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Combination of sugars, lipoproteins and centrifugation improve dairy goat sperm viability submitted to cryopreservation

Combinação de açúcares, lipoproteínas e centrifugação melhoram a viabilidade de espermatozoides de caprinos leiteiros pós-criopreservação

La combinación de azúcares, lipoproteinas y centrifugación mejora la viabilidad de los espermatozoides de macho cabrio lechera pos-congelácion

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Abstract
The aim of the present study was to evaluate the effects of different cryoprotectants and semen centrifugation on the kinetic parameters and membrane integrity of cryopreserved goat semen. Four buck goats were used, and their semen was collected using an artificial vagina. After semen collection and approval, six pools were formed, and each pool was divided into eight aliquots. The plasma was removed from four aliquots by centrifugation (1200 g/10 min) and subsequently diluted; the remaining four aliquots were diluted in standard Tris-egg yolk (SYE), standard milk (SME), test Tris-egg yolk (TYE) and test milk (TME) extenders without removing the seminal plasma. After dilution, samples were loaded into straws (0.25 mL), frozen and stored at -196 °C. The samples were thawed (37 °C/30 s) and evaluated immediately and two hours after thawing to determine the kinetics and integrity of plasma and mitochondrial membranes. A difference ($p < 0.05$) in the maintenance of sperm kinetics, plasma membrane integrity and mitochondrial potential was observed between the centrifuged and non-centrifuged groups and between the extenders at different time points. We concluded that the use of centrifugation to remove seminal plasma positively affects the semen evaluation parameters and that the yolk extender is more efficient when applied with different cryopreservation techniques, preserving the desirable traits after the cryopreservation of goat semen.
Keywords: Freezing; Mitochondria; Phospholipase A; Seminal plasma.

Resumo
O objetivo do presente estudo foi avaliar os efeitos de diferentes crioprotetores e da centrifugação do sêmen sobre os parâmetros cinéticos e integridade da membrana do sêmen caprino criopreservado. Quatro bodes foram utilizados, e o sêmen foi colhido por meio de uma vagina artificial. Após colheita e aprovação do sêmen, seis *pools* foram formados,

e cada *pool* foi dividido em oito alíquotas. O plasma foi retirado de quatro alíquotas por centrifugação (1200 g/10 min) e posteriormente diluído; as quatro alíquotas restantes foram diluídas em Tris-gema de ovo padrão, leite padrão, Tris-gema de ovo teste e diluente a base de leite, sem remoção do plasma seminal. Após a diluição, as amostras foram colocadas em palhetas (0,25 mL), congeladas e armazenadas a -196 °C. As amostras foram descongeladas (37 °C/30 s) e avaliadas imediatamente e duas horas pós-descongelação para determinar a cinética e integridade das membranas plasmática e mitocondrial. Foi observada diferença ($p < 0,05$) na manutenção da cinética espermática, integridade da membrana plasmática e potencial mitocondrial entre os grupos centrifugados e não centrifugados e entre os extensores em diferentes momentos. Concluímos que o uso da centrifugação para remoção do plasma seminal afeta positivamente os parâmetros de avaliação do sêmen e que o extensor de gema é mais eficiente quando aplicado com diferentes técnicas de criopreservação, preservando as características desejáveis após a criopreservação do sêmen caprino.
Palavras-chave: Congelação; Fosfolipase A; Mitocôndria; Plasma seminal.

Resumen

El objetivo de este estudio fue evaluar los efectos de diferentes crioprotectores y la centrifugación del semen sobre los parámetros cinéticos y la integridad de la membrana del semen del macho cabrio criopreservado. Se utilizaron cuatro machos y el semen se recogió a través de una vagina artificial. Después de la recolección y aprobación del semen, se formaron seis grupos y cada grupo se dividió en alícuotas. O se extrajo plasma de cuatro alícuotas por centrifugación (1200 g/10 min) y se diluyó adicionalmente; Las cuatro alícuotas restantes se diluyen en Tris-yema de huevo estándar, Leche, Tris-yema de huevo de prueba y diluyente a base de leche, sin eliminar el plasma seminal. Después de la dilución, las muestras se colocaron en paletas (0.25 mL), se congelaron y se pelaron a -196 °C. Las muestras se descongelaron (37 °C/30 s) y se evaluaron inmediatamente durante dos horas después de la descongelación para determinar la cinética y la integridad del plasma y las mitocondrias. membranas. Se observaron diferencias ($p < 0,05$) en el mantenimiento de la cinética de los espermatozoides, la integridad de la membrana plasmática y el potencial mitocondrial entre los grupos centrifugado y no centrifugado y entre los dilutores en diferentes momentos. Concluimos que el uso de la centrifugación para eliminar el plasma seminal afecta positivamente los parámetros de evaluación del semen y que el dilutor de yema es más eficiente cuando se aplica con diferentes técnicas de criopreservación, conservando las características de criopreservación del semen caprino.
Palabras clave: Congelación; Fosfolipasa A; Mitocondria; Plasma seminal.

