http://dx.doi.org/10.1590/1678-4162-11354

Arq. Bras. Med. Vet. Zootec., v.72, n.2, p.295-304, 2020

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Sorting and cryopreservation of goat sperm with or without phenolic compounds

[*Seleção e criopreservação de espermatozoides caprinos com ou sem compostos fenólicos*]

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ABSTRACT

The objectives of this study were to evaluate goat sperm sorting in continuous Percoll® density gradients and gamete freezability, in the presence or absence of phenolic antioxidants. For this, semen *pools* were sorted, frozen, and evaluated. The non-selected group (NSg) presented lower progressive motility (PM), linearity (LIN), straightness (STR), and wobble (WOB) than the selected groups, and straight line velocity (VSL) compared to those with catechin or resveratrol. The amplitude of lateral head displacement (ALH) was higher in NSg, and quercetin reduced the mitochondrial membrane potential (MMP). After thawing, the NSg presented lower PM than the selected groups, VSL and VAP (average path velocity) than the selected group with or without catechin, LIN and WOB than the selected with or without catechin or resveratrol, and STR than the selected with catechin. Moreover, NSg presented higher ALH and BCF than the samples selected with or without catechin. Plasma membrane integrity and intact and living cells were higher in the selected groups, and MMP was lower in the NSg and the selected group with quercetin. Thus, centrifugation in Percoll® continuous density gradients is a viable methodology to select goat sperm compatible with the freezing, especially in the presence of catechin or resveratrol.

Keywords: Flavonoids, oxidative stress, semen freezing, sperm selection, stilbenes

RESUMO

Objetivou-se avaliar a separação de espermatozoides caprinos em gradientes de densidade contínuos de Percoll® e a congelabilidade espermática, com ou sem antioxidantes fenólicos. Para tal, pools seminais foram selecionados, congelados e avaliados. O grupo não selecionado (gNS) apresentou menor motilidade progressiva (MP), linearidade (LIN), retilinearidade (STR) e oscilação (WOB) do que os selecionados, bem como menor velocidade linear progressiva (VSL) do que os com catequina ou resveratrol. A amplitude de deslocamento lateral de cabeça (ALH) foi maior no gNS e a quercetina reduziu o potencial de membrana mitocondrial (PMM). Após a descongelação, o gNS manifestou menor MP do que os selecionados, menor VSL e VAP (velocidade média da trajetória) do que os com ou sem catequina, menor LIN e WOB do que os com ou sem catequina ou resveratrol, e menor STR do que os com catequina, além de maior ALH e BCF do que os com ou sem catequina. A integridade da membrana plasmática e as células intactas e vivas foram maiores nas amostras selecionadas e o PMM foi inferior no gNS e no selecionado com quercetina. Portanto, a centrifugação em gradientes contínuos de densidade de Percoll® é uma metodologia viável para selecionar espermatozoides caprinos compatíveis com a congelação, especialmente na presença de catequina ou resveratrol.

Palavras-chave: flavonoides, estresse oxidativo, congelação de sêmen, seleção espermática, estilbenos

Recebido em 28 de fevereiro de 2019
Aceito em 3 de julho de 2019
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INTRODUCTION

The success of assisted reproduction techniques depends on methods that enable selection of sperm cells with high quality and fertilization capacity (Olivares *et al*., 2017). Moreover, the possibility of pre-determining the sex of progeny through sperm sexing is a long-standing desire in the livestock industry, aiming to improve productive efficiency (Promthep *et al*., 2016). In goats, the use of sexed semen, in combination with other biotechnologies for reproduction, is considered a means to increase reproductive and productive efficiency. The large difference in the DNA content of goat spermatozoa, carrying an X versus a Y chromosome (4.4%), makes the sexing technique attractive for use in this species (Parrilla *et al*., 2004).

A satisfactory result was obtained after sexing goat spermatozoa via flow cytometry, which presented acuity of separation greater than 90% (Bathgate *et al*., 2013). However, the quality and fertility of the sexed and frozen semen after artificial insemination was low (Bathgate *et al*., 2013). Thus, alternative methods are required that are simpler, cheaper (Promthep *et al*., 2016), less harmful to sperm (Evans *et al*., 2004), and allow the gametes to be frozen without reducing fertility (Hossepian de Lima *et al*., 2011).

