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Sperm Cell Capacitation Status of Ram Semen after Cooling*

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ABSTRACT

***Background*:** The use of conventional artificial insemination (AI) in sheep production is usually associated with lower fertility rates when frozen semen is used. Cooled ram semen has been an alternative over frozen semen due to the higher viability, seminal quality and fertility rates following AI. The semen preservation process promotes sperm cell modifications similar to capacitation (capacitation-like) that causes cell damage affecting viability and seminal quality, but such effects are unclear for cooled semen. The aim of this study was to determine the status of sperm cell capacitation (CA) and acrosome reaction (AR) during ram semen processing and cooling under different extenders, dilution factors, and aerobiosis conditions as a function of storage time at 5ºC.

***Materials, Methods & Results*:** Two consecutive ejaculates per day per male were collected from 2 adult rams by artificial vagina at 48-72 h intervals, in three replications. After macro- and microscopic evaluations, semen was segregated into groups under 3 extenders (Tris-egg yolk or TY, citrate-egg yolk or CY, skimmed milk or SM), 2 dilution factors (1 x 10^9 or Bi, 100 x 10^6 or Mi cells/mL), and 2 aerobiosis conditions (aerobic or A, semi-anaerobic or SA). Diluted semen was cooled to 5ºC and stored for up to 72 h, with evaluations every 24 h. Aliquots of fresh ejaculates and of each cooled diluted subgroup, according to extender, dilution, and aerobiosis, were collected at times T0 and T72 for determination of acrosome status and membrane integrity by the chlortetracycline (CTC) and trypan blue-Giemsa stainings, respectively. No differences were detected in sperm cell motility (M) and motility vigor (V) between fresh and diluted semen. After cooling, a significant decrease in M was observed after 48 h in CY and SM compared with fresh semen and 0 h of cooling, while V started to decrease after 24 h in CY compared with TY. Likewise, M/V from different dilutions and aerobic conditions decreased more significantly after 48 and 24 h of cooling, respectively. The sperm capacitation status did not show differences in the proportion of non-capacitated (NCA), CA and AR sperm cells between TY, CY, and SM extenders (NCA: 75.0%, 71.3%, 74.0%; CA: 15.7%, 17.2%, 15.9%; AR: 9.3%, 11.5%, 10.2%) or between Bi and Mi dilutions (NCA: 74.0%, 72.9%; CA: 15.9%, 16.6%; AR: 10.1%, 10.5%), respectively. However, differences ($P < 0.05$) were observed between A and SA aerobic conditions, with CA (17.0% *vs*. 15.5%) and AR (11.9% *vs*. 8.7%) rates being higher in A than SA, respectively, with no differences in NCA (71.1% *vs*. 75.8%), irrespective of the storage time. Sperm cell viability decreased after 48 h, especially in CY ($P < 0.05$).

***Discussion*:** Ram sperm cells can suffer irreversible damage due to thermal shock during cooling. Egg yolk-based extenders provide phospholipids and cholesterol to protect the sperm cell membrane during the thermal shock caused by the change in temperature. In this study, sperm cells had irreversible decreases in M/V, with increase in acrosome and plasma membrane damage after cooling to 5ºC. The largest and smallest decreases in M and V over time were observed in the CY and TY extenders, respectively. In addition to the extender type, the semen preservation method and storage time promoted changes in the capacitation status, AR and in sperm cell viability, which *per se* were associated with a decrease in semen fertility. In fact, the proportions of CA and/or AR sperm cells gradually increased over time after dilution and storage at 5ºC, with a negative correlation between sperm cell viability and M/V over time. In summary, extender and cooling time affected mostly M/V, while aerobiosis condition and dilution factor were more associated with acrosome status and sperm survival, with the extender having less impact on the acrosome status as a function of time.

Keywords: sperm cell viability, acrosome status, cooled semen, extenders, dilution factor, aerobiosis, sheep.

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INTRODUCTION

The success of artificial insemination (AI) in mammals is associated with semen quality, which *per se* depends on semen processing and the extender ability to maintain ultrastructural, biochemical, and functional features of the sperm cells [60]. Ovine semen quality decreases after freezing, turning conventional AI inefficient due to low conception rates [11]. Such reduction in fertility has been attributed to cell damage caused by cryopreservation, leading to biochemical, functional and structural changes in the plasma membrane, and promoting capacitation-like changes, or cryocapacitation, and early acrosome reaction [54,61,65]. Alternatively, diluted cooled semen has been used for sheep conventional AI for decades [41,69]. Yet, gradual reduction in sperm cell viability occurs during cooling as storage days increase [41].

The capacitation process starts from ejaculation, continuing during dilution, cooling and freezing. Such process promotes changes and potential damage to the plasma membrane, reducing the fertility potential [24,51]. Cryopreservation induces loss of phospholipids from the cell membrane and increases intracellular calcium concentrations [30], which lead to susceptibility to cryocapacitation, acrosome reaction (AR) and loss of cell viability, irrespective of the species [18,23,25,65]. To counteract such effects, extenders are used to stabilize and protect the cell membrane against thermal shock during processing, cooling and/or freezing, and storage [64].

Frozen ram semen has lower sperm cell viability and higher proportion of cryocapacitation and AR after thawing [27,66]. However, the capacitation status of the ram sperm cells after dilution, cooling and storage time at 5ºC are still unknown. Thus, this study aimed to evaluate the status of sperm cell capacitation in cooled ram semen, comparing extenders, dilution factors, aerobiosis condition, and storage time at 5°C for up to 72 h, relating it to the viability of the sperm cells.

MATERIALS AND METHODS

Chemicals

All chemicals were embryo- or cell culture-tested from Sigma Chemicals[1], unless stated otherwise. All culture media were prepared with purified water using the Direct-Q3/Milli-Q Synthesis system[2].

Animals and semen collection

Semen was collected from two adult rams (one Texel, one Ile de France) by artificial vagina (AV) at 42ºC [43]. Rams were housed at the School of Veterinary Medicine (FAVET) at Federal University of Rio Grande do Sul (UFRGS), Brazil (30° 4' 33.6'' S, and 51° 7' 35.9'' W), during the spring season (September-December).

Two consecutive ejaculates were collected per day from each adult ram, with three days of collections at 48 to 72 h intervals. Upon each collection, each ejaculate was kept at 30ºC in a water bath for up to 15 min until evaluation. Macroscopic/physico-chemical evaluations included volume (mL), color, appearance/density, gross macroscopic motility (1 to 5 scale), and odor. Then, microscopic evaluations included analysis of gross microscopic motility (1 to 5 scale) on a slide glass, and total motility (TM; in %) and motility vigor/intensity (MV; 1 to 5 scale) between a warm glass slide and a coverslip, diluted in warm phosphate-buffered saline (PBS) at 37ºC under phase contrast microscopy (100 to 400x magnification). In addition, after diluting semen samples in warm formalin-citrate solution (4% formalin in a 2.94% sodium citrate solution, pH 7.2) at 1:400 dilution, sperm cell concentration (per mm^3 and per mL) and sperm morphology were determined under phase contrast microscopy (400 to 1000x magnification), according to Mies Filho [43] and as recommended by the Colégio Brasileiro de Reprodução Animal (CBRA) [16]. Total sperm cell number per ejaculate was determined by the product of sperm cell concentration (billions per mL) and total ejaculate volume (mL). Finally, semen samples from each ejaculate were used for the analyses of membrane integrity by trypan blue-Giemsa staining, and sperm cell capacitation status by chlortetracycline (CTC) staining, as described below.

