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(+)-Catechin and (-)-epigallocatechin gallate: are these promising antioxidant therapies for frozen goat semen?

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[*(+)-Catequina e (-)-epigalocatequina galato: essas são promissoras terapias antioxidantes para a congelação de sêmen caprino?*]

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ABSTRACT

The aim of this study was to evaluate the effects of different concentrations of (+)-catechin or (-)-epigallocatechin gallate (EGCG) on goat semen freezability. *Pools*of semen were processed (Experiment 1: 0, 15, 25, 50, 75, or 100µM (+)-catechin; Experiment 2: 0, 15, 25, 50, 75, or 100µM EGCG) and frozen. After thawing, the samples were evaluated for kinematics, plasma membrane (PMi) and acrosome integrity, morphology, and oxidative stress, at 0 and 1h. In Experiment 1, at 0h, VSL and VAP were greater (P<0.05) with 15µM than with 50 and 100; WOB was lower (P<0.05) with 100µM than with 0, 15, and 25; and BCF was higher (P<0.05) with 75 and 100µM than with 0. In turn, in Experiment 2, progressive motility was higher (P<0.05) with0 and 15µM than with50 and 75; LIN was lower (P<0.05) with75 and100µM than with0 and 15; WOB was higher (P<0.05) with0 and 15µM; and PMi was greater (P<0.05) with100µM than 0. Thus, (+)-catechin or EGCG at higher concentrations inhibits the kinematics of frozen goat sperm, in a transitory way, and 100µM of EGCG preserves the PMi.

Keywords: antioxidants, cryopreservation, flavonoids, oxidative stress, semen

RESUMO

Objetivou-se avaliar o efeito de diferentes concentrações de (+)-catequina ou (-)-epigalocatequina galato (EGCG) sobre a congelabilidade do sêmen caprino. Poolsseminais foram processados (experimento 1: 0, 15, 25, 50, 75 ou 100µM de (+)-catequina; experimento 2: 0, 15, 25, 50, 75 ou 100µM de EGCG) e congelados. Após a descongelação, foram avaliadas a cinética, a integridade de membrana plasmática (iMP) e acrossomal, a morfologia e o estresse oxidativo, a zero e a uma hora. No experimento 1, a zero hora, VSL e VAP foram maiores (P<0,05) com 15µM do que com 50 e100; WOB foi menor (P<0,05) com 100µM do que com 0, 15 e 25; e BCF foi maior (P<0,05) com 75 e 100µM do que com 0. No experimento 2, a motilidade progressiva foi maior (P<0,05) com 0 e 15µM do que com 50 e 75; LIN foi menor (P<0,05) com 75 e 100µM do que com 0 e 15; WOB foi maior (P<0,05) com 0 e 15µM; e iMP foi maior (P<0,05) com 100µM do que com 0. Assim, (+)-catequina ou EGCG em altas concentrações inibem, transitoriamente, a cinética de espermatozoides congelados caprinos, e 100µM de EGCG preserva a iMP.

Palavras-chave: antioxidantes, criopreservação, flavonoides, estresse oxidativo, sêmen

INTRODUCTION

The catechin isomers, (+)-catechin and epigallocatechin gallate (EGCG), are flavanols from the flavonoid family, found in plant-based foods and beverages (Weinreb *et al*., 2009; Li, 2011). Because of their chemical structures, these phytochemical compounds are more powerful antioxidants than other agents (Weinreb *et al*., 2009). The protective effects of catechins occur throughhydrogen-donation, the regulatory effect on enzymatic antioxidant expression, inhibition of pro-oxidant enzymes,chelation of metallic ions (Li, 2011), and protection fromother antioxidants (Lotito and Fraga, 2000).

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Some of the beneficial effects of catechins *in vitro* and *in vivo* are antibacterial (Mabe *et al*., 1999), antiviral (Song *et al*., 2005), antifungal (Hirasawa and Takada, 2004), antitumor (Gu *et al*., 2013), and anti-obesity (Nagao *et al*., 2005) activities. Moreover, these flavanolsactas neuroprotectors (Weinreb *et al*., 2009; Nath *et al*., 2012) and protectors of testicular parenchyma (Ding *et al*., 2015)and sperm (Boonsorn *et al*., 2010; De Amicis *et al*., 2012).