The technique of sperm sex sorting in Percoll® density gradients has shown excellent results, enabling the separation of X and Y sperm at a lower cost and without compromising gamete viability (Horokhovatskyi *et al*., 2018), despite its lower acuity in bulls (Promthep *et al*., 2016). However, this technique can also generate injuries to sperm, with consequent decreases in resistance to freezing (Hossepian de Lima *et al*., 2015).

The main reason for reduced sperm survival during sorting (Klinc and Rath, 2007) and freezing (Bansal and Bilaspurig, 2011) seems to be the increased generation of reactive oxygen species (ROS) and subsequent oxidative damage. Thus, antioxidant therapies have been applied during sorting in flow cytometry and/or cryopreservation to minimize the damage caused by these processes on the gametes (Klinc and Rath, 2007).

In the same way, the development of strategies to improve the quality of semen sexed using density gradients and subjected to cryopreservation is also necessary. Based on the high antioxidant power of phenolic compounds (Sarlós *et al*., 2002; Simos *et al*., 2012), the aim of this study was to evaluate the effect on gamete quality of goat sperm sorting by centrifugation in continuous Percoll® density gradients and subsequent sperm freezing, with or without added (+)-catechin, (-)-epigallocatechin gallate (EGCG), quercetin, or resveratrol.

MATERIALS AND METHODS

All reagents used were purchased from Sigma-Aldrich ® (St. Louis, MO, USA), except for those described below. The study was approved by the Ethics Committee for Animal Experimentation of the Universidade Federal Rural de Pernambuco (UFRPE - Brazil), under process number CEEUA / UFRPE 014/2012. Five sexually mature and fertile male goats were used (two Saanen, two Toggenburg, and one British Alpine), aged between one and four years. The animals were raised at UFRPE, in Pernambuco, Brazil (08° 03' 14" S, 34° 52' 52" W), and fed with Tifton hay and concentrate, with water and mineral salts *ad libitum*.

The semen collection was performed three times a week, using an artificial vagina and a female as a dummy, totalizing 30 ejaculates (six per male). The fresh semen samples were subjectively evaluated for mass movement (0-5), sperm motility (0-100%), and vigor (0-5), under an optical phase-contrast microscope (Olympus, Tokyo, Japan; 100x). Only approved ejaculates (mass movement≥3, sperm motility≥70%, vigor≥3) were used to form the seminal *pools*, totaling six *pools* (n= 6).

Percoll® density gradients were performed as described by Oliveira *et al*. (2011) and Resende *et al*. (2011) for sex sorting of bovine semen, with modifications. An isotonic solution of 90% Percoll® was prepared diluting commercial Percoll® (9:1, v:v) (Percoll® TM; GE Healthcare Bio-Sciences AB, Rapsgatan, Uppsala, Sweden) in 10x concentrated DMEM solution (135g DMEM, 0.3% BSA, 10mg/L antibiotic, 6mM HEPES, 1L Milli-Q q.s.ad water). This solution was used to form the 85 and 80%, through new dilutions in 1x concentrated

DMEM (13.5g DMEM, 0.3% BSA, 10mg/L antibiotics, 6mM HEPES, 1L water Milli-Q q.s.ad).

The 90, 85, and 80% Percoll® solutions were fractionated into six aliquots and treated according to the experimental groups [S: selected control (without antioxidant); SC75: selected with 75µM catechin; SE100: selected with 100µM EGCG; SQ25: selected with 25µM quercetin; SR25: selected with 25µM resveratrol; and SR75: selected with 75µM resveratrol]. After adjustment of the pH and osmolarity to approximately 7.4 and 300mmol/kg H_2O (290-320mmol/kg H_2O), respectively, the 90, 85, and 80% Percoll® solutions presented densities corresponding to 1.121, 1.111, and 1.101g/mL.

To form the Percoll® continuous density gradient, the three Percoll® solutions were deposited in 15mL polystyrene plastic tubes, from the most to the least dense, such that each gradient was composed of three layers of 2mL each. The gradients were stored at 5°C for 24h to become continuous, and prior to use they were placed at 37°C for 3h. Simultaneously, the semen *pools* (NS: non-selected; control) were evaluated for sperm kinematics, plasma membrane integrity, mitochondrial membrane potential, acrosome integrity, morphology, and sperm concentration, as described below. Subsequently, the semen samples were fractionated into seven aliquots, which were centrifuged in Percoll® gradients, with or without antioxidants, or conventionally processed to freezing.

