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Cholesterol and desmosterol incorporation into ram sperm membrane before cryopreservation: effects on membrane biophysical properties and sperm quality

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ABSTRACT

Ram sperm are particularly sensitive to freeze-thawing mainly due to their lipid composition, limiting their use in artificial insemination programs. We evaluated the extent of cholesterol and desmosterol incorporation into ram sperm through incubation with increasing concentrations of methyl-ß-cyclodextrin (MβCD)-sterol complexes, and its effect on membrane biophysical properties, membrane lateral organization and cryopreservation outcome. Sterols were effectively incorporated into the sperm membrane at 10 and 25 mM MβCD-sterols, similarly increasing membrane lipid order at physiological temperature and during temperature decrease. Differential ordering effect of sterols in ternary-mixture model membranes revealed a reduced tendency of desmosterol of segregating into ordered domains. Live cell imaging of fluorescent cholesterol showed sterol incorporation and evidenced the presence of sperm sub-populations compatible with different sterol contents and a high concentration of sterol rich-ordered domains mainly at the acrosome plasma membrane. Lateral organization of the plasma membrane, assessed by identification of GM1-related rafts, was preserved after sterol incorporation except when high levels of sterols (25 mM MβCD-desmosterol) were incorporated. Ram sperm incubation with 10 mM MβCD-sterols prior to cryopreservation in a cholesterol-free extender improved sperm quality parameters after cooling and freezing. While treatment with 10 mM MβCD-cholesterol increased sperm motility, membrane integrity and tolerance to osmotic stress after thawing, incorporation of desmosterol increased the ability of ram sperm to overcome osmotic stress. Our research provides evidence on the effective incorporation and biophysical behavior of cholesterol and desmosterol in ram sperm membranes and on their consequences in improving functional parameters of sperm after temperature decrease and freezing.

Keywords: Cholesterol; Desmosterol; Ovine sperm; Membrane lipid order; Cryopreservation

1. Introduction

Cryopreservation of mammalian sperm is a widely used biotechnological tool in artificial insemination breeding programs. However, pregnancy rates in sheep inseminated via cervical with frozen-thawed sperm are markedly low and therefore are not feasible for the animal production industry [1].

Spermatozoa undergo structural and functional alterations related to cryodamage. It is noteworthy that cryopreservation modifies the lipid composition of the sperm membrane [2,3] Temperature decrease induces phase transitions and clustering of sperm membrane lipids that alter lipid-lipid and lipid-protein interactions [4]. Different sperm sensitivity to cryopreservation among species is closely related to lipid composition of the membrane. Particularly, sterol content has been associated with cryotolerance of sperm [5]. Ram sperm membrane is characterized by low cholesterol to phospholipid ratio and therefore is more sensitive to temperature decrease than those with higher cholesterol to phospholipid ratios such as rabbit or human sperm membranes [6].

Cholesterol is an important lipid component of membranes with a well-known role in modulating membrane lipid order [7]. Sterols are major components of membrane rafts, small domains that compartmentalize cellular processes and play important roles in cell signaling and gamete interaction [8,9]. In sperm cells, the efflux of sterols has a physiological role on the extra testicular sperm maturation process known as capacitation. However, sterol loss produced by cryopreservation has a significant impact on sperm viability by inducing a premature capacitation-like state that shortens the life span of the spermatozoa [6]. The major sterols in mammalian sperm membranes are cholesterol and its immediate precursor, desmosterol [10]. Both sterols are known to prevent human sperm capacitation *in vitro* [11]. Desmosterol is a cholesterol isomer with an extra double bond at carbon 24 that diminishes its ordering potential in lipid bilayers [7]. In ram sperm, desmosterol is converted into cholesterol during *epididymis* transit and therefore it is barely

detected in mature sperm collected from the *cauda epididymis* [12]. In this respect, increasing sterol content of sperm membrane to minimize lipid phase transition and thus stabilize the membrane at low temperatures is a widespread strategy to broaden sperm cryotolerance. By interacting with the acyl chains of phospholipids, sterols maintain the phospholipids in a lamellar arrangement as temperature decrease. Interestingly, it was recently shown that incorporation of desmosterol into chicken sperm improves motility and viability after freezing [13].

Methyl-β-cyclodextrin (MβCD) is a cyclic oligosaccharide which has a high affinity for inclusion of cholesterol and other sterols in its hydrophobic cavity and therefore, it is used to add cholesterol to the cell membranes [14]. Early studies by Graham and co-workers demonstrated that incubation of bull sperm with MβCD complexed with cholesterol (MβCD-Chol) prior to cryopreservation improves sperm viability and motility after thawing [15,16]. Similarly, studies performed in ram sperm showed that treatment with MβCD-Chol before cryopreservation improves sperm motility and osmotic stress tolerance after thawing [17–20]. However, incorporation of cholesterol into ram sperm cells after MβCD treatment has not been quantitatively analyzed except for two previous studies in which different experimental conditions lead to non-comparable results [17,20]. Moreover, pretreatment of cryopreserved ram sperm with MβCD-Chol did not increase pregnancy rates after artificial insemination [18,20]. The presence of lipids, including sterols, in egg yolk or skim milk-based extenders usually used as semen diluents may interact with MβCD or the sperm, either by interfering with the availability of the sterol binding drug or by incorporating variable amounts of cholesterol into the sperm cells. To date, there is no evidence for the effective incorporation of cholesterol into sperm cells after MβCD-Chol treatment, nor of its impact on membrane biophysical properties or in plasma membrane organization. Furthermore, the addition of desmosterol as a key intermediate in cholesterol biosynthesis has not been explored in ram sperm cryopreservation.

The aim of the present work was to study the molecular and biophysical basis of cholesterol and desmosterol incorporation into ram sperm and its implications for sperm cryopreservation in a cholesterol-free extender. We evaluated: (1) the extent and distribution of cholesterol and desmosterol incorporation into the sperm cell membrane; (2) the biophysical properties of model membranes prepared from sperm lipids; (3) the organization of the sperm membrane by analysis of a raft marker; and, (4) the effect of sterol incorporation on sperm functional parameters after cooling and freezing.

2. Materials and methods

2.1. Chemicals and reagents

Cholesterol and MβCD were purchased from Sigma Chemical Company (St. Louis, MO, USA). Desmosterol and BODIPY-Cholesterol (BPY-Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Laurdan and cholera toxin subunit B-Alexa Fluor 488 (CTB-AF^{488}) were obtained from Molecular Probes (Eugene, OR USA). All solvents used in this study were HPLC grade (JT Baker, USA; UVE, Argentina).

2.2. Preparation of methyl-β-cyclodextrin-sterol complexes

MβCD-sterol complexes were prepared according to Christian and co-workers [21]. A volume of cholesterol or desmosterol from a stock solution (10 μg/μL) in chloroform:methanol 1:1 (v/v) was added to a glass tube and the solvent was evaporated under a gentle stream of nitrogen for 1 h. A volume of 0.5 M stock solution of MβCD in Tris-citric acid buffer (300 mM Tris, pH 7.4, 95 mM citric acid) was subsequently added to the dried material (MβCD:sterol, 4:1 molar ratio) to reach the concentrations 10, 40 and 100 mM MβCD:sterol. The mixture was clarified by vigorous mixing, subjected to bath sonication for 5 min and incubated in a rotating water bath at 37˚C overnight. Before using the solution, it was centrifuged at 2000 × g for 10 min to remove excess of sterol crystals.

2.3. Semen collection and processing

Twenty mature Texel rams were kept on a ray grass, fescue and white clover pasture (37° 450 S, 58° 180 W), with water *ad libitum.* Animals used in this study were handled in strict accordance with Good Animal Welfare Practices approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE-Resolution 046/2016). Semen was obtained using an artificial vagina during the breeding season in the southern hemisphere (March-June) following a routine collection of twice per week with at least two days of sexual abstinence. Only semen samples with mass motility ≥ 4 were processed. In order to avoid individual effects, semen from four randomly-selected rams was pooled by collection date, with each male contributing an equal number of spermatozoa to the pool. Pooled semen was divided into aliquots to conform each experimental group. The number of repetitions (number of pools) for each evaluation is indicated in the figure legend. Ejaculates were directly placed in a water bath at 32°C until processing. The sperm concentration was assessed in a Neubauer hemocytometer under light microscopy. Subsequently, semen was pooled and diluted to 3000×10^6 sperm/mL in pre-warmed (32°C) Tris-citric acid buffer and incubated for 15 min with the corresponding MβCD-sterol concentration to achieve a final concentration of 2.5, 10 and 25 mM. In order to remove MβCD molecules after incubation, spermatozoa were washed by centrifugation (1000 × g, 5 min) with pre-warmed extender or Tris-citric acid buffer followed by sperm cryopreservation or lipid extraction, respectively.

2.4. Lipid extraction

Sperm were washed by centrifugation in citrate-EDTA buffer (35.5 mM sodium citrate, pH 7.4, 2.5 mM EDTA) at 2500 × g for 10 min. Lipids were extracted with chloroform:methanol (1:2, v/v) according to Bligh and Dyer [22]. The total amount of phospholipids was measured from each lipid extract through phosphorus quantification [23].

2.5. Quantification of sperm sterols

Total sterols were quantified by an analytical method using a commercially available enzymatic assay (Colestat Wiener Lab, Rosario, Argentina). An aliquot from the lipid extract, equivalent to 5 μg of phosphorous (approximately 140×10^6 spermatozoa), was placed into a glass tube and dried under a nitrogen stream. The dried extract was resuspended in isopropyl alcohol and vigorously mixed for 1 min. In this procedure, 1 mL of Working Reagent was combined with 100 μL of isopropyl alcohol extract and incubated at room temperature (25°C) for 30 min. The absorbance was measured in a spectrophotometer at 505 nm and compared to a standard curve.