Semen cryopreservation is a reproductive bio-technique that has an important role in the animal industry, contributing to the expansion of other reproductive techniques (Barbas and Mascarenhas, 2009), and intensifying the productive system and genetic improvement (Leboeuf *et al*., 2000). Nonetheless, during this process, especially during freezing-thawing, the sperm cell is frequently injured (Leboeuf *et al*., 2000), with consequent reductions in its viability and function (Bansal and Bilaspuri, 2011).

Among the factors related to sperm injuries generated during cryopreservation, the high production of reactive oxygen species (ROS) is noteworthy (Watson, 2000; Bansal and Bilaspuri, 2011). ROS have detrimental effects on biological molecules, especially lipids (Saleh and Agarwal, 2002). Thus, mammalian sperm cell membranes are susceptible to oxidants attack, once that are rich in polyunsaturated fatty acids (Bansal and Bilaspuri, 2011).Therefore, the aim of this study was to evaluate the effects of different concentrations of (+)-catechin or (-)-epigallocatechin gallate on goat semen freezability.

MATERIALS AND METHODS

Except when specified, all reagents used in the experiment were obtained from Sigma-Aldrich Company (St Louis, MO, USA).

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Federal Rural University of Pernambuco (UFRPE - Brazil), under process number CEUA/UFRPE 014/2012. Six mature and fertile goats (three Saanen, two Toggenburg, and one British Alpine), from one to fouryrsold, were used. The animals were raised at UFRPE, Pernambuco, Brazil (08° 03’ 14’’ S; 34° 52’ 52’’ W), and fed with hay and commercial chow, as well as mineral salt and water *ad libitum*.

The semen was collected from six mature male goats, six times per experiment, at 48h intervals, using an artificial vagina and a female as the dummy, totalling 72 ejaculates (36 ejaculates per experiment). Fresh semen samples were subjectively examined for mass movement (0-5), motility, and vigour (0 to 100% and 0 to 5, respectively; magnification, 100x). Samples were also analysed in a Neubauer chamber for concentration (400x) and in a moist chamber for sperm morphology (1000x), after dilution in formol citrate solution (1:400; v:v), using phase-contrast microscopy (Olympus, Tokyo, Japan). Ejaculates collected on the same day from the six male goats and approved (mass movement≥3, motility≥70%, vigor≥3, concentration≥2 x 10^9 sperm/ml, and total sperm pathologies≤20%) were pooled, totalling six *pools* (n=6) per experiment.

Each one of the six goat semen *pools* per experiment were diluted (1:9; v:v) in Tris solution (3,605g Tris-hydroxymethyl aminomethane, 2,024g citric acid, 1,488g fructose and 100mlMilli-Q water, pH 6.8) and centrifuged (250 X g for 10min) twice. Subsequently, semen samples were divided into six equal aliquots, and diluted with a skim milk-based extender [10g skim milk powder, 194mg D-(+)-glucose, 100mlMilli-Q water, 7% glycerol, pH 6.8] containing (+)-catechin or EGCG, according to the experiment or experimental groups (Experiment 1: 0, 15, 25, 50, 75, and 100µMcatechin; Experiment 2: 0, 15, 25, 50, 75, and 100µM EGCG).The final sperm concentration was 200 x 10^6 sperm/ml.

Extended semen samples were packed into 0.25ml straws, and frozenusing an automated system (TK-3000®, TK Tecnologia em congelação Ltd, Uberaba, Brazil), in a slow curve specific for goats. During the positive step, the temperature drop was 0.25°C per min until reaching 5°C, at which temperature the samples were maintained for 120min for stabilisation. The negative step was started with a temperature drop of 20°C per min until the temperature reached -120°C, when the straws were immersed and stored in liquid nitrogen (-196ºC). The (+)-catechin and EGCG stock solutions (10mM) were prepared in DMSO and stored at -20°C.

After a minimum interval of 24h of frozen storage, four straws per experimental group were

thawed (37°C for 30s). Aliquots of semen were analysed after 0 and 1h of incubation at 34°C, according to the descriptions below. This procedure was repeated six times for each experiment and experimental group (n=6).