For sperm sorting, six aliquots of the semen *pool* were diluted in 1x DMEM (pH 6.8), with or without antioxidant, according to the selected experimental groups, to the concentration of $800x10^6$ sperm/mL. Aliquots (500µL) of diluted semen were deposited on the corresponding density gradients, which were centrifuged (2000rpm) in a horizontal rotor (Baby®I, Fanem® Ltda., SP, Brazil), for 20min, at room temperature (24°C). The supernatants were removed and the sperm pellets recovered, setting the volume retrieved at 1mL per gradient. The sorting spermatozoa were washed twice in 1x DMEM (250xg/5min) and evaluated in the same way as the fresh semen *pools*.

For freezing, aliquots of fresh semen were conventionally processed, which were considered the non-selected frozen (NSf) control group. In this case, the semen was diluted with Tris buffer (3.605g tris-hydroxymethyl aminomethane, 2.024g citric acid, 1.488g fructose, and 100mL Milli-Q water, pH 6.8; 1:9, v:v) and centrifuged twice, to remove seminal plasma. Next, the semen was diluted with skimmed milk extender (10g skim milk powder, 194mg D-(+)-glucose, 100mLMilli-Q water, 7% glycerol, pH 6.8) to a final concentration of $200x10^6$ sperm/mL. However, to select spermatozoa for freezing, these were washed in 1x DMEM (pH 6.8), and diluted to $40x10^6$ sperm/mL in skimmed milk extender, with or without antioxidant, according to the experimental group.

The diluted semen were stored in straws (0.25mL) and frozen in an automatic system (TK-3000®, TK Technology freezing Ltda, Uberaba, Brazil), using a slow curve specific for goat semen (-0.25°C/min until reaching 5°C, stabilization time of 120min at 5°C, -20°C/min down to -120°C). The straws were immerged and stored at -196°C. After one week of storage, two semen straws per experimental group were thawed (37°C/30s) and analyzed, as described below. This process was performed six times per experimental group (n= 6), in both fresh and thawed semen.

For sperm kinematic analysis, aliquots of semen were diluted in 1x DMEM to an approximate concentration of $50x10^6$ sperm/mL. To perform the analysis, an aliquot of semen (5µL) was deposited on a pre-warmed slide (37°C), covered with a cover slip, and subsequently analyzed under a phase-contrast microscope (100x; NikonTM H5505, Eclipse 50i, Tokyo, Japan). The images were captured using a video camera (Basler Vision TecnologieTM A312FC, Ahrensburg, Germany). Five non-consecutive, randomly selected microscopic fields per sample were scanned, recording at least 2000 sperm. The parameters evaluated using Sperm Class Analyzer - SCA^{TM} software v. 5.1 (Microptics, SL, Barcelona, Spain) were: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µM), and beat cross frequency (BCF, Hz).

Plasma membrane integrity assessment was determined through double staining with carboxyfluorescein diacetate and propidium iodide (Silva *et al*., 2012). A total number of 200 cells were analyzed per slide under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany, 400x), using a DBP 485-520nm excitation filter and a DBP 580-630nm emission filter. Stained sperm showing green or red fluorescence were interpreted as having intact and damaged plasma membranes, respectively.

The mitochondrial membrane potential analyses were performed using a fluorescent probe, JC-1 (Silva *et al*., 2012). On an epifluorescence microscope (Carl Zeiss, Göttingen, Germany; 400x), using the same filters cited above, a total of 200 cells were analyzed per slide. These were classified as having a high or low mitochondrial membrane potential, when the midpieces were stained, respectively, with orange or green.

Acrosome integrity was analyzed using fluorescein isothiocyanate-conjugated agglutinin (FITC-PNA) (Silva *et al*., 2012). A total of 200 spermatozoa per slide were examined using an LP 515nm emission filter and a BP 450-490nm excitation filter, under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany, 1000x). The gametes were classified as having intact acrosome when this region was fluoresced in green, or reacted acrosome, when the equatorial region of the head displayed a band of fluorescent green but the green fluorescence was absent from the entire head of the cell.

The semen samples were diluted in formalin citrate (1:400 for fresh semen, 1:10 for selected and thawed semen; v:v) and the moist chamber and Neubauer chamber methods were used to access the morphology of the gametes and sperm concentration, respectively (Oliveira *et al*., 2013). During the morphology analysis, 200 cells per slide were classified as morphologically normal or abnormal, using a phase-contrast microscope (Olympus, Tokyo, Japan, 1000x); also used to determine the sperm concentration. The rate of sperm recovery was calculated by the mathematical formula Rr=(fVxfC/iCxiV) x 100 (Rr: recovery rate, fV: volume recovered after centrifugation of the sample; fC: sperm concentration recovered after centrifugation; iV: initial volume of the sample; iC: initial sperm concentration in the sample) (Oliveira *et al*., 2011).