1. Introduction

Cryopreservation coupled with artificial insemination (AI) are techniques that aid breeding programs. However, despite the advances in the field of biotechnology, the number of damaged and dead cells increases substantially compared to fresh semen, regardless of the freezing and thawing techniques used (Barbas et al., 2018) and the species studied.

For fertility, the spermatozoa must exhibit motility, an appropriate mitochondrial potential, membrane integrity and highly condensed nuclei (Graham and Mocé, 2005). Alterations to these parameters potentially result in reduced cell fertility (Mocé et al, 2020).

The quality of the cryopreservation extenders represents one of the main factors related to the maintenance of the viability of goat cryopreserved spermatozoa and influences the quality and fertility of the frozen samples (Vidal et al., 2013). Egg yolk and skim milk are routinely used as semen extenders because they protect cells during the freezing and thawing stages by mainly acting on the plasma membrane of spermatozoa (Purdy, 2006). However, due to the interactions of goat semen phospholipases with some substances present in egg yolk and milk that exert adverse effects on the spermatozoa, researchers recommend the removal of the seminal plasma (Roof et al., 2012).

The sugars used in the extender medium for the cryopreservation of animal semen include simple sugars, such as glucose and fructose, which, in addition to serving as nutrition that provides the necessary energy for sperm motility, also possess a cryoprotectant function (Naing et al., 2010).

According to Celeghini et al. (2008), a better understanding of the mechanisms of action of extenders in the sperm cell and the use of techniques with high accuracy, greater reproducibility and objectivity in the evaluation of the effects of cryopreservation on spermatozoa is essential to assist in the development of the ideal extender. Thus, substantial emphasis has been placed on automated motility assessment techniques, such as computer-assisted semen analysis (CASA) and specific

labeling with fluorescent probes due to the possibility of a more thorough analysis of the structural integrity of the spermatozoa (Cunha et al., 2015) and the role of seminal plasma on the sperm cell.

Therefore, based on an evaluation of the effects of cryopreservation on the plasma, acrosomal and mitochondrial membranes using epifluorescence microscopy and computer-assisted sperm analysis, as well as semen centrifugation techniques, the composition of extenders and efficient techniques for goat semen cryopreservation will be able to be determined.

2. Methodology

Four buck goats, two of the Saanen breed and two of the Alpine breed, were used. The animals were two to four years old and weighed an average of 50 kg. The animals were maintained on an intensive regime, and offered elephant grass (*Pennisetum purpureum*), feed composed of soybean, corn, wheat bran and cotton cake and water *ad libitum*. The semen was collected in an artificial vagina with the aid of a dummy female in estrus. Collections were performed three times a week for two weeks (n = 6). The study was approved by the Ethics Committee for Animal Use (License n°. 095/2017/CEUA-UFPB).

Initially, the microscopic parameters were separately evaluated in each buck goat, and after approval [gross motility ≥ 3 (0-5); motility ≥ 70% (0-100%)], the samples were *pooled*, and a new evaluation of motility and vigor was conducted. The plasma membrane integrity was analyzed using eosin-nigrosin staining and the plasma membrane function was analyzed using the hypoosmotic swelling test (HOST) (CBRA, 2013). The sperm number was obtained by counting a 1:400 dilution of each sample in a formaldehyde-saline solution with a Neubauer chamber using a microscope (BA300, Motic, Kowloon, Hong Kong, China).

Four different extenders were prepared in this study. The standard Tris-egg yolk (SYE) extender (3.605 g of Tris, 2.024 g of citric acid, 1.488 g of fructose, 100 mL of distilled water, 20% of egg yolk; 3 mg of antibiotic (Pentabiotic Agrovet ®), and 5% glycerol, pH 6.8) was prepared using the methodology reported by Hafez and Hafez (2004). The standard milk (SME) extender (10 g of skim milk powder, 194 mg of glucose; 100 mL of distilled water, 3 mg of antibiotic (Pentabiotic Agrovet ®), and 7% glycerol, pH 6.8). The following test extenders were also prepared: test Tris-egg yolk (TYE; 3.605 g of Tris, 2.024 g of citric acid, 0.5 g of glucose, 100 mL of distilled water, 20% egg yolk, 3 mg of antibiotic (Pentabiotic Agrovet ®) and 5% glycerol, pH 6.8) and test milk (TME; 10 mg of skim milk powder, 578 mg of fructose, 100 mL of distilled water, 3 mg of antibiotic (Pentabiotic Agrovet ®), and 7% glycerol, pH 6.8). For each extender, the substances were homogenized using a magnetic stirrer. The milk-based extenders were placed in a water bath at 90 °C for 10 minutes. The extenders were aliquotted into Falcon tubes and frozen (-20 °C).