For sperm kinematic analysis, aliquots of semen from each experimental group were diluted in a skim milk-based extender (1:4; v:v) to reduce the sperm concentration (50 x 10^6 sperm/ml) and facilitate the capture of images. The analysis was performed by placing 5µl of each sample on a previously heated slide (37ºC) and depositing a cover slip over the drop. The slide was placed under a phase-contrast microscope (Nikon™ H5505, Eclipse 50i, Tokyo, Japan) and the images captured using a video camera (Basler Vision Tecnologie™ A312FC, Ahrensburg, Germany). Five non-consecutive, randomly selected microscopic fields per sample were scanned, recording at least 2000 sperm. The parameters assessed using the Sperm Class Analyzer - SCA™ software v. 5.1 (Microptics, S.L., Barcelona, Spain) were: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral movement of the head (ALH, µm), and beat cross frequency (BCF, Hz).The values of the CASA system were measured for the goat species, with the following configurations: 37°C temperature, 100x magnification, number of images 25, 25images per second,20 to 70µm^2 particle area, VAP 10µ/s < slow < 45µ/s < medium <75µ/s rapid, progressivity> 80% of STR, circular< 50% LIN, connectivity 12, 5 VAP points, 5 fields, 2000 sperms, 20% concentration.

Plasma membrane integrity assessment was determined using the double staining method with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI), as described by Silva *et al*. (2012). Aliquots (50µl) of semen were diluted in 150µlTris solution containing 5µl CFDA (0.46mg/ml in DMSO) and 20µl PI (0.5mg/ml in PBS), incubated for 10min at 37°C and fixed with PBS containing 0.5% glutaraldehyde. Using DBP 485/20nm excitation and DBP 580 to 630-nm emission filters, 200 cells were examined per slide in an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) at a magnification of 400x. Green fluorescence was interpreted as an intact membrane and red fluorescence as a damaged membrane.

Acrosome integrity was analysed using fluorescein isothiocyanate-conjugated agglutinin (FITC-PNA) (Silva *et al*., 2012). Aliquots (10µl) of semen were prepared for smearing and air dried. An aliquot of 30µl FITC-PNA working solution (40µg/ml in PBS) was placed on each slide to stain the cells, incubated in a moist chamber at 4°C for 20min, rinsed in PBS, and dried in the dark. At the time of assessment, 5µl of the mounting medium (4.5ml glycerol, 0.5ml PBS, 5mg sodium azide, and 5mg p-phenylenediamine) was placed on the slide and covered with a cover slip. A total of 200 sperm per slide were examined using an LP 515-nm emission and 450-490 BP nm excitation filter, in an epifluorescence microscope (Carl Zeiss, Göttingen, Germany; 1000x). Sperm were classified as having an intact acrosome when this region was stained with green fluorescent and ashaving a reacted acrosome when the green fluorescent was present in the sperm equatorial region or when it was absent from the head.

The moist chamber method was used for the analysis of sperm morphology (Oliveira and Silva, 2013). Using this method, semen samples were diluted in a formol citrate solution (1:5) and a 10µl aliquot was placed on the slide and covered with a cover slip. A total of 200 cells were analysed per slide using a phase-contrast microscope (Olympus, Tokyo, Japan; 1000x) and classified as morphologically normal or not.

The nitroblue tetrazolium (NBT) test was employed to study sperm oxidative stress as described by Saleh and Agarwal (2002). Semen samples were diluted (1:1; v:v) in NBT solution (0.1% in PBS), incubated for 30min at 37ºC and for an additional 30min at room temperature. Next, the samples were centrifuged (250 X g for 5min) and the pellets re-suspended in Tris solution. Aliquots (10µl) were prepared for smearing and air dried. One hundred sperm were evaluated per slide in a phase-contrast microscope (Olympus, Tokyo, Japan; 1000x). Sperm were classified as positive oxidative stress when they presented formazan deposition in the sperm head and/or mid-piece and as negative oxidative stress when the formazan deposition was absent.