The oxidative stress rate was conducted using the nitroblue tetrazolium test (Saleh and Agarwal, 2002), only for the thawed semen (either selected or not). In total, 100 spermatozoa per slide were examined under a phase contrast microscope (Olympus, Tokyo, Japan, 1000x). The gametes were classified as having, or not, oxidative stress, when formazan deposits were present or absent in the head or midpiece, respectively.

The translocation of phosphatidylserine to outside the plasma membrane in live or dead cells was determined using Annexin V-Cy3 associated with CFDA (Annexin V- Cy3^{TM} kit for the apoptosis detection) (Martí *et al*., 2006), under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany; BPD 485-520nm excitation filter and DBP 580-630nm emission filter; 400x), in thawed semen. In total, 200 cells per slide were examined and classified as; CFDA+/AnV-, when the cells were stained green, indicating that they were alive and did not show phosphatidylserine translocation (intact cells); CFDA+/AnV+, when the cells where stained green and red (alive and apoptotic cells); or CFDA-/AnV+, when the cells were stained red (dead cells).

The results are expressed as mean and standard deviation (mean±SD). Prior to the statistical analysis, the percentage data were arcsine transformed. Comparisons between experimental groups were performed using one-way ANOVA, followed by a multiple comparison Tukey-Kramer test (INSTAT for Windows, version 3.01) to detect differences between groups. For all analyses, the significance level was 5%.

RESULTS

Regarding the sperm kinematics of fresh semen, the goat spermatozoa selected in Percoll® continuous density gradients showed higher ($P< 0.05$) progressive motility, LIN, STR, and WOB than non-selected spermatozoa, independent of the antioxidant use. On the other hand, VSL was higher ($P< 0.05$) in the selected groups treated with catechin (75µM) and resveratrol (75µM) than in the non-selected groups. ALH was higher ($P< 0.05$) in the non-selected than in the selected

samples, regardless of the antioxidant treatment (Table 1).

Quercetin (25µM) reduced (P< 0.05) the mitochondrial membrane potential of goat spermatozoa selected in Percoll® continuous density gradients, compared with the other groups. The recovery rate did not differ (P> 0.05) among the groups subjected to sorting, regardless of the antioxidant (Table 2).

Table 1. Kinematic parameters (mean±SD) of fresh goat semen samples, either selected in Percoll® continuous density gradients or not, with or without antioxidants

	NS	S	SC75	SE100	SQ25	SR25	SR75
TM (%)	86.3±4.5	83.9±6.2	89.2±7.8	88.8±5.1	83.8±8.2	84.4±5.5	84.6±7.2
PM (%)	41.1±4.6^{b}	63.8±11.7^{a}	73.2±9.8^{a}	66.3±5.8^{a}	67.5±7.9^{a}	66.4±5.4^{a}	67.6±7.5^{a}
VCL (µm/s)	132.8±12.5	125.2±22.6	139.3±10.8	130.6±13.9	132.8±22.0	134.1±15.7	138.5±11.8
VSL (µm/s)	87.9±14.8^{b}	110.7±24.6^{ab}	123.4±9.7^{a}	115.0±12.6^{ab}	117.7±22.5^{ab}	119.2±16.9^{ab}	122.2±11.5^{a}
VAP (µm/s)	113.3±15.9	119.3±23.6	133.5±10.8	125.4±14.2	126.8±23.1	128.7±16.5	133.2±11.3
LIN (%)	65.8±4.9^{b}	88.0±4.3^{a}	88.7±3.8^{a}	88.0±2.8^{a}	88.4±3.5^{a}	88.7±3.3^{a}	88.3±2.7^{a}
STR (%)	77.3±2.3^{b}	92.5±3.0^{a}	92.5±3.0^{a}	91.8±1.9^{a}	92.8±1.8^{a}	92.5±2.6^{a}	91.7±2.3^{a}
WOB (%)	85.1±3.9^{b}	95.1±1.8^{a}	95.8±1.5^{a}	95.9±1.4^{a}	95.3±2.2^{a}	95.9±1.13^{a}	96.2±0.6^{a}
ALH (µm)	2.8±0.2^{a}	1.8±0.2^{b}	1.8±0.2^{b}	1.8±0.4^{b}	1.8±0.2^{b}	1.8±0.1^{b}	1.8±0.1^{b}
BCF (Hz)	8.1±0.2	8.2±1.1	8.3±0.3	8.3±0.5	8.3±0.4	8.5±0.5	8.4±0.8

