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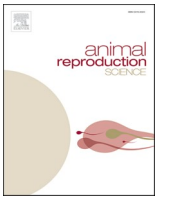

Association between different soy lecithin-based extenders and freezing rates in ram semen cryopreservation

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ABSTRACT

The aim of the study was to evaluate the effect of the association between glycine-milk (GM) based extenders made with different concentrations of soy lecithin (SL) and freezing rates (FR) on semen quality after thawing. Pooled semen from rams (n = 12) were diluted in GM extenders with 20% egg yolk (EY–20%) or with different concentrations of SL: 0.5% (SL–0.5%), 1.0% (SL–1.0%), and 2.0% (SL–2.0%). The diluted semen (150 $\times 10^6$ spermatozoa/0.25 mL) was frozen at three FR of − 10, − 20, and − 60 °C/min, and subsequently thawed and analyzed. Results revealed that EY–20% and SL–2.0% had better kinetic parameters, and showed higher proportions of viable, non-apoptotic, plasma-membrane-intact spermatozoa (A^-/PI^-) and non-capacitated spermatozoa (F), and had lower acrosome-reacted spermatozoa (AR) in the EY–20% and satisfactory values for SL-2.0% compared to SL–0.5% and SL–1.0% ($P < 0.05$). The FR at − 20 and − 60 °C/min maintained higher A^-/PI^- and viable spermatozoa compared to − 10 °C/min. The combination EY–20% and − 60 °C/min showed the highest A^-/PI^- and F ($P < 0.05$) and the lowest AR, and it did not differ from the combinations EY–20% at − 20 °C/min and SL–2.0% at − 20 °C/min ($P > 0.05$). In conclusion, the combination EY–20% and − 60 °C/min, showed the best cryoprotective effects on ram spermatozoa. Changes in spermatozoa after thawing were related to the use of the type of extender, the amounts of the same compound in the extender, and the freezing rates to which they were subjected.

1. Introduction

Cryopreserved spermatozoa, especially from rams, have poor quality, with damage to their structure and loss of their functionality (Keskin et al., 2020). In cryopreservation, the plasma membrane phospholipids undergo structural and distribution changes, affecting their composition, quantity, and symmetry (Hinkovska-Galcheva et al., 1989), which impair the enzymatic activity (Marti et al., 2008)

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required to ensure the survival and fertilizing capacity of spermatozoa after thawing.

To counteract the undesired effect of cryopreservation on the phospholipids of the spermatozoa plasma membrane, different components are added to the freezing extenders of the semen for their protection and restoration, including egg yolk (EY), with low density lipoproteins (LDL), which fulfills this function (Moussa et al., 2002). Despite its several advantages, EY is of animal origin and represents a potential risk of microbiological contamination of spermatozoa, affecting their quality (Bousseau et al., 1998; Perumal, 2018). The use of plant-based substances, such as soy lecithin (SL), may be an alternative to EY as a source of protection and restoration of spermatozoa phospholipids (Forouzanfar et al., 2010). Soy lecithin is composed of polyunsaturated phospholipids, which has shown effectiveness in protecting and restoring the plasma membrane of spermatozoa from bucks (Vidal et al., 2013), bulls (El-Sisy et al., 2016) and rams (Forouzanfar et al., 2010).

Freezing rate is also one of the main factors that determine the success of semen cryopreservation. It should be slow enough for spermatozoa water to flow out by osmosis, preventing the formation of intracellular ice, and at the same time, fast enough to minimize the damage to cells caused by exposure to high concentrations of solutes (Watson, 2000). Thus, the structure and kinetics of thawed spermatozoa from males of various species, such as bulls, boars, rams (Kumar et al., 2003), rats (Varisli et al., 2013), and roosters (Madeddu et al., 2016), are strongly affected by the freezing rate used in semen cryopreservation. These freezing rates are achieved both manually, allowing the use of higher freezing rates than − 50 °C/min but with less temperature control (less constant temperatures), and automatically or semi-automatically, using machines that have the advantage of allowing more constant temperature control but that, for the most part, especially for the cheapest machines, have limitations regarding the maximum freezing rate less than − 50 °C/min (Kumar et al., 2003; Salamon and Maxwell, 2000).

Most strategies for improving cryopreservation of ram spermatozoa have tested the effects of factors such as extenders (Gogol et al., 2019) and freezing rates (Iraj et al., 2011) individually and separately. A better understanding of the effect of factors that interact on biological processes (Scholtens et al., 2004) under different scenarios leads us to high-efficiency multifactorial studies. We hypothesized that the cryoprotective effect of soy lecithin is dependent on its concentrations in the extender and on the freezing rates to which the spermatozoa are subjected. The aim of this study was to evaluate the effect of replacing the egg yolk in the glycine milk (GM) - based extender with soy lecithin and to define, through association studies, what the most suitable freezing rate for these extenders is in the process of cryopreservation of ram semen.

2. Materials and methods

All experimental procedures were approved by the Ethics Committee on Animal Use (CEUA) of the Brazilian Agricultural Research Company (EMBRAPA), Embrapa Tabuleiros Costeiros (Embrapa Coastal Tablelands), under registration number 0035/2020.

2.1. Preparation of the extenders

Four cryopreservation extenders were prepared from a standard GM-based medium (Gonzalez, 1996), with EY (control) or EY being replaced by different concentrations of SL. First, a standard solution was prepared (19.64 mM of glycine, 14.59 mM of sodium citrate, 17.52 mM of fructose, and 2.31 g of gentamicin sulfate / L of Milli-Q water). In a second step, 15 mL (30%) of the standard solution was combined with 10 mL (20%) of EY, forming the extender EY–20%, or with 0.5%, 1.0%, and 2.0% of SL in 10 mL of Milli-Q water, forming the extenders SL–0.5%, SL–1.0%, and SL–2.0%, corresponding to the amounts of 0.27, 0.54, and 1.08 g (m/v), to correct the concentration of 90% lecithin used (Lecithin, Alpha Aesar, India). In a third step, all four extenders received 7.5 mL (15%) of reconstituted skimmed milk powder at a ratio of 11% (v/v), preheated to 92–95 °C for 10 min, 0.20 mL (0.4%) of orvus es paste (OEP – v/v), 2.5 mL (5%) of glycerol (v/v), and Milli-Q water until completing 50 mL. The extenders were evaluated for pH (Calibration Check™ HI 2221; Sao Paulo, Brazil) and osmolality (VAPRO® 5520; Utah, USA) in two steps, before the addition of glycerol and after the addition of glycerol, with the following results, respectively: EY–20% (7.03 and 7.05 pH; 310 and 1320 mOsm/kg), SL–0.5% (7.05 and 7.19 pH; 230 and 1034 mOsm/kg), SL–1.0% (7.10 and 7.11 pH; 242 and 1061 mOsm/kg), and SL–2.0% (7.04 and 7.05 pH; 262 and 1100 mOsm/kg). Finally, the four extenders were properly identified and stored at − 20 °C until their use after thawing in a water bath at 37 °C.

2.2. Animals and semen collection

We used 12 sexually mature Santa Inês rams, with an average of 60 kg live weight, previously approved for breeding soundness in examination according to the recommendations of the Colégio Brasileiro de Reproduç ão Animal (Brazilian College of Animal Reproduction) (CBRA, 2013). The rams were kept in the Pedro Arle experimental farm of Embrapa Tabuleiros Costeiros in the municipality of Frei Paulo (37º38'30.41" W, 10º36'12.63" S), state of Sergipe, Brazil, and were managed in a semi-intensive regime. The rams consumed native and cultivated fodder composed of Pangola grass *(Digitaria decumbens L*), star grass (*Cynodon nlemfuensis*), buffel grass (*Cenchrus ciliares*), and the *Panicum maximum* cultivars aruana and green-panic. They remained confined in a covered shed overnight from 16:00 until 07:00, where they received food supplementation with corn silage-based roughage (Ro) and concentrate based on soybean meal and cornmeal (C) in the ratio 50% Ro:50% C, with the availability of mineral salt and water ad libitum.