Semen sample dilution and treatment groups

Upon analysis of fresh semen samples from each ejaculate, within up to 10 min after collecting the second ejaculate, a pool of the 2 ejaculates from each ram (per day of collection) was performed in a 15-mL centrifuge tube, which was kept in a water bath, at 27ºC. Then, the final volume and the total number of sperm cells in each pool were determined. Each pool was divided into 3 groups with equal volumes in new 15-mL centrifuge tubes. A 1:2 (V/V) pre-dilution was

carried out isothermically with 3 different extenders: (a) skimmed milk (SM) prepared by dissolving 10 g skimmed powdered milk (W/V; CCGL®[3]) in 100 mL distilled water[2] heated for 15 min at 95ºC [17]; (b) sodium citrate at 2.94% (1.53 mM sodium citrate[1]) supplemented with 20% egg yolk (V/V; Citrate-Egg yolk; CY) [21]; and (c) Tris-based solution (2.99 mM Tris[1], 1.03 mM sodium citrate[1], 0.27 mM fructose[1]) supplemented with 20% Egg yolk (V/V; Tris-Egg yolk, TY) [55,56], all isothermal to each semen fraction (27ºC), and with osmolarity and pH adjusted to 290 mOsm/L and to 6.5-7.0, respectively (prior to the addition of egg yolk in extenders CY and TY). Each pre-diluted semen fraction was evaluated for M and V. Then, each extender group (SM, CY, TY) was subdivided by dilution with the respective extenders at concentrations of 100 x 10^6 (Million group; Mi) or 1 x 10^9 (Billion group; Bi) sperm cells/mL, in new 15-mL centrifuge tubes, for each extender, with another M/V analysis performed afterwards. Finally, each sample for each extender and each dilution was again subdivided into 2 new centrifuge tubes, with a top layer of sterile mineral oil (300 µL) being added to 1 of the tubes to obtain a semi-anaerobic (SA) environment, while the other centrifuge tube remained oil-free (aerobic; A), directly exposed to atmospheric air. For each pool of 2 ejaculates, three extenders (SM, CY, TY), two concentrations (Mi, Bi), and two aerobic conditions (SA, A) were evaluated in a 3 x 2 x 2 factorial design, in a total of 12 samples per semen pool (6 semen pools), per ram (2 rams), per replication (3 replications), with each tube being properly identified.

Semen cooling

Properly identified tubes according to rams, extenders, concentration dilutions and aerobiosis conditions were gradually cooled in a water bath from 27°C to 5°C (T0 h) in a refrigerator for approximately 3 h (mean cooling curve rate of 0.12ºC/min). Tubes were kept under controlled cooling at 5ºC for 72 h. Cooled semen samples were evaluated for M/V at times 0 h (T0), 24 h (T24), 48 h (T48), and 72 h (T72). Time in which samples were stabilized at 5ºC was set as t= 0 h (T0) for cooling exposure up to 72 h (T72). Cooled semen samples were also analyzed for sperm cell capacitation status and sperm cell viability at times T0 and T72 by sperm cell staining, as below.

Assessment of capacitation status and sperm cell viability

To assess the *in vitro* sperm cell capacitation status, the chlortetracycline (CTC) staining procedure was performed as described previously [48], with modifications. Aliquots of 10 µL were collected from each fresh ejaculate (pool of 2 ejaculates per male per collection day) and a semen sample of each sub-group, according to the ram, replication, extender, concentration dilution, and aerobiosis conditions, prior to and after cooling, at times t = 0 h (T0) and t = 72 h (T72). To remove the extenders, semen samples from each extender subgroup were centrifuged at 650 *g* for 10 min in Mini-Percoll® gradients [37]. Upon the centrifugation, pellets were resuspended (individually) in Sperm-TALP and centrifuged again at 160 *g* for 5 min. Then, in a dark room, 5 µL of each sample were placed in a microcentrifuge tube with 10 µL CTC solution (1:3, v/v; 835 mM CTC[1], 5 mM DL-cysteine[1], 130 mM NaCl[1], and 20 mM Tris–HCl[1] at pH 7.8, prepared immediately prior to use) and incubated for 1 min at 37°C. Soon, 8 µL of a 12% paraformaldehyde solution (in 0.5 M Tris–HCl; pH 7.8) was added to fix sperm cells in each sample, followed by homogenization and placement onto a slide, covered with a coverslip afterwards. Then, the properly identified samples in slides were stored in boxes for protection from light and refrigerated at 4-5ºC for a minimum of 4 h. Slides were evaluated under epifluorescence microscopy (Zeiss[4]), under a 355/425-nm (em/ex) filter (RKP 455, Zeiss[4]) exposure, counting 200 cells at 1000x magnification, under oil immersion. Parameters for interpretation of sperm cell capacitation patterns were based on the Atlas Embrapa [39], in 3 categories: (1) non-capacitated sperm cells (NCA), showing a bright fluorescence throughout the whole sperm cell head; (2) capacitated sperm cells (CA), showing absence or low fluorescence in the post-acrosomal region, and bright fluorescence in the acrosomal region; and (3) acrosome-reacted (AR) sperm cells, showing no or low fluorescence in the head, with a fluorescent ring in the sperm equatorial region.

Sperm cell viability was performed using the trypan blue and Giemsa staining method, as previously described [31], with modifications. Aliquots (10 µL) of each semen sample were diluted in 10 µL trypan blue[1] solution at 0.4% in a 1.5-mL tube, and incubated at 37°C for 15 min. Then, 10 µL of each stained suspension was placed on glass slides, in duplicates,

and air-dried in vertical position. Samples were fixed in absolute alcohol for 5 min and air-dried in vertical position. Finally, samples were stained in a 4% Giemsa solution[1] for 1 h in an incubator at 38°C. Then, slides were rinsed in running water and left to dry at room temperature (RT). Samples were evaluated under a bright field microscope, by visualizing 200 sperm cells per slide, under oil immersion, at 1000× magnitude. The assessment of the sperm cell viability was based on Atlas Embrapa [39], as (1) dead sperm cells with intact acrosome, which presented a blue-stained head and a dark pink-stained acrosome pattern; (2) dead sperm cells with acrosome reaction, which presented a blue-stained head and a non-visible acrosome (discolored) pattern; (3) live sperm cells with intact acrosome, which presented a light pink-stained head and a dark pink-stained acrosome pattern; and (4) live sperm cells with acrosome reaction, which presented a light pink-stained head and a non-visible acrosome (discolored) pattern.

Statistical analysis

Proportion of sperm cell total motility, motility vigor, capacitation status (NCA, CA, AR) and viability (live/dead) were evaluated by the Anderson Darling normality test. All data were normally distributed, being evaluated by analysis of variance (ANOVA) and by factorial analysis by the General Linear Model of Minitab (Minitab[5]), using as factors the extenders (SM, CY, TY), concentrations (Mi, Bi), aerobic condition (SA, A), and cooling times (0 h, 24 h, 48 h, 72 h for M/V, and 0 h and 72 h for capacitation status and sperm cell viability), with pairwise comparisons by the Tukey test, for $P < 0.05$. The proportion of live and dead, and non-capacitated (NCA), capacitated (CA), and acrosome-reacted (AR) sperm cells were also compared by the X^2 test, for $P < 0.05$. A simple Pearson correlation test was also used to evaluate relationships between variables, for $P < 0.05$.

RESULTS

Macroscopic and microscopic parameters for fresh ram semen, from 2 ejaculates per day per male, in 3 days of collection, from 2 adult rams were similar between rams, ejaculates and days of collection. All ejaculates were white in color, with a creamy aspect, and odorless. Mean values for volume (mL) and gross macroscopic motility (1 to 5) were 1.06 ± 0.61 and 3.90 ± 0.32, and for gross microscopic motility (1 to 5), total sperm cell motility (%), motility vigor (1 to 5), and sperm cell concentration (10^9 sperm cells/mL) were 3.76 ± 0.72, 71.0 ± 10.1, 4.00 ± 0.57, and 3.95 ± 1.50, respectively. The mean morphological values (%) for total abnormal forms, major defects, and minor defects were 23.0 ± 16.9, 11.3 ± 7.4, and 11.7 ± 11.8, respectively. The mean proportions of the capacitation status observed in fresh ejaculates were 74.5 ± 14.3%, 15.7 ± 7.7% and 9.8 ± 8.5% for NCA, CA, and AR sperm cells, respectively. Fresh ejaculates showed mean proportions of 69.5 ± 9.6% for live cells and 30.5 ± 9.6% for dead cells.

Sperm motility and motility vigor from fresh and diluted ram semen during the cooling time for 72 h

No differences were observed in sperm cell M and V patterns between fresh and diluted ram semen prior to cooling. However, upon cooling, sperm cell M and V decreased significantly ($P < 0.05$) over storage time at 5ºC.

Percentage means of sperm cell M and V in each extender, concentration dilutions and aerobic conditions during the cooling times, from 0 h to 72 h, are shown in Figure 1. Sperm cell M decreased significantly ($P < 0.05$) in each extender from 48 h to 72 h, especially in the CY extender (Figure 1A, left panel). Similarly, sperm cell V of diluted ram semen in CY and SM extenders decreased after 24 h of cooling, mainly in the CY extender (Figure 1A, right panel). Sperm cell M and V patterns for both dilution factors (Mi and Bi) and aerobic conditions (SA and A) during cooling decreased significantly after 48 h and 24 h of cooling, respectively (Figure 1B & C).