Different letters in the same line indicate differences between the groups (P< 0.05). NS: non-selected; S: selected; SC75: selected with 75µM catechin; SE100: selected with 100µM EGCG; SQ25: selected with 25µM quercetin; SR25: selected with 25µM resveratrol; SR75: selected with 75µM resveratrol. TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: oscillation; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

Table 2. Plasma membrane integrity, mitochondrial membrane potential, acrosome integrity, sperm morphology and sperm recovery rate (mean±SD) in fresh goat semen samples, either selected in Percoll® continuous density gradients or not, with or without antioxidants

	NS	S	SC75	SE100	SQ25	SR25	SR75
iPM (%)	79.1±6.1	83.7±5.3	76.0±12.8	80.9±9.7	84.8±4.0	85.3±6.9	82.1±4.4
hMMP (%)	86.5±10.4^{a}	93.0±5.0^{a}	91.8±4.4^{a}	82.8±5.8^{a}	0.8±2.0^{b}	88.9±4.4^{a}	80.0±4.1^{a}
iAC (%)	74.6±13.3	58.3±7.0	62.0±6.6	58.3±9.9	69.9±10.7	68.2±14.8	63.5±6.8
nMOR (%)	88.8±4.5	95.3±4.2	95.3±4.5	95.4±4.4	94.6±4.8	94.8±5.6	95.7±4.8
Rr (%)	100.0±0.0^{a}	26.3±7.8^{b}	20.2±9.0^{b}	20.3±17.0^{b}	15.2±10.0^{b}	27.3±10.5^{b}	22.6±12.8^{b}

Different letters in the same line indicate differences between the groups (P< 0.05). NS: non-selected; S: selected; SC75: selected with 75µM catechin; SE100: selected with 100µM EGCG; SQ25: selected with 25µM quercetin; SR25: selected with 25µM resveratrol; SR75: selected with 75µM resveratrol. iPM: intact plasma membrane; hMMP: high mitochondrial membrane potential; iAC: intact acrosome; nMOR: normal morphology; Rr: sperm recovery rate.

After thawing, higher PM (P< 0.05) was observed in the selected spermatozoa samples than in the non-selected samples, regardless of the antioxidant applied. VSL and VAP were higher (P< 0.05) in samples that were either selected without an antioxidant or treated with catechin (75µM) than in those non-selected. LIN and WOB were higher (P< 0.05) in selected groups without antioxidants and added catechin (75µM) or resveratrol (25 or 75µM) than in non-selected groups. The selected group with catechin (75µM) showed a higher STR (P< 0.05) than the non-selected group. On the other hand, ALH was lower (P< 0.05) in the selected groups without antioxidant and in those treated with catechin (75µM) or resveratrol (25 or 75µM) than in the non-selected groups. Likewise, the selected group without an antioxidant showed a lower BCF (P< 0.05) than the non-selected group (Table 3).

Quercetin (25µM) reduced (P< 0.05) the mitochondrial membrane potential of goat spermatozoa selected in Percoll® continuous density gradients, compared with the other groups. The recovery rate did not differ (P> 0.05) among the groups subjected to sorting, regardless of the antioxidant (Table 2).

The percentages of spermatozoa with an intact plasma membrane after thawing were higher (P< 0.05) in the selected groups without an antioxidant and those with added catechin (75µM), quercetin (25µM), and resveratrol (25 or 75µM) than in the non-selected groups. Moreover, non-selected samples and selected samples with quercetin (25µM) showed, after thawing, a lower (P< 0.05) percentage of gametes with a higher mitochondrial membrane potential than the other groups (Table 4).