Cholesterol and desmosterol were quantified by reverse high-pressure liquid chromatography (HPLC). Lipid extracts from spermatozoa were spotted on thin-layer chromatography (TLC) plates (500 μm, silica gel G) along with commercial standards. The sterol fraction was resolved using hexane:ether:ammonia (45:65:1, v/v). Lipid bands were located under ultraviolet light after spraying the TLC plates with 2,7-dichlorofluorescein in methanol and exposing them to ammonia vapor. The sterol fraction was eluted with chloroform:methanol:water (5:5:1, v/v) and partitioned by the addition of 0.8 volumes of water. Reverse phase HPLC was performed at 40ºC with a C18 HPLC column (Agilent Technologies, USA; 100 cm × 4.6 mm, 3.5 μm) equilibrated with methanol (100%) at a flow rate of 1 mL/min. The detection of sperm sterols along with the commercial standards was monitored by the absorbance at 205 nm. The percentages of each sterol after MβCD-sterol treatment were calculated based on the cholesterol and desmosterol content quantified by HPLC for fresh spermatozoa.

2.6. Biophysical studies

Membrane lipid order was measured by fluorescence spectroscopy in small unilamellar vesicles (SUVs) prepared from total lipids extracted from sperm. Liposomes were prepared by mixing appropriate aliquots of the lipid extracts and the fluorescent probe Laurdan [6-dodecanoyl-2-

(dimethylamino) naphthalene] to a final phospholipid:Laurdan molar ratio of 100:1. The mixtures were placed under a stream of nitrogen in the dark for 60 min. The dried lipid films were then hydrated by adding buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.25 mM $MgCl_2$), vigorously mixed for 1 min and sonicated in an ultrasonic bath for 30 min. Each sample was diluted with buffer A to reach a final lipid concentration of 100 µM.

Fluorometric measurements were performed in a SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using a vertically polarized light beam from a Hannovia 200 W Hg/Xe arc obtained with a Glan-Thompson polarizer (4 nm excitation and emission slits) and 5- × 5-mm quartz cuvettes. Emission spectra were corrected for wavelength-dependent distortions. Temperature was set with a thermostatic-controlled circulating water bath (Haake, Darmstadt, Germany). Excitation generalized polarization (exGP) [24] values were calculated as $exGP= (I_{434} - I_{490})/(I_{434} + I_{490})$, where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel phase (434 nm) and the liquid-crystalline phase (490 nm), respectively. The exGP values were obtained from the emission spectra at one specific excitation wavelength (360 nm).

To compare lipid biophysical state associated with the increase of cholesterol or desmosterol, Laurdan-containing SUVs were prepared from sperm lipids extracted under control conditions (0.4 sterol/phospholipid ratio) and cholesterol or desmosterol exogenously added to the lipid extracts at final sterol/phospholipid ratio of 0.6, 0.7, 0.8 and 1.0.

2.7. In situ *imaging of BODIPY-cholesterol*

2.7.1. Studies in giant unilamellar vesicles

Giant unilamellar vescicles (GUVs) containing POPC:SM:sterol (cholesterol or desmosterol) were prepared by electroformation [25] in a molar proportion of 1:1:1 to obtain vesicles with coexisting liquid-ordered (*lo)* and liquid-disordered (*ld)* lipid domains [26]. The chamber (Nanion Technologies GmbH, Germany) is equipped with two slides coated with optically transparent and

electrically conductive indium tin oxide (ITO). Briefly, 5 μL of the lipid mixture POPC:SM:sterol (1:1:1 molar ratio, 10 μg/μL) was spread on an ITO-coated glass slide. The solvent was evaporated and the dried lipid film was hydrated with 200 μl of a 450 mM sucrose solution and then the chamber was sealed with a second ITO slide. Running conditions were set at 4.33 h, 1.3 V, 500 Hz and 45ºC [27]. The fluorescent probe 1,1'-dyoctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiIC12) and BPY-Chol were incubated with GUVs in a probe:lipid ratio of 1:1000 to visualize liquid disordered (*ld)* and liquid ordered (*lo)* domains, respectively. GUVs were directly observed in a TCS-SP2 confocal microscope (Leica Mycrosystems CMS GmbH, Mannheim, Germany). DiIC12 was excited by a 514 nm Ar^{++} laser line and BPY-Chol by a 488 nm laser. Fluorescence intensity of confocal images was quantified with the software Fiji [28] by outlining regions of interest (ROI). Mean gray value (MGV) was quantified as the fluorescence intensity relativized by the area of each GUV. To quantify total fluorescence of a ROI, the integrated density was calculated as the sum of all the gray values of each pixel.

2.7.2. *Imaging of BODIPY-Cholesterol in living sperm*

A stock solution (1 mM) of BPY-Chol was prepared in ethanol and stored in a dark glass tube at -20°C protected from light. A sperm suspension was diluted to a final concentration of 100×10^6 spermatozoa/mL in IVF-SOF medium supplemented with 1 μM of BPY-Chol and 0.5 μM propidium iodide and incubated at 38.5°C under 5% CO_2 in humidified air for 1 h in the dark. After incubation, 20 μL of the sperm suspension was placed for 10 min on a slide previously coated with poly-L-lysine for sperm immobilization. Adhered cells were washed with IVF-SOF medium and observed using an epifluorescence inverted microscope (Nikon TE-300; Nikon, Tokyo, Japan). The fluorescent dye BPY-Chol was excited at 450-490 nm and digital photographs were taken with a DSfi1 camera (Nikon, Tokyo, Japan) connected to the microscope. Fluorescence intensity of sperm from randomly selected microscope fields was measured using the software Fiji [28] in background corrected images. A mask was created to locate each sperm-associated fluorescence in the images

and measure the MGV from 5 fields at 40 × magnification, counting at least 200 spermatozoa for each experimental condition. To study differences in fluorescence intensity among spermatozoa within a population of cells, relative frequency histograms were performed by grouping MGV in 5-arbitrary fluorescence units (AFU) ranges. Additionally, the distribution of the probe among the different sperm compartments was studied in control and 25 mM MβCD-sterol treated sperm. Linear fluorescence profiles were generated by outlining a line-selection of 25 μm along each spermatozoon from the head to the end of the middle piece to quantify the MGV in each pixel of the image linear profile.

2.8. Localization of the raft marker lipid GM1

The glycosphingolipid GM1 was detected in living sperm by using the fluorescent-labeled cholera toxin B subunit (CTB-AF^{488}), which binds specifically to the ganglioside. A sperm suspension was diluted to a final concentration of 100×10^6 spermatozoa/mL in IVF-SOF medium and 100 μL of the cell suspension were incubated with 20 μg/mL of CTB-AF^{488} and 0.5 μM propidium iodide for 30 min in the dark at 38.5°C under 5% CO_2 in humidified air. After incubation, adhered cells to poly-L-lysine were washed with IVF-SOF medium, excited at 450-490 nm and imaged in an epifluorescence inverted microscope (Eclipse TE- 300; DS-Fi1c camera, Nikon, Tokyo, Japan). Sperm mean fluorescence intensity (MGV) from randomly selected microscope fields was measured using the software Fiji [28] in background corrected images using a mask created to locate the sperm fluorescence. Quantitative fluorescence analysis was performed from at least 200 spermatozoa from 5 fields at 40 × magnification for each experimental condition.

2.9. Semen cryopreservation

Semen pre-incubated with Tris-citric acid buffer (control) or MβCD-sterol (treatment) was diluted in three steps with a soy lecithin-based commercial extender (Andromed© Minitüb, Germany) to a final concentration of 400×10^6 sperm/mL and packaged into 0.25 mL straws (Minitüb, Germany). Temperature was progressively decreased from 32°C to 5°C with a 0.1°C/min rate in a water bath

and equilibrated for 2 h at this temperature. The straws were frozen in liquid nitrogen vapors (4 cm above the liquid level) for 10 min and finally stored in liquid nitrogen tanks. Thawing was performed by immersion of the straws in a water bath at 37°C for 30 sec.

2.10. Sperm quality analysis

Semen quality parameters were analyzed in 5ºC-cooled and frozen-thawed sperm. Sperm total motility was scored on a 0 to 100% scale in samples placed under a coverslip using a phase contrast microscope (Zeiss, Germany) fitted with a 38°C warm stage. Membrane integrity was evaluated by the eosin-nigrosin exclusion test [29]. A smear was made on the slide, and a total of 200 cells were counted under optical microscopy at 40 × magnification, recording the non-stained cells as membrane-intact sperm and the reddish-pink stained cells as sperm with damaged membranes. Plasma membrane functional integrity at the acrosome and tail regions was evaluated using the Hypoosmotic Swelling test (HOSt) by exposing spermatozoa to hypoosmotic conditions [30]. The spermatozoa were observed under pase contrast microscopy and classified as HOSt-positive or -negative based on the presence or absence of a coiled tail, respectively.

2.11. ***Statistical analysis***

Statistical analysis was carried out using InfoStat software [31]. Sterol content, biophysical studies and fluorescence intensity were analyzed through analysis of variance (ANOVA), followed by *post hoc* test analysis of multiple comparisons Bonferroni or Fisher's Least Significant Difference (LSD Fisher). When two mean values were compared, Student´s *t* test was used. Seminal quality variables (binomial distribution) were compared using Generalized Linear Mixed Models (GLMM) with Binomial family, *logit* link function and LSD Fisher contrast. Fluorescence profile of sperm incubated with BPY-Chol was analyzed through ANOVA for each pixel of the profile using a slighting window in software R [32]. Differences were considered significant at $p<0.05$. All experiments were repeated at least three times. The number of repetitions for each evaluation is indicated in the figure legend.