In general, no differences in sperm cell M and V were observed overtime between groups, irrespective of the interaction between factors (extender, dilution factor, aerobiosis, time) and time (data not shown). Specifically, the SA condition at the Bi sperm concentration over time (SA/Bi *vs* time) resulted in a gradual and significant decrease in sperm cell M during 72 h of storage, especially after 48 h (Fresh: 68.2 ± 2.1%; 0 h: 66.3 ± 3.2%; 24 h: 61.7 ± 3.2%; 48 h: 54.7 ± 3.2%; 72 h: 51.3 ± 3.2%), with no differences to the other conditions of interaction, which were similar to one another and over time. Negative effects on V over cooling time were also observed within each group for SA/Bi *vs* time, also being more evident after 48 h (Fresh: 3.8 ± 0.1; 0 h: 3.8 ± 0.2; 24 h: 3.7 ± 0.2; 48 h: 3.2 ± 0.2; 72 h: 2.9 ± 0.2). Semen diluted and cooled in the CY

extender over time, at both sperm cell concentrations (Mi and Bi), and under both aerobic conditions (A an SA), showed a decrease in V over time from fresh semen (3.8 ± 0.1) and diluted cooled semen under the A/Bi, A/Mi, SA/Bi, and SA/Mi groups at t= 0 h (3.7 ± 0.3; 3.8 ± 0.3; 4.2 ± 0.3; 3.6 ± 0.3;), 24 h (3.5 ± 0.3; 3.5 ± 0.3; 3.8 ± 0.3; 3.2 ± 0.3), 48 h (2.9 ± 0.3; 3.0 ± 0.3; 2.9 ± 0.3; 2.8 ± 0.3), and 72 h (2.6 ± 0.3; 2.7 ± 0.3; 2.4 ± 0.3; 2.7 ± 0.3), respectively, especially from 48 to 72 h of incubation at 5ºC. Conversely, semen diluted in TY extender demonstrated better performance in V over time for A/Bi, A/Mi, SA/Bi, and SA/Mi at t= 0 h (3.7 ± 0.3; 3.6 ± 0.3; 3.7 ± 0.3; 3.9 ± 0.3), 24 h (3.8 ± 0.3; 3.9 ± 0.3; 3.6 ± 0.3; 3.9 ± 0.3), 48 h (3.5 ± 0.3; 3.7 ± 0.3; 3.5 ± 0.3; 3.7 ± 0.3), and 72 h (3.3 ± 0.3; 3.3 ± 0.3; 3.2 ± 0.3; 3.3 ± 0.3), respectively.

The effects of interactions between the different (i) extenders *vs* concentration dilutions, (ii) extenders *vs* aerobiosis conditions, and (iii) concentration dilutions *vs* aerobiosis conditions on sperm cell M and V of diluted and cooled ram semen at 5ºC for up to 72 h, irrespective of storage time, are shown in Figure 2. The sperm cell V in the TY extender, especially at the Mi sperm cell concentration, and independent of the aerobic condition (SA or A), was maintained higher over time than the other extenders, especially the CY extender (Figure 2A & B). The use of SM extender showed intermediate proportions of sperm cell M and V than the other 2 extenders, being more similar to the TY extender. No differences were seen for both sperm cell concentration dilutions (Mi and Bi) and aerobic conditions (SA and A) in relation to storage time (Figure 2C).

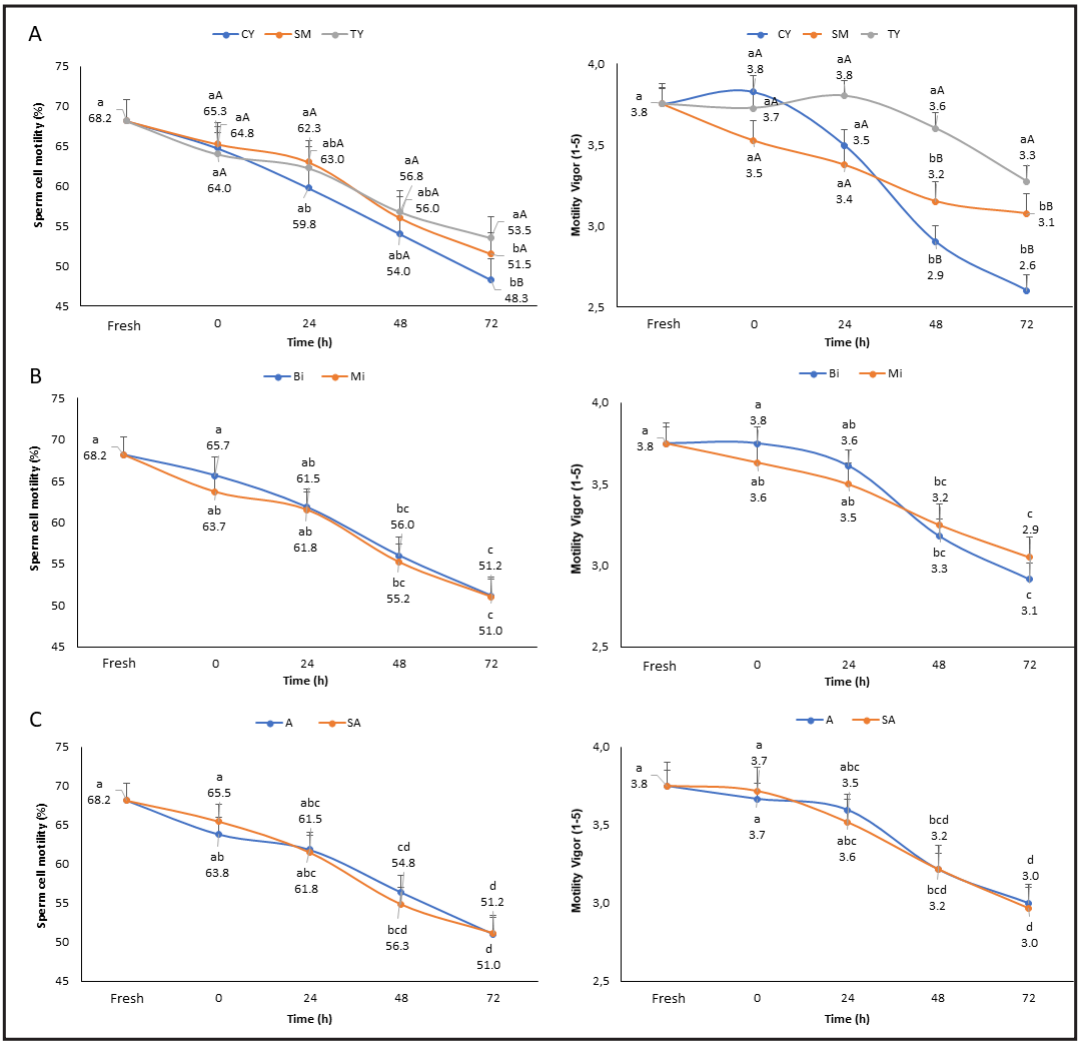

Figure 1. Effects of storage time on sperm cell motility (left panels, in %) and motility vigor (right panels, from 1 to 5 scale) in ram semen at collection and after dilution, cooling and storage at 5°C for 72 h in different extenders (A), dilution factors (B), and aerobic conditions (C). CY: citrate-egg yolk. SM: skimmed milk. TY: tris-egg yolk. Bi: 1 x 10^9 cells/mL dilution. Mi: 100 x 10^6 cells/mL dilution. A: aerobiosis. SA: semi-aerobiosis. $^{a,b,c,d}P < 0.05$, within each group. $^{A,B}P < 0.05$, between groups.

