



# Can centrifugation force compromise the plasmatic membrane, acrosome and DNA integrity of goat spermatozoa?

*A força de centrifugação pode comprometer a integridade de membrana plasmática, acrossomal e DNA de espermatozoides caprinos?*

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## Abstract

Protocols for cooling or freezing goat semen usually recommend centrifugation for seminal plasma removal. However, little is known about the effect of this process on goat sperm viability and functionality. The present study evaluated the effects of centrifugation force on the plasma membrane, acrosomes, and DNA integrity of goat semen. Four ejaculates from each of the four different Anglo Nubian male goats were used. Semen samples were obtained using artificial vagina, and immediately after collection, ejaculates were diluted using Ringer's sodium lactate solution and split into three groups: Control (CG, without centrifugation), G1 (centrifugation 600 x g/10 min), G2 (centrifugation 1200 x g/10 min). After centrifugation, seminal plasma was removed, the sperm pellets were resuspended using Tris-egg yolk extender (80 x 10<sup>6</sup> spermatozoa/mL) and the sperm morphology was analyzed. Samples were cooled at 5°C for 5, 24, 36, and 48 h and then sperm plasma membrane and acrosome integrity (PMAI, %) and sperm DNA fragmentation index (SDF, %) were evaluated at each time-point, using a flow cytometer. Additionally, sperm movement was determined using computer semen analysis (CASA) after 5, 24, and 48 h of refrigeration period. The semen centrifugation did not induce additional sperm morphology defect or reduction in sperm kinetics in the experimental groups. Differences were not observed (p > 0.05) in PMAI and SDF among different groups, in any of each time-point of the cooling process. In conclusion, centrifugation, even at high speeds, did not affect goat sperm integrity and functionality when submitted to refrigeration process.

**Keywords:** Centrifugation. Goat. Integrity. Semen. Viability.

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## Resumo

A maior parte dos protocolos de refrigeração e criopreservação do sêmen caprino recomenda o uso de centrifugação para remoção do plasma seminal. No entanto, não existe consenso sobre o risco que esse tipo de processamento pode ocasionar à viabilidade espermática. Nesse contexto, o presente trabalho investigou os possíveis efeitos deletérios da centrifugação sobre a integridade estrutural e DNA de espermatozoides caprinos. Para a pesquisa foram selecionados quatro reprodutores para colheita de sêmen (n = 4 ejaculados/bode). Cada ejaculado foi fracionado em três alíquotas iguais, diluídas em ringer e divididas em três grupos: Controle (GC, não centrifugado), G1 (centrifugação a 600 g/10 minutos) e G2 (centrifugação a 1200 g/10 minutos). As amostras seminais por grupo foram diluídas em meio Tris gema respeitando-se a concentração final de 80 milhões de espermatozoides/mL e foram submetidas à avaliação de morfologia espermática. Todas as amostras foram acondicionadas a 5°C, sendo analisadas nos momentos 5, 24, 36 e 48 horas do processo de refrigeração por meio da avaliação da integridade de membrana plasmática e acrossomal (MPAI, %) e índice de fragmentação de DNA (IDF, %). Adicionalmente, a cinética espermática foi avaliada com o emprego de um sistema computadorizado de análise (CASA) nos momentos 5, 24 e 48 horas da refrigeração. A centrifugação não induziu a manifestação de defeitos morfológicos ou redução significativa da cinética de

espermatozoides caprinos. Não foram observadas diferenças para a integridade de membrana plasmática e para o índice de fragmentação de DNA quando comparados, respectivamente, GC, G1 e G2 em cada um dos quatro momentos experimentais. Conclui-se que mesmo quando empregadas altas forças de rotação não ocorre lesão à ultraestrutura dos espermatozoides caprinos submetidos ao processo de refrigeração.

**Palavras-chave:** Caprino. Centrifugação. Integridade. Sêmen. Viabilidade.

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## Introduction

Cooled or frozen goat semen can be used for artificial insemination (BISPO et al., 2011), but specific extenders should be used to protect sperm cells during the biotechnology processes. However, some peculiarities related to the enzymatic composition of goat semen can lead to deleterious interactions between spermatozoa and extenders components.