The semen *pool* was divided into eight aliquots with equal volumes; four samples were directly diluted in the different extenders without removing the seminal plasma - NC (noncentrifuged). The seminal plasma was removed from the other four samples - C (centrifuged) - through double centrifugation (1200 g/10 min) with a saline solution (0.9% NaCl, pH 6.0) at a ratio of 1:10. The supernatant was discarded and the pellet was resuspended in the different extenders.

At the end of the dilution process, eight experimental groups were formed: SMC (standard milk + glucose, centrifuged); SYC (standard egg yolk + fructose, centrifuged); TMC (test milk + fructose, centrifuged); TYC (test yolk + glucose, centrifuged); SMNC (standard milk + glucose, noncentrifuged); SYNC (standard egg yolk + fructose, noncentrifuged); TMNC (test milk + fructose, noncentrifuged); TYNC (test egg yolk + glucose, noncentrifuged). For each experimental group, four 0.25-µL straws were filled with an insemination dose of 100 x 106 spermatozoa per straw, as recommended by the Brazilian College of Animal Reproduction, for a total of 32 straws per *pool*.

After filling, the straws were refrigerated in a refrigerator with an internal temperature of 5 °C using the refrigeration curve characterized by Bispo et al. (2011) for three hours and thirty minutes, as proposed by Jiménez-Rabadán et al. (2013).

Next, the straws were horizontally placed in liquid nitrogen vapor (N2) at 5 cm above the liquid nitrogen for 20 minutes inside a styrofoam box, subsequently immersed in N2 and stored in a cryovessel at -196 °C until thawing.

After thawing (37 °C/30 s) and transfer of the samples to 1.5 mL microtubes, aliquots of samples from the experimental groups were evaluated for kinetic parameters, plasma membrane integrity (PMi), plasma membrane functionality (PMf) and mitochondrial membrane potential (MMP) at 0 and 2 hours after thawing (Soares et al., 2014).

Sperm kinetics were evaluated using the computer-assisted sperm analysis (CASA) system (SCATM; Microptics, SL, Version 5.1, Barcelona, Spain). An aliquot (10 µL) of the sample was diluted in 100 µL of PBS and placed on a preheated slide (37 °C), covered with a cover slip and evaluated under a phase-contrast microscope (Eclipse 50i, Nikon, Japan) coupled to video camera with 100x magnification; images were captured and analyzed using imaging software. For each sample, images of six randomly selected and nonconsecutive fields were captured, and the trajectory of each cell was reconstructed.

The following variables were evaluated: total motility (TM; %), progressive motility (PM; %), linearity (LIN; %), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s) and average path velocity (VAP; µm/s). The CASA values were measured with the following configurations: temperature of 37 °C; 100x magnification; number of images, 25; images per second, 25; head area, 20 to 70 µm; VAP: slow 10 µ/s, medium 45 µ/s, and fast 75 µ/s; progressivity, 80% straightness coefficient (STR); and 50% LIN (Verstegen et al., 2002).

Plasma membrane integrity was evaluated with the double staining method using the fluorochromes carboxyfluorescein diacetate (CFDA; 0.46 mg/mL in DMSO) and propidium iodide (PI; 0.5 mg/mL in PBS). Then, 150 µL of PBS + 50 µL of the sample were added, and stained with 5.0 µL of CFDA and 5.0 µL of PI through a five-minute incubation at room temperature (25 °C), and then incubated with 5.0 µL of paraformaldehyde. Two hundred spermatozoa were evaluated under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) using a 485/20-nm DBP excitation filter and a 580-630-nm emission filter.

The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide monomer (JC-1; 0.15 mM in DMSO) was used to evaluate the mitochondrial membrane potential. For each treatment, one aliquot (50 µL) of the sample was stained with 5.0 µL of JC-1 for five minutes and then treated with 5.0 µL of paraformaldehyde. Two hundred spermatozoa were evaluated using a 485/20-nm DBP excitation filter and a 580-630-nm emission filter.