Three semen samples were collected from each ram using a heated artificial vagina (45 °C) and an ewe in natural estrus as sexual stimulation in the interval of approximately 36 h between them. The ejaculates were kept in a water bath (37 °C) until initial analysis and processing for freezing.

2.3. Semen selection, freezing, and thawing

The motility, vigor, and spermatozoa concentration of fresh semen were analyzed using a phase-contrast microscope at 400x magnification (Olympus Optica Co., Tokyo, Japan). To assess motility and vigor, an aliquot of 10 μL from the ejaculate of each ram was transferred and mixed with 500 μL of X-Cell® evaluation solution (IMV-Technologies, L'Aigle, France) and incubated at 37 °C for 5

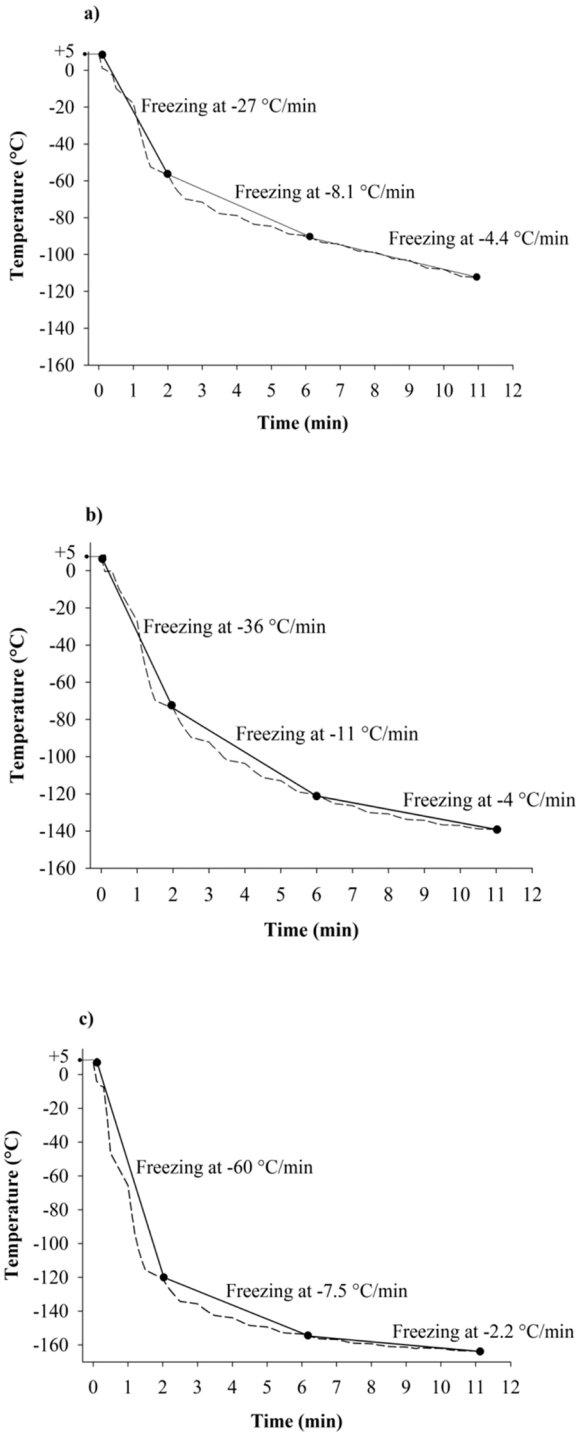

Fig. 1. Characteristics of the curves that comprised the average freezing rates of − 10, − 20 and − 60 °C/min obtained from the exposure of straws containing the ram semen to the respective heights of 7.5 (a), 4.5 (b) and 1.5 cm (c) above the level liquid nitrogen.

min. Subsequently, 12 μL of the mixture was placed on a slide and covered with a heated coverslip (37 °C) for evaluation. Spermatozoa concentration was measured by adding an aliquot of fresh semen to distilled water at a dilution of 1/400, placing this mixture in a Neubauer chamber, and counting the number of spermatozoa. All ejaculates whose semen samples showed spermatozoa with mass motility ≥ 70%, vigor ≥ 3 (score from 0 = absent movement to 5 = vigorous and fast movement) (CBRA, 2013), and spermatozoa concentration ≥ 4.5×10^9 spermatozoa/mL were approved. Ejaculates from each of three different approved rams were mixed to form pooled semen, generating four pools from the 12 rams used in the study.

The semen pools were kept in a water bath at 37 °C and gently homogenized before removal of an initial sample of each containing approximately 5.0×10^9 spermatozoa. Each of these samples was divided into four equal aliquots to be diluted (1/6) and mixed with its respective extender (Ex) that had previously been heated (37 °C), representing the experimental groups (EY–20%, SL–0.5%, SL–1.0% and SL–2.0%) of the first factor tested (Factor 1). The semen samples diluted in their respective extenders were transferred to room temperature (approximately 25–28 °C) where they were placed in French straws of 0.25 mL (600×10^6 spermatozoa/mL) previously identified and subsequently sealed with polyvinyl alcohol powder. Each group of straws of semen diluted in each extender was divided into three subgroups of straws to be distributed and arranged horizontally in three stainless-steel racks to allow the cold to act directly on the straws.

Refrigeration was carried out by placing the three racks in the refrigerator (Minitub® Minitube do Brasil, Porto Alegre, Brazil), previously adjusted to the temperature of 5 °C, for 90 min. Under these conditions, the average cooling rate was − 0.30 °C/min in the period between initial (30 °C) and final (5 °C) temperatures, with a subsequent equilibrium time at the final temperature. This rate was achieved using plastic bags (48 cm long × 32 cm wide × 0.3 mm thick) containing distilled water, and the procedure followed a technique that was adapted from one previously used in the refrigeration stage of freezing the ram semen (Rodello, 2006).

The three racks containing the semen straws were simultaneously removed from the refrigerator and transferred to a rectangular stainless-steel tub (55 cm long × 40 cm wide × 35 cm high, with walls internally filled with 3.5 cm thick polyurethane) that had previously received 7.2 liters of liquid nitrogen (LN_2) (supplementary figure). The racks were arranged in the tub at three different heights: 7.5, 4.5, and 1.5 cm above the LN_2 level. They remained under its vapors for 11 min, the time required for all semen-containing straws to reach a minimum temperature of − 110 °C. This procedure generated curves with three different freezing rates (FR) of approximately − 10, − 20, and − 60 °C/min (Factor 2) until reaching a temperature of − 110 °C, as previously performed (Fig. 1) using a digital thermometer (Dual Input Thermometer Model 710. BK precision, USA) with the sensor in contact with the sample inside the straws. Soon after, all the racks were submerged immediately in the LN_2 at − 196 °C, and the semen straws were then transferred to a cryogenic tank to be conserved and stored in LN_2 until thawing for evaluation.

The frozen semen straws were thawed after one year of storage by transferring them to heated water at 40 °C for 20 s. After thawing, the straws were dried for cutting, and their content was transferred to microtubes maintained at 37 °C for spermatozoa evaluations. The average of each sperm parameter was obtained from evaluation of 48 semen straws.