Previous studies (PURDY, 2006; SARIÖZKAN et al., 2010; JIMÉNEZ-RABADÁN et al., 2012) reported that bulbourethral glands of male goats secrete large amounts of enzymes, such as phospholipase A, which constitutes the seminal plasma of these animals (SANTIAGO-MORENO et al., 2017). The detergent action of these enzymes is observed on cell membrane lipids and lipids from egg yolk, which is a common component of semen extenders (BEZERRA, 2010). This enzymatic action produces toxic substances that induce cellular death (PURDY, 2006). Thus, centrifugation demonstrated a beneficial effect for plasma removal, prior to dilution, on goat sperm viability and integrity (ISLAM et al., 2006; PURDY, 2006; CASTELO et al., 2008; SANTIAGO-MORENO et al., 2017).

Usually, centrifugation is recommended for goat semen at speeds ranging from 500 to 1000 x g for 10 to 15 min, before dilution (NUNES et al., 1982;

PURDY, 2006; RITAR; SALAMON, 1982). However, centrifugation can damage goat sperm structure, decreasing sperm motility and plasma membrane integrity (AZERÊDO et al., 2001). These deleterious effects of centrifugation were also reported for equine sperm, with more damage occurring when higher-speed rotations (> 600 xg) are used (WAITE et al., 2008). Using high rotational forces for bull semen submitted to Percoll density gradient centrifugation, Guimarães et al. (2014) observed an increase in reactive oxygen species (ROS) generation and decrease in sperm viability and motility. Additionally, in the same study, low centrifugation speeds were associated with an increase in sperm penetration and *in vitro* fertility rates.

While several studies have reported that centrifugation can cause irreversible damage to sperm cells, there are few reports about the effect of this process on sperm DNA ultrastructure. DNA fragmentation may alter embryo development, inducing apoptosis and cell fragmentation (TWIGG et al., 1999). Therefore, evaluation of sperm DNA is essential to understand male sub- or infertility (BELETTI; MELLO, 1996; RUI et al., 2017).

Centrifugation is an important biotechnological process that is commonly used for goat semen processing. Therefore, the aim of this study was to investigate the effect of centrifugal force on sperm DNA integrity and plasma membrane and acrosome viability by flow cytometry.

## Materials and Methods

### *Animals*

Four Anglo Nubian goats from one private farm in São Paulo, SP, Brazil (Latitude 23°43'55"S and

longitude 46°42'28"W), with a mean age of 30 months, were used. These animals were chosen based on clinical and reproductive history and andrological evaluation based on the Brazilian Breeding Soundness Evaluation Manual (CBRA, 2013): at least 28 cm of scrotal circumference; total sperm concentration  $> 2 \times 10^9$  cells; total sperm motility  $\geq 70\%$ ; major morphological defects  $< 20\%$  and total proportion of morphologically abnormal cells  $< 20\%$ . This study was approved by the Ethics Committee on Animal Use of Santo Amaro University (CEUA-UNISA, protocol n. 6/2015).

### ***Semen Collection and Experimental Design***

Four ejaculates from each of the four goats were used in this study. Semen samples were obtained using artificial vagina, during the Southern Hemisphere breeding season (April to June). Immediately after collection, the ejaculates were evaluated for fulfilment of the experimental criteria, and then each ejaculate sample was split into three aliquots to be divided into the following groups: 1) Control group (CG) – semen was not centrifuged, but diluted (1:9) using Tris-egg yolk extender (TRIS) without cryoprotectants; 2) G1 – semen was centrifuged (Fanem Baby II®, Fanem Ltda, Guarulhos, São Paulo, Brazil) at  $600 \times g$  for 10 min; 3) G2 – centrifuged at  $1200 \times g$  for 10 min. For G1 and G2, the semen sample was diluted (1:9) using prewarmed (37°C) Ringer's sodium lactate solution as per the protocol used by Jiménez-Rabadán et al. (2012), before centrifugation.