3. Results

3.1. Quantification of cholesterol and desmosterol incorporation into ram sperm membrane

To identify and quantify free sterol classes, neutral lipids were isolated from ejaculated sperm lipid extracts and the fraction corresponding to free sterols was resolved by reverse HPLC (Fig. 1A). Cholesterol was the major sterol component in ram sperm, accounting for 93.5% ± 2.3 of the total sterols, while desmosterol represented 6.5% ± 0.14 of the sterol fraction (Fig. 1B). Total sterols were quantified by an enzymatic assay after incubation of sperm with increasing concentrations of MβCD-Chol or -Des (0, 2.5, 10, 25 mM) (Fig. 1C). An increase in sperm total sterols was observed when the cells were incubated with 10 and 25 mM of MβCD-Chol or –Des. Incubation of spermatozoa with 2.5 mM MβCD-sterols did not increase sperm sterol content, irrespective of the sterol used in the complex (Chol or Des). With 10 mM MβCD-Chol or -Des, cholesterol and desmosterol content both increased similarly to almost double the original sterol level. At the highest concentration of MβCD-Chol assayed (25 mM), cholesterol seems to have reached a *plateau* since no further increase was observed relative to 10 mM. However, 25 mM MβCD-Des incubation did further increase total sterol content to almost triple that of the control, resulting in the main sterol in this experimental group compared to the sperm that incorporated cholesterol (Fig. 1D-E).

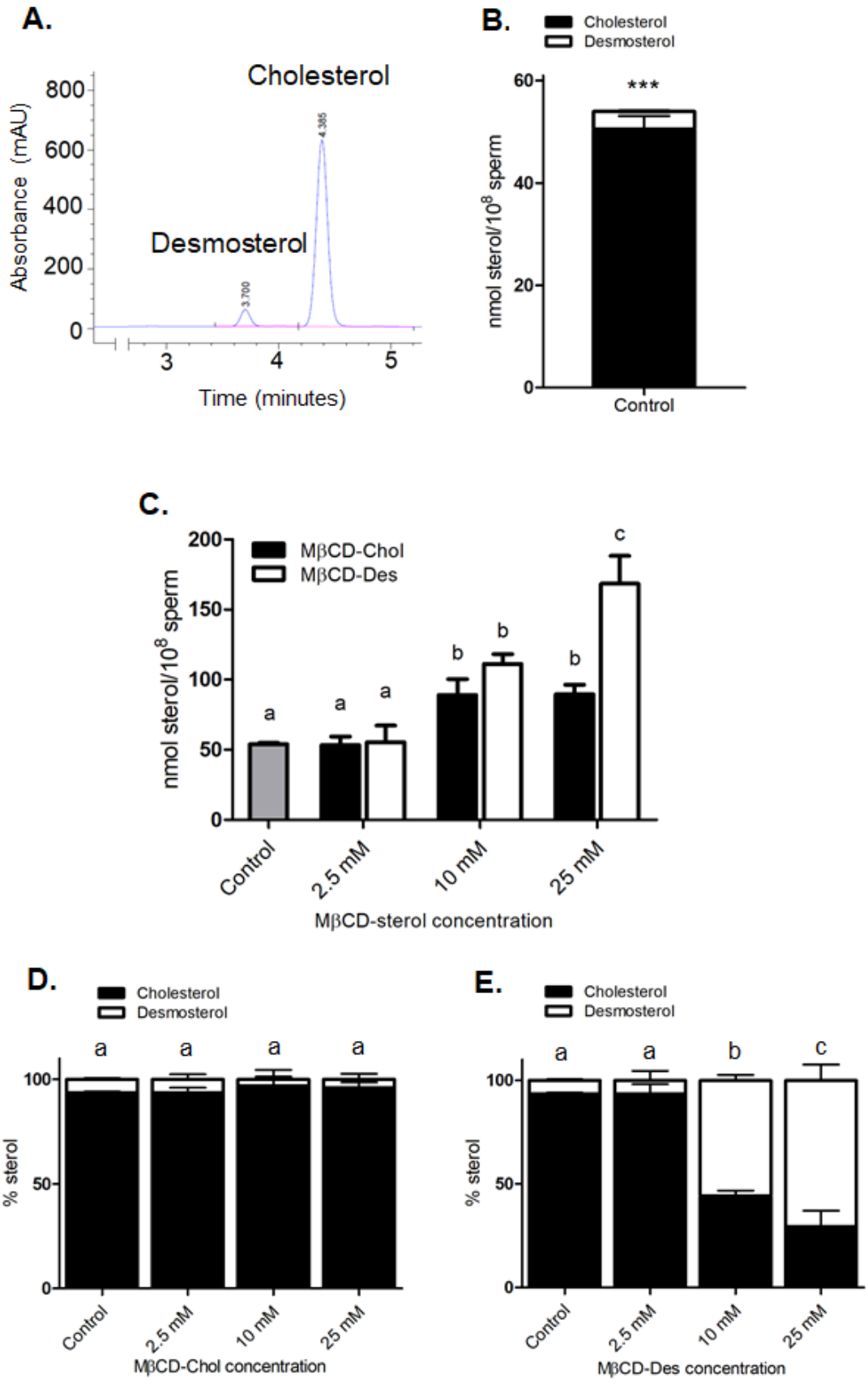
A.
Cholesterol
Desmosterol
Absorbance (mAU)
800
600
400
200
0
3.700
4.385
3
4
5
Time (minutes)
B.
Cholesterol
Desmosterol
***
nmol sterol/10^8 sperm
60
40
20
0
Control
C.
MβCD-Chol
MβCD-Des
nmol sterol/10^8 sperm
200
150
100
50
0
a
a
a
b
b
b
c
Control
2.5 mM
10 mM
25 mM
MβCD-sterol concentration
D.
Cholesterol
Desmosterol
a
a
a
a
% sterol
100
50
0
Control
2.5 mM
10 mM
25 mM
MβCD-Chol concentration
E.
Cholesterol
Desmosterol
a
a
b
c
% sterol
100
50
0
Control
2.5 mM
10 mM
25 mM
MβCD-Des concentration

Fig 1. Quantification of cholesterol and desmosterol in ovine spermatozoa. (A) Identification of cholesterol and desmosterol by HPLC in the ovine sperm sterol fraction. The peaks correspond to each sterol with the indicated retention times. (B) Quantification of cholesterol and desmosterol in ovine spermatozoa by HPLC. The results are expressed as percentage of sterols and represent the mean values ± SD (N=3). Data were analyzed using the Student's *t* test. Asterisks (***) indicate significant differences (****p*<0.001). (C) Effect of MβCD-Chol or -Des treatment on total sterol content of ram sperm. Results are expressed as nmol of sterols per 100 million sperm and represent mean values ± SEM (N=4). Data were analyzed by ANOVA and mean values were compared using the *post hoc* test LSD of Fisher. Different letters (a-c) indicate significant differences ($p<0.05$). (D-E) Percentage distribution of cholesterol and desmosterol in ovine spermatozoa after treatment with MβCD-Chol (D) or -Des (E). The results are expressed as a percentage of sterols and represent the mean values ± SEM (N=4). Desmosterol content among the experimental groups was compared by MLGM with binomial distribution. The different letters (a-c) indicate significant differences ($p<0.05$).

3.2. Effect of sterol enrichment on the biophysical state of model membranes prepared from sperm lipids

Generalized polarization (GP) was calculated from Laurdan emission spectra obtained by direct excitation of the probe. Higher GP values indicate higher membrane lipid order (lower membrane fluidity, fewer water molecules). To evaluate any possible differential biophysical behavior between cholesterol and desmosterol, the lateral ordering effect of each sterol was measured in liposomes prepared from sperm lipids extracted under control conditions and after adding cholesterol or desmosterol exogenously (Fig. 2A). At equivalent concentrations of each sterol, no statistical difference was found between GP values in relation to the temperature (Fig. 2B).

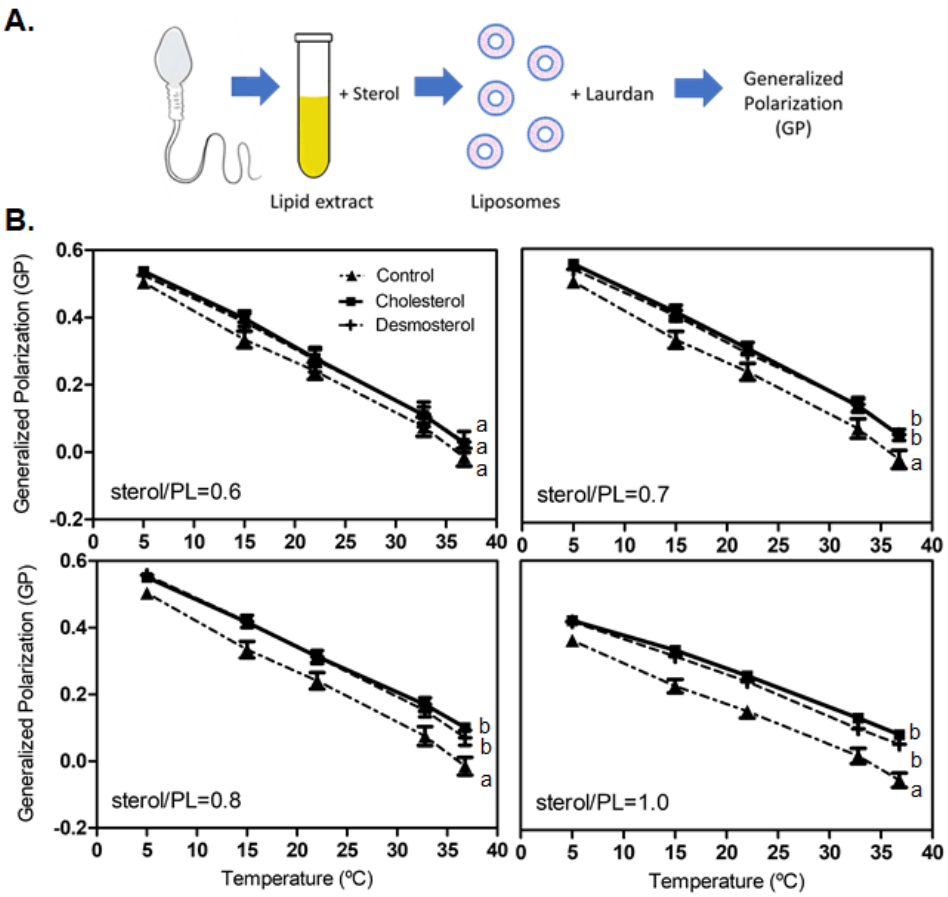

Fig 2. Comparative biophysical properties of model membranes prepared from sperm lipids containing increasing contents of cholesterol or desmosterol. (A) Schematic diagram of the experimental approach used to evaluate the biophysical behavior of each sterol under controlled conditions. (B) Thermotropic profiles of Laurdan generalized polarization (GP) assessed in model membranes prepared from ram sperm lipids with increasing sterol to phospholipids ratio. Results represent mean values ± SEM (N=3). Data were analyzed by ANOVA and mean values were compared using the *post hoc* test LSD of Fisher. Different letters (a-b) indicate significant differences ($p<0.05$) among experimental groups. No differences were found between sterols at any of the conditions assayed ($p>0.05$). PL: Phospholipids.