3. Spermatozoa evaluations after semen thawing

3.1. Motility and vigor

The motility (Mot) values, expressed as a percentage from 0 to 100 regarding the proportion of spermatozoa that show movement, and the vigor (Vig) values, representing the intensity of spermatozoa movement with a score from 0 to 5, were subjectively determined following the procedures described below. We removed 100 μL of thawed semen to be mixed with 150 μL of X-Cell® evaluation solution in a microtube previously heated to 37 °C, obtaining a dilution with a concentration of 240×10^6 spermatozoa/mL. An aliquot of 10 μL of the mixture was placed between previously heated (37 °C) glass slides and coverslips to be evaluated in a phase-contrast optical microscope (Olympus Optica Co., Tokyo, Japan) using 400x magnification. Both motility and spermatozoa vigor were evaluated by a single previously-trained technician.

3.2 Computerized kinetics

Samples of thawed semen were previously diluted in X-Cell® evaluation solution preheated to 37 °C (Azevedo, 2006), with a concentration of 20×10^6 spermatozoa/mL. After the samples were homogenized and maintained at 37 °C for 5 min, aliquots of 2 μL of the diluted samples were pipetted into a Makler chamber (Couting Chamber Makler® 0.01 sq.mm 10 μm deep Sefi-Medical Instruments, Haifa, Israel) preheated to 37 °C to capture the image and analyze the sample using a video camera (Basler ace acA1300–200 μm, Ahrensburg, Germany) connected to a phase-contrast microscope (Nikon® 50i, Japan), under 100x magnification. Images were captured at a speed of 50 frames/s, with manual selection of five fields equidistant from the same center point, capturing at least 500 cells per sample and an average of 100 cells per field. The captured images were analyzed by Computer Assisted Sperm Analysis (CASA-motility) (Microptics, S.L. Version 6.5, Barcelona, Spain) using the factory setup as default for the sheep species, which was modified using the following settings: box size of 150 acquired frames and frame rate of 50 Hz, with minimum particle size of 30 μm and maximum of 70 μm. The following kinetic parameters were analyzed: total motility (TM), progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and wobble index (WOB). Spermatozoa were also grouped, based on their VCL, into four subpopulations as follows: rapid (RAP), with VCL > 200 μm/s; medium (MED), with VCL ≥ 100 to ≤ 200 μm/s; slow (SLO), with VCL ≥ 20 to < 100 μm/s; and static (STA), with VCL < 20 μm/s. Spermatozoa with STR > 80% were considered progressive, and those with STR > 40%, with linear motion.

3.3 Functionality of the plasma membrane

The functionality of the spermatozoa plasma membrane was evaluated using the hypoosmotic swelling test (HOST) (Jeyendran et al., 1984) based on the coiled or swollen tails of spermatozoa after subjecting them to a low osmolarity solution of 100 mOsm/L [49.95 mM of fructose and 22.88 mM of sodium citrate/L of Milli-Q water]. The technique was performed by incubating 10 μL of semen with 100 μL of hypoosmotic solution at 37 °C for 60 min. After incubation, 20 μL of the mixture was placed between slides to be viewed under a phase-contrast microscope (Nikon 50i, Japan) at 1000x magnification in immersion oil in order to count 200 spermatozoa per sample, considering those with a coiled or swollen tail as having a functional plasma membrane.

3.4 Viability

Spermatozoa viability was evaluated by flow cytometry related to detection of phosphatidylserine externalization in the plasma membrane of the spermatozoa. Two fluorescent dyes were used, Annexin V and Propidium Iodide (PI) (Kit–Alexa Fluor® 488 Molecular Probes/Invitrogen, Brazil), with high affinity to phosphatidylserine and double-stranded DNA, respectively. First 1.6 μL of semen (0.96×10^6 spermatozoa) was mixed in 98.4 μL of Annexin V buffer solution (1/5 – Annexin V Binding Buffer / deionized water). In a dark environment, 5 μL of Annexin V (25 mM) was added to this mixture, which remained under incubation for 5 min. Subsequently, 2 μL of PI (1.5 mM) was added, and the solution was incubated for another 10 min. At the end of the incubation cycle, 300 μL of Annexin V buffer solution was added to every sample. The samples were analyzed in a flow cytometer (Attune NxT Acoustic Cytometer, Thermo Fisher Scientific) equipped with a 488 nm argon-ion laser. Fluorescence was photo detected in the green BL1 channel using the 530/30 nm band pass filter and in the red BL3 channel using the 695/40 nm band pass filter for Annexin V and PI, respectively. Calibration was performed periodically using standard beads (Calibrites® Becton Dickson). The volume acquired for analysis was 200 μL with a flow rate of 100 μL/min, event rate was under 4000 events/s and 10.000 events were collected for each sample. The spermatozoa were gated through the scatter plot according to the relative size (FSC-A axis) and granularity (SSC-A axis) of spermatozoa cells suspension to eliminate debris. Finally, the gated spermatozoa were analyzed in the BL1 axis and BL3 axis four categories of spermatozoa were distinguished: i) non-apoptotic with intact plasma membrane = negative for Annexin V and PI (A^-/PI^-), ii) at the beginning of apoptosis = positive for Annexin V and negative for PI (A^+/PI^-), iii) late apoptosis = positive for Annexin V and PI (A^+/PI^+), and vi) necrotic = negative for Annexin V and positive for PI (A^-/PI^+). Data obtained from flow cytometry were analyzed on a logarithmic scale and compensated using the FlowJo™ v10.5.3 Software (BD Biosciences, San Jose, CA, USA). The proportion of viable spermatozoa (Viab) was measured by the sum of the proportions of A^-/PI^- and A^+/PI^- spermatozoa (Masoudi et al., 2016).

3.5 Capacitation and acrosome reaction

The proportion of the capacitation and of acrosome reaction-like change in spermatozoa after freezing-thawing were evaluated following the chlortetracycline (CTC) technique, according to protocols described by Gillan et al. (1997) and Azevedo (2006). An aliquot of 11 μL of semen was added to 150 mL of buffered Dulbecco solution (PBS) (Barth and Oko, 1989), obtaining a solution with approximately 40×10^6 spermatozoa/mL. We removed 20 μL from this mixture, which was transferred to a microtube to which 20 μL of CTC (1 mM) was added. After homogenization, 10 μL of this mixture was transferred to a slide and coverslip and refrigerated at 4 °C for 10 min until its evaluation with the count of 200 cells in immersion in oil at 1000x magnification under a microscope with epifluorescent illumination (CARL ZEISS, AX10 Cam MRc Göttingen, Germany) using filters with excitation and emission wavelengths of 450–490 nm and 514 nm, respectively. Three categories of spermatozoa were classified according to greenish-yellow fluorescence: F = non-capacitated without acrosome-reacted spermatozoa (uniform fluorescence throughout the head), B = capacitated spermatozoa (uniform fluorescence in the anterior acrosome portion of the head), and AR = with acrosome-reacted spermatozoa (anterior region of the head with little or no fluorescence).

3.6 Experimental design and statistical analyses

The data were analyzed using the SAS 9.1® computer program and R software. Initially, the normality and homoscedasticity of the spermatozoa parameters were verified by the Hartley (Hc>H) and Shapiro-Wilk ($P > 0.05$) tests, respectively, and when necessary, were transformed by the arc sine of the square root. The data that showed normal distribution naturally (VAP, LIN, SLO, STA, A^+/PI^-, Viab, and AR) or after transformation (TM, VSL, WOB, MED, A^-/PI^-, A^+/PI^+, A^-/PI^+, and F) were analyzed by a randomized block design in a 4 × 3 factorial arrangement, considering the extenders (Factor 1) and freezing rates (Factor 2) as fixed effects, following the model described: $Yij = \mu + Ti + \beta j + (T\beta)\ ij + \varepsilon ij$, where Yij is the dependent variable or spermatozoa parameter, μ is the mean of all observations, Ti represents the extender effect, βj is the effect of the freezing rate, (Tβ) ij represents the interaction between the extender and the freezing rate, and εij represents the experimental error.