The viscosities of the standard (skim milk + glucose and, tris + egg yolk + fructose) and test extenders (skim milk + fructose and, tris + egg yolk + glucose) were determined using the rheometer (Brookfield LVDVIII Ultra, São Paulo) located at the Petroleum Laboratory of the Center of Technology at the Federal University of Paraíba, Campus I. Five hundred microliters of the sample were placed in the rheometer container and subjected to a torque sufficient to maintain the rotation of the CP51 spindle immersed in the sample at temperatures of 10 ºC and 37 ºC. 30 points were obtained for each sample and the average viscosity given in centipoise (cP) was performed. This study was carried out to verify and compare the viscosity of the different extenders as a function of temperature variation (Farias et al., 2019).

Statistical analysis

The kinetics, plasma membrane integrity and function and mitochondrial potential data were analyzed using the Kolmogorov-Smirnov test. Next, the data were analyzed using analysis of variance (ANOVA) followed by the Tukey post hoc test. The t-test was used compare data collected at 0 and 2 h after thawing. All tests adopted a significance level of 5%.

3. Results

The TM values of the SMNC and TMNC groups differed from the SYNC group immediately after thawing (0 h); the

SMNC and TMNC groups exhibited the lowest percentage of TM. Differences were observed ($p< 0.05$) between the SMC group and the SYC and TYC groups two hours after thawing; the SYC and TYC groups maintained greater motility. After considering viable samples, the samples preserved in the milk extenders also maintained motility. Regarding the evaluation times, a difference ($p< 0.05$) was observed between the SMC, TMC, SYNC and TYNC groups, with a significant reduction in TM.

A difference ($p< 0.05$) in the PM was observed between the GPC and TMC groups, which differed from the SMNC and TMNC groups after thawing (0 h). A lower percentage of PM was observed in the noncentrifuged SM and TM groups. The values of the SYC and TYC groups differed from the SMC, SYNC and TYNC groups two hours after thawing ($p< 0.05$). Regarding the evaluation time, a difference ($p< 0.05$) was observed between the SMC, SYC, TMC, SYNC and TYNC groups, where the treatments behaved similarly to TM, with a greater than 50% reduction in PM. Thus, the presence of yolk in the extender composition and the centrifugation process exert a positive effect on the maintenance of TM and PM. Total and progressive motility average are shown in Table 1.

Table 1. Percentage of total and progressive motility of goat sperm cells cryopreserved with different extenders and analyzed at 0 and 2 hours after thawing

	Total motility (%)		**Progressive motility (%)**	
Groups	0 h	2 h	0 h	2 h
SMC	32.62±11.77^{ab*}	8.05±4.37^{b*}	16.89±0.06^{ab*}	2.10±0.02^{b*}
SYC	40.60±7.03^{ab}	34.00±6.49^{a}	24.29±0.06^{a*}	13.10±0.08^{a*}
TMC	41.63±11.44^{ab*}	17.13±8.65^{ab*}	22.30±0.09^{a*}	6.00±0.04^{ab*}
TYC	32.27±18.61^{ab}	33.20±25.16^{a}	18.10±0.13^{ab}	12.70±0.11^{a}
SMNC	22.38±9.12^{b}	16.6±6.00^{ab}	6.90±0.04^{b}	5.30±0.03^{ab}
SYNC	52.58±10.26^{a*}	11.8±10.25^{ab*}	16.56±0.05^{ab*}	2.20±0.03^{b*}
TMNC	21.53±11.33^{b}	17.8±8.20^{ab}	6.10±0.06^{b}	6.60±0.04^{ab}
TYNC	42.67±13.02^{ab*}	17.2±15.28^{ab*}	14.90±0.07^{ab*}	2.59±0.02^{b*}

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

Lower values for the parameters VAP, VSL and VCL were obtained ($p< 0.05$) in the SMNC and TMNC groups than the SMC, SYC, TMC and TYC groups at the time of thawing. Regardless of the extender used, differences in the VCL and VAP ($p< 0.05$) were not observed between treatments at two hours after thawing; the only difference was observed for the VSL parameter, where the SMC and TMNC groups differed from the TYNC group.

A correlation between the extender and evaluation time ($p< 0.05$) was observed, with a reduction in the spermatozoa velocity of the SMC group, as evidenced by the decreases of 66.6%, 58% and 59.2% in the VCL, VSL and VAP, respectively. In the TMC group, the VCL and VAP were reduced by 68% and 64.3%, respectively; the SYNC group showed a decrease of 53.4% in the VCL and the TYNC group showed a 45.3% decrease in the VSL at two hours after thawing. Spermatozoa velocities average are shown in Table 2.