To study the effect of sterol incorporation into sperm after treatment with increasing concentrations of MβCD-Chol or -Des (Fig. 3A), membrane lipid order was analyzed in liposomes prepared from sperm lipids. GP values gradually increased when temperature was varied from 38°C to 5°C, without a thermotropic phase change. At physiological temperature (38°C), the control showed the lowest GP value, and this condition was maintained all along the thermic decrease (Fig. 3B and Fig. 3C). Treatment of sperm with 2.5, 10 and 25 mM MβCD-Chol or -Des increased membrane lipid order relative to the control, at all temperatures evaluated. Liposomes prepared from sperm treated with 10 or 25 mM MβCD-Chol showed the same thermal behavior. At 38°C liposomes from sperm treated with 10 mM MβCD-Chol showed GP values significantly higher than those from sperm treated with 25 mM. On the other hand, the liposomes prepared from sperm treated with 2.5 and 10 mM MβCD-Des showed similar GP values in the range of temperatures 25-5°C, while in the range 32-38°C liposomes from sperm treated with 10 mM MβCD-Des showed higher values of GP. Liposomes from sperm treated with 25 mM MβCD-Des showed the highest GP values at all temperatures evaluated except at 5°C, the temperature at which GP values among MβCD-Des concentrations became similar. At the lowest concentration assayed (2.5 mM MβCD-sterol), an increase in membrane lipid order was detected with the probe Laurdan with respect to controls.

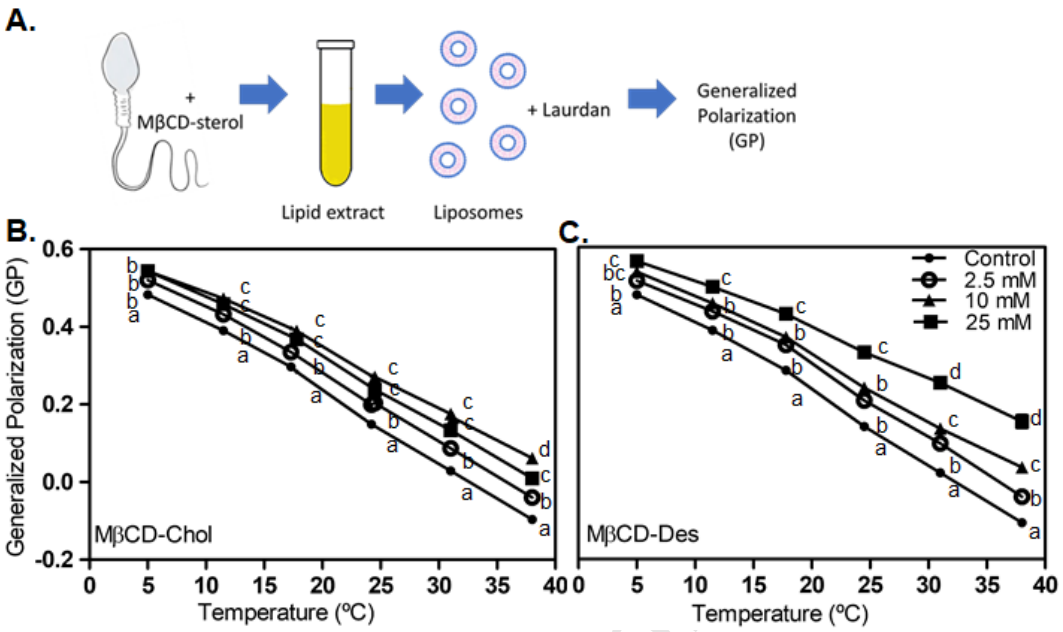

Fig 3. Effects of sterol incorporation on membrane lipid order of ram sperm. (A) Schematic diagram of the experimental approach used to evaluate the biophysical behavior of lipids from sperm after MβCD-sterol treatment. (B-C) Thermotropic profiles of Laurdan generalized polarization (GP) in protein-free model membranes prepared from ram spermatozoa after incubation with increasing concentrations of MβCD-Chol or -Des. Results represent mean values ± SEM (N=3). Data were analyzed by ANOVA and mean values were compared using the *post hoc* Bonferroni test. Different letters (a-c) indicate significant differences among experimental groups at each temperature evaluated (p<0.05).

3.3. In situ *imaging of BODIPY-Cholesterol*

To explore whether BPY-Chol can also be used as a desmosterol estimator and tracer, we evaluated BPY-Chol-associated fluorescence in ternary-mixture GUVs composed of SM, POPC and either cholesterol or desmosterol. BPY-Chol was used in combination with the fluorescent probe DiIC12 to visualize phase segregation in this artificial membrane system. BPY-Chol and DiIC12

differentially partition into liquid-ordered (*lo*) or liquid-disordered (*ld*) domains, respectively. BPY-Chol was found to partition mainly into *lo* domains in GUVs containing either cholesterol or desmosterol (Fig. 4A). Mean fluorescence intensity did not differ between *lo* domains from cholesterol-containing GUVs compared to those containing desmosterol, indicating that BPY-Chol partitions into both *lo* domains with the same equilibrium density (Fig. 4B). On the other hand, total fluorescence ratio was lower in desmosterol-containing GUVs compared to cholesterol-containing GUVs, thus indicating that the area occupied by *ld* domains is larger than the area occupied by *lo* domains.

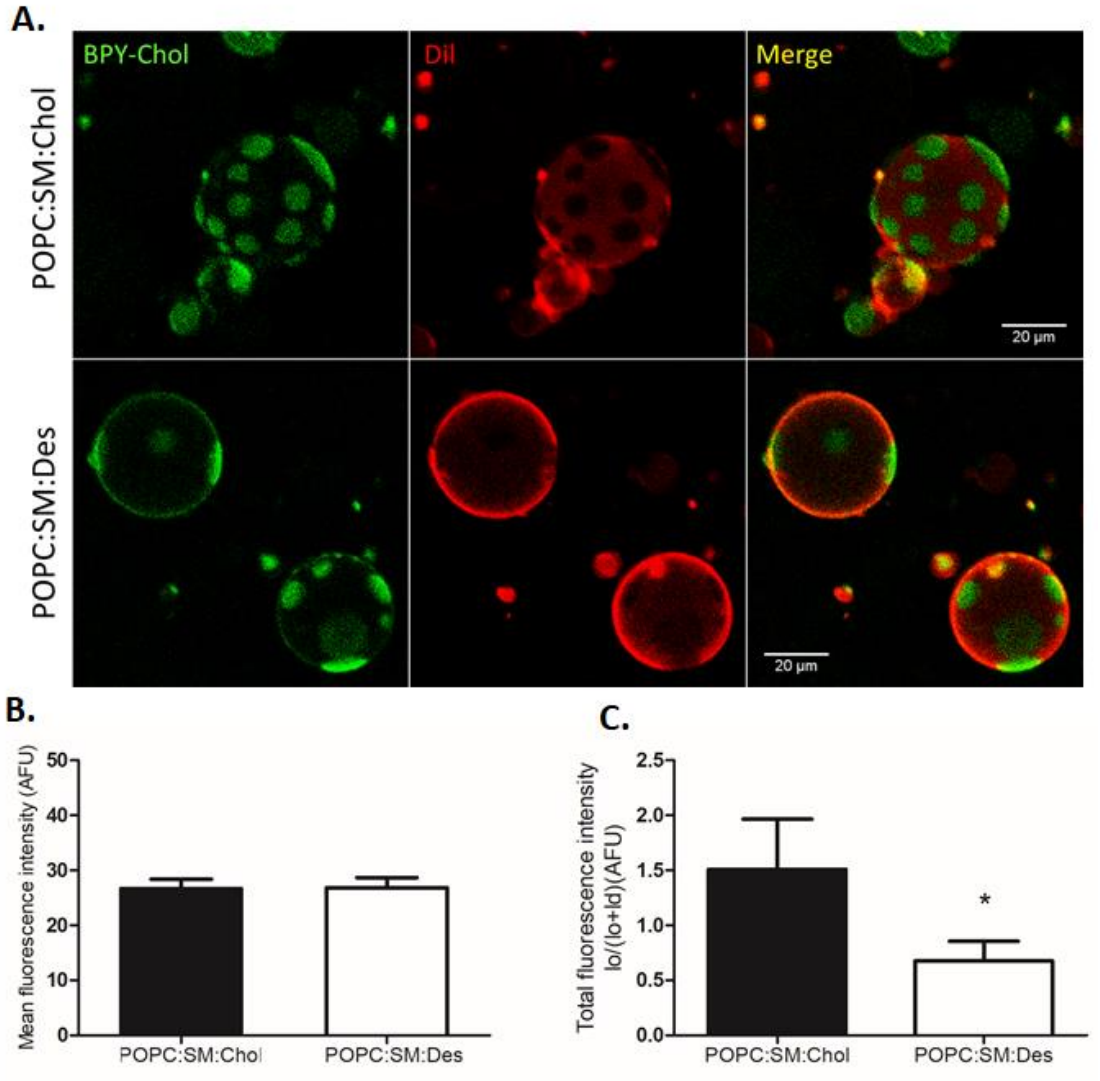

Fig 4. Partitioning of BPY-Chol into cholesterol or desmosterol containing giant unilamellar vescicles with *lo-ld* phase coexistence. (A) Confocal images of cholesterol or desmosterol containing giant unilamellar vescicles (GUVs) prepared from SM, POPC, and cholesterol or desmosterol (upper and lower rows, respectively). (B) Quantification of mean fluorescence intensity of BPY-Chol in cholesterol- or desmosterol-containing GUVs. (C) Quantification of total fluorescence intensity of BPY-Chol in *lo*/(*lo*+*ld*) domains from cholesterol- or desmosterol-containing GUVs. The bars represent the mean ± SEM of a total of (N=110 cholesterol containing

GUVs, N=170 desmosterol containing GUVs). Data were analyzed by Student´s *t* test. The asterisk indicates significant differences ($p<0.05$).