After centrifugation, the supernatants were discarded, and the sperm pellets resuspended with TRIS without cryoprotectants (ISLAM et al., 2006), to a final sperm concentration of  $80 \times 10^6$  sperm/mL. Thereafter, samples were placed in 1.5 ml disposable cryotubes, transferred to Botutainer® (Botupharma, Botucatu, SP, Brazil), a passive cooling and semen storage container, and maintained at a rate of  $-0.13^\circ\text{C}/\text{min}$  until the stabilization temperature of  $5^\circ\text{C}$  was reached, according to the refrigeration curve

described by Crespilho et al. (2014). Samples of the three experimental groups were evaluated during 48 h of cooling process by the same analyzer.

### ***Morphology and kinetics analysis***

The proportion of morphological normal spermatozoa was evaluated immediately before the cooling process and examined under phase-contrast microscopy at 1000x of magnification. The sperm defects were classified in major (*MD*), minor (*md*) and total (*TD*) defects, according to Blom (1973).

Additionally, samples were analyzed at 5, 24 and 48 h following the refrigeration process for total (TM, %) and progressive motility (PM, %) using the computer system ISAS® V.1.2. (Proiser®, Valencia, Spain). Before the kinetic evaluation, each sperm sample was warmed in a dry bath at  $37^\circ\text{C}$  for 10 min. Computer assisted semen analysis (CASA) was performed depositing 5  $\mu\text{L}$  aliquots of semen in a SpermTrack® chamber (Proiser®, Valencia, Spain) with a height of 20  $\mu\text{m}$ , pre-warmed to  $37^\circ\text{C}$ . The software settings were those recommended by the manufacturer for analysis of goat semen motility, namely: frames per second: 30 Hz; number of frames: 30; minimum contrast: 50; minimum resolution of cell size: 4 pixels; slow-static cells with average path velocity (VAP) cut-off: 10  $\mu\text{m}/\text{s}$ ; VAP cut-off: 50  $\mu\text{m}/\text{s}$ ; straightness (VSL) threshold: 70%; Connectivity: 12; Temperature:  $37^\circ\text{C}$ .

### ***Flow cytometry evaluation***

Flow cytometric acquisition was performed using a BD LSR® Fortessa (Becton Dickinson, Mountain View, CA, USA) equipped with three laser excitation sources (violet: 405 nm 50 mw; blue: 488 nm 50 mw; red: 640 nm 40 mw). The filter configurations for PMTs measuring fluorescence emission of the applied fluorochromes used for the experiment were 530/30 nm (FITC); 694/50 nm (PI); 660/20 nm (APC); and 450/50nm (Hoescht). Auto-fluorescence and single-color controls were acquired to perform spectral overlap compensation using the automated

compensation matrix feature in FACS DiVA® software. Flow cytometry data was plotted using bi-exponential plots that include axes < 0t to assure all data was visible and properly compensated. At least 10,000 cells per sample were analyzed.

Sperm DNA fragmentation index (SDF, %) was assessed at 5, 24, 36, and 48 h after cooling, using orange acridine (A6014, Sigma Aldrich, USA) technique. Briefly, 100 µL of the semen from each group was diluted in a buffer solution (0.186 g of disodium EDTA, 0.790 g of Tris-HCl, 4.380 g NaCl in 500 mL of deionized water, pH 7.4). The final sperm concentration was fixed at 1 x 10<sup>6</sup> sperm/mL. Each

sample was incubated with 400 µL of acid detergent solution (2.19 g NaCl, HCl 1.0 mL of 2N solution, 0.25 mL Triton-X, in q.s.p. 250 ml of deionized water, pH 1,8) for 30 min at room temperature (24°C). After incubation, 1 mL of orange acridine dye solution (AO; 3.8869 g citric acid monohydrate, 8.9429 g Na<sub>2</sub>HPO<sub>4</sub>, 4.3850 g NaCl, 0.1700 g disodium EDTA, 4 µg/ml acridine orange solution, diluted to 1 mg/mL in 500 ml of deionized water, pH 6.0) was mixed with the samples, which were then finally analyzed by flow cytometry. The generated data (Figure 1) were analyzed using WinList 6.0 (Verity Software House).