The data of spermatozoa parameters that did not show normality after the transformation (Mot, Vig, PM, VCL, STR, BCF, ALH, RAP, HOST, and B) were analyzed with nonparametric statistics first using the transformation of aligned posts of each parameter by the ARTool package (Wobbrock et al., 2011). Finally, all data on spermatozoa parameters were analyzed by ANOVA to verify the effect of factors and their interaction by the F test. The differences between the means of the experimental groups were determined by Tukey's test (HSD). A significance level of 5% ($P < 0.05$) was considered for all analyses.

4. Results

4.1. Motility and vigor

Spermatozoa motility (Mot) and vigor (Vig) were strongly affected ($P < 0.01$) by the extender and freezing rate factors and the interaction between them (Table 1), and the results are shown in Fig. 2. The interaction within extenders each level of the freezing rates shows that among all the extenders in the study, the means of Mot and Vig of SL–1.0% extender samples were the only ones influenced by the interaction with the freezing rate, obtaining higher means in the interactions between SL–1.0% with − 20 and − 60 °C/min ($P < 0.05$).

The effect of the interaction within freezing rates each level of the extenders on the value of Mot and Vig, it is observed that the interactions between − 60 °C/min and EY–20%, as well as − 20 and − 60 °C/min and SL–2.0%, showed higher Mot values than the interactions between − 10, − 20, and − 60 °C/min and SL–0.5% and between − 10 and − 60 °C/min and SL–1.0% ($P < 0.05$). However, they did not differ from the values of the interactions between − 10 and − 20 °C/min and EY–20%, − 10 °C/min and SL–2.0%, and between − 20 °C/min and SL–1.0% ($P > 0.05$). The Vig value obtained in the interaction between − 20 °C/min and EY–20% was greater than the Vig values from the interactions between − 10 °C/min and EY–20%; − 10, − 20, and − 60 °C/min and SL–0.5%; − 10, − 20, and − 60 °C/min and SL–1.0%; and − 10 °C/min and SL–2.0% ($P < 0.05$). However, the Vig values did not differ from the interactions between − 60 °C/min and EY–20%, and between − 20 and − 60 °C/min and SL–2.0% ($P > 0.05$). In summary, the lowest values of Mot and Vig were derived from the interactions between − 10, − 20, and − 60 °C/min and SL–0.5% and between − 10 °C/min and SL–1.0% ($P < 0.05$).

4.2. Computerized sperm kinetics

The extender factor had a significant effect on most parameters of computed spermatozoa kinetics (Table 1). Table 2 shows higher means of TM, PM, BCF, WOB, and MED and lower mean STA ($P < 0.05$) in the extenders EY–20% and SL–2.0%, which did not differ from each other ($P > 0.05$), but were significantly different from SL–0.5% and SL–1.0% ($P < 0.05$), which also did not differ from each

Table 1
Statistical analysis of the effects of factors extender (Ex), freezing rate (FR) and the interaction between them (Ex*FR) on spermatozoa parameters in thawed ram semen (n = 48).

Spermatozoa parameters	Factors								
	Ex			FR			Ex*FR		
	df	*F*-valor	*P*-valor	df	*F*-valor	*P*-valor	df	*F*-valor	*P*-valor
Mot	3	44.84	0.0001 ***	2	15.63	0.0005 ***	6	4.18	0.0050 **
Vig		48.11	0.0007 ***		8.58	0.0010 ***		4.57	0.0030 **
MT		15.00	0.0001 ***		0.89	0.4200 ns		0.37	0.8800 ns
MP		13.25	0.0003 ***		0.28	0.7500 ns		0.80	0.5700 ns
VCL		7.47	0.0010 **		2.59	0.0970 ns		2.09	0.0940 ns
VAP		44.65	0.0001 ***		4.31	0.0200 *		4.39	0.0046 **
VSL		39.34	0.0001 ***		2.93	0.0742 ns		4.68	0.0030 **
LIN		21.49	0.0001 ***		0.74	0.4907 ns		2.62	0.0454 *
STR		15.44	0.0001 ***		21.47	0.0006 ***		6.31	0.0005 ***
ALH		2.53	0.0800 ns		0.59	0.5500 ns		0.61	0.7100 ns
BCF		11.49	0.0009 ***		2.72	0.0800 ns		1.91	0.1200 ns
WOB		30.95	0.0001 ***		2.74	0.0869 ns		0.98	0.4629 ns
RAP		15.37	0.0010 ***		0.84	0.4400 ns		0.92	0.4900 ns
MED		15.79	0.0001 **		0.08	0.9261 ns		0.98	0.4985 ns
LEN		8.98	0.0004 ***		0.26	0.7758 ns		1.62	0.1881 ns
EST		13.75	0.0001 ***		0.56	0.5811 ns		0.38	0.8825 ns
HOST		127.60	0.0004 ***		0.07	0.9200 ns		0.54	0.7600 ns
A^-/PI^-		15.83	0.0001 ***		50.71	0.0001 ***		2.85	0.0331 *
A^+/PI^-		4.38	0.0108 *		3.40	0.0530 ns		0.91	0.4244 ns
A^+/PI^+		45.77	0.0001 ***		6.39	0.0065 **		0.49	0.8089 ns
A^-/PI^+		25.89	0.0001 ***		0.36	0.6998 ns		0.45	0.8343 ns
Viab		26.52	0.0001 ***		13.29	0.0002 ***		0.91	0.5054 ns
F		57.06	0.0001 ***		0.92	0.4134 ns		7.56	0.0002 ***
B		0.33	0.8000 ns		0.39	0.6700 ns		1.21	0.3300 ns
AR		24.70	0.0001 ***		0.41	0.6660 ns		5.21	0.0018 **

Mot = motility, Vig = vigor, TM = total motility, PM = progressive motility, VCL = curvilinear velocity, VAP = average path velocity, VSL = straight line velocity, LIN = linearity, STR = straightness, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, WOB = wobble index, RAP = rapid spermatozoa, MED = medium speed spermatozoa, SLO = slow spermatozoa, STA = static spermatozoa, HOST = hypoosmotic test, A^-/PI^- = non-apoptotic spermatozoa with intact plasma membrane, A^+/PI^- = spermatozoa in the beginning of apoptosis, A^+/PI^+ = late apoptosis spermatozoa, A^-/PI^+ = necrotic spermatozoa, Viab = viable spermatozoa, F = non-capacitated spermatozoa without acrosome reaction, B = capacitated spermatozoa and AR = spermatozoa with acrosome reaction. df = degrees of freedom. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = non-significant ($P > 0.05$).