To further investigate the effect of MβCD-sterol treatment on ram spermatozoa, we incubated sperm from each experimental condition with BPY-Chol and estimated the extent and cellular localization of sterol incorporation. Analysis of BPY-Chol fluorescence revealed that treatment of ram sperm with 2.5 mM MβCD complexed either with cholesterol or desmosterol did not increase mean fluorescence intensity of spermatozoa (Fig. 5A). An increase of fluorescence intensity was observed after treatment with 10 and 25 mM MβCD, irrespective of the sterol class incorporated. However, no significant difference in terms of BPY-Chol fluorescence level was detected between both concentrations. Living sperm incubated with BPY-Chol displayed differences in fluorescence intensities among cells (Fig. 5B). As can be observed in fluorescence frequency histograms (Fig. 5C), this finding is compatible with the existence of different sterol content among cells within the same population. However, all conditions showed a unimodal frequency distribution, being the most frequent values within 15-20 AFU, except for the control group (5-10 AFU range) and 25 mM MβCD-Chol treated sperm (20-25 AFU). In the control group, more than 80% of the sperm showed low fluorescence intensities (less than 10 AFU). At 2.5 mM of MβCD, BPY-Chol fluorescence did not increase (Fig. 5A) but, unlike the control group, the most frequent values of fluorescence intensity were found in the range 15-20 AFU. Regardless of the type of sterol incorporated, as the sterol content increased after incubation with MβCD 10 and 25 mM, spermatozoa with low fluorescence intensities decreased and the percentage of sperm with higher mean fluorescence intensities increased (up to 60 AFU).

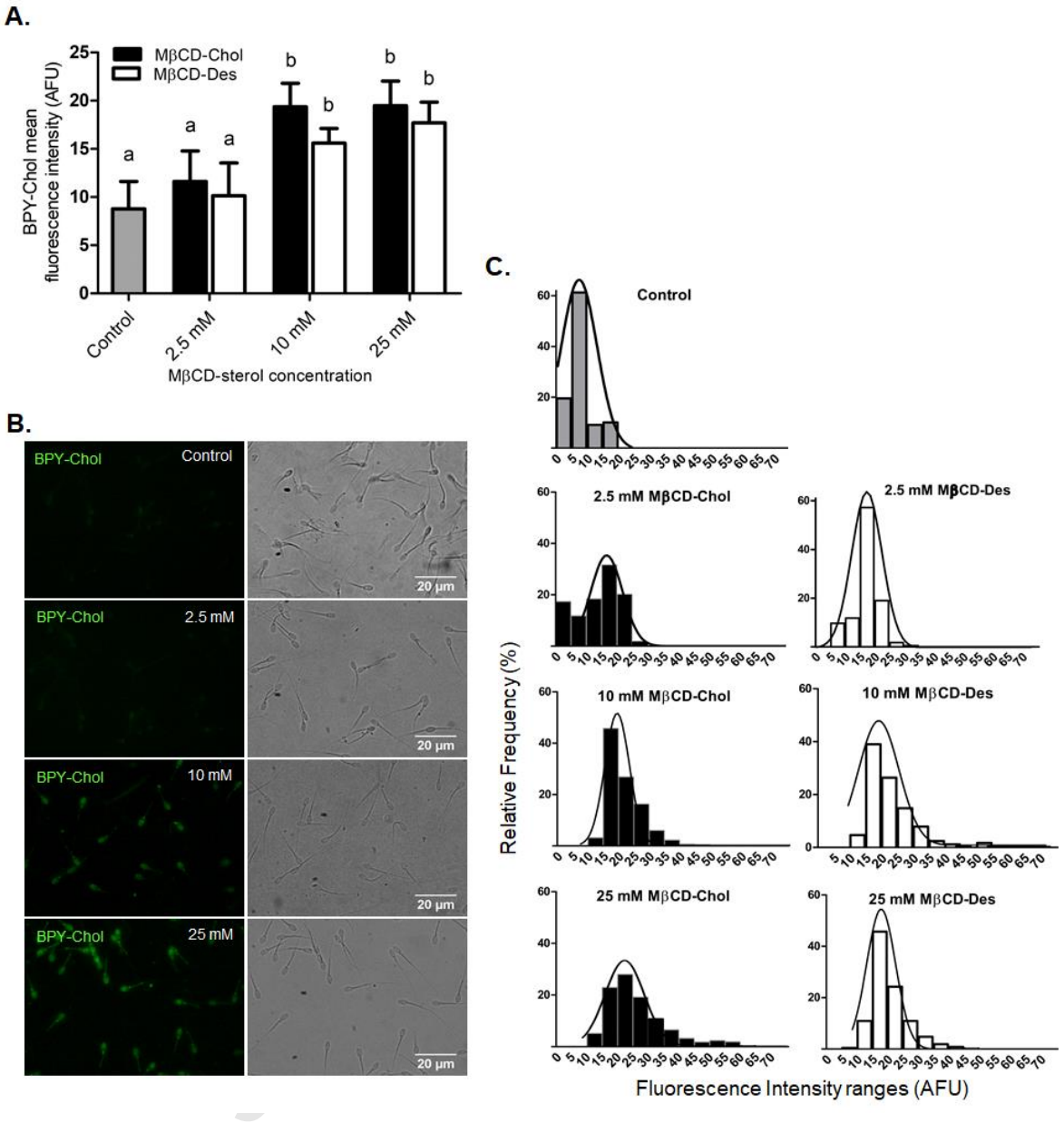

Fig 5. Incorporation and distribution of sterols in ovine spermatozoa. (A) Incorporation of sterols into ram spermatozoa estimated by BPY-Chol labeling of living cells. Results are expressed as arbitrary fluorescence units (AFU) and represent mean values ± SEM (N=4).Data were analyzed by ANOVA and mean values were compared using the *post hoc* Bonferroni test. Different letters (a-b) indicate significant differences (p<0.05). (B-C) Distribution of sub-populations of spermatozoa compatible with different sterol contents. (B) Left: Representative images of ram spermatozoa incubated with BPY-Chol after sterol incorporation at different MβCD-Chol concentrations. Right:

Bright fields. (C) Histograms of the relative frequencies of BPY-Chol mean fluorescence intensity measured per spermatozoa. Lines estimate unimodal distributions of each experimental group.

In addition, membrane distribution of BPY-Chol in ram sperm compartments was studied by analyzing fluorescence profiles of spermatozoa that incorporated either cholesterol or desmosterol at 25 mM MβCD-sterol according to the increased level of sterol incorporation at this concentration (Fig. 6). The ram sperm showed higher intensity of BPY-Chol fluorescence in the acrosomal region compared to the post-acrosomal region and the mid-piece (Fig. 6). After MβCD-Chol treatment, fluorescence intensity significantly increased in all sperm compartments compared to the control but the same distribution profile among compartments was maintained. On the other hand, after MβCD-Des treatment, BPY-Chol fluorescence increased significantly only in the mid-piece region.