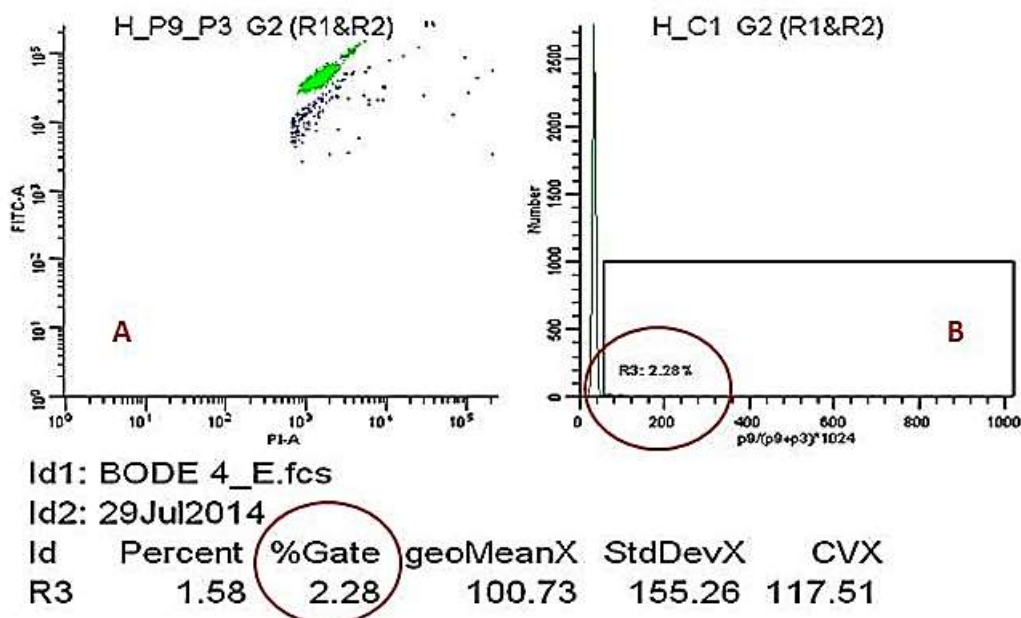


Figure 1 – Histogram example performed during flow cytometry analysis of DNA fragmentation index using orange acridine dye solution (AL). A – Cellular content differentiation in samples (green – cell DNA; blue – cellular debris and particles). B – Determination of sperm DNA fragmentation based on analyses of 10.000 sperm cells stained with AL (2.28%)

The determination of the DNA fragmentation index was based on an analysis of 10,000 cells stained with acridine orange. The DNA fragmentation index, which is expressed as a percentage of sperm DNA, corresponds to the amount of fragmented DNA (red fluorescence) divided by total fluorescence, i.e., the sum of fragmented and non-fragmented DNA (red and green fluorescence, respectively; Figure 1).

Proportions of sperm with intact plasma membrane and acrosomes (PMAI, %) were evaluated at 5, 24, 36, and 48 h after cooling using the association of the fluorescent probes propidium iodide (IP, P4170, Sigma Aldrich, St. Louis, MO, USA), FITC-PSA (L0770, Sigma Aldrich, St. Louis, MO, USA), and Hoechst 33342 (14533, Sigma Aldrich, St. Louis, MO, USA), according to the

method by Freitas-Dell'Aqua et al. (2012). A total of 200  $\mu\text{L}$  of semen was used to obtain a final semen sample concentration of  $5 \times 10^6/\text{mL}$  with TALP-PVA; the samples were then mixed with 5  $\mu\text{L}$  of Hoechst (100  $\mu\text{L}/\text{mL}$ ), 5  $\mu\text{L}$  of IP (50  $\mu\text{g}/\text{mL}$ ), and 1  $\mu\text{L}$  of FITC-

PSA (100  $\mu\text{g}/\text{mL}$ ). The mixtures were incubated at 37°C for 5 min and immediately evaluated by flow cytometry. Only cells with intact plasma membrane and acrosome (MPAI, %; Figure 2) were considered for the statistical analyses.