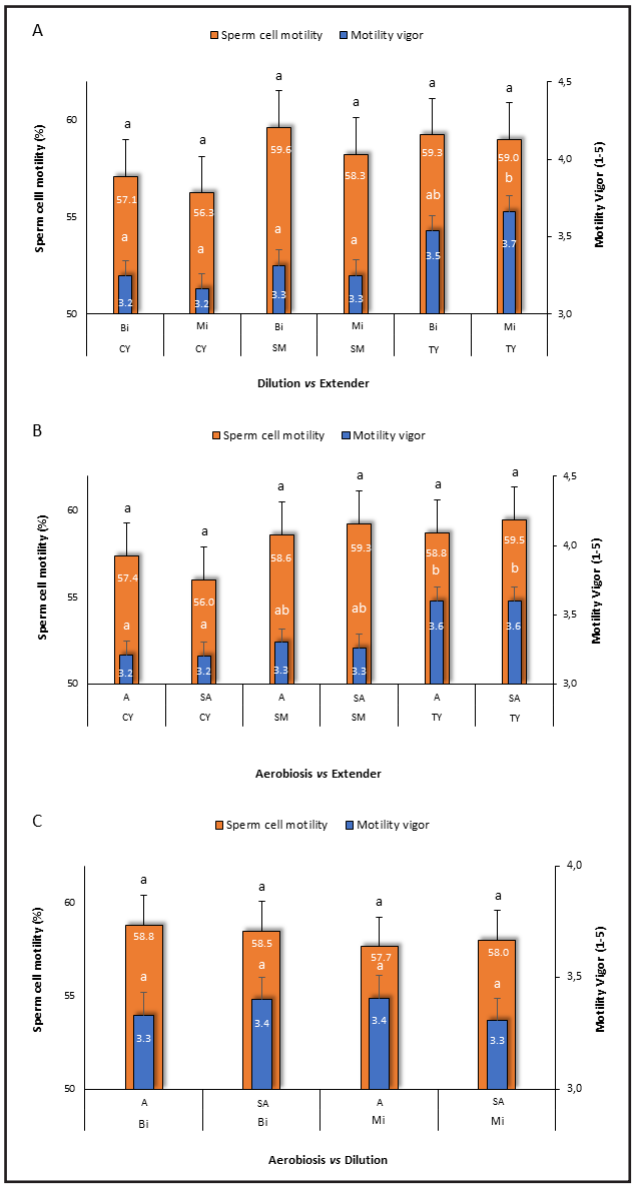

Figure 2. Effects of extenders and dilution factors (A), extenders and aerobiosis conditions (B), and dilution factors and aerobiosis conditions (C) on sperm motility (%) and motility vigor (1 to 5 scale) in ram semen after dilution and cooling at 5ºC for 72 h. CY: citrate-egg yolk. SM: skimmed milk. TY: tris-egg yolk. Bi: 1 x 10^9 cells/mL dilution. Mi: 100 x 10^6 cells/mL dilution. A: aerobiosis. SA: semi-aerobiosis. $^{a,b}P$ <0.05.

Patterns of sperm cell capacitation status and acrosome reaction of fresh and cooled ram semen during cooling time at 0 h and 72 h of incubation

Sperm cell capacitation and acrosome reaction profiles from fresh and refrigerated ram semen samples over storage time at 5ºC in different extenders (SM, CY, TY), concentration dilutions (Mi, Bi), and aerobic conditions (SA, A) are shown in Figure 3. No differences were observed in relation to the proportion of NCA, CA, and AR sperm cells, either after dilution with distinct extenders (SM, CY, TY; Figure 3A) or in both dilution factors (Mi, Bi; Figure 3B), regardless of the storage time at 5ºC (from 0 h to 72 h). The SA condition maintained NCA and AR sperm cells at higher and lower rates, respectively, when compared to the A condition (Figure 3C), with CA sperm cell rates being similar for both aerobic conditions, irrespective of the storage time at 5ºC (Figure 3C). However, sperm cell capacitation status was influenced by storage time, with a decrease in NCA sperm cell proportions ($P < 0.05$), for an increase in CA and AR cell proportions during storage time at 5ºC, as shown Figure 3D.

The proportions of sperm cell survival, sperm cell capacitation and acrosome reaction of ram semen samples that were submitted to dilution and cooling in different extenders for 72 h are shown in Figure 4A. Sperm cell mortality rates increased after semen dilution and refrigeration for 72 h when compared with fresh semen (control), especially for semen diluted in CY extender ($P < 0.05$). Furthermore, the proportion of NCA sperm cells decreased ($P < 0.05$) after semen dilution in all 3 extenders, when compared with fresh semen, being similar between all of the extenders within the 0 h and 72 h storage times. In the SM extender, such reduction in the proportion of NCA sperm cells was significant at 72 h of storage compared to 0 h (Figure 4A). Proportions of CA and AR sperm cells were higher in the diluted semen in TY extender at time 0 h compared with fresh semen. At 72 h, the sperm cell capacitation and acrosome reaction rates were higher in all groups compared with fresh semen, especially in SM and TY extenders. Such observations correlated with sperm cell survival parameters evaluated in this study, such as M, V and acrosome status. In general, positive correlations were observed between sperm M and V (R=0.778, $P < 0.001$), and between the proportions of NCA sperm cell and cell M (R=0.538, $P < 0.001$) and V (R=0.528, $P < 0.001$). Also, a positive correlation was observed between the proportions of CA and AR (R=0.526, $P < 0.001$) sperm cells. On the other hand, CA and AR sperm cells showed a negative correlation with cell M (R=-0.537, $P < 0.001$; R=-0.413, $P < 0.001$), V (R=-0.520, $P < 0.001$; R=-0.412, $P < 0.001$), and NCA sperm cells (R=-0.856, $P < 0.001$; R=-0.890, $P < 0.001$), respectively.

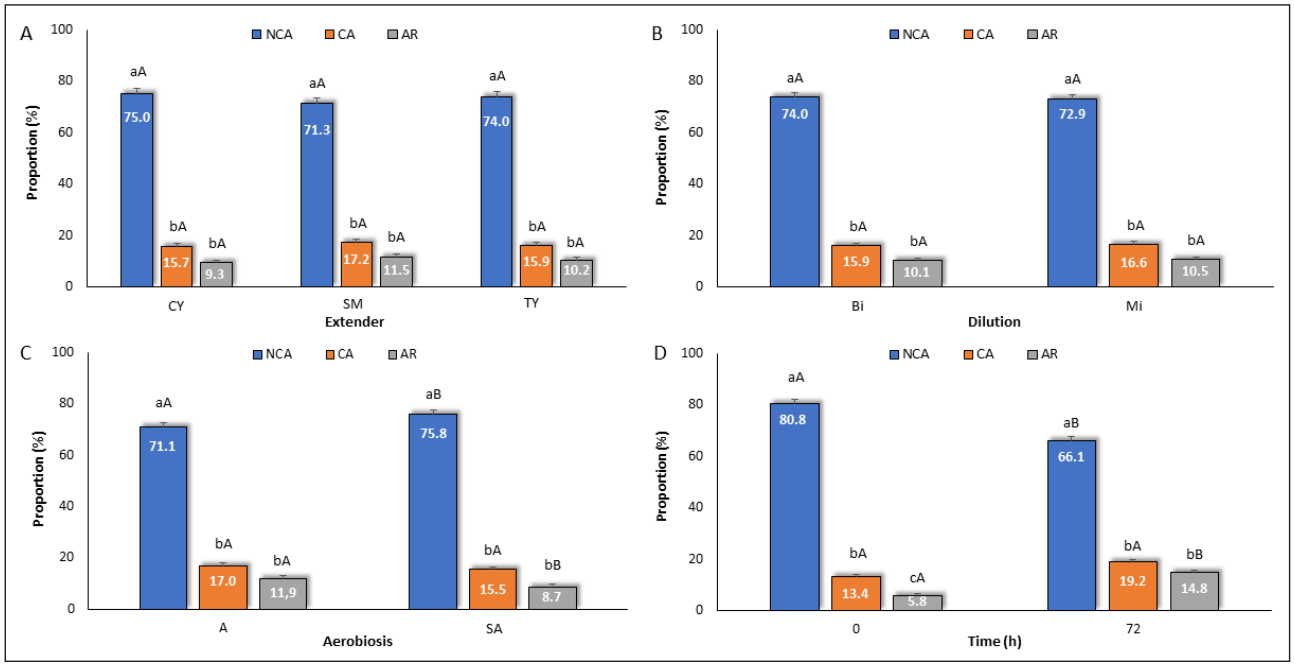

Figure 3. Effects of extender (A), dilution factors (B), aerobiosis conditions (C), and storage time (D) on the proportion (%) of non-capacitated (NCA), capacitated (CA) and acrosome reaction (AR) in sperm cells from ram semen after dilution and cooling at 5°C for 72 h. CY: citrate-egg yolk. SM: skimmed milk. TY: tris-egg yolk. Bi: 1 x 10^9 cells/mL dilution. Mi: 100 x 10^6 cells/mL dilution. A: aerobiosis. SA: semi-aerobiosis. [a,b]Different letters within the same groups for extender, dilution, aerobiosis, and time, differ, for $P < 0.05$, [A,B]Different letters between each status of capacitation (NCA, CA or AR) for each group (extender, dilution, aerobiosis, or time), differ, for $P < 0.05$.