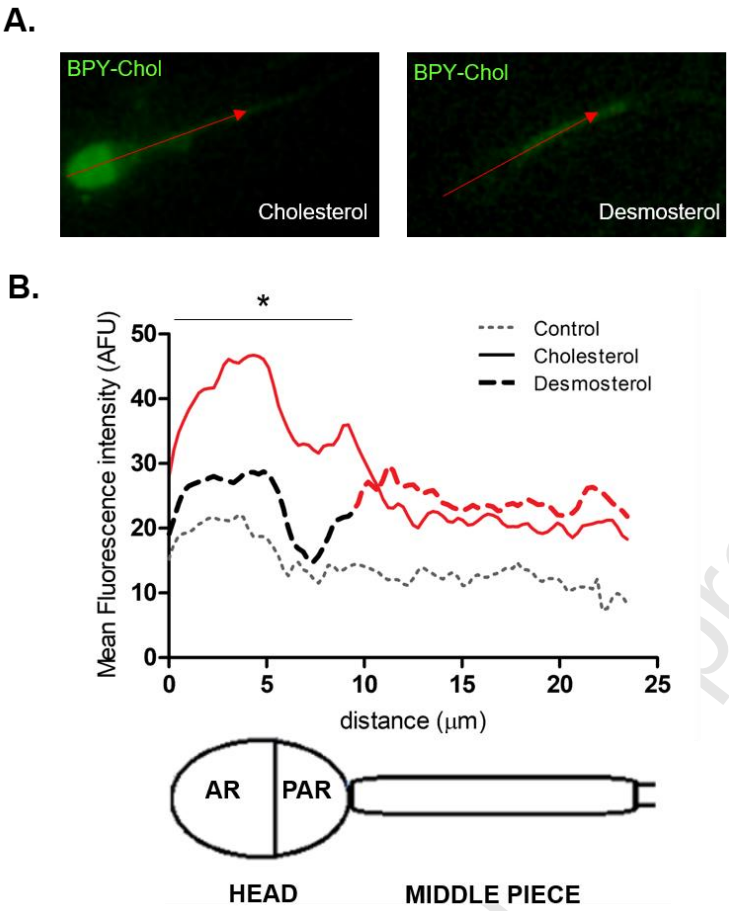

Fig 6. Line fluorescence intensity profiles of BPY-Chol in control sperm and 25 mM MβCD-sterol treated sperm. (A) Fluorescence images of representative sperm incubated with BPY-Chol after MβCD-sterol treatment. The red arrow in the upper images shows the location and direction used for generating the line intensity profile in sperm that incorporated cholesterol (left) or desmosterol (right). (B) Mean fluorescence intensity of BPY-Chol along sperm compartments. The values represent the mean fluorescence intensity expressed as AFU of each pixel measured on the line selection (N=4). Data of each pixel was analyzed with ANOVA using a sliding window. Profile lines in red represent pixels with fluorescence intensity significantly higher (p<0.05) than the control. The asterisk indicates significant differences between sterol groups (p<0.05). Below: schematic diagram showing sperm compartments crossed by the line intensity profile. AFU: arbitrary units of fluorescence; AR: acrosomal region; PAR: post-acrosomal region.

3.4. Effect of sterol incorporation on the raft marker lipid GM1 in living sperm

To study the effect of cholesterol or desmosterol incorporation on microdomain organization of the sperm plasma membrane, both fluorescence intensity and distribution of the raft marker, ganglioside GM1, were evaluated. Live ovine spermatozoa displayed different fluorescence patterns, with GM1 mainly located in the sperm head (Fig. 7A). When each experimental group was compared to the control, treatment with increasing concentrations of either MβCD-Chol or -Des did not change the distribution of GM1-related patterns (data not shown). Total fluorescence intensity of CTB-AF^{488} was quantified in control and experimental groups (Fig 7C). Incorporation of sterols at 2.5 and 10 mM of MβCD did not alter GM1-associated fluorescence intensity, irrespective of the sterol incorporated. At 25 mM MβCD-Chol, no changes of GM1-associated fluorescence were observed, according to the *plateau* in sterol incorporation previously registered at 25 mM MβCD-Chol. However, at 25 mM MβCD-Des, a decrease in the intensity of GM1-associated fluorescence was observed.

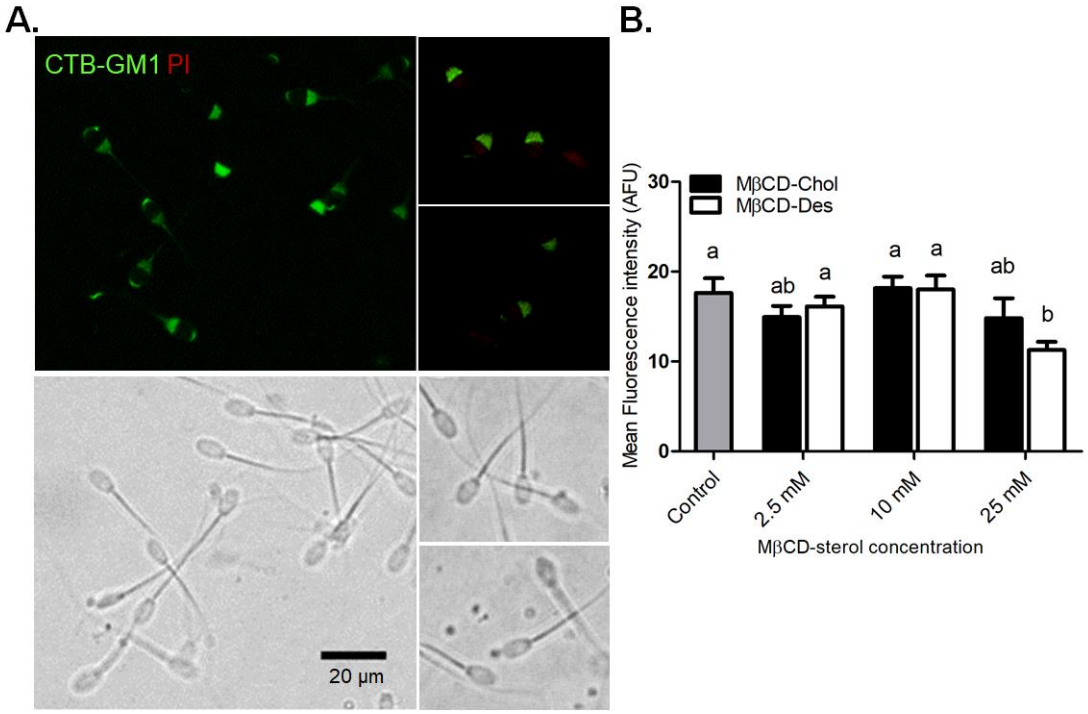

Fig 7. GM1-associated fluorescence in living ram sperm. (A) Representative fluorescence images of CTB-AF^{488} bound to GM1 in ram sperm with bright fields below. (B) Effect of treatment of ram spermatozoa with increasing concentrations of MβCD-sterol on GM1-associated fluorescence intensity. Results are expressed as arbitrary units of fluorescence (AFU) and represent mean values ± SEM (N=4). Data were analyzed by non-parametric Kruskall-Wallis test. The different letters (a-b) indicate significant differences (p<0.05). PI: propidium iodide.

3.5. Outcome of MβCD-sterol treatment in ram sperm cryopreservation

Incubation of fresh sperm with 10 mM MβCD complexed either with cholesterol or desmosterol increased sperm sterol content to the same extent, with a concomitant increase in membrane lipid order and no alteration of GM1-associated fluorescence. For this reason, this concentration of MβCD-sterol was selected for further cryopreservation analyses. Seminal quality parameters were evaluated in refrigerated and frozen-thawed spermatozoa pretreated with 10 mM of MβCD-sterol (Figs. 8 and 9). Incorporation of cholesterol or desmosterol increased total motility and the ability of the sperm membrane to overcome osmotic stress after refrigeration (Fig. 8A and 8C). No effect on the integrity of the membrane was observed in refrigerated sperm (Fig. 8B). Moreover, treatment with 10 mM MβCD-Chol increased sperm total motility, membrane integrity and HOSt positive cells after freezing and thawing (Fig. 9). However, treatment with 10 mM MβCD-Des only increased the percentage of positive cells for the HOSt.

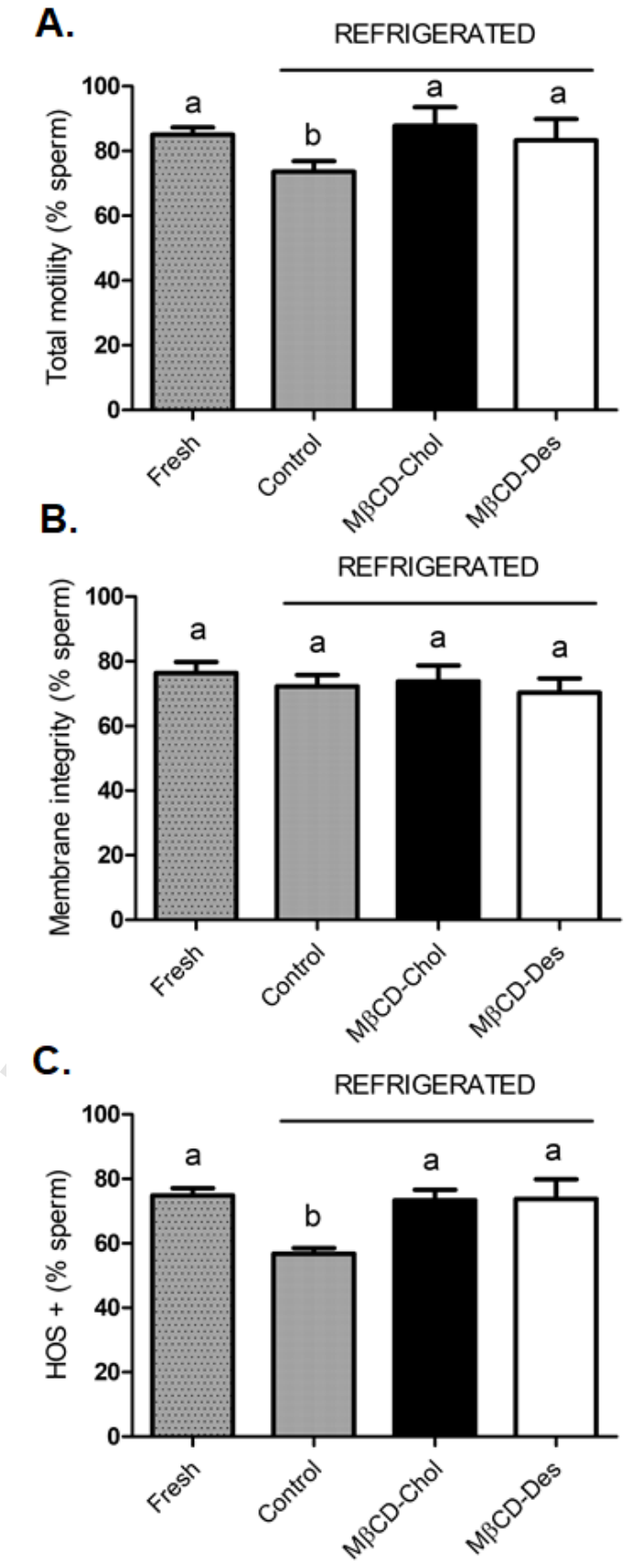
A.
REFRIGERATED
Total motility (% sperm)
100
80
60
40
20
0
a
b
a
a
Fresh
Control
MβCD-Chol
MβCD-Des
B.
REFRIGERATED
Membrane integrity (% sperm)
100
80
60
40
20
0
a
a
a
a
Fresh
Control
MβCD-Chol
MβCD-Des
C.
REFRIGERATED
HOS + (% sperm)
100
80
60
40
20
0
a
b
a
a
Fresh
Control
MβCD-Chol
MβCD-Des

Fig 8. Seminal quality parameters in fresh and refrigerated spermatozoa pretreated with 10 mM MβCD-sterol. (A) Total Motility. (B) Membrane integrity. (C) HOSt-positive cells. Results are shown as percentages of spermatozoa and represent mean values ± SEM (N=3). Data were analyzed using Generalized Linear Mixed Models (GLMM) with Binomial family. The different letters (a-b) indicate significant differences (p<0.05).