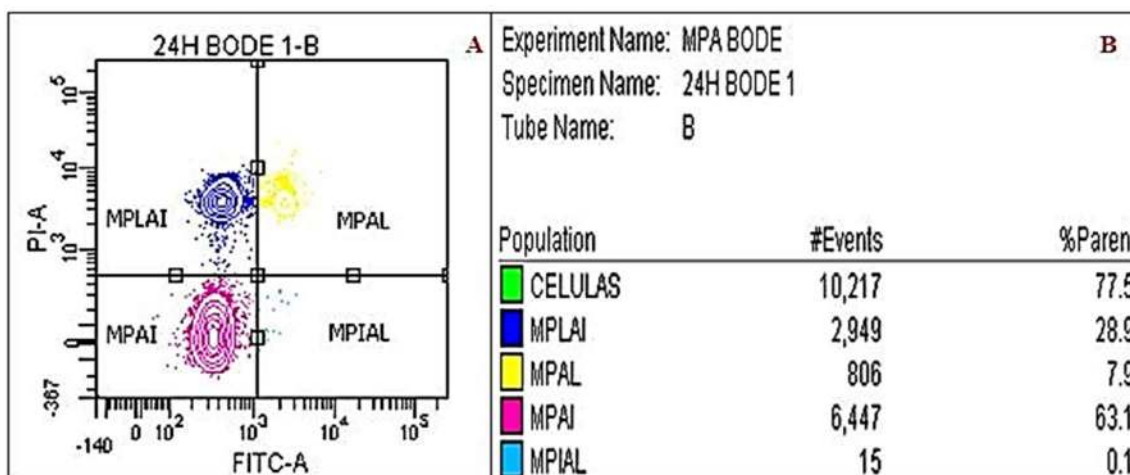


Figure 2 – Histogram example performed during plasma membrane and acrosomes analysis by flow cytometry (n = 10.217 sperm tested in this analysis). Identification (A) and quantification (B). MPLAI – damaged plasma membrane and intact acrosome; MPAL – damaged plasma membrane and acrosome; MPAI – intact plasma membrane and acrosome; MPIAL – intact plasma membrane and damaged acrosome

### Statistical Analysis

Data were evaluated using the program Statistical Analysis System (SAS Institute Inc., 1999; Cary, NC, USA). Primarily, the Shapiro-Wilk test (Proc-Univariate) was used to evaluate the data normality, and the Chi-Square test (Proc-GLM) was used to test the homogeneity of variances. Means of the experimental groups, as well as their relation to semen incubation times, were evaluated using analysis of variance (Proc-GLM). Additionally, the interaction of centrifugation methodologies and different incubation times at 5°C under acrosome and DNA integrity was analyzed using least squares means (LS-Means SAS) for linear models. Significance was defined as  $P \leq 0.05$ .

### Results

Samples of the control group did not differ in *MD* ( $P = 0.9121$ ) or *TD* ( $P = 0.1318$ ) in relation to centrifuged

treatments, independent of the centrifugation force (Table 1). However, the proportion of minor defects (represented especially by normal detached heads and distal cytoplasmic droplets) were  $7.35^a \pm 3.37$ ,  $5.00^b \pm 1.60$  and  $4.38^b \pm 1.30$ , respectively for Control, G2 and G3 ( $P = 0.0115$ ).

Table 1 – Mean values ( $\pm$ SD) for major (*MD*), minor (*md*) and total (*TD*) goat sperm morphological defects according to each experimental group: GC, Control Group; G1, centrifugation (600  $\times\text{g}/10$  minutes); G2, centrifugation (1200  $\times\text{g}/10$  minutes)

Groups	<i>MD</i> (%)	<i>md</i> (%)	<i>TD</i> (%)
CG	$3.75 \pm 2.66^a$	$7.75 \pm 3.37^a$	$11.50 \pm 5.56^a$
G1	$4.13 \pm 2.23^a$	$5.00 \pm 1.60^b$	$9.13 \pm 3.09^a$
G2	$3.88 \pm 2.42^a$	$4.38 \pm 1.30^b$	$8.25 \pm 2.92^a$

Columns with different superscript (<sup>a</sup> <sup>b</sup>) represent statistically significant differences ( $P < 0.05$ )

Table 2 – Mean values ( $\pm$ SD) for total (TM) and progressive motility (PM) of goat sperm in each experimental group (Control Group: CG; G1: centrifugation for 10 minutes at 600  $xg$  (G1) and 1200  $xg$  (G2), over the refrigeration time.