Sperm cell survival rates, and sperm cell capacitation and acrosome reaction proportions in ram semen samples diluted at both dilution factors (Mi, Bi) after 72 h of storage at 5ºC are shown in Figure 4B. No differences were observed at the onset of storage time (0 h) at 5ºC in terms of sperm cell viability in both concentration dilutions (Mi and Bi). However, the Mi group showed higher sperm cell mortality after 72 h of cooling than the Bi dilution, showing that the lower dilution factor (Bi) conferred a favorable environment to sperm cell survival when stored for up to 72 h at 5ºC than the higher dilution factor (Mi). The status of sperm cell capacitation showed different patterns according to the dilution factor, as shown in Figure 4B, with a significant reduction ($P < 0.05$) in the proportion of NCA sperm cells for both concentrations at time 0 h, being reduced even more at 72 h afterwards ($P < 0.05$). Such pattern was associated with an increase in the proportions of CA and AR sperm cells, especially in the lower concentration (Mi) at 0 h, and for both (Mi and Bi) at 72 h, compared with the fresh semen, with higher proportion of non viable sperm cells. Correlations between such parameters have already being described above.

Aerobic conditions resulted in higher mortality at 0 h, while mortality rates in the SA conditions were higher at 72 h (Figure 4C). Both conditions maintained sperm mortality proportions similar at 0 h and 72 h. The reduction in proportions of NCA sperm cells was more significant under A conditions after 72 h. No differences were observed in the proportion of AR sperm cells between aerobic conditions and with fresh semen at 0 h. However, sperm cell CA was higher in the A group, with the SA condition being similar to fresh semen. At 72 h, sperm cell CA and AR rates increased under the A condition compared with fresh semen and 0 h, with the SA condition presenting similar rates to both groups at 0 h, but with higher CA and AR sperm cell rates than fresh semen.

DISCUSSION

In this study, the evaluation of M/V served as a secondary parameter for the evaluation of associations with changes in the acrosome status over time, as a function of semen dilution *per se*, concentration dilutions and type of extender. The aerobiosis conditions were also verified, as such conditions are known to be associated with biochemical processes on sperm cell capacitation and acrosome reaction status [29,68], and generation of ROS [28]. Variations in sperm cell M and V were observed over storage time at 5ºC, depending on the extender.

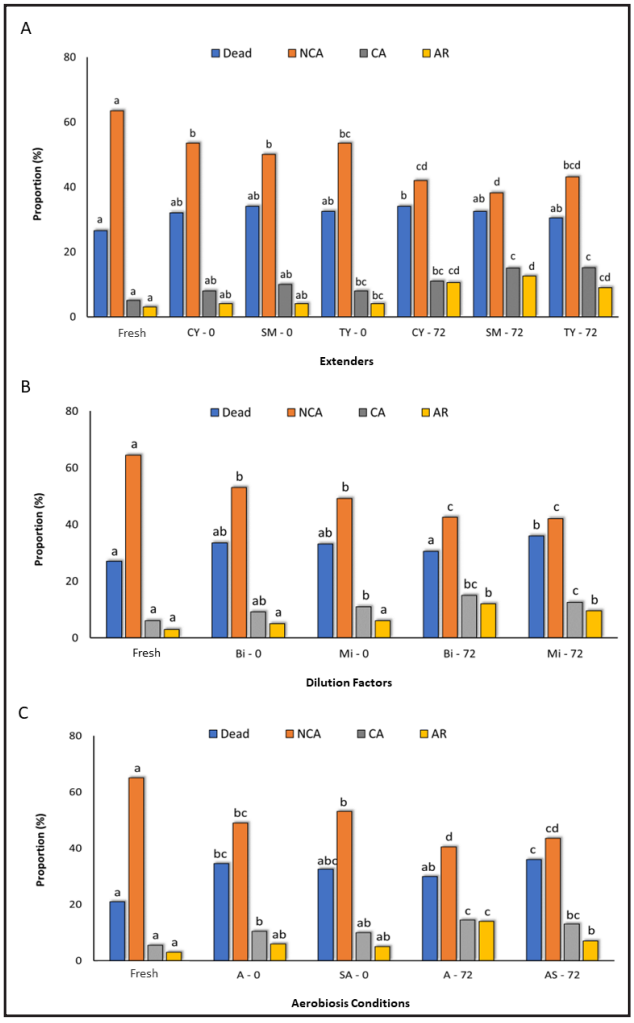

Figure 4. Proportion (%) of dead, non-capacitated (NCA), capacitated (CA) and acrosome reacted (AR) sperm cells in fresh ram semen upon collection (Fresh) and after dilution and cooling in different extenders (A) [CY, SM, TY], dilutions factors (B) [Bi, Mi], and aerobiosis conditions (C) [A, SA] over time (T0 h and T72 h). CY: citrate-egg yolk. SM: skimmed milk. TY: tris-egg yolk. Bi: 1 x 10^9 cells/mL dilution. Mi: 100 x 10^6 cells/mL dilution. A: aerobic. SA: semi-aerobic. T0: time 0 h, stabilization at 5ºC. T72: 72 h after stabilization at 5ºC. $^{a,b,c,d}P < 0.05$, within each column of the same color.

The kinetics patterns of M and V observed in ram semen samples were similar prior to and after dilution, but declined after the cooling process, from time 0 h, irrespective of the factors under analysis. Such decline may have been associated with potential heat shock occurring in cooled ram sperm cells. The susceptibility of sperm cells to heat shock is linked to a high ratio of unsaturated:saturated fatty acids in sperm cell membrane phospholipids and a reduced cholesterol content, which turn sperm cell membranes less stable [67].

In ram semen, when sperm cells are cooled near to the freezing point (5ºC), motility and metabolic activity may be irreversibly depressed and the acrosome and plasma membranes may suffer disruptions [8,50,67]. For extenders based on egg yolk, phospholipids present in the yolk protect the sperm membrane by restoring phospholipids lost during heat shock caused by the change in temperature during cooling [27,50]. Then, during a potential heat shock phase, the egg yolk lipoproteins interact with the lipid structure of the sperm cell plasma membrane, providing membrane protection [20]. In milk-based extenders, it has been shown that casein micelles isolated from milk can protect sperm cell membranes during storage at 4-5°C [12,38,45].

Ram semen samples diluted in citrate-based and Tris-based extenders containing 20% egg yolk were expected to maintain sperm motility and show minor membrane damage during storage time compared to skimmed milk-based extenders. However, in this experiment, ram semen samples diluted in TY maintained sperm cell M for a longer period with apparent minor damage to sperm cell membranes after 72 h of storage at 5°C, while semen samples extended with SM extender was intermediate between the TY and CY extenders. Moreover, sperm cell M was evaluated immediately after semen samples were collected (fresh) and after reaching 5°C (0 h), with the observation of a reduction, in all sub-groups, of around 3.5% for M and 0.1 for V (1 to 5) in relation to fresh semen. The greatest immediate losses after dilution and during cooling to 5°C in terms of M occurred in the TY extender (4.2% *vs* 3.4 in the CY and 2.9% in the SM extenders), and in terms of V, occurred in the SM extender (0.3 *vs* 0.0% in the CY and 0.1 in the TY extenders). Such trends in changes in M and V may have been caused by potential thermal shock during cooling, even though it could also be caused by damage to sperm cell membranes, corroborating with observations by Paulenz *et al*. [47], who did not observe differences in sperm cell M and V in diluted ram semen maintained for 0, 6, 24 and 30 h at 5ºC.

Significant reduction in sperm cell M observed during storage time for 72 h, especially after 48 h to 72 h of cooling compared to 0 h, corroborates with previous studies in the literature [5,22,36]. Furthermore, Falchi *et al*. [22] also observed that the cooling process affected significantly the parameters for total and progressive motility of diluted and cooled ram semen at 4°C, maintained for 0, 24, 48, 72 and 96 h. In this study, the largest reductions in M and V were observed in the CY extender (16.5% in M, and 1.2 in

V, from 0 to 72 h), and the smallest in the TY extender (10.5% in M, and 0.4 in V, from 0 to 72 h), with the fall in M and V in the SM extender showing an intermediate pattern between the other 2 extenders (13.8% in M, and 0.4 in V, from 0 to 72 h).