Table 3. Kinematic parameters (mean±SD) after thawing goat semen samples, either selected in Percoll® continuous density gradients or not, with or without antioxidants and frozen in skimmed milk extender (7% glycerol) in the presence or absence of antioxidants

	NSc	Sc	SC75c	SE100c	SQ25c	SR25c	SR75c
TM (%)	41.6±20.3	66.1±12.8	63.6±13.0	59.7±15.8	58.9±13.5	60.7±13.5	57.9±12.7
PM (%)	17.3±11.9[b]	40.9±7.4[a]	43.1±11.9[a]	35.7±13.0[a]	35.9±10.7[a]	37.6±6.0[a]	36.6±5.6[a]
VCL (μm/s)	69.1±21.1	91.2±13.2	90.2±11.9	73.9±6.0	76.8±11.6	85.2±9.6	85.7±8.5
VSL (μm/s)	44.7±23.1[b]	75.1±16.9[a]	76.0±13.1[a]	57.3±5.5[ab]	60.5±12.4[ab]	70.1±15.6[ab]	70.3±9.7[ab]
VAP (μm/s)	54.4±24.1[b]	83.9±15.1[a]	83.3±12.9[a]	63.9±6.6[ab]	67.8±12.6[ab]	77.4±12.8[ab]	78.2±9.3[ab]
LIN (%)	62.0±14.6[b]	81.7±8.5[a]	84.0±4.7[a]	77.6±3.8[ab]	78.3±6.4[ab]	81.4±9.5[a]	81.9±4.9[a]
STR (%)	80.9±8.1[b]	89.0±5.4[ab]	91.0±2.8[a]	89.8±2.3[ab]	89.0±3.2[ab]	89.7±5.5[ab]	89.8±2.7[ab]
WOB (%)	76.1±11.4[b]	91.6±4.2[a]	92.2±2.6[a]	86.4±4.0[ab]	87.9±5.1[ab]	90.4±5.3[a]	91.2±2.9[a]
ALH (μm)	2.4±0.5[a]	1.8±0.2[b]	1.8±0.2[b]	2.0±0.2[ab]	1.9±0.2[ab]	1.8±0.3[b]	1.8±0.14[b]
BCF (Hz)	10.5±2.6[a]	7.7±0.6[b]	8.3±0.9[ab]	9.4±2.3[ab]	8.8±1.6[ab]	8.0±0.3[ab]	7.9±0.3[ab]

Different letters in the same line indicate differences between the groups (P< 0.05). NSc: non-selected and cryopreserved; Sc: selected and cryopreserved; SC75c: selected and cryopreserved with 75μM catechin; SE100c: selected and cryopreserved with 100μM EGCG; SQ25c: selected and cryopreserved with 25μM quercetin; SR25c: selected and cryopreserved with 25μM resveratrol; SR75c: selected and cryopreserved with 75μM resveratrol. TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: oscillation; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

Table 4. Plasma membrane integrity, mitochondrial membrane potential, acrosome integrity, sperm morphology, negative oxidative stress, intact, apoptotic and dead cells (mean±SD) after thawing goat semen samples, either selected in Percoll® continuous density gradients or not, with or without antioxidants, and frozen in skimmed milk extender (7% glycerol), in the presence or absence of antioxidants

	NSc	Sc	SC75c	SE100c	SQ25c	SR25c	SR75c
iPM	19.3±5.7[b]	42.0±11.5[a]	44.7±4.6[a]	35.1±8.0[ab]	40.0±9.3[a]	43.3±10.1[a]	48.1±14.8[a]
hMMP	0.0±0.0[b]	53.2±17.2[a]	52.3±10.1[a]	49.6±15.5[a]	0.3±0.6[b]	49.3±8.5[a]	39.7±12.4[a]
iAC	44.3±6.2	40.8±7.5	45.2±12.3	46.8±14.9	48.2±11.3	46.3±8.3	54.1±11.3
nMOR	95.6±3.3	92.7±5.4	93.8±4.3	91.8±5.2	92.8±2.7	90.6±3.1	91.2±6.4
-OS	73.8±7.7	81.8±8.5	77.5±9.2	82.3±7.6	83.0±5.9	83.2±8.5	80.0±4.1
CFDA+/AnV-	35.0±4.9[b]	54.3±5.1[a]	56.3±8.6[a]	55.9±9.0[a]	55.2±5.8[a]	59.3±9.3[a]	58.9±8.5[a]
CFDA+/AnV+	16.8±2.8	18.3±7.0	21.8±3.7	22.9±5.2	19.8±3.8	18.3±7.1	18.8±7.0
CFDA-/AnV+	48.3±3.9[a]	27.4±7.4[b]	25.2±5.1[b]	21.1±10.2[b]	25.2±5.8[b]	22.5±7.3[b]	23.0±7.3[b]

Different letters in the same line indicate differences between the groups (P< 0.05). NSc: non-selected and cryopreserved; Sc: selected and cryopreserved; SC75c: selected and cryopreserved with 75μM catechin; SE100c: selected and cryopreservedwith 100μM EGCG; SQ25c: selected and cryopreservedwith 25μM quercetin; SR25c: selected and cryopreservedwith 25μM resveratrol; SR75c: selected and cryopreserved with 75μM resveratrol. iPM: intact plasma membrane; hMMP: high mitochondrial membrane potential; iAC: intact acrosome; nMOR: normal morphology; -OS: negative oxidative stress; CFDA+/AnV-: intact cell; CFDA+/AnV+: apoptotic cell; CFDA-/AnV+: dead cell.