Sperm Parameters	Treatments	5 hrs	24 hrs	48 hrs
TM (%)	CG	87.63 $\pm$ 9.76 <sup>aA</sup>	86.63 $\pm$ 6.95 <sup>aA</sup>	85.88 $\pm$ 8.15 <sup>aA</sup>
	G1	83.88 $\pm$ 6.58 <sup>aA</sup>	81.75 $\pm$ 10.42 <sup>aA</sup>	84.38 $\pm$ 5.24 <sup>aA</sup>
	G2	85.00 $\pm$ 7.39 <sup>aA</sup>	83.25 $\pm$ 9.71 <sup>aA</sup>	81.00 $\pm$ 10.39 <sup>aA</sup>
PM (%)	CG	32.63 $\pm$ 8.09 <sup>aA</sup>	32.88 $\pm$ 6.75 <sup>aA</sup>	25.88 $\pm$ 10.01 <sup>aA</sup>
	G1	32.00 $\pm$ 10.41 <sup>aA</sup>	28.50 $\pm$ 10.76 <sup>aA</sup>	23.38 $\pm$ 11.76 <sup>aA</sup>
	G2	27.25 $\pm$ 7.11 <sup>aA</sup>	27.88 $\pm$ 9.42 <sup>aA</sup>	26.63 $\pm$ 12.59 <sup>aA</sup>

Columns with different superscript (<sup>a,b</sup>) represent statistically significant differences ( $P < 0.05$ ). Upper case letters indicate the possible differences presented within each treatment, during the 48 hours of refrigeration ( $P < 0.05$ )

The influence of centrifugation force on cooled goat semen kinetics are presented in Table 1. The percentage of total and progressive motility were very similar ( $P > 0.05$ ) among the different experimental groups during the 48 h cooling process (Table 2).

No significant differences were observed in MPAI and SDF using grouped results (i.e., when seminal plasma was or was not removed, regardless of the

experimental time among the different groups; Table 3), or when sperm integrity (Table 4) and functionality (Table 5) was evaluated among groups at each experimental time-point during the 48 h cooling process. Likewise, interactions between treatments and evaluation time-points, and treatments with goat identity, were not detected ( $P = 0.5603$  and  $P = 0.8678$ , respectively).

Table 3 – Mean values ( $\pm$ SD) for intact plasma membrane and acrosomes (MPAI) and sperm DNA fragmentation index (SDF) of goat sperm in each experimental group: Control Group (CG), G1 (600  $xg$ ) and G2 (1200  $xg$ ), regardless the experimental time among the different groups

Groups	MPAI (%)	SDF (%)
CG	78.30 $\pm$ 3.92 <sup>a</sup>	3.05 $\pm$ 0.39 <sup>a</sup>
G1	80.81 $\pm$ 1.46 <sup>a</sup>	3.76 $\pm$ 0.34 <sup>a</sup>
G2	77.47 $\pm$ 3.99 <sup>a</sup>	3.60 $\pm$ 0.32 <sup>a</sup>

Columns with different superscript (<sup>a,b</sup>) represent statistically significant differences ( $P < 0.05$ )

Table 4 – Mean values ( $\pm$ SD) for intact plasma membrane and acrosomes (MPAI, %) of goat sperm evaluated among groups in each experimental moment during the 48 hours of cooling process

MPAI	5 hrs	24 hrs	36 hrs	48 hrs
CG	82.17 $\pm$ 36.65 <sup>aA</sup>	80.63 $\pm$ 36.93 <sup>aA</sup>	76.98 $\pm$ 36.34 <sup>aA</sup>	73.40 $\pm$ 38.27 <sup>aA</sup>
G1	81.12 $\pm$ 36.89 <sup>aA</sup>	81.71 $\pm$ 37.78 <sup>aA</sup>	78.65 $\pm$ 38.29 <sup>aA</sup>	81.74 $\pm$ 37.13 <sup>aA</sup>
G2	82.02 $\pm$ 35.52 <sup>aA</sup>	76.63 $\pm$ 39.71 <sup>aA</sup>	78.97 $\pm$ 37.32 <sup>aA</sup>	76.27 $\pm$ 35.98 <sup>aA</sup>