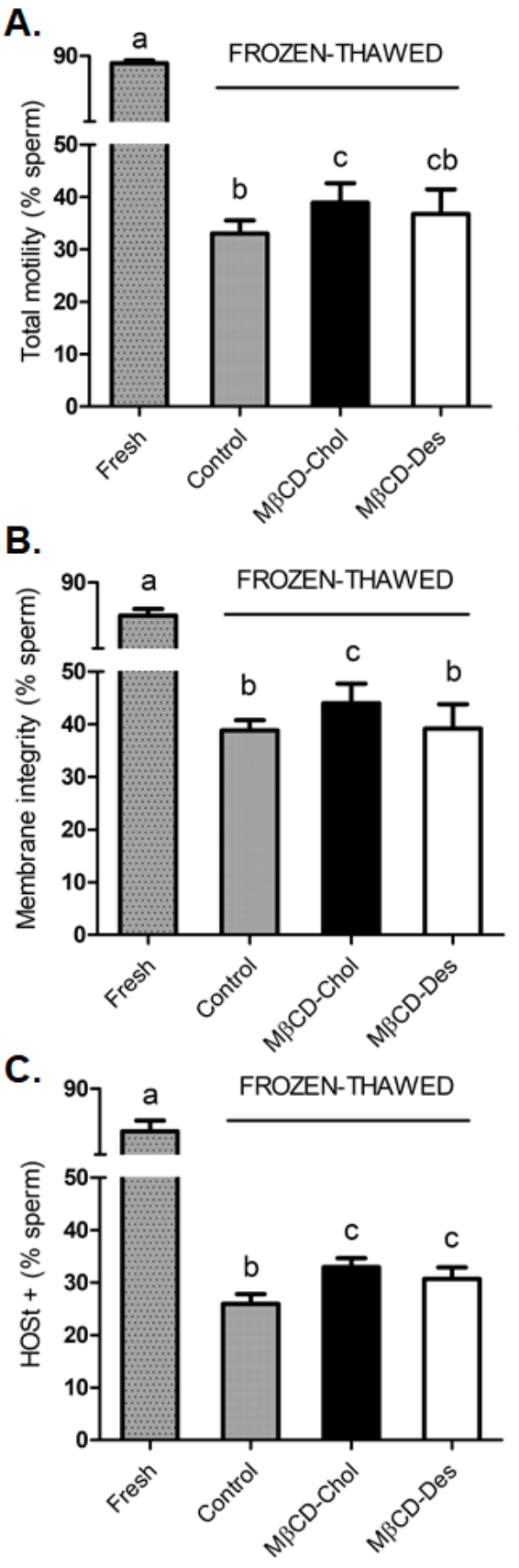
A.
FROZEN-THAWED
Total motility (% sperm)
a
b
c
cb
B.
FROZEN-THAWED
Membrane integrity (% sperm)
a
b
c
b
C.
FROZEN-THAWED
HOSt + (% sperm)
a
b
c
c
Fresh
Control
MβCD-Chol
MβCD-Des

Fig 9. Effect of 10 mM MβCD-sterol pretreatment on seminal quality of frozen-thawed spermatozoa. (A) Total Motility. (B) Membrane integrity. (C) HOSt-positive cells. Results are shown as percentages of spermatozoa and represent mean values ± SEM (N=9). Data were analyzed using Generalized Linear Mixed Models (GLMM) with Binomial family. The different letters (a-b) indicate significant differences (p<0.05).

4. Discussion

This study provides new insights into the extent of sterol incorporation in ram sperm, its effects on membrane biophysical properties and organization, and its role in preventing cryodamage. When membranes are rich in unsaturated phospholipids, such as sperm membranes, increasing cholesterol content mostly results in an increase in the order of the lipid bilayer. The planar structure of cholesterol confers special biophysical properties that influence membrane fluidity and hence a wide variety of membrane regulated processes. Interestingly, the ability of desmosterol to modulate the biophysical properties of the sperm membrane and its role in sperm functionality are still understudied topics. In the present work, we analyzed cholesterol and desmosterol incorporation into the ram sperm membrane by incubation with MβCD-sterol complexes prior to cryopreservation in a cholesterol-free extender. This is the first report in which MβCD-sterol complexes were used in combination with a sterol-free extender of defined composition based on soy lecithin. Under these experimental conditions, MβCD mediated the incorporation of cholesterol and desmosterol with different efficacy depending on the concentration. Both sterols were incorporated into ram sperm membrane at the concentration of 10 mM MβCD-sterol. However, at the highest concentration assayed (25 mM MβCD-sterol), the cholesterol level seems to have reached a *plateau* while desmosterol continued to be incorporated until it tripled the basal sterol level. This phenomenon could be explained by the possibility that a cholesterol saturation equilibrium point at the membrane reduced its capacity to incorporate more cholesterol beyond this point. Physiological content of

desmosterol in the ram sperm membrane is minor and for this reason when cells are treated with high concentrations of MβCD-sterol, the membrane is more prompt to incorporate a larger extent of this sterol compared to cholesterol (which represents more than 90% of total ram sperm sterols).

When biophysical properties of cholesterol and desmosterol were compared in liposomes prepared from sperm lipids, both sterols similarly increased membrane lipid order at equivalent controlled concentrations of each sterol and according to total sterol increase. The ability of these sterols to increase membrane lipid order was previously shown by other authors in model membranes [33]. In very disordered lipid bilayers, such as PUFA-rich sperm membranes, the effect of desmosterol on the lipid order is very similar to that of cholesterol [33,34]. The *plateau* in the incorporation of cholesterol registered by means of the enzymatic assay was reflected in similar lipid order profiles between 10 and 25 mM MβCD-Chol. As expected and recently well-documented by other authors [35], at higher temperatures the difference between GP values of different cholesterol content conditions has been shown to increase. Consequently, small differences in lipid order are evidenced. In this respect, at 38°C, GP values of liposomes from sperm treated with 10 mM MβCD-Chol were statistically higher than those from sperm treated with 25 mM MβCD-Chol. As discussed above, membrane cholesterol incorporation reached a *plateau* at 10 mM MβCD-Chol, resulting in a dynamic equilibrium between membrane and MβCD with very small differences in effective cholesterol incorporation. Therefore, this difference in GP between 10 and 25 mM MβCD-Chol can be explained by a very small difference in cholesterol incorporation between both conditions, being subtly higher in the former. However, it was not reflected as a significant difference by the colorimetric method, reaffirming the high sensitive of Laurdan. It is known that Laurdan probe, due to its molecular structure and location, is very sensitive to the presence of cholesterol molecules in liquid phases. In addition to sensing the packaging of the acyl chains, this probe is also directly influenced by additional membrane-condensing effects of cholesterol [35]. On the other hand, we did not observe a main thermotropic phase transition in liposomes containing lipids from ram sperm

at the evaluated temperature range. However, small deviation from linearity can be observed between 20°C and 30°C, according with a previous study [36]. Using DPH, these authors described three phase transitions at 35-38 °C, 24-26 °C and 15-17 °C in isolated ram sperm membranes.

BPY-Chol is a fluorescent cholesterol analog that mimics cholesterol behavior in the lipid bilayer and enables relative estimation of sterol levels by direct monitoring of living cells [37]. In this respect, differential partition of BPY-Chol among cell populations with different cholesterol content has been previously demonstrated [37]. By incubating a mixed population of primary brain cells in culture with BPY-Chol, the label mostly highlighted neurons (with high sterol level) whereas the accompanying glial cells were labeled to a much lesser extent. In addition, BPY-Chol was found to partition preferentially into ordered (*lo)* domains in model membranes [37,38]. Interestingly, incubation with BPY-Chol has been recently cross-validated by mass spectrometry to quantify cholesterol efflux during capacitation of boar sperm [39]. Here, incubation of ram sperm with BPY-Chol revealed the existence of different sperm sub-populations compatible with different sterol contents. [40] Similarly, the identification of porcine sperm sub-populations based on filipin labeling and cholesterol content has been previously reported [40]. In the present study, we also found that BPY-Chol partitioned heterogeneously among ram sperm compartments with higher fluorescence in the acrosomal region with respect to the mid-piece and tail. It is likely that BPY-Chol signal was mainly associated with the plasma membrane since the acrosome derives from the Golgi apparatus which displays a low sterol:phospholipid ratio [41]. The presence of sterols in the acrosome plasma membrane exerts a stabilizing effect by increasing membrane lipid order. Cholesterol efflux from the apical region of the head towards its acceptor in the female reproductive tract destabilizes the membrane and triggers capacitation signaling in the sperm cell [42]. The post-acrosomal region showed lower BPY-Chol fluorescence intensity than the acrosomal region, compatible with lower content of sterols, as has been shown in sperm of other mammalian species [39,43–45]. Considering that membrane fusion events between the sperm and the oocyte initially

take place from the equatorial membrane, the lower sterol content at the posterior region of the head seems to be needed to increase membrane fluidity and favor this interaction. Fluorescence intensity of BPY-Chol was consistent with measured total sterol content, except for 25 mM MβCD-Des treated sperm that showed lower fluorescence intensity than initially expected. Indeed, fluorescence intensity in the head region of MβCD-Des treated sperm showed no differences with respect to the control and was lower than the fluorescence in 25 mM MβCD-Chol treated sperm. However, a significant increase of BPY-Chol fluorescence with respect to control sperm was observed in the middle piece region of sperm incubated with cholesterol or desmosterol. These results suggest that at high levels of sterols at the membrane, as observed at 25 mM MβCD-Des (also with higher desmosterol than cholesterol content in the membrane), desmosterol behaves as a raft-domain disruptor dispersing endogenous cholesterol and BPY-Chol molecules through the sperm membrane. Interestingly, in other species, such as monkey and rabbit, desmosterol has been found to be mainly concentrated in the sperm tail [46,47]. More information is needed to identify a possible differential spatial localization between sterols in the ram sperm.