Regarding phosphatidylserine translocation, the goat semen samples that were selected and frozen, with or without antioxidants, showed higher (P< 0.05) percentages of intact cells (CFDA+/AnV-) than the non-selected frozen group. However, the number of apoptotic cells (CFDA+/AnV+) did not differ (P> 0.05) between the experimental groups after semen thawing, whereas the percentage of dead cells (CFDA-/AnV+) was higher (P< 0.05) in the non-selected frozen group than in the other groups (Table 4).

DISCUSSION

Semen centrifugation in Percoll® continuous density gradients was shown to be a viable technique to select goat spermatozoa with higher quality, especially in the presence of catechin (75μM) or resveratrol (25 or 75μM), as, after sorting, the best values of PM, LIN, STR, and WOB were obtained, parameters positively correlated with fertility rate (Matos *et al*., 2008). In opposition, ALH, which is associated with sperm hyperactivation (Cancel *et al*., 2000), was reduced. Furthermore, VSL increased only in the selected groups with catechin (75μM) or resveratrol (75μM), revealing the protective effect (although slight) of these agents, as previously reported (Sarlós *et al*., 2002).

The results regarding sperm kinematics, post-centrifugation in Percoll® gradients, corroborated with previous studies with sex sorting in other species (Oliveira *et al*., 2011; Horokhovatskyi *et al*., 2018) and demonstrated the ability of this technique to select sperm with greater competence to move. This could be associated with the absence of negative effects determined by damaged cells and the presence of debris (Oliveira *et al*., 2011; Horokhovatskyi *et al*., 2018), reflecting the best sperm kinematics in selected semen after thawing. However, STR only increased in thawed semen with the catechin (75µM) treatment, which demonstrates that this agent shows the best ability to maintain the activity of cryopreserved spermatozoa (Boonsorn *et al*., 2010), probably as it minimizes oxidative damage (Klinc and Rath, 2007).

The lack of differences observed between the non-selected and selected groups for plasma membrane integrity, mitochondrial membrane potential, acrosome integrity, and sperm morphology of fresh semen, agrees with previous reports (Lee *et al*., 2009; Oliveira *et al*., 2011). Thus, as demonstrated by Horokhovatskyi *et al*. (2018) with *Acipenserruthenus*, the sperm sex sorting in Percoll® density gradients did not compromise the gamete parameters. Moreover, regarding the quercetin group, the decrease in percentage of sperm with a high mitochondrial membrane potential can be attributed to the action of these compounds as an enzyme inhibitor (Breitbart *et al*., 1985). Therefore, as noted by Gibb *et al*. (2013) in equine semen sexed by flow cytometry, in the presence of quercetin, this antioxidant did not demonstrate beneficial effects on the described sperm parameters.

Regarding the sperm recovery rate after goat semen centrifugation in Percoll® continuous density gradients, relatively high values were observed for all the selected experimental groups, with an average overall percentage of 21.99%. A similar value (25%) was obtained by Hossepian Lima *et al*. (2000) in bovine sperm, sexed in discontinuous Percoll® density gradients. Nevertheless, in most studies, a lower recovery rate was reported, ranging from 4.12 to 5.7% (Oliveira *et al*., 2011; Resende *et al*., 2011).

The high values of sperm recovery may result from the use of fresh rather than thawed semen in the Percoll® gradients centrifugation. Cryopreserved semen present more damaged cells, which are retained in the upper layers of the reported density gradient (Oliveira *et al*., 2011), reducing the recovery rate. Moreover, the difference in the quantity of DNA between sperm carrying X and Y chromosomes is high in goats (4.4%) (Parrilla *et al*., 2004), in comparison with other species (Garner, 2001), and this may have facilitated the separation of the two populations and gamete recovery. Therefore, this result could demonstrate the compatibility of this sorting method with goat sperm, enabling the obtention of a higher number of seminal doses.