Columns with different superscript (<sup>a,b</sup>) represent statistically significant differences ( $P < 0.05$ ). Upper case letters indicate the possible differences presented within each treatment. during the 48 h of refrigeration ( $P < 0.05$ ). Values are expressed as mean  $\pm$  standard deviation. GC. Control Group; G1. centrifugation (600  $xg/10$  min); G2. centrifugation (1200  $xg/10$  min)

## Discussion

Goat semen differs from that of other domestic species in the enzymatic composition of the seminal

plasma, which causes deleterious interactions between extenders and spermatozoa (MAIA, 2014). Enzymatic synthesis in seminal

plasma is performed by the bulbourethral glands, with EYCE (egg yolk coagulant enzyme) or phospholipase

A being the main secreted molecule (BISPO et al., 2011; SANTIAGO-MORENO et al., 2017).

Table 5 – Mean values ( $\pm$ SD) for sperm DNA fragmentation index (SDF, %) of goat sperm evaluated among groups in each experimental moment during the 48 h of cooling process

SDF	5 hrs	24 hrs	36 hrs	48 hrs
CG	2.53 $\pm$ 2.39 <sup>aA</sup>	3.51 $\pm$ 2.91 <sup>aA</sup>	3.32 $\pm$ 2.69 <sup>aA</sup>	2.84 $\pm$ 1.94 <sup>aA</sup>
G1	3.25 $\pm$ 2.74 <sup>aA</sup>	3.85 $\pm$ 3.25 <sup>aA</sup>	4.21 $\pm$ 3.93 <sup>aA</sup>	3.71 $\pm$ 3.51 <sup>aA</sup>
G2	3.24 $\pm$ 3.08 <sup>aA</sup>	4.13 $\pm$ 3.89 <sup>aA</sup>	3.58 $\pm$ 3.38 <sup>aA</sup>	3.50 $\pm$ 2.85 <sup>aA</sup>

Columns with different superscript (<sup>a,b</sup>) represent statistically significant differences ( $P < 0.05$ ). Upper case letters indicate the possible differences presented within each treatment. During the 48 h of refrigeration ( $P < 0.05$ ). Values are expressed as mean  $\pm$  standard deviation. GC. Control Group; G1. Centrifugation (600  $xg/10$  min); G2. centrifugation (1200  $xg/10$  min)

Phospholipase A is an enzyme that hydrolyzes the lecithin and phospholipids present in egg yolk and in the sperm plasma membrane, transforming these substances into fatty acids and lysolecithins that are toxic to sperm cells by reacting with the acrosomes and fragmenting the chromatin (CASTELO et al., 2008; BEZERRA, 2010). Therefore, several previous studies have indicated the separation of seminal plasma as a strategy to ensure greater sperm quality during goat semen processing (ISLAM et al., 2006; PURDY, 2006; SALVADOR et al., 2006; MAIA, 2014).

The effect of centrifugal force on the kinetic patterns of goat sperm has already been reported by some authors (CAMPOS et al., 2004; ROOF et al., 2012; SANTIAGO-MORENO et al., 2017); however, limited research has objectively evaluated the deleterious effects of centrifugation on sperm integrity and viability, especially using highly sensitive techniques like flow cytometer. Thus, although previous studies have indicated a beneficial effect on sperm kinetics when seminal plasma is removed, no differences were observed ( $P > 0.05$ ) in the present work when MPAI (Table 3) and SDF (Table 4) were evaluated among groups at each experimental time-point during the 48 h of cooling. Furthermore, other research studies have reported similar results, indicating that centrifugation may not

represent an indispensable process to guarantee greater preservation of goat sperm cells subjected to cooling (BUCAK et al., 2009) or cryopreservation (ROOF et al., 2012).

Medrano et al. (2010) described an important interaction effect of male and seasons on cryopreserved goat semen, suggesting that seminal plasma removal may not be necessary for all goats, and justifying our results. In addition, Aguiar et al. (2013) reported lower phospholipase production in seminal plasma of crossbred goats during breeding season, the same season during which our study was performed. Regarding to the season of ejaculates were collected, previous studies reported better results in post-thawed semen for some kinetics and structural parameters when semen were obtained during the breeding season, suggesting that seminal plasma proteins may influence seasonal resistance of sperm damage (JIMÉNEZ-RABADÁN et al., 2012). This may explain no deleterious effect observed in cooled semen submitted to high centrifugation forces before cooling process.