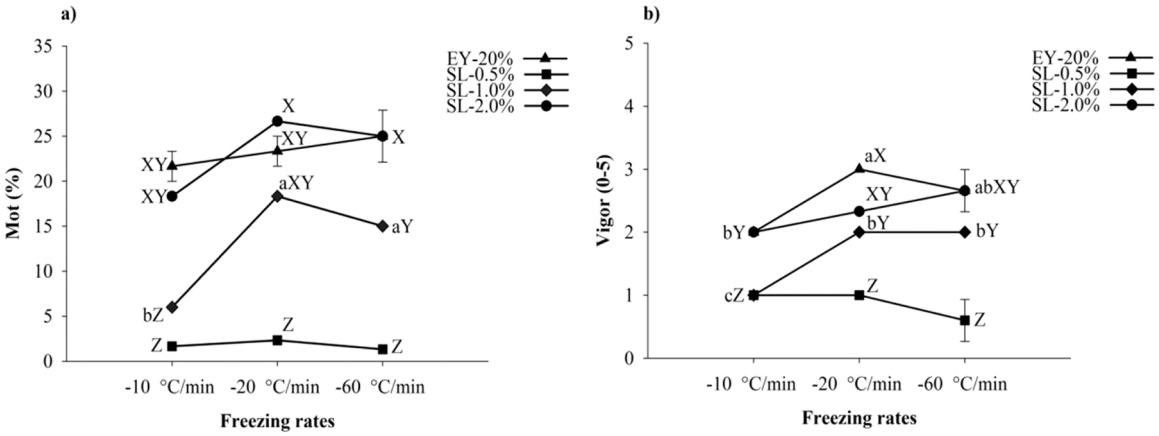

Fig. 2. Effects of interactions between different extenders based on egg yolk (EY) and soy lecithin (SL) and freezing rates on spermatozoa motility (a) and vigor (b) in thawed ram semen. [a,b,c] Different lowercase letters indicate differences between interactions inside the extender, and [X,Y,Z] different capital letters indicate differences between interactions inside the freezing rates ($P < 0.05$).

Table 2
Means ± standard errors of spermatozoa parameters in semen from rams submitted to freezing after dilution in different extenders based on egg yolk (EY) and soy lecithin (SL) (n=48).

Spermatozoa parameters	Extenders			
	EY–20%	SL–0.5%	SL–1.0%	SL–2.0%
TM (%)	30.27 ± 3.99 a	9.64 ± 1.37 b	13.75 ± 2.00 b	25.04 ± 2.22 a
PM (%)	13.01 ± 2.25 a	1.52 ± 0.36 b	3.03 ± 1.07 b	10.16 ± 1.66 a
VCL (μm/s)	192.49 ± 2.53 a	148.32 ± 14.31 c	167.52 ± 9.99 bc	190.03 ± 2.53 ab
ALH (μm)	2.73 ± 0.07 ab	0.72 ± 0.47 c	2.12 ± 0.24 bc	2.99 ± 0.15 a
BCF (Hz)	30.12 ± 1.37 a	19.64 ± 3.96 b	16.07 ± 3.25 b	31.67 ± 0.90 a
WOB (%)	76.98 ± 1.89 a	42.39 ± 8.09 b	34.99 ± 5.47 b	67.31 ± 1.88 a
RAP (%)	5.08 ± 1.05 a	0.32 ± 0.19 c	0.50 ± 0.25 bc	2.17 ± 0.71 ab
MED (%)	9.91 ± 1.42 a	1.42 ± 0.35 b	2.93 ± 0.87 b	8.70 ± 1.07 a
SLO (%)	15.30 ± 1.97 a	7.42 ± 1.07 b	10.33 ± 1.29 ab	14.85 ± 1.06 a
STA (%)	69.71 ± 4.00 b	90.84 ± 1.39 a	86.24 ± 2.02 a	74.28 ± 2.02 b
HOST (%)	14.16 ± 1.10 a	0.72 ± 0.12 d	2.00 ± 0.27 c	6.50 ± 0.87 b
A^+/PI^- (%)	6.50 ± 1.24 ab	7.49 ± 1.03 a	3.66 ± 0.52 b	7.38 ± 0.58 a
A^+/PI^+ (%)	30.03 ± 5.40 c	80.25 ± 1.92 a	75.55 ± 2.50 a	67.46 ± 1.97 b
A^-/PI^+ (%)	47.50 ± 5.74 a	8.31 ± 1.10 c	15.31 ± 1.97 b	15.50 ± 1.49 b
Viab (%)	22.37 ± 2.02 a	11.43 ± 1.47 c	9.06 ± 1.09 c	17.02 ± 1.36 b
B (%)	25.13 ± 2.20	25.93 ± 3.12	28.23 ± 2.30	25.90 ± 2.59

TM = total motility, PM = progressive motility, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, WOB = wobble index, RAP = rapid spermatozoa, MED = medium speed spermatozoa, SLO = slow spermatozoa, STA = static spermatozoa, HOST = hypoosmotic test, A^+/PI^- = spermatozoa in the beginning of apoptosis, A^+/PI^+ = late apoptosis spermatozoa, A^-/PI^+ = necrotic spermatozoa, Viab = viable spermatozoa, B = capacitated spermatozoa. [a,b,c] Different lowercase letters on the same line indicate differences between extenders ($P < 0.05$).

other ($P > 0.05$) for any of these kinetic parameters. Higher means of VCL and RAP were found in EY–20% compared to SL–0.5% and SL–1.0% ($P < 0.05$), but did not differ from the means of VCL and RAP observed in SL–2.0%. SL–2.0% had higher means for these parameters than SL–0.5% ($P < 0.05$), but did not differ from SL–1.0% ($P > 0.05$), which remained in an intermediate position. The SL–2.0% extender showed higher mean of ALH than SL–1.0% and SL–0.5% did ($P < 0.05$), but it did not differ from EY–20% for this parameter ($P > 0.05$). Higher means of SLO were found in the EY–20% and SL–2.0% samples than in SL–0.5% ($P < 0.05$); but it did not differ from the mean of this parameter observed in SL–1.0% ($P > 0.05$).

The interaction between extender and freezing rate significantly affected ($P < 0.001$ to < 0.05) VAP, VSL, STR, and LIN (Table 1, Fig. 3). Analysis only of interactions formed by the same extender showed that the interactions within extenders between EY–20%, SL–2.0%, or SL–0.5% and each level the freezing rates of − 10, − 20, and − 60 °C/min did not differ in terms of VAP, VSL, STR, and LIN ($P > 0.05$). Unlike that result, comparison among the interactions formed by the SL–1.0% extender showed that the freezing rate of − 20 °C/min had higher VAP, VSL, and STR than at the freezing rate of − 60 °C/min ($P < 0.05$), although it did not differ from − 10 °C/min ($P > 0.05$). Furthermore, consideration of only the interactions formed by SL–1.0% showed that higher LIN values were obtained between the interaction of this extender and the freezing rates of − 20 and − 60 °C/min compared to − 10 °C/min ($P < 0.05$).