In mammals, gangliosides are characteristic components of membrane rafts and therefore, GM1 ganglioside is widely used as a genuine marker of these microdomains [48,49]. The existence of large domains in the sperm plasma membrane has been associated with functional compartmentalization [50]. CTB fluorescence revealed a selective localization of GM1 in the ram sperm head, both in the apical acrosomal region and in the post-acrosomal region. These patterns have been observed in murine [51], rat [52] and ovine [53] sperm, however, it has been reported that CTB fluorescence patterns may differ depending on the species, type of sperm (epididymal or ejaculate) and on incubation and fixation conditions [49]. Moreover, it has been shown that CTB induces a relocation of GM1 from the plasma membrane in the acrosomal region to sterol-poor domains in the post-acrosomal region [54]. Hence, the biological relevance of this diversity of patterns is still unclear. In this scenario, and taking into account that chemical fixatives can alter *in*

situ localization of certain membrane molecules, in particular lipids [48], we quantified total intensity of CTB fluorescence in non-fixed ram sperm, regardless of fluorescence patterns. Interestingly, live cell imaging by BPY-Chol evidenced not only the existence of sperm sub-populations compatible with different sterol contents but also a high concentration of sterol-rich ordered domains mainly at the acrosome plasma membrane. Under these experimental conditions, only high desmosterol incorporation disorganized GM1-rich domains, a fact that correlates with the known desmosterol raft disruptive effect [7]. On the contrary, the level of sterols incorporated at 10 mM MβCD complexed either with cholesterol or desmosterol or at 25mM MβCD-Chol did not perturb the membrane domain organization. These results are in accordance with the fluorescence intensity observed after BPY-Chol incubation.

In the present study, incubation of ram sperm with 10 mM MβCD-sterol increased total sterol content and membrane lipid order without disturbing membrane lateral organization. Under these conditions, treatment of sperm prior to cryopreservation decreased ram sperm sensitivity to cooling and freezing, which was mainly reflected by an increase of seminal quality parameters after thawing. Treatment with 10 mM MβCD-Chol increased sperm motility and membrane integrity, while treatment with 10 mM MβCD complexed either with cholesterol or desmosterol increased the ability of ram sperm to overcome osmotic stress after thawing. Previously, it has been shown that temperature decrease induces cold shock damage in sperm, causing reduced motility and alterations in membrane permeability [55]. In our study, cholesterol or desmosterol incorporation increased membrane lipid order at supra-zero temperatures, leading to an increase in sperm tolerance to cooling. This effect may be due to an increase of the cell resistance to leaking or rupture as a response to the volume changes imposed by cooling. Interestingly, studies in model membranes showed that cholesterol increases the mechanical strength of lipid bilayers [56,57]. In the present study, MβCD molecules were removed from the cryopreservation base-medium before cryopreservation. After sterol transfer to the membrane, unsaturated MβCD may act as a sterol

acceptor instead of a donor. Indeed, MβCD has been used not only to generate cholesterol inclusion complexes that donate cholesterol to the membrane but also to remove cholesterol from biological membranes when used free of cholesterol [14]. Semen extenders based on egg yolk or skim milk contain lipoproteins that may act as sterol donors, preventing sterol loss from sperm membranes or interfering with controlled sterol incorporation mediated by MβCD.

Several mechanisms by which cholesterol exerts its effects on sperm cryotolerance have been proposed. Increase in sperm cryotolerance after cholesterol enrichment is often explained in terms of its ability to increase lipid packing and minimize the thermodynamic phase separation in the plane of the plasma membrane during cooling, thus preventing large-scale rearrangement of lipids. Studies in model membranes suggest that the protective effect of cholesterol arises from its capacity to reduce thermotropic phase transitions [58], which are thought to be intimately related to membrane leakage during temperature decrease [59]. Indeed, several authors found a positive correlation between low temperature lipid phase segregation and chilling resistance of gametes [60,61]. Considering the observed differences in seminal quality between cryopreserved sperm that incorporated cholesterol versus desmosterol, the protection mechanism seems to be more efficient with cholesterol. Lower affinity of desmosterol compared to cholesterol for segregating into ordered domains [7] may exert different local biophysical properties at low temperatures.

5. Conclusions

Cholesterol and desmosterol were effectively incorporated by ram sperm membrane, similarly increasing membrane lipid order at physiological temperature and during temperature decrease. Even when cholesterol and desmosterol incorporation exerted similar effects on global membrane lipid order, imaging of a fluorescent cholesterol analog in model membranes revealed a reduced tendency of desmosterol compared to cholesterol of segregating into ordered domains. Interestingly, live cell imaging by BPY-Chol evidenced not only the existence of ram sperm sub-populations with different sterol content but also a high concentration of sterol-rich ordered domains mainly at the

acrosome plasma membrane. Lateral organization of the plasma membrane, assessed by the identification of GM1-related rafts, was preserved after sterol incorporation except when high levels of desmosterol were incorporated. Finally, incubation of ram sperm with 10 mM MβCD-sterols prior to cryopreservation improved sperm quality parameters both, after cooling and freezing, with different efficiency in the protective effect of sterols. Future studies will be focused on determining whether this strategy for improving seminal quality after cryopreservation has a consequence on ram sperm fertilizing competence *in vitro* and *in vivo*.

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7. Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

8. Author contributions

María de las Mercedes Carro: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing-Original Draft. **Daniel A. Peñalva:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing-Original Draft. **Silvia S. Antollini:** Conceptualization, Formal analysis, Resources, Writing-Original Draft. **Federico A. Hozbor:** Conceptualization, Resources, Project administration, Funding acquisition. **Jorgelina Buschiazzo:**

Conceptualization, Methodology, Formal analysis, Resources, Writing-Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

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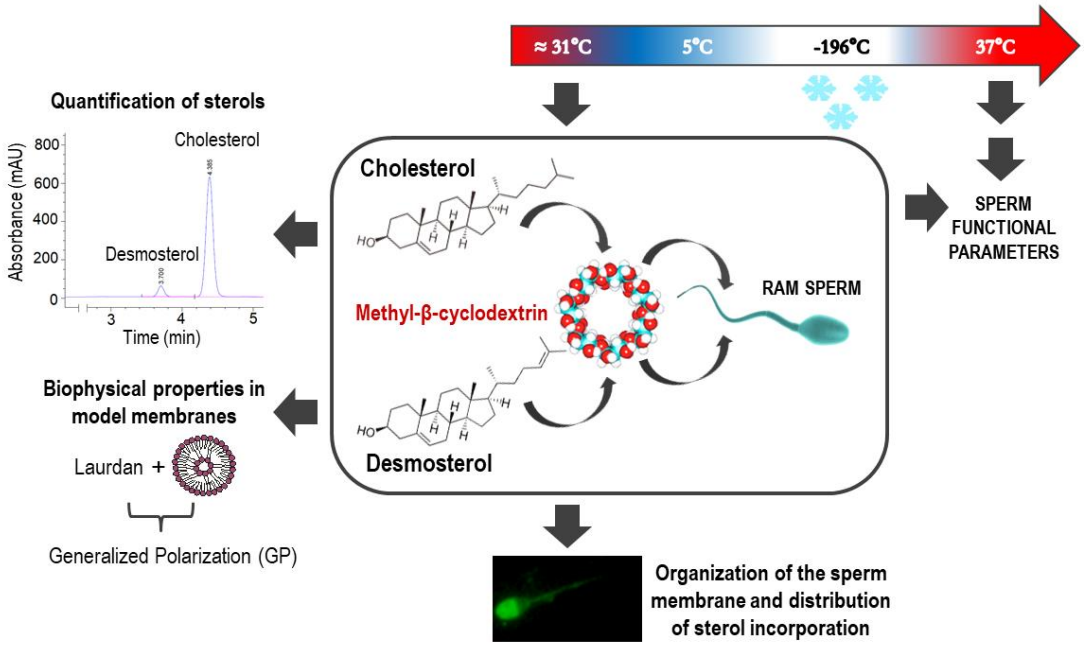
10. Footnotes

Abbreviations: MβCD-Chol and MβCD-Des, methyl-β-cyclodextrin bound to cholesterol and desmosterol, respectively; BPY-Chol, BODIPY-Cholesterol; CTB, cholera toxin subunit b; HPLC, high-pressure liquid chromatography; TLC, thin layer chromatography.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Graphical abstract

Highlights

- Cholesterol and desmosterol incorporated by ram sperm increased membrane lipid order.
- Differential ordering effect of sterols was shown in ternary-mixture model membranes.
- BPY-Chol imaging revealed sperm sub-populations with different sterol contents.
- Lateral organization of the plasma membrane was preserved after sterol incorporation.
- Incubation with sterols prior to cryopreservation improved post-thawed sperm quality.