The results were expressed as means and standard deviations (mean±SD). Before statistical analyses, the percentage data were arcsine transformed.To detect differences among concentrations of (+)-catechin (0, 15, 25, 50 75, and 100µM) or EGCG (0, 15, 25, 50 75, and 100µM) and the times of evaluation (0 and 1h after thawing) from each experiment, were used the one-way ANOVA test and Student-Newman-Keuls (SNK) multiple comparison test. Moreover, regression analysis was performed from semen parameters, according to antioxidant concentrations (linear, quadratic, and cubic effect). For all analyses, values were considered significant at P<0.05. Data were analyzed using the GLM (General Linear Model) from the Statistical Analysis System (SAS, 2009), as repeated measures in time.

RESULTS

Regarding the sperm kinematics of the cryopreserved goat semen samples, in Experiment 1, immediately after semen thawing (0h; Table 1), VSL and VAP were higher (P< 0.05) in 15µM than 50 and 100µM (+)-catechin; WOB was higher (P<0.05) in 0, 15 and 25µM than 100µM (+)-catechin; and BCF was higher (P<0.05) in 25, 75 and 100µM than 0µM (+)-catechin.

Similarly, in Experiment 2, at 0h after thawing (Table 2), a higher (P< 0.05) PM was observed in the control (0µM) and 15µM treatments than with 50 and 75µM EGCG; LIN was greater (P< 0.05) in the control group than the 75 and 100µM EGCG, as well as in the 15µM than in 100µM EGCG; and WOB was higher (P< 0.05) in 0 and 15µM than in 50, 75 and 100µM EGCG.

Table 1. Kinematic parameters of goat semen frozen with different concentrations of (+)-catechin

Parameters	Time	Concentrations of (+)-catechin						Regression assay*		
								P>F		
		0µM	15µM	25µM	50µM	75µM	100µM	L	Q	C
TM	0h	68.2±7.0	71.0±13.8	71.2±9.4	69.4±7.2	67.2±10.3	72.1±10.7	Ns	ns	Ns
	1h	59.5±11.5	54.3±15.8	56.0±14.6	57.9±8.6	57.1±14.4	56.0±15.1	Ns	ns	Ns
PM	0h	23.7±3.7	27.7±4.1	24.2±2.6	21.8±2.3	23.5±3.2	21.2±4.6	Ns	ns	Ns
	1h	25.1±3.6	23.3±9.6	21.8±5.5	23.1±8.8	23.3±6.4	21.0±12.2	Ns	ns	Ns
VCL	0h	81.2±10.1	86.0±4.6	80.9±2.5	75.7±7.0	80.3±10.8	77.4±8.1	Ns	ns	Ns
	1h	78.1±7.5	80.4±15.2	77.1±13.1	76.8±14.6	79.0±11.4	73.8±12.2	Ns	ns	Ns
VSL	0h	41.7±5.3^{ab}	45.4±5.3^{a}	41.5±3.7^{ab}	36.9±1.4^{b}	39.7±3.5^{ab}	35.2±3.1^{b}	0.0008	ns	Ns
	1h	42.4±4.0	43.2±6.1	41.5±7.2	40.4±8.0	41.3±2.4	38.0±8.8	Ns	ns	Ns
VAP	0h	55.4±7.2^{ab}	59.2±4.4^{a}	54.7±3.1^{ab}	49.3±2.6^{b}	52.2±6.3^{ab}	48.0±4.5^{b}	0.0012	ns	Ns
	1h	52.2±4.6	53.8±9.2	52.4±9.1	50.5±9.9	51.4±5.2	47.6±9.4	Ns	ns	ns
LIN	0h	51.4±2.9	52.9±7.0	51.3±3.9	49.2±5.1	49.9±4.7	45.6±2.2	0.0139	ns	ns
	1h	54.5±5.0	54.1±4.2	54.2±7.2	53.0±6.8	52.8±4.9	51.2±5.8	Ns	ns	ns
STR	0h	75.3±2.5	76.7±6.4	75.9±2.8	75.0±4.5	76.4±4.6	73.4±3.0	Ns	ns	ns
	1h	81.2±3.4	80.6±3.6	79.3±5.0	80.2±6.0	80.8±4.1	79.6±6.3	Ns	ns	ns
WOB	0h	68.2±2.5^{a}	68.8±4.2^{a}	67.5±2.8^{a}	65.4±3.2^{ab}	65.2±2.3^{ab}	62.1±1.2^{b}	<.0001	ns	ns
	1h	67.0±3.9	67.1±3.3	68.1±5.7	65.9±4.7	65.3±3.4	64.2±3.0	ns	ns	ns
ALH	0h	3.1±0.2	3.2±0.2	3.1±0.2	3.1±0.3	3.2±0.4	3.3±0.2	ns	ns	ns
	1h	3.2±0.2	3.2±0.4	3.1±0.4	3.1±0.5	3.2±0.3	3.2±0.3	ns	ns	ns
BCF	0h	11.1±0.8^{c}	11.4±0.6^{abc}	11.3±0.4^{ab}	11.7±0.5^{abc}	12.4±0.5^{a}	12.2±0.8^{ab}	0.0003	ns	ns
	1h	13.0±1.0	12.8±1.2	12.5±0.8	13.2±0.9	13.1±1.1	13.4±1.3	ns	ns	ns