For each extender, the largest decrease in M and V were observed between 24 and 48 h, regardless of the dilution factor or aerobic condition (5.8, 7.0 and 5.5% in M, and 0.6, 0.2 and 0.2 in V, for CY, SM, and TY, respectively). Therefore, most of such effects occurred during storage at 5°C, especially between 48 and 72 h, when differences between the extenders could be clearly detected. This shows that the TY extender could maintain sperm cell M and V with greater physicochemical-biological properties than the CY extender, despite the slightly greater initial reduction, with the SM extender again showing an intermediate behavior. Such findings demonstrate that the Tris-egg yolk association is beneficial, with Tris acting as a buffer, fructose contributing to sperm cell oxidative metabolism [52], while egg yolk serves as a membrane protector and as a protein source, contributing to the preservation of sperm cell energy and membrane integrity more efficiently over time and during cooling [59]. The loss of phospholipids from the sperm cell plasma membrane that may be caused by thermal shock [7] is probably related to the phase transitions of the lipids in sperm cells membranes during the cooling process [28]. Such changes caused by the lipid phase transitions can directly interfere with protein channels, for instance, since membrane proteins are embedded in specific areas of the plasma membrane and might be separated by the presence and/or the changes in lipid composition under different phases, thus resulting in phase separations and loss of selective permeability [57,66].

Regardless of the type of extender or sperm concentration dilutions and storage temperature used for reducing cellular metabolism, any storage method causes damage to a significant portion of sperm cells that results in reduction in sperm cell motility, viability and fertility over time [7,34]. To obtain energy for cell metabolism and function, sperm cells need substrates that are easily metabolized, to which both skimmed milk and egg yolk sufficiently provide after dilution [44]. In this study, the TY extender better maintained sperm cell M and V than the CY and SM extenders, corroborating with findings by others for ram semen [46,26]. However, contrary to that, other studies [32,36] reported better motility rates in diluted semen using SM-based extenders. The SM extender has a buffering capacity due to its protein fraction, also having bactericidal action, adequate viscosity for maintaining sperm cells in the liquid medium, and abundance of carbohydrates that can be used by sperm cells as energy source [19,62]. Moreover, lactose contained in SM-based extenders, in addition to its osmotic function, can act as an energy substrate as well, if cleaved into glucose and galactose, with caseins being also able to increase the kinetic activity of sperm cells [20].

In this study, when considering the interaction between factors, no differences in M and V were seen between experimental groups, regardless of the interaction between extenders (SM, CY, TY), concentration dilutions (Mi, Bi), or aerobic conditions (SA, A). Interestingly, fresh semen presented a proportion of around 25% of CA and AR sperm cells, and around 75% to NCA sperm cells upon semen collection. A higher proportion of NCA and lower proportions of CA or AR sperm cells were correlated with higher sperm cell M and V in fresh and in diluted semen prior to cooling. However, after dilution and cooling, a gradual decrease in sperm cell M and V was observed, with a reduction in the proportion of NCA sperm cells, and an increase in CA and AR. In other words, live sperm cells and the NCA sperm cell proportion decreased during storage time, for a parallel increase in CA and AR sperm cell proportions. In fact, sperm viability of CA and AR sperm cells has been shown to be reduced in horses and humans upon the induction of cell capacitation and storage time at 5ºC [3,58,60], with similar findings observed in this study.

Semen quality decreases throughout the storage period, regardless of the extender, concentrations or storage conditions [44], even though there is a decrease in sperm cell metabolism, fructolysis rates and oxygen consumption by the reduction in storage temperature [13]. Furthermore, once ejaculated, the sperm cell is an aerobic cell, which uses oxygen in its metabolism, producing, under physiological conditions, basal levels of ROS molecules, which are essential in several processes involved in sperm capacitation, hyperactivation, acrosomal reaction and sperm-oocyte interaction [15,33]. Thus, during the sperm cell capacitation process, an increase in intracellular calcium, ROS and tyrosine kinase levels promote a higher production of

cyclic adenosine monophosphate (cAMP), culminating in a hyperactivation status and, finally, in the acrosome reaction that allows for fertilization [2,33,53]. Therefore, under conditions with high ROS concentrations, sperm cell quality and survival are compromised, especially in sheep and horses, which are the species known to generate high ROS levels that may cause damage to the chromatin, cell membranes, and sperm proteins [9,14,33,35,40,63].

The semi-aerobic condition at the highest dilution (Mi, 100 x 10^6 cells/mL) in this study resulted in lower sperm motility over storage time. Interestingly, however, it also resulted in a higher proportion of NCA and a lower proportion of AR, which was expected, potentially due to oxygen deprivation and lower ROS generation to promote capacitation and acrosome reaction. Furthermore, it has been shown that sperm oxygen deprivation is directly related to sperm cell motility, with a reduction in motility rates over a 5-h incubation time when under anaerobic conditions [57]. Then, the primary causal factor of the lower sperm motility observed in the group with the higher concentration in this study (Bi, 1 x 10^9 cells/mL) may have been the oxygen deprivation to the sperm cells. Then, a secondary factor may possibly be related to the high number of sperm cells that possibly consume more oxygen present in the extender, generating and accumulating metabolites into the suspension, including ROS, that may compromise survival overtime.

In this study, the proportion of NCA decreased significantly from 0 h to 72 h in all groups, with an increase in cell mortality and the proportion of CA and AR sperm cells. The reduction in NCA and increase in CA and AR were lower under the SA condition, which, paradoxically, had a higher mortality rate at 72 h. The most significant decrease in the proportion of NCA occurred in the A conditions at 72 h of storage, which was related to a higher rate of CA and AR under the same conditions, with a significant increase in dead sperm cells and lower CA and AR in the SA condition. Such condition seems to be related to the early mortality of sperm cells, which may be caused by oxygen deprivation and the increase in ROS in the extracellular medium, mostly from dead cells, thus leading to toxicity and impairing cell viability [4]. The role of oxidation for energy metabolism is prime for sperm motility, which is lower when glucose is present as a substrate for glycolysis [41]. Under the A condition, a higher proportion of CA was observed when compared to the SA condition, which was increased in the highest dilution factor (Mi, 100 x 10^6/mL), an aspect potentially associated with a higher dilution of disabling factors of the seminal plasma and the lower capacity of membrane protection and stabilization of the different extenders used in this study. This corroborates with Pérez-pe *et al*. [49], who demonstrated that in the ram, seminal plasma has decapacitating properties that prevent tyrosine phosphorylation, which is related to capacitation. As the survival of CA and AR sperm cells is lower [42], an increase in the proportion of CA and AR sperm cells is observed over storage time, with a concomitant decrease in sperm M and V. Based on the results above, it can be inferred that semen samples under A conditions may have undergone higher capacitation and AR over time potentially due to higher ROS levels, while the SA conditions appeared to have been more cytotoxic to sperm cells, causing higher mortality rates. An increase in ROS in the medium could induce lipid peroxidation, which in turn, could alter membrane properties that are critical for the maintenance of sperm function [1].

Higher sperm cell mortality was seen in this work in the highest dilution group (Mi) after 72 h of cooling compared with the other group (Bi), which indicates that the dilution factor did not compromise survival after cooling at 0 h, but caused increase in mortality after 72 h. Conversely, the lowest dilution factor (Bi) provided more favorable conditions for cell survival when stored for up to 72 h at 5°C. Possibly, the lower dilution allowed the protection of cell membranes by the components of the seminal plasma and decapacitation factors, which were still present in biologically active equimolar amounts [6,33]. The reduction of such factors and components of the seminal plasma as a function of semen dilution led to a significant reduction in NCA already at 0 h, being even more noticeable at 72 h. This feature was associated with an increase in the proportion of CA and AR, evident at time 0 h at the highest dilution with respect to capacitation, and for both traits at 72 h, compared with fresh semen. Such findings corroborate with the fact that, in ram semen, the higher dilution of seminal plasma, and consequent higher protein dilution, has a disabling action, due to the higher destabilization and restoration of the membrane of intact or damaged sperm cells [10].