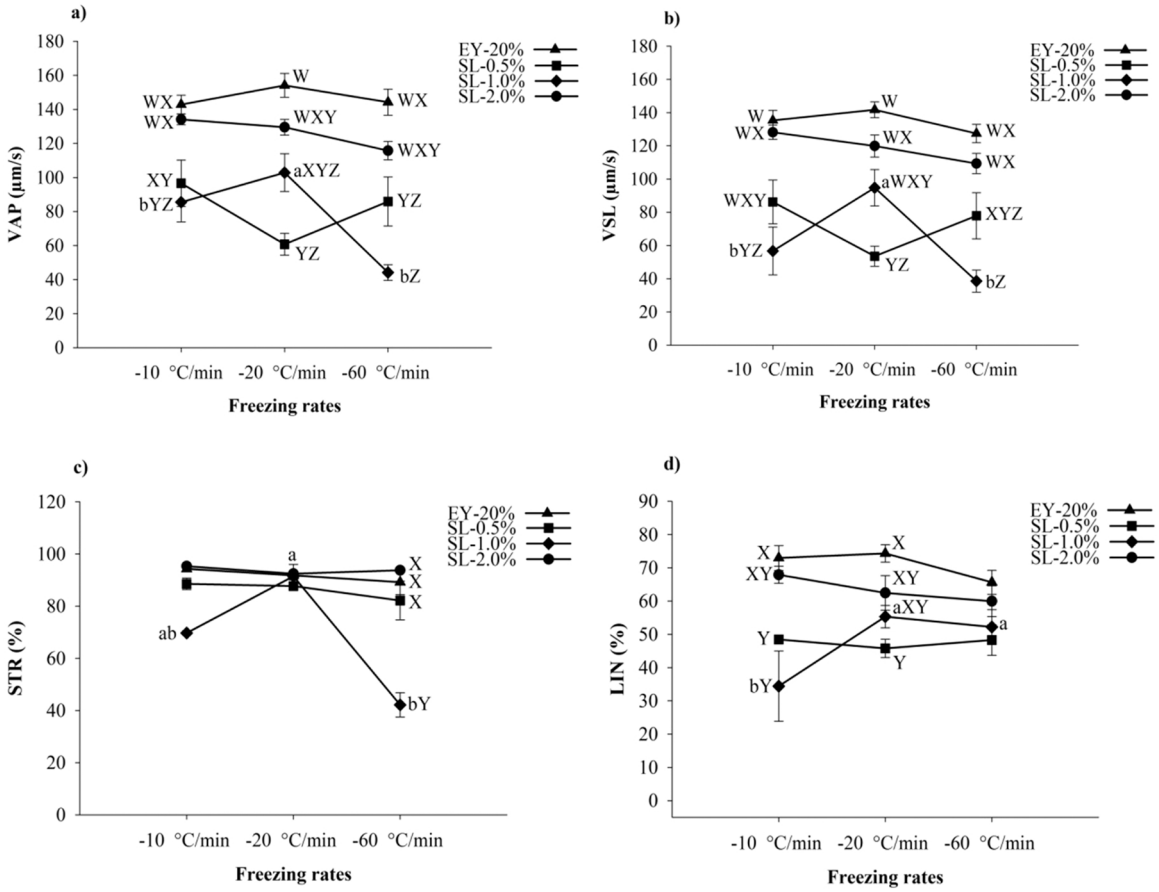

Fig. 3. Effects of interactions of different extenders based on egg yolk (EY) and soy lecithin (SL) and freezing rates on computerized spermatozoa kinetics parameters: average path velocity (VAP); straight line velocity (VSL); straightness (STR) and linearity (LIN). [a,b] Different lowercase letters indicate differences between interactions inside the extender, and [X,Y,Z] different capital letters indicate differences between interactions inside the freezing rates ($P < 0.05$).

4.3. Functionality of the plasma membrane

The functionality of the spermatozoa plasma membrane (HOST) was strongly affected by the extender factor ($P = 0.0004$), whereas the freezing rate and the interaction of both factors had no significant affect ($P > 0.05$) on this variable (Table 1). All extenders differed from each other ($P < 0.05$) regarding the proportion of spermatozoa with functional plasma membranes, with values progressively decreasing from EY–20%, SL–2.0%, SL–1.0%, and SL–0.5% (Table 2).

4.4. Viability

The extender factor had a significant ($P < 0.05$) or highly significant ($P < 0.0001$) effect on the proportions of spermatozoa categories and viable spermatozoa determined by flow cytometry (Tables 1 and 2). EY–20% had higher proportions of non-apoptotic spermatozoa with intact plasma membrane (A^-/PI^-) and viable spermatozoa ($A^-/PI^- + A^+/PI^-$) compared to the SL–0.5%, SL–1.0%, and SL–2.0% extenders ($P < 0.05$).

Comparing only the soy lecithin-based extenders, SL–2.0% had a higher proportion of A^-/PI^- spermatozoa than SL–0.5% and SL–1.0% did ($P < 0.05$), which showed no significant differences between them for this parameter ($P > 0.05$). The spermatozoa at the beginning of apoptosis (A^+/PI^-) were more frequent in the SL–0.5% and SL–2.0% extenders compared to SL–1.0% ($P < 0.05$). The A^+/PI^- proportions of all the lecithin-based diluents did not differ ($P > 0.05$) from the proportion of EY–20%, which ranked midway among the extenders for this parameter. A lower proportion of spermatozoa with late apoptosis (A^+/PI^+) was found in EY–20% compared to the other extenders ($P < 0.05$); SL–2.0% had a lower proportion of this category compared to SL–0.5% and SL–1.0% ($P < 0.05$); and the latter two did not differ from each other ($P > 0.05$). The highest proportion of necrotic spermatozoa (A^-/PI^+) was found in EY–20%, which differed from the other extenders ($P < 0.05$). SL–0.5% had the lowest proportion of necrotic spermatozoa, differing from all the other extenders ($P < 0.05$), while SL–2.0% and SL–1.0% did not differ in regard to this spermatozoa type ($P > 0.05$).

The freezing rate factor strongly affected ($P < 0.001$ to $P < 0.01$) the proportion of some categories of spermatozoa viability

Table 3
Means ± standard errors of spermatozoa parameters in ram semen submitted to different freezing rates (n=48).

Spermatozoa parameters	Freezing rates		
	−10 °C/min	−20 °C/min	−60 °C/min
TM (%)	18.62 ± 2.26	21.95 ± 3.99	18.45 ± 3.42
PM (%)	6.56 ± 1.57	7.96 ± 2.22	6.26 ± 1.93
VCL (μm/s)	184.72 ± 8.98	168.75 ± 9.99	170.3 ± 8.19
ALH (μm)	2.55 ± 0.29	2.73 ± 0.13	2.50 ± 0.30
BCF (Hz)	22.58 ± 3.48	27.49 ± 1.80	23.05 ± 3.34
WOB (%)	52.02 ± 7.85	62.78 ± 4.59	51.46 ± 6.86
RAP (%)	1.97 ± 0.62	2.59 ± 0.97	1.51 ± 0.71
MED (%)	5.13 ± 1.18	6.43 ± 1.80	5.04 ± 1.48
SLO (%)	13.69 ± 1.46	11.67 ± 1.92	11.02 ± 1.76
STA (%)	79.22 ± 2.15	79.43 ± 4.39	82.54 ± 3.61
HOST (%)	5.62 ± 1.43	6.33 ± 1.94	5.58 ± 1.67
A^+/PI^- (%)	4.69 ± 0.63	7.21 ± 0.93	6.87 ± 0.90
A^+/PI^+ (%)	69.19 ± 6.26 a	62.7 ± 5.70 b	58.07 ± 7.23 b
A^-/PI^+ (%)	19.84 ± 5.03	21.17 ± 5.04	23.95 ± 5.78
Viab (%)	10.90 ± 1.84 b	16.04 ± 1.66 a	17.96 ± 1.93 a
B (%)	26.72 ± 1.81	27.08 ± 2.42	27.69 ± 2.32

TM = total motility, PM = progressive motility, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, WOB = wobble index, RAP = rapid spermatozoa, MED = medium speed spermatozoa, SLO = slow spermatozoa, STA = static spermatozoa, HOST = hypoosmotic test, A^+/PI^- = spermatozoa in the beginning of apoptosis, A^+/PI^+ = late apoptosis spermatozoa, A^-/PI^+ = necrotic spermatozoa, Viab = viable spermatozoa, B = capacitated spermatozoa. [a,b,c] Different lowercase letters on the same line indicate differences between extenders ($P < 0.05$).