Table 2. Absolute values of the parameters curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) of goat spermatozoa cryopreserved with different extenders and analyzed at 0 and 2 hours after thawing.

	VCL (µm/s)		VSL (µm/s)		VAP (µm/s)	
	0 h	2 h	0 h	2 h	0 h	2 h
SMC	106.3±11.1^{a*}	70.9±13.4*	81.2±11.0^{a*}	47.1±12.1^{a*}	99.1±11.4^{a*}	58.7±12.2*
SYC	95.2±21.9^{a}	83.0±27.6	70.0±20.2^{a}	49.9±21.1^{ab}	86.1±23.3^{ab}	68.2±26.0
TMC	100.8±16.4^{a*}	68.7±16.8*	70,8±17.3^{ab}	47.5±17.3^{ab}	87.7±20.6^{ab*}	56.4±17.9*
TYC	93.5±21.8^{ab}	74.3±27.5	72.5±22.6^{ab}	44.2±12.7^{ab}	84.0±22.8^{ab}	61.8±23.5
SMNC	62.7±18.8^{b}	58.3±12.7	43.8±11.1^{b}	43.4±11.6^{ab}	56.0±16.2^{b}	52.2±12.2
SYNC	103.5±10.7^{a*}	55.3±44.4*	46.8±10.6^{b}	23.0±19.3^{ab}	69.3±12.0^{ab}	35.7±29.5
TMNC	69.8±16.7^{b}	75.1±11.5	43.9±14.0^{b}	50.3±9.6^{a}	59.6±6.2^{b}	65.2±8.2
TYNC	89.1±12.3^{ab}	50.0±39.9	46.3±14.6^{b*}	21.0±16.4^{b*}	62.1±17.7^{b}	31.4±24.8

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

The LIN of the spermatozoa from the noncentrifuged SY and TY groups differed ($p< 0.05$) from the other groups at 0 and 2 hours after thawing. The same trend was observed for STR, in which the SYNC, TMNC and TYNC groups had lower values than the other groups at 0 h. The wobble coefficient (WOB) showed the same patterns for LIN and STR, with lower values for the spermatozoa cryopreserved in the SYNC and TYNC extenders. An increase in velocity was also observed in the groups containing fructose in the extender. Considering the relationship between the extender and time, a difference ($p< 0.05$) in LIN was only observed the TYC group and a difference in WOB was observed in the SMC group. Kinetics parameters average are shown in Table 3.

Table 3. Percent values of the parameters linearity (LIN), straightness coefficient (STR) and wobble coefficient (WOB) of goat sperm cells cryopreserved with different extenders and analyzed at 0 and 2 hours after thawing.

	LIN (%)		STR (%)		WOB (%)	
	0 h	2 h	0 h	2 h	0 h	2 h
SMC	76.3±6.4^{a}	65.8±9.7^{a}	81.9±6.1^{a}	79.4±7.3	93.1±1.5^{a*}	82.9±9.0^{ab*}
SYC	77.2±5.9^{a}	61.8±17.6^{ab}	85.9±3.0^{a}	74.9±16.3	89.7±4.6^{a}	81.6±8.8^{ab}
TMC	69.5±5.9^{a}	68.2±11.8^{a}	80.6±2.1^{a}	83.6±7.2	86.2±6.8^{a}	81.1±8.9^{ab}
TYC	76.4±6.5^{a*}	62.0±11.2^{ab*}	85.4±4.0^{a}	74.5±11.4	89.3±4.4^{a}	83.1±6.8^{ab}
SMNC	70.8±5.7^{a}	74.1±5.9^{a}	79.0±4.7 ^{a}	82.7±4.2	89.6±3.5^{a}	89.5±3.8^{a}
SYNC	45.0±10.5^{b}	35.3±18.1^{b}	66.6±6.5^{b}	52.5±26.2	67.0±10.2^{b}	56.5±29.9^{b}
TMNC	62.7±8.6^{a}	67.3±10.9^{a}	73.3±6.3^{b}	77.0±9.5	85.3±6.5^{a}	87.1±5.2^{a}
TYNC	51.2±10.8^{b}	41.7±26.3^{b}	74.0±4.5^{b}	61.0±33.2	68.7±11.5^{b}	55.6±28.6^{b}

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

The SYNC and TYNC groups showed a higher amplitude of lateral head displacement (ALH) values ($p< 0.05$); a similar result was observed for the beat-cross frequency (BCF), where the values for the SYNC and TYNC groups differed ($p< 0.05$) from the other experimental groups in the evaluation performed immediately after thawing (0 h). In the evaluation performed two hours after thawing, a difference ($p< 0.05$) in the BCF was only noted between the SMC and SYC groups, where the SYC group showed higher values. A significant difference ($p< 0.05$) in the BCF was observed between the SMC,

TYC and SYNC groups. ALH and BCF average are shown in Table 4.