Factors of positive or negative variations in M and V in this study were more related to the extender used and to the storage time at 5ºC, whereas the acrosome and sperm survival status were more associated with the aerobic condition and concentration dilutions, with the extenders having a lower impact as a function of storage time. Based on the observations of M/V and acrosome status over storage time, it can be inferred that the CY extender, especially the buffering/isosmotic solution of sodium citrate, was not efficient for the maintenance of M and V for longer periods (over 24 h), with the TY extender showing greater ability to maintain M and V at 5ºC for up to 72 h, although TY was more effective up to a maximum of 48 h. A reduction in NCA and an increase in CA and AR in sperm cells were observed in all extenders up to 72 h of storage, especially in the SM extender. However, higher proportions of CA and AR were seen after dilution in TY at 0 h. Thus, based on our observations, the CY is an extender that proved to be appropriate for more immediate use after dilution and refrigeration, with indication for more immediate use in AI. The SM extender showed properties suitable for refrigeration for up to 24 h, and may be of interest for collection, dilution, cooling and transport for use in AI the day after collection. Finally, the diluted semen in TY extender allowed semen to be properly stored for up to 48 h, corroborating with several reports in the literature [7,36,17]. Nevertheless, under the conditions of this study, none of the extenders was suitable for cooling for up to 72 h, and the SA condition did not present benefits for semen conservation. Potentially, but not definitively conclusive, the lower dilution factor appears to be less negative for ram sperm cell acrosome viability and status. Finally, all conditions and factors evaluated were related to a gradual decrease in the proportion of NCA sperm and sperm viability of the diluted ram semen refrigerated at 5ºC for up to 72 h.

In this study, a positive correlation was detected between viability and the proportion of non-capitated sperm cells, and a negative correlation was observed between the aforementioned factors and the proportion of capacitated or acrosome-reacted sperm cells, which has a potential impact on fertility and for AI use, depending mostly on the extender, dilution factor, and storage time. As it is known that capacitated or acrosome-reacted sperm cells have lower viability over time, the protection/stabilization of the sperm cell plasma membrane after dilution, cooling and storage at 5ºC should maintain a high proportion of non-capacitated sperm cells in the semen sample, which may, in turn, lead to higher pregnancy rates after conventional AI. Studies in this area should result in an improvement in the efficiency of ram semen preservation and in the development of more efficient protocols for the preservation of sheep genetics, with the aim of optimizing the use of inseminating doses and reducing reproductive costs and management for sheep meat and milk production.

CONCLUSIONS

Our study described that the *in vitro* ram semen manipulation procedures, including extender types, dilution factors, cooling temperature, and aerobic conditions, decreased sperm cell viability and the proportion of non-capacitated sperm cell as a function of storage time at 5ºC. Thus, all conditions and factors analyzed determined gradual changes in the status of the sperm cell capacitation, with a reduction in the proportion of non-capacitated sperm cells and sperm cell survival upon collection, and after dilution, cooling to 5ºC, and storage for up to 72 h. The most significant fall in sperm cell motility and motility vigor occurred after 24 and 48 h of storage at 5ºC, respectively. The proportion of capacitated and/or acrosome reacted sperm cells gradually increased after semen dilution and storage at 5ºC, with a negative correlation with sperm viability, sperm cell motility and motility vigor as a function of time. Moreover, the destabilization of the acrosome associated with the process of sperm capacitation and acrosome reaction was already detected at significant levels (25%) immediately after the collection of fresh ram semen.

MANUFACTURERS

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REFERENCES

1 **Aitken R.J. 2017.** Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*. 84(10): 1039-1052.

2 **Aitken R.J. & Baker H.G. 1995.** Seminal leukocytes: passengers, terrorists or good samaritans? *Human Reproduction*. 10(7): 1736-1736.

3 **Aitken R.J. & Clarkson J.S. 1987.** Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *Reproduction*. 81(2): 459-469.

4 **Alberts B., Johnson A., Lewis J., Morgan D., Raff M., Roberts K. & Walter P. 2015.** The innate and adaptive immune systems. In: Alberts B., Johnson A., Lewis J., Morgan D., Raff M., Roberts K. & Walter P. (Eds). *Molecular Biology of The Cell*. 6th edn. New York: Garland Publishing, pp.1297-1342.

5 **Al-Mutary M.G., Al-Ghadi M.Q., Ammari A.A., Al-Himadi A.R., Al-Jolimeed A.H., Arafah M.W. & Swelum A.A.A. 2020.** Effect of different concentrations of resveratrol on the quality and *in vitro* fertilizing ability of ram semen stored at 5°C for up to 168 h. *Theriogenology*.152: 139-146.

6 **Andrade A.F.C.D. 2009.** Efeito da adição do plasma seminal nas mudanças semelhantes à capacitação (Criocapacitação) em espermatozoides criopreservados de equinos. 133f. São Paulo. SP. Tese (Doutorado em Reprodução Animal) - Universidade de São Paulo (USP), Faculdade de Medicina Veterinária e Zootecnia (FMVZ/SBD).

7 **Anel L., Alvarez M., Martinez-Pastor F., Garcia-Macias V., Anel E. & Paz P. 2006.** Improvement strategies in ovine artificial insemination. *Reproduction in Domestic Animals*. 41: 30-42.

8 **Bailey J.L., Bilodeau J.F. & Cormier N. 2000.** Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *Journal of Andrology*. 21(1): 1-7.

9 **Ball B.A. 2008.** Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal Reproduction Science*. 107(3-4): 257-267.

10 **Barrios B., Fernández-Juan M., Muiño-Blanco T. & Cebrián-Pérez J.A. 2005.** Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. *Journal of Andrology*. 26(4): 539-549.

11 **Bergeron A. & Manjunath P. 2006.** New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Molecular Reproduction and Development*. 73(10): 1338-1344.

12 **Bittencourt R.F., Oba E., Ribeiro Filho A.D.L., Chalhoub M., Azevedo H.C. & Bicudo S.D. 2013.** Advances in cryopreservation of ram semen I: extenders and cryoprotectants. *Ciência Animal Brasileira*. 14(4): 522-536.

13 **Blackshaw A.W., Salisbury G.W. & Van Demark N.L. 1957.** Factors influencing metabolic activity of bull spermatozoa. I. 37, 21, and 5ºC. *Journal of Dairy Science*. 40(9): 1093-1098.

14 **Brummer M., Hayes S., Dawson K.A. & Lawrence L.M. 2013.** Interrelationships among selenium status, antioxidant capacity and oxidative stress in the horse. *Journal of Equine Veterinary Science*. 5(33): 332. DOI: 10.1016/j.jevs.2013.03.035

15 **Burnaugh L., Sabeur K. & Ball B.A. 2007.** Generation of superoxide anion by equine spermatozoa as detected by dihydroethidium. *Theriogenology*. 67(3): 580-589.

16 **CRBA - Colégio Brasileiro de Reprodução Animal. 2013.** *Manual para exame andrológico e avaliação de sêmen animal*. 3.ed. Belo Horizonte: CBRA, 104p.

17 **Colas G. 1975.** Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. *Reproduction*. 42(2): 277-285.

18 **Cormier N., Sirard M.A. & Bailey J.L. 1997.** Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *Journal of Andrology*. 18(4): 461-468.

19 **Cunha I.C.N.D. 2002.** Criopreservação do sêmen de cães. 149f. Botucatu. SP. Tese (Doutorado em Reprodução Animal) - Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Universidade Estadual Paulista.

20 **De Souza Castelo T., Rodrigues Frota T. & Rodrigues Silva A. 2008.** Considerations on goat semen cryopreservation. *Acta Veterinaria Brasilica*. 2(3): 67-75.

21 **Evans G. & Maxwell W.M. 1990.** *Inseminación Artificial de Ovejas y Cabras*. Zaragoza: Acribia, 192p.

22 **Falchi L., Galleri G., Zedda M.T., Pau S., Bogliolo L., Ariu F. & Ledda S. 2018.** Liquid storage of ram semen for 96 h: Effects on kinematic parameters, membranes and DNA integrity, and ROS production. *Livestock Science*. 207: 1-6. DOI: 10.1016/j.livsci.2017.11.001

23 Fuller S.J. & Whittingham D.G. 1996. Effect of cooling mouse spermatozoa to 4ºC on fertilization and embryonic development. *Reproduction*. 108(1): 139-145.

24 Gao D., Mazur P. & Critser J.K. 1997. Fundamental cryobiology of mammalian spermatozoa. In: Armand M. & Karow J.K.C. (Eds). *Reproductive Tissue Banking*. London: Academic Press, pp.282-331.

25 Gillan L., Evans G. & Maxwell W.M.C. 1997. Capacitation status and fertility of fresh and frozen–thawed ram spermatozoa. *Reproduction, Fertility and Development*. 9(5): 481-488.