Different letters in the same line denote significant differences between groups (P<0.05). TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (µm/s); VSL: straight linear velocity(µm/s); VAP: average path velocity(µm/s); LIN: linearity (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral movement of sperm head(µm); BCF: beat cross frequency (Hz). *L: linear; Q: quadratic; C: cubic.

No differences (P> 0.05) were observed between the experimental groups related to the other kinematic parameters analysed at 0h, or those analysed at 1h of incubation, in both experiments (Table 1 and 2). In addition, in Experiment 1, no differences (P< 0.05) were observed in plasma membrane and acrosome integrity, percentage of morphologically normal sperm, and sperm without oxidative stress between the experimental groups, independent of the time of evaluation (0 and 1h) after semen thawing (Table 3). In Experiment 2 (Table 4), the percentage of sperm cells with intact plasma membrane was higher (P< 0.05) in 100µM than in 0µM EGCG at 0h, but no statistical difference (P> 0.05) was observed for the other parameters at 0 and 1h of incubation.

Table 2. Kinematic parameters of goat semen frozen with different concentrations of EGCG

Parameters	Time	Concentrations of EGCG						Regression assay* P>F		
		0µM	15µM	25µM	50µM	75µM	100µM	L	Q	C
TM	0h	68.8±9.0	66.4±11.5	67.4±11.8	60.0±11.0	59.7±10.7	69.1±8.3	ns	ns	ns
	1h	59.5±7.4	49.8±16.1	51.3±7.3	57.6±15.2	55.4±19.5	54.4±17.4	ns	ns	ns
PM	0h	27.1±2.6^{a}	27.2±4.3^{a}	24.4±2.6^{ab}	19.4±2.8^{b}	19.9±2.0^{b}	24.6±6.6^{ab}	0.0035	ns	ns
	1h	24.2±9.1	19.7±6.2	18.4±3.6	19.7±6.3	21.1±7.4	19.8±5.9	ns	ns	ns
VCL	0h	80.4±14.6	84.3±9.6	81.0±7.7	78.1±11.7	82.7±9.4	84.1±6.7	ns	ns	ns
	1h	75.6±12.2	71.3±6.3	68.7±5.9	72.4±13.7	75.1±9.8	78.0±12.8	ns	ns	ns
VSL	0h	43.9±6.1	45.8±5.9	41.8±3.7	38.3±6.1	39.6±6.2	39.6±5.4	0.0269	ns	ns
	1h	42.0±12.8	38.5±6.2	35.9±3.3	35.8±4.6	38.8±6.0	39.0±4.5	ns	ns	ns
VAP	0h	56.1±9.0	58.6±7.7	55.1±5.8	50.8±8.6	53.3±7.5	53.5±4.9	ns	ns	ns
	1h	52.0±12.1	47.0±5.0	45.3±2.9	45.9±7.4	48.6±6.2	49.9±7.0	ns	ns	ns
LIN	0h	55.1±5.2^{a}	54.4±3.5^{ab}	51.7±3.2^{abc}	49.1±3.2^{abc}	47.8±3.7^{bc}	47.1±5.9^{c}	<.0001	ns	ns
	1h	54.6±8.6	54.1±7.9	52.4±5.4	50.1±5.3	51.8±7.1	50.6±5.5	ns	ns	ns
STR	0h	78.7±4.2	78.5±3.8	76.1±4.2	75.5±3.8	74.3±3.4	73.8±4.7	0.0085	ns	ns
	1h	79.7±6.2	81.5±6.0	79.2±5.1	78.4±4.1	79.6±5.4	78.6±5.7	ns	ns	ns
WOB	0h	70.0±3.1^{a}	69.2±2.0^{a}	67.9±1.6^{ab}	65.0±2.3^{b}	64.3±2.5^{b}	63.7±3.8^{b}	<.0001	ns	ns
	1h	68.2±5.8	66.0±5.4	66.1±2.8	63.8±3.5	64.9±4.8	64.3±2.3	ns	ns	ns
ALH	0h	3.0±0.4	3.0±0.2	3.1±0.2	3.3±0.2	3.2±0.1	3.2±0.3	0.0271	ns	ns
	1h	3.0±0.2	3.0±0.3	3.1±0.3	3.1±0.4	3.0±0.4	3.2±0.3	ns	ns	ns
BCF	0h	11.5±0.9	12.2±1.0	12.1±0.7	12.1±1.0	12.2±0.8	12.4±0.6	ns	ns	ns
	1h	12.5±1.3	13.1±0.8	12.4±0.9	13.1±0.6	13.0±1.0	13.4±0.7	ns	ns	ns