Table 4. Absolute values of the parameters amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF) of goat spermatozoa analyzed at 0 and 2 hours after thawing.

	ALH (µm)		BCF (Hz)	
	0h	2h	0h	2h
SMC	2.0±0.1[b]	1.3±0.9	7.4±0.7[b]*	4.6±2.5[b]*
SYC	2.2±0.3[b]	2.2±0.3	8.4±0.3[b]	9.2±1.1[a]
TMC	2.4±0.4[b]	2.1±0.2	8.3±0.2[b]	8.6±1.9[ab]
TYC	2.4±0.4[b]	1.9±0.8	8.7±0.6[b]*	7.1±1.8[ab]*
SMNC	2.0±0.3[b]	1.9±0.4	7.4±0.4[b]	7.3±0.9[ab]
SYNC	3.6±0.6[a]	1.7±1.8	11.3±2.5[a]*	5.3±4.6[ab]*
TMNC	2.1±0.5[b]	1.9±0.7	7.0±0.9[b]	7.7±1.7[ab]
TYNC	3.1±0.6[a]	2.1±1.7	10.0±1.7[a]	7.9±6.3[ab]

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

The subjective evaluations of PMi revealed a difference ($p < 0.05$) between the extenders, and the SYNC and TYNC groups were superior to the SMC, SMNC and TMNC groups, which showed fewer numbers of viable cells in the analysis performed immediately after thawing (0 h). A difference ($p > 0.05$) in the integrity evaluated using fluorescent probes was not observed. Spermatozoa plasma membrana integrity average are shown in Table 5.

Table 5. Percentage of plasma membrane integrity of goat sperm cells cryopreserved with different extenders and analyzed at 0 and 2 hours after thawing.

	Eosin/Nigrosin (%)		Fluorescent Probes (%)	
	0 h	2 h	0 h	2 h
SMC	27.9±16.2[b]*	17.3±13.1*	37.5±18.2	36.1±16.1
SYC	43.0±18.1[ab]	32.9±15.6	46.5±10.0	45.5±15.3
TMC	43.7±9.4[ab]*	20.7±15.7*	53.4±11.6	49.6±15.5
TYC	45.5±9.1[ab]	33.5±16.2	33.6±14.2	30.9±13.3
SMNC	33.9±5.9[b]	26.6±9.9	36.6±12.3	31.1±9.8
SYNC	64.0±10.6[a]*	31.4±23.6*	48.5±9.9	27.3±23.1
TMNC	28.5±7.9[b]*	17.7±7.3*	35.8±11.2	35.5±6.8
TYNC	58.5±13.9[a]*	30.7±15.1*	43.0±8.1	28.0±23.4

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

In the evaluation of the PMi, this parameter was not altered in the evaluation performed immediately after thawing; however, two hours after thawing, lower values ($p < 0.05$) were observed for the SYNC and TYNC groups, which differed from the SYC, TYC and SMNC groups. A change in the plasma membrane function was not observed, regardless of the group tested and the evaluation time. Mitochondrial potential and plasma membrana functionality average are shown in Table 6.

Table 6. Percentage of high mitochondrial potential and plasma membrane functionality of goat sperm cells cryopreserved with different extenders and analyzed at 0 and 2 hours after thawing.

	Mitochondrial Potential (%)		Plasma membrane functionality (%)	
	0 h	2 h	0 h	2 h
SMC	39.0±18.4	29.5±24.3^{ab}	37.5±18.2	36.1±16.1
SYC	55.0±6.6	37.9±26.6^{a}	46.5±10.0	45.5±15.3
TMC	38.9±15.4	24.5±25.0^{ab}	53.4±11.6	49.6±15.5
TYC	53.8±20.4	44.4±24.3^{a}	33.6±14.2	30.9±13.3
SMNC	31.3±18.2	47.9±13.7^{a}	36.6±12.3	31.1±9.8
SYNC	49.7±28.0*	0.0±0.0^{b}*	48.5±9.9	27.3±23.1
TMNC	28.9±19.6	33.1±13.8^{ab}	35.8±11.2	35.5±6.8
TYNC	37.0±23.3*	1.1±2.8^{b}*	43.0±8.1	28.0±23.4

Data are represented as the mean ± standard desviation. Legend: SMC = centrifuged standard milk group; SYC = centrifuged standard egg yolk group; TMC = centrifuged test milk (milk+fructose) group; TYC = centrifuged test egg yolk (egg yolk+glucose) group; SMNC = non-centrifuged standard milk group, SYNC = non-centrifuged standard egg yolk group; TMNC = non-centrifuged test milk (milk+fructose) group; TYNC = non-centrifuged test egg yolk (egg yolk+glucose) group. Different lowercase letters in the same column indicate differences between groups. Asterisks in the same row indicate difference between the evaluation times. Source: Authors.