26 Gündoğan M. 2009. Short term preservation of ram semen with different extenders. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. 15(3): 429-435.

27 Hammerstedt R.H., Graham J.K. & Nolan J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of Andrology*. 11(1): 73-88.

28 Holt W.V. 2000. Basic aspects of frozen storage of semen. *Animal Reproduction Science*. 62(1-3): 3-22.

29 Jimenez T., Sánchez G. & Blanco G. 2012. Activity of the Na,K-ATPase α4 isoform is regulated during sperm capacitation to support sperm motility. *Journal of Andrology*. 33(5): 1047-1057.

30 Kadirvel G., Kumar S. & Kumaresan A. 2009. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Animal Reproduction Science*. 114(1-3): 125-134.

31 Kovacs A. & Foote R.H. 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotechnic & Histochemistry*. 67(3): 119-124.

32 Kulaksız R., Bucak M.N., Akcay E., Sakin F., Daşkın A. & Ateşşahin A. 2011. The effects of different extenders and myo-inositol on post-thaw quality of ram semen. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. 17(2): 217- 222.

33 Lamirande E., Jiang H., Zini A., Kodama H. & Gagnon C. 1997. Reactive oxygen species and sperm physiology. *Reviews of Reproduction*. 2(1): 48-54.

34 Leahy T. & Gadella B.M. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction*. 142(6): 759. DOI: 10.1530/REP-11-0310

35 Leboeuf B., Guillouet P., Batellier F., Bernelas D., Bonne J.L., Forgerit Y., Renaud G. & Magistrini M. 2003. Effect of native phosphocaseinate on the *in vitro* preservation of fresh semen. *Theriogenology*. 60(5): 867-877.

36 Lopez Saez A., Ortiz N., Gallego L. & Garde J.J. 2000. Liquid storage (5ºC) of ram semen in different diluents. *Archives of Andrology*. 44(2): 155-164.

37 Machado G.M., Carvalho J.O., Siqueira Filho E., Caixeta E.S., Franco M.M., Rumpf R. & Dode M.A.N. 2009. Effect of Percoll® volume, duration and force of centrifugation, on *in vitro* production and sex ratio of bovine embryos. *Theriogenology*. 71(8): 1289-1297.

38 Makler A., Makler-Shiran E., Stoller J., Lissaak A., Abramovici H. & Blumenfeld Z. 1992. Use of a sealed minichamber to investigate human sperm motility in real time under aerobic and anaerobic conditions. *Archives of Andrology*. 29(3): 255-261.

39 Martins C.F., Dode M.A.N. & Silva A.E.D.F. 2016. *Atlas de Morfologia Espermática Bovina*. Brasília: Embrapa Cerrados-Livro Científico (ALICE), pp.30-31.

40 Mata-Campuzano M., Álvarez-Rodríguez M., Alvarez M., Anel L., Paz P., Garde J.J. & Martínez-Pastor F. 2012. Effect of several antioxidants on thawed ram spermatozoa submitted to 37ºC up to four hours. *Reproduction in Domestic Animals*. 47(6): 907-914.

41 Maxwell W.M. & Salamon S. 1993. Liquid storage of ram semen: a review. *Reproduction, Fertility and Development*. 5(6): 613-638.

42 Medeiros C.M.O., Forell F., Oliveira A.T.D. & Rodrigues J.L. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*. 57(1): 327-344.

43 Mies Filho A. 1987. *Reprodução dos Animais e Inseminação Artificial*. v.2. 6.ed. Porto Alegre: Sulina, 423p.

44 O'Hara L., Hanrahan J.P., Richardson L., Donovan A., Fair S., Evans A.C.O. & Lonergan P. 2010. Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. *Theriogenology*. 73(4): 541-549.

45 O'shea T. & Wales R.G. 1966. Effect of casein, lecithin, glycerol, and storage at 5ºC on diluted ram and bull semen. *Australian Journal of Biological Sciences*. 19(5): 871-882.

46 Paulenz H., Söderquist L., Ådnøy T., Fossen O.H. & Berg K.A. 2003. Effect of milk- and TRIS-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid semen. *Theriogenology*. 60(4): 759-766.

47 Paulenz H., Söderquist L., Pérez-Pé R. & Berg K.A. 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. *Theriogenology*. 57(2): 823-836.

48 Pérez L.J., Valcarcel A., Las Heras M.A., Moses D.F. & Baldassarre H. 1996. *In vitro* capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. *Theriogenology*. 45(5): 1037-1046.

49 Pérez-Pé R., Cebrián-Pérez J.A. & Muiño-Blanco T. 2001. Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. *Theriogenology*. 56(3): 425-434.

50 Purdy P.H. 2006. A review on goat sperm cryopreservation. *Small Ruminant Research*. 63(3): 215-225.

51 Ricker J.V., Linfor J.J., Delfino W.J., Kysar P., Scholtz E.L., Tablin F., Crowe J.H., Ball B.A. & Meyers S.A. 2006. Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. *Biology of Reproduction*. 74(2): 359-365.

52 Rodrigues B.A. 1997. Efeito do diluidor à base de albumina sérica bovina (BSA) sobre a viabilidade *in vitro* do sêmen canino criopreservado. 176f. Porto Alegre. RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

53 Saalu L.C. 2010. The incriminating role of reactive oxygen species in idiopathic male infertility: an evidence-based evaluation. *Pakistan Journal of Biological Sciences*. 13(9): 413-422.

54 Salamon S. & Maxwell W.M.C. 1995. Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science*. 38(1-2): 1-36.

55 Salamon S. & Maxwell W.M.C. 2000. Storage of ram semen. *Animal Reproduction Science*. 62(1-3): 77-111.

56 Salamon S. & Visser D. 1972. Effect of composition of Tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Australian Journal of Biological Sciences*. 25(3): 605-618.

57 Samo M.U., Axford R.F.E., Qureshi T.A., Memon A.A. & Memon M.M. 2006. Effects of aerobic and anaerobic incubation on the quality of ram semen. *Pakistan Journal of Biological Sciences*. 9(1): 123-127.

58 Samper J.C., McKinnon A.O. & Pycock J. 2007. *Current Therapy in Equine Reproduction*. St. Louis: Saunders Elsevier, 492p.

59 Santos I.W. 2004. Albumina sérica bovina como fonte proteica do diluidor Tris (hidroximetil amino metano) para congelação do sêmen canino. 63f. Jaboticabal. SP. Tese (Doutorado em Medicina Veterinária) - Programa de Pós-Graduação em Medicina Veterinária na Área de Reprodução Animal, Universidade Estadual Paulista.

60 Squires E.L., Carnevale E.M., McCue P.M. & Bruemmer J.E. 2003. Embryo technologies in the horse. *Theriogenology*. 59(1): 151-170.

61 Storey K.B. & Storey J.M. 2017. Molecular physiology of freeze tolerance in vertebrates. *Physiological Reviews*. 97(2): 623-665.

62 Ubah S.A., Sule M., Chibuogwu I.C., Columbus P.K., Abah K.O., Agbonu O.A., Ejiofor C.E., Mshelbwala P.P. & Bankole S.A. 2019. Comparative study of chicken egg yolk and quail egg yolk in two chilled canine semen extenders. *Sokoto Journal of Veterinary Sciences*. 17(4): 62-69.

63 Valença R.M.B. & Guerra M.M.P. 2007. Espécies reativas ao oxigênio (ROS) e a utilização de antioxidantes na criopreservação do sêmen suíno. *Revista Brasileira de Reprodução Animal*. 31(1): 47-53.

64 Verstegen J.P., Onclin K. & Iguer-Ouada M. 2005. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris–glucose extender: *in vitro* and *in vivo* studies. *Theriogenology*. 64(3): 720-733.

65 Watson P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development*. 7(4): 871-891.

66 Watson P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*. 60: 481-492.

67 White I.G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reproduction, Fertility and Development*. 5(6): 639-658.

68 Yanagimachi R. 1970. The movement of golden hamster spermatozoa before and after capacitation. *Reproduction*. 23(1): 193-196.

69 Yániz J., Marti J.I., Silvestre M.A., Folch J., Santolaria P., Alabart J.L. & López-Gatius F. 2005. Effects of solid storage of sheep spermatozoa at 15 degrees C on their survival and penetrating capacity. *Theriogenology*. 64(8): 1844-1851.