(Table 1). Table 3 shows higher proportions of A^-/PI^- and viable spermatozoa at − 60 °C/min compared to − 10 °C/min ($P < 0.05$). The rate of − 20 °C/min did not differ from − 60 °C/min for either parameter ($P > 0.05$) and did not differ significantly ($P > 0.05$) from − 10 °C/min for the proportion of A^-/PI^- and was higher ($P < 0.05$) than − 10 °C/min for the proportion of viable spermatozoa. The highest proportion of A^+/PI^+ spermatozoa was obtained at − 10 °C/min, differing significantly from − 20 °C/min and − 60 °C/min ($P < 0.05$); and the latter two freezing rates did not differ from each other ($P > 0.05$). The interaction between the extender and the freezing rate affected ($P < 0.05$) only the proportion of A^-/PI^- spermatozoa (Table 3). The Fig. 4 the interaction within extenders each level of the freezing rates shows that the interaction between EY–20% and − 60 °C/min had the highest proportion of A^-/PI^- spermatozoa, differing from all the other interactions ($P < 0.05$).

4.5. Capacitation and acrosome reaction

Factor 1, represented by the extender, and its interaction with factor 2, represented by the freezing rate, had a strong effect ($P < 0.001$ to $P < 0.01$) on the proportion of spermatozoa with changes like to non-capacitated (F) and acrosome reaction status (AR) spermatozoa (Table 1). The freezing rate did not affect any parameter of this evaluation, and the capacitated spermatozoa (B) were not affected by any of the factors tested or by the interaction between them ($P > 0.05$).

The Fig. 5 the interaction within extenders each level of the freezing rates shows among all the interactions, the EY–20% extender samples were the only ones influenced by the interaction with the freezing rate exhibited the highest proportion of spermatozoa

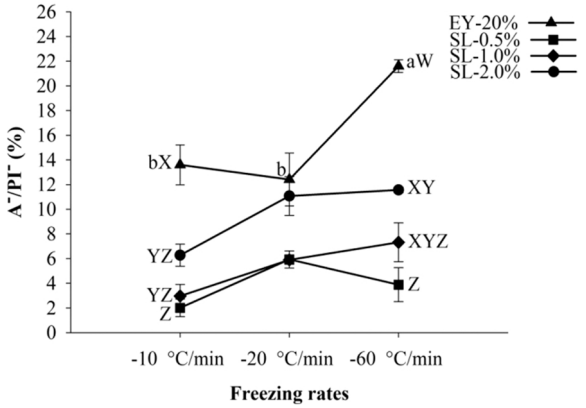

Fig. 4. Effects of interactions between different extenders based on egg yolk (EY) and soy lecithin (SL) and freezing rates on the proportion of non-apoptotic spermatozoa with intact plasma membrane (A^-/PI^-). [a,b] Different lowercase letters indicate differences between interactions inside the extender, and [W,X,Y,Z] different capital letters indicate differences between interactions inside the freezing rates ($P < 0.05$).

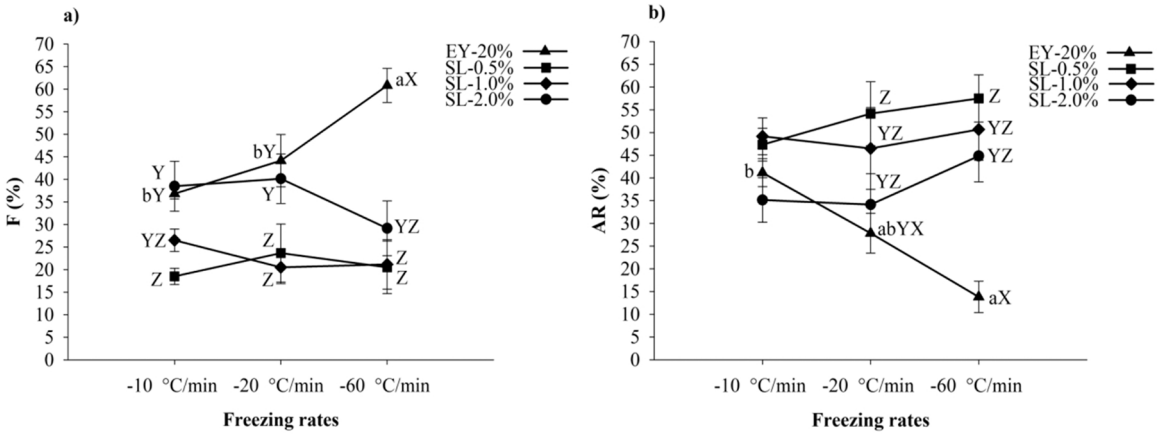

Fig. 5. Effects of interactions between different extenders based on egg yolk (EY) and soy lecithin (SL) and freezing rates on the proportions of spermatozoa non-capacitated without acrosome-reacted (F) and with acrosome-reacted (AR). [a,b] Different lowercase letters indicate differences between interactions inside the extender, and [X,Y,Z] different capital letters indicate differences between interactions inside the freezing rates ($P < 0.05$).

classified as F in the interaction between EY–20% and − 60 °C/min ($P < 0.05$). The interaction within freezing rates each level of the extenders based on soy lecithin, those between − 10 and − 20 °C/min and SL–2.0% had a higher proportion of spermatozoa classified as F compared to the interactions between − 10, − 20, and − 60 °C/min and SL–0.5%, and between − 20 and − 60 °C/min and SL–1.0% ($P < 0.05$), but they did not differ from the interactions between − 10 °C/min and SL–1.0% and between − 60 °C/min and SL–2.0% ($P > 0.05$). The interaction within extenders each level of the freezing rates shows among all the interactions, the EY–20% extender samples were the only ones influenced by the interaction with the freezing rate exhibited lower proportions of spermatozoa classified as AR in the interaction between the EY–20% extender and the freezing rate at − 60 °C/min compared to the interaction between EY–20% and − 10 °C/min ($P < 0.05$). The interaction within freezing rates each level of the extenders based on soy lecithin, those between − 10 and − 20 °C/min and SL–2.0% had lower AR values compared to the interaction between − 60 °C/min and SL–0.5% ($P < 0.05$), but they did not differ from the interactions between − 10 and − 20 °C/min and SL–0.5% and between − 10, − 20, and − 60 °C/min and SL–1.0% ($P > 0.05$).

5. Discussion

Our results showed that spermatozoa with greater total and progressive motility, as well as straight and linear spermatozoa with medium to fast velocity in CASA-motility were obtained from extenders (EY–20% and SL–2.0%), these same extenders in combination with freezing rates (−20 and −60 °C/min) also showed high Mot and Vig characteristics desirable for reproduction. The samples of thawed ram semen of the Zell and Zandi breeds diluted in the extender based on egg yolk at 20% had spermatozoa kinetic values that did not differ significantly from those diluted in extenders containing 1.0% (Masoudi et al., 2019) and 2.0% soy lecithin (Emamverdi et al., 2013). The results already shown on the effect of the freezing rate showed higher performance of VAP and STR was obtained between − 10 °C/min to − 20 °C/min freezing rate, with higher values at − 10 °C/min, and lower at −60 °C/min. In the thawed semen of roosters, freezing rates did not affect the value of VAP, but the STR had a response similar to that of our study, where higher values of this parameter were obtained at the slow freezing rate than at the fast freezing rate (Madeddu et al., 2016).

The best spermatozoa kinetics from semen samples diluted in EY–20% and SL–2.0% extenders, regardless of the freezing rates (−10, −20 and 60 °C/min) can be attributed to a possible better cryoprotective effect of these extender come from their greater contribution as non-permeable solutes in the spermatozoa extracellular space related to their higher osmolalities before glycerol addition (EY–20% = 310 mOsm/kg and SL–2.0% = 262 mOsm/kg), compared to the others (SL–1.0% = 242 mOsm/kg and SL–0.5% = 230 mOsm/kg). Regarding the soy lecithin extenders, it was also observed that the increase in the quality kinetics of the thawed spermatozoa was better as the osmolality of the extenders was closer to the isosmotic osmolality. After thawing, electroejaculated samples spermatozoa of red deer showed greater progressive motility and viability in the extender with 320 mOsm/kg compared to the extender with 430 mOsm/kg, not differing from of 380 mOsm/kg (Martínez-Pastor et al., 2006). The authors concluded that the ideal is extensors should be isosmotic and slightly hyperosmotic.