The viscosity values of the different extenders are reported as means and standard deviations in Table 7. The viscosity differed between the experimental groups; however, the SYE and TYE extenders were more viscous ($p< 0.05$) than the TME group, obtaining a higher viscosity at 10 °C. At 37 °C, the viscosities of the SYE and TYE groups were greater than the SME and TME groups.

Table 7. Viscosity values of standard and test extenders (Egg yolk and skim milk based) at 10 ºC and 37 ºC.

Extenders	10 °C (cP)	37 °C (cP)
Skim milk standard	3.19±0.40^{ab}	1.12±0.15^{c}*
Egg yolk standard	3.67±0.18^{a}*	1.60±0.11^{a}*
Skim milk Test	2.55±0.12^{c}*	1.19±0.11^{c}*
Egg Yolk Test	2.91±0.22^{b}*	1.41±0.10^{b}*

Legend: cP = centipoise unit. Source: Authors.

4. Discussion

In this experiment, the percentage of motility differed according to the extender used, evaluation time and centrifugation. The TM in all centrifuged groups and the noncentrifuged egg yolk groups remained above the standards considered acceptable for frozen semen, namely, greater than 30% (CBRA, 2013), although a noticeably greater maintenance of motility was observed in the groups where semen was centrifuged and diluted in egg yolk. Based on this observation, substrates present in the cryoprotectants acted differently on the sperm cell, particularly on the structures involved in cell motility, such as membrane integrity, flagellar structures and mitochondrial function (Celeghini et al., 2008).

A reduction in PM was observed in most experimental groups, with lower results obtained for the noncentrifuged groups, showing that the lack of centrifugation negatively affected sperm motility and velocity, particularly in the groups containing milk in the extender. These groups showed a greater interaction with the seminal plasma, leading to a decrease in the VCL, VSL and VAP of the spermatozoa in the evaluations performed immediately after thawing; however, the percentage of the velocity parameters LIN, STR and WOB showed that the milk was able to maintain these parameters for a longer period.

This observation is related to the presence of a characteristic enzyme in the semen of goats, phospholipase A, which interacts with the egg yolk and milk present in the extenders to produce substances that compromise sperm viability (Pellicer-

Rubio and Combarnous, 1998). These enzymes are postulated to interact with milk more extensively during the cryopreservation process and hydrolyze the triglycerides of the sperm plasma membrane, and skim milk produces oleic acid, a fatty acid that is toxic to the spermatozoa (Pellicer-Rubio et al., 1997), causing a substantial reduction in sperm velocity.

This effect may also be related to the high concentration of calcium present in milk, which may be responsible for increasing the activity of phospholipase A, because this enzyme requires calcium and subsequently increases the permeability of the membrane to ions (Martins, 2006). However, after stabilizing its biological activities, milk efficiently preserves spermatozoa. This adaptation may be possible because milk has a certain buffering capacity, bactericidal action and carbohydrates that would increase the kinetic activity (Menezes et al., 2016).

A different behavior was observed for the centrifuged groups. In the evaluations performed immediately after thawing, the semen cryopreserved in extenders containing egg yolk exhibited lower velocities than semen treated with the milk-based extenders, although egg yolk can maintain the viability of the velocity parameters for a longer period, even in the absence of seminal plasma.

According to Aboagla and Terada (2004), the addition of egg yolk to goat semen extenders increases the proportion of mobile sperm recovered after thawing by restoring the phospholipids that provide greater stability to the plasma membrane, improving the velocity parameters and maintaining cell viability for longer periods.

Capacitation consists of a series of changes that prepare the spermatozoa to bind to the zona pellucida of the oocyte. After this process, the sperm becomes responsive to the stimulus that induces the acrosomal reaction and develops a particular motility pattern known as hyperactivation or hypermobility (Luconi et al., 2005).

