane stability and modification of the cell response to the temperature capacitation inducers [51,52].

5. Conclusion

Cholesterol-loaded cyclodextrin added to semen extender prior to cryopreservation improved the maintenance of sperm motility, integrity of plasma and acrosome membranes, and mitochondrial membrane potential. In addition, improvement was greater in low and intermediate freezability ejaculates.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2020.01.002.

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