The great plasma membrane integrity, after thawing, of goat spermatozoa selected in Percoll® gradients can also be justified by the removal of damaged cells, debris, and, consequentially, its negative actions (Horokhovatskyi *et al*., 2018). As this parameter is positively correlated with fertility (Lee *et al*., 2009), these results favor the use of semen centrifugation in Percoll® continuous density gradients, without restriction to freezing gametes. Only the selected group with EGCG did not present an improvement in plasma membrane integrity, compared with the non-selected group, demonstrating that this antioxidant had no beneficial effect on goat spermatozoa subjected to sorting and freezing.

After thawing, the mitochondrial membrane potential was totally inhibited in the non-selected samples, demonstrating the adverse effect of the freezing on cellular respiration (Horokhovatskyi *et al*., 2018), as well as the ability to select spermatozoa by Percoll® density gradient (Oliveira *et al*., 2011). In the semen treated with quercetin this parameter was also committed, although the inhibition pathway may have been differentiated, as the inhibitory effect was generated before freezing. Quercetin is an enzyme inhibitor and, thereby, interferes with mitochondrial activity (Breitbart *et al*., 1985). Despite this mitochondrial inhibition, quercetin, intriguingly, did not affect the remaining sperm parameters evaluated in this study, as reported previously (Silva *et al*., 2012).

Regardless of whether the samples had been selected, the freezing process did not affect the sperm morphology or oxidative stress level. This was also noted by Matás *et al*. (2011), who suggested that the lack of reduction in oxidative stress arises from the existence of different routes to ROS generation between the selected and unselected groups. In this context, ROS generated after centrifugation in Percoll® density gradients would result from the stimulation of sperm capacitation, while in non-centrifuged semen, ROS would be derived from dead cells and residual cytoplasm (Matás *et al*., 2011).

After thawing, the higher percentage of intact cells (CFDA+/AnV-), and lower values of dead cells (CFDA-/AnV+) in selected goat semen compared to non-selected semen favor the use of Percoll® gradients for semen sorting, as it has been demonstrated that this technique is not harmful to goat gametes. However, the effect of quercetin on sperm cells was even more intriguing, because apoptosis and the loss of mitochondrial membrane potential are related events (Câmara and Guerra, 2008). This supports the idea that the mitochondrial inhibition caused by quercetin is different, and should be studied further.

It is known that the success of assisted reproduction techniques depends on methods that select sperm cells with high quality and fertilization capacity (Olivares *et al*., 2017), and that sperm sexing is a long-standing desire of the livestock industry (Promthep *et al*., 2016). Thus, the results described in this study indicated that the sperm centrifugation in Percoll® continuous density gradients is an interesting technique for use in goats, as it was not harmful to the gametes and was compatible with the semen cryopreservation process, which are market requirements (Hossepian de Lima *et al*., 2011). Moreover, because the gradient used is continuous, it can be stored, facilitating its use commercially (Resende *et al*., 2011). Despite this, it is necessary to determine if the methodology is efficient to separate goat X and Y sperm populations, which is a determining factor for its application in sperm sexing (Hossepian de Lima *et al*., 2011).

Regarding antioxidant therapies, only catechin and resveratrol showed a slight beneficial effect, which was exclusively observed compared with the non-selected group. It is noteworthy that, despite their demonstrated antioxidant strength (Simos *et al*., 2012), phenolic compounds act differently according to the cell type (Bianch and Antunes, 1999). Moreover, these agents (especially catechins) become unstable with increases in pH (pH 4 to 8) (Chobot *et al*., 2009) and form stable complexes with proteins such as those present in milk (Parrilla *et al*., 2004), which compromises their activity.

Therefore, the experimental conditions applied in this study may have affected the phenolic compound activity, since the pH ranged from 6.8 to 7.4 and the freezing extender had a skimmed milk base. It is possible that in other conditions, the antioxidants tested, and particularly catechin, would show a protective effect on the sperm, representing a stimulating subject for new studies. Hence, it would be interesting to use these polyphenols in semen samples submitted to sexing by flow cytometry, as in centrifugation in Percoll® density gradients, the pH is determinant to the technique success.

CONCLUSION

Semen centrifugation in Percoll® continuous density gradients is a viable methodology to select goat sperm with higher quantity and quality, especially in the presence of catechin or resveratrol, demonstrating compatibility with the freezing process. However, quercetin is a powerful inhibitor of the mitochondrial potential, without manifestations of other cellular alterations.

ACKNOWLEDGEMENTS

The authors are grateful to FACEPE, CNPq, CAPES, and RENORBIO.

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