Mortimer et al. (1998) postulated that spermatozoa with a linearity < 50% are considered hyperactivated. For Robertson et al. (1988), a BCF of 8 Hz characterizes hyperactivity. The present study recorded values similar to the data presented by these authors: LIN between 45% and 51.1%, BCF between 11.3 Hz and 10.0 Hz and ALH of 3.6 and 3.1 for the SYNC and TYNC groups, respectively, in the evaluation performed immediately after thawing. These values characterize a process of early hyperactivation in the spermatozoa from these groups.

The effect of the previously mentioned phospholipases associated with the cryopreservation process triggers biological processes that may have led to sperm hyperactivation in these groups. However, in addition to phospholipases, goat semen also contains proteins analogous to BSP (bovine seminal plasma) proteins that have a direct role in sperm capacitation and may have potentiated this effect (Manjunath and Therien, 2002; Menezes et al., 2016).

The affinity of these proteins for low-density lipoprotein (LDL) of the egg yolk may have caused these proteins to interact with the extender, reducing the amount of lipids available to exert their functions in the sperm membrane (Bergeron and Manjunath, 2006) and in the removal of this steroid from the sperm membrane, suggesting a possible destabilization that increased the permeability of the membrane to Ca^{2+} and the intracellular pH (Villemure et al., 2003). Calcium activates different enzymes, including phospholipase $A_{2,}$ in addition to acting as a trigger for capacitation, the acrosome reaction, and hyperactivated sperm motility, among others (Suarez et al., 1993).

Based on these data, without centrifugation, the yolk interacts with plasma enzymes and proteins, preventing LDL from being integrated into the cell membrane, causing membrane disorganization and increased fluidity to subsequently trigger intracellular calcium influx, capacitation and a premature acrosomal reaction.

Suarez et al. (1991) provided a new perspective on this finding, namely, that hyperactivated motility represents an increase in the flagellar beat frequency in response to the increase in viscosity. Thus, the higher viscosity of the yolk extenders (Table 7) corresponds to a mechanical advantage that facilitates the passage of the sperm through the viscous fluid of the oviduct and the zona pellucida.

In the evaluation performed immediately after thawing (0 h) of the samples labeled with the combination of DCF and PI probes, a difference was not observed in the percentage of spermatozoa with an intact plasma membrane. However, the yolk-based groups contained a greater number of viable cells. This protective effect results from the binding of LDL from the egg yolk to the plasma membrane, which is associated with the osmotic profile of the sugar and the viscosity pattern of the yolk. At a temperature of 10 °C (Table 7), the yolk increases its viscosity, particularly in the presence of fructose, contributing to the stabilization of the lipid bilayer when the membrane begins to transition from the fluid phase to the gel state.

Bergeron et al. (2007) hypothesized that the yolk exerts a greater protective effect than milk because it contains lipids instead of proteins. This fact was confirmed by the changes in viscosity observed at 37 °C, where the yolk maintains a higher viscosity at body temperature because it contains a large amount of lipids, in contrast to the skim milk used in the extenders, which is depleted of fats.

The reduction in the number of viable cells observed after two hours of incubation, particularly in the yolk groups, revealed a decrease in stiffness of the plasma membrane, which is compatible with fertilization because the cell must decouple and decrease the membrane integrity to perform the acrosomal reaction.

ATP production was positively correlated with membrane integrity and motility. The mitochondrial potential of the yolk-based extenders was higher at the first evaluation (Table 6); however, a substantial decrease was observed after two hours of incubation in the noncentrifuged groups. At the moment when a decrease in the integrity was detected, the mitochondrial potential and motility were also reduced, as the latter depends on energy production. In this context, spermatozoa from the SYNC and TYNC groups were rapidly hyperactivated by depleting their mitochondrial potential and/or the negative interaction of the plasma with the yolk may have hindered the mechanism of energy consumption, regardless of the type of sugar used.

According to Celeghini et al. (2008), the reduction in these parameters during the incubation may also be related to the gradual decrease in the ability of spermatozoa to produce ATP through mitochondrial respiration as a consequence of the toxic effects of reactive oxygen species (ROS), which compromise the mitochondrial membrane potential.

5. Conclusion

Although skim milk is the basic component of the extenders recommended for routine use in the preservation of goat semen, the egg yolk based extender and the removal of seminal plasma better preserve the goat semen, as evidenced by the changes induced by cryopreservation, maintaining the characteristics desired for fertility after thawing. Milk-based extenders maintain the sperm cell velocity parameters for a longer period, although they do not preserve motility. Although these results are positive to sperm goat cryopreservation, researchs are needed to replace substitute for egg yolk-based extenders to possible the international germplasm trade.

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