The optimum VAP and VSL values of the SL–0.5% and SL–1.0% extenders, and the best of SRT value of the SL–1.0% extender can be obtained a freezing rate of around −20 °C/min, with the rates of − 10 °C and −60 °C/min presenting values of these parameters below ideal. Probably the suboptimal behavior of spermatozoa kinetics when subjected to freezing rates of − 10 and − 60 °C/min may be related to the low amounts of solutes reflected in the hyposmotic osmolalities of the SL–1.0% and SL–0.5% extenders. These extenders may not have been able to protect the spermatozoa from cryogenic damage that negatively alters the membrane structure and mitochondrial bioenergetic metabolism of this cell (O'Connell et al., 2002; Oberoi et al., 2014).

We found higher means of spermatozoa with a functional plasma membrane analyzed by HOST in the EY–20% extender than in the

extenders containing soy lecithin. A similar result was obtained in the thawed semen of the Zandi breed, which had a higher proportion of functional spermatozoa in the extender that contained 20% egg yolk than in the extenders that contained soy lecithin at 2.0% and 2.5% (Emamverdi et al., 2013). The intact plasma membrane is characterized by the maintenance of a stable lipid-protein bilayer, providing spermatozoa with protection and fertilization capacity (Parks and Lynch, 1992). Thawed spermatozoa that show apoptotic-like changes in the plasma membrane with the externalization of phosphatidylserine (Martin et al., 2004) are susceptible to being phagocytosed in the female tract, decreasing their availability (Anzar et al., 2002). The extender containing soy lecithin was not able to overcome the EY–20% extender to preserve a higher proportions of spermatozoa with membrane functionality (HOST), non-apoptotic and intact plasma-membrane (A^-/PI^-) and viable (Viab). The good performance of egg yolk has been attributed to the action of non-penetrating molecules such as sugars, lipoproteins (α and β-lipovitelins), phospholipids and cholesterol present in its composition (Martin et al., 1963). A greater protective effect of membrane integrity on the thawed spermatozoa of bulls was also obtained with extenders that contained egg yolk compared to extenders that contained soy lecithin (Thun et al., 2002).

The greater protection offered by the SL–2.0% extender represented by an satisfactory performance of proportions of spermatozoa with membrane functionality (HOST), non-apoptotic and intact plasma-membrane (A^-/PI^-), and viable (Viab) was also probably due to the better combination of its higher osmolality and availability of non-permeable molecules compared to the SL–0.5 and SL–1.0% extenders. The protective effect of soy lecithin varies depending on their composition and concentration (Rydhag and Wilton, 1981). The faster freezing rates (− 20 and − 60 °C/min) showed higher proportions of non-apoptotic spermatozoa with intact plasma membrane and viable spermatozoa compared to the slower rate (− 10 °C/min). Non-significant differences were observed between fast freezing rates of − 30 and − 50 °C/min in sheep and pigs (Kumar et al., 2003) and between − 40 and − 70 °C/min in roosters (Madeddu et al., 2016), which increase the preservation of spermatozoa structures and viability compared to slow freezing rates. In cervical artificial insemination in sheep, spermatozoa with a non-capacitated status are required in greater proportion because they have the greater longevity needed to reach the site of fertilization in the uterus and have a positive correlation with fertility (Gil et al., 2003; Mendoza et al., 2021). Our results showed higher proportions of non-capacitated spermatozoa after freezing-thawing in EY–20% extender, which were higher than those reported in the thawed semen of Santa Inês rams diluted in another extender containing egg yolk at 20% (Sicherle et al., 2011). The low cryoprotection performance of the extenders SL–0.5% and SL–1.0% subjected to freezing rates (− 10, − 20, and − 60 °C/min) was reflected in the high proportions of spermatozoa with early acrosome reacted, as a consequence of the cryodamage to the acrosomal membrane (Khan et al., 2021), which was greater than that found in thawed semen of rams of the Zandi breed diluted in extender containing 1.0% soy lecithin (Najafi et al., 2013).

The combination of the EY–20% extender and the freezing rate of − 60 °C/min exhibited the highest proportions of non-capacitated spermatozoa, such as those found in fresh ram semen (Gillan et al., 1997). In addition, it showed a lower mean of acrosome-reacted spermatozoa, lower than the values found in the thawed semen of Mehraban breed rams diluted in the extender that contained 1.5% soy lecithin and subjected to a moderate freezing rate obtained at 5 cm above the LN_2 (Najafi et al., 2014). This better combination may be related to the positive effects of the different solutes present in egg yolk and the rapid freezing rates on spermatozoa membrane stability (Kumar et al., 2003; Mocé et al., 2010). Studies have also shown that different extenders based on egg yolk and commercial extenders in combination with glycerol influence the quality of thawed semen from rams (Álvarez et al., 2012) and camels (Malo et al., 2021) respectively. In our study, the extenders based on egg yolk (EY–20%) and soy lecithin (SL–0.5%, SL–1.0%, and SL–2.0%) combined with 5% glycerol and subjected to freezing rates (− 10, − 20, and − 60 °C/min) had different cryoprotective effects on thawed spermatozoa.

The differences between the results of our study and those obtained by the authors cited can be mainly related to the extenders osmolalities. All authors used and tested only isosmotic extenders while our study tested extenders with different osmolalities, some hypoosmotic (eg. SL–0.5%, SL–1.0% and SL–2.0%) and some isosmotic (eg. EY–20%). Differences can also be attributed to such spermatozoa concentrations by dose, freezing rates, species, breed, etc. In this respect, any change in factors involved in semen cryopreservation protocols must be accompanied by adjustments. We propose that soy lecithin-based extenders can be tested with higher osmolality by adding salts or sugars before adding glycerol, in order to confirm their potential as substitutes for egg yolk-based extenders used for freezing ram semen. This study was the first to test replacement of egg yolk by soy lecithin in the glycine-milk (GM) extender that has been used by us and by other research teams for conservation of ram semen with proven performance in vitro and in vivo (Bandeira et al., 2017; Duenhas Monreal et al., 2014; Gonzalez, 1996).

6. Conclusions

The present study showed the superior effect of 20% egg yolk and the promising effect of 2.0% soy lecithin as components of the glycine-milk (GM) extenders and the fast-freezing rates of − 60 °C/min and − 20 °C/min in the preservation of the structure and functionality of ram spermatozoa subjected to cryopreservation. The GM extender based on egg yolk at 20% has its best performance when the semen is subjected to a freezing rate of − 60 °C/min, while the GM extender with the concentration of 2.0% soy lecithin (SL) has the best performance compared to other SL concentrations (0.5% and1.0%), regardless of the freezing rate used. The satisfactory performance of 2.0% soy lecithin, obtained in this work indicates the need to test higher concentrations of soy lecithin as the main source of phospholipids, in the GM extender formulated with higher osmolality for freezing ram semen.

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CRediT authorship contribution statement

Julio Constantino Jeri Molina: Conceptualization, Methodology, Software, Investigation, Writing – original draft, Writing – review & editing, Project administration, Formal analysis. **Matheus Batista de Oliveira**: Investigation, Data curation. **Paulo Henrique Conceição Costa**: Data curation. **Edmilson Willian Propheta dos Santos**: Data curation. **Cristiane Bani Correa**: Resources. **Hymerson Costa Azevedo**: Resources, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2023.107234.

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