Usually, centrifugation protocols for goat semen involve rotation forces between 550 and 950  $xg$  over 10 to 15 min, in one or two steps (PURDY, 2006; RITAR; SALAMON, 1982). However, even after using high centrifugation speeds, no deleterious effects were observed on the structural integrity of goat

spermatozoa in our study. Similar results were reported by Jiménez-Rabadán et al. (2012), Cunha and Lopes (2009), and Resende et al. (2015), in goat, canine, and ram semen, respectively.

The structural resistance of goat spermatozoa to centrifugation can be explained by the large amount of membrane lipids present in goat sperm cells, which contain phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, as the most prevalent structural phospholipids (RANA et al., 1991). The cholesterol/phospholipids proportion in these membrane cells is also a determinant, not only of plasma membrane fluidity to maintain two-dimensional lamellar arrangement, but also of cell structural integrity and stability, as the temperature drops during the cooling or freezing process (MOCÉ et al., 2010).

This structural stability presented by goat spermatozoa may justify the similarity on total and progressive motility observed in the 3 experimental groups over the 48 h cooling period. For total motility, reduction of less than 5% were observed between the first evaluation (immediately after stabilization at 5°C) and analysis after 48 h of the refrigeration period, which illustrates the great resistance of goat semen to cooled temperatures. Similar conclusions were reported by previous studies involving cooled (VIANA et al., 2006) and frozen-thawed goat semen (BATISTA et al., 2011; JIMÉNEZ-RABADÁN et al., 2012), demonstrating that the sperm motility was not significantly modified after 48 h of storing at 5°C, even when submitted to centrifugation.

In the present study, the SDF was also evaluated. Gutiérrez-Cepeda et al. (2012) reported the influence of the centrifugation protocol on SDF results of cryopreserved equine semen. SDF may occur as a consequence of sperm chromatin compaction, cell apoptosis, and oxidative stress. It is due to imbalance between the antioxidant agents and reactive oxygen species in seminal plasma, with temperature changes being primarily responsible (HAMILTON, 2014). Severe temperature changes are responsible for

increased SDF and also decreased sperm cell viability (LÓPEZ-FERNANDEZ et al., 2008). In the present study, goat semen samples were not frozen, justifying similar results among MPAI and SDF, with or without the use of centrifugation to process the cooled semen. Additionally, Qiu et al. (2016) observed an increase in ROS only when goat semen was cooled for more than three days, justifying our findings of low cellular stress in buck semen cooled for a shorter period at 5°C.

There were reports that centrifugation process may cause prominent enhance in abnormal goat sperms count (ŞEN et al., 2015). However, in the present study no effect of the different centrifugal forces was observed on the frequency of major and total sperm defects. These results demonstrate that even when high rotational forces are used (1200  $\times$  g), no significant morphological alterations were observed in structural scale (expressed by *DM* and *DT* percentage) or microstructural scale (IPMA and DNA fragmentation) of sperm.

An interesting finding in the present study was the decrease in the percentage of minor sperm defects in G2 and G3 (Table 1) samples. Among the most prevalent *md* in the ejaculates obtained were the distal cytoplasmic drops (about 40% of the minor defects observed), structures identified as a regularly shaped remnants of cytoplasm under the sperm tail (CARREIRA et al., 2012). In the present study it was observed that the centrifugation may reduce the proportion of sperm with distal cytoplasmic droplets, making significative influence in the minor defects count. Kato et al. (1983) observed that when goat sperm were centrifuged for 10 min at 700 or 1600  $\times$  g, the percentage of proximal droplets markedly decreased, and some droplets moved further and reached the distal part of the principal piece. In this sense, is possible to conclude that the centrifugation in G2 and G3 was responsible for the elimination of distal cytoplasmic droplets, justifying a decrease observed on minor defects.

In conclusion, although previous studies have associated centrifugation with damage to sperm



structure in several species, we observed that even with high centrifugal forces, no significant morphological and structural damages were caused to goat semen cooled at 5°C during 48 h.

## Reference

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