Different letters in the same line denote significant differences between groups (P<0.05). TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity(µm/s); VSL: straight linear velocity(µm/s); VAP: average path velocity(µm/s); LIN: linearity (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral movement of sperm head(µm); BCF: beat cross frequency (Hz). *L: linear; Q: quadratic; C: cubic.

According to the regression analysis, higher concentrations of (+)-catechin resulted in lower values of VSL, VAP, LIN, and WOB, as well as higher values of BCF at 0h (Table 1). Likewise, higher concentrations of EGCG led to a reduction in PM, VSL, LIN, STR, and WOB, as well as higher ALH and percentage of gametes with intact plasma membrane at 0h (Table 2 and 4).

DISCUSSION

The findings of the present study showed that the treatments with (+)-catechin or EGCG, at higher concentrations (50 to 100µM), had an inhibitory effect on goat sperm kinematics, immediately after semen thawing. Thus, in a dose-dependent manner, (+)-catechin, especially, reduced the sperm velocity parameters (VSL and VAP), whereas EGCG inhibited the sperm progressive motility and LIN; parameters directly linked with fertility rates and the progression of sperm displacement (Matos *et al*., 2008). However, after 1h of incubation, the kinematic parameters of the experimental groups in both experiments were similar, revealing the reversible character of catechin treatments.

Table 3. Plasma membrane, acrosome, morphology, and oxidative stress of goat semen frozen with different concentrations of (+)-catechin

Parameters	Time	Concentrations of (+)-catechin						Regression assay* P>F		
		0µM	15µM	25µM	50µM	75µM	100µM	L	Q	C
PMi	0h	39.6±6.5	44.6±5.6	47.4±8.4	43.5±7.2	48.0±7.8	47.1±4.0	ns	ns	ns
	1h	28.1±6.7	32.8±8.7	34.0±7.3	33.4±5.6	37.2±12.2	33.6±7.4	ns	ns	ns
ACi	0h	50.9±9.3	48.3±4.3	48.8±4.3	53.2±10.1	52.5±5.5	49.7±9.0	ns	ns	ns
	1h	51.5±14.9	48.5±10.6	44.3±10.9	50.8±13.8	46.6±7.2	48.9±7.3	ns	ns	ns
nMOR	0h	79.9±3.2	79.6±3.8	78.4±2.5	75.8±4.9	78.3±4.0	77.0±2.7	ns	ns	ns
	1h	76.3±8.4	74.9±3.1	73.5±2.6	76.6±5.3	70.8±5.4	75.2±1.8	ns	ns	ns
-OE	0h	78.8±4.0	81.2±4.9	82.5±3.5	83.0±3.4	82.3±6.5	82.3±4.3	ns	ns	ns
	1h	76.3±4.8	76.5±3.3	75.2±5.1	78.8±4.1	77.0±4.5	80.2±5.9	ns	ns	ns

PMi: plasma membrane integrity (%); ACi: acrosome integrity (%); nMOR: normal morphology (%); −OS: negative oxidative stress (%). *L: linear; Q: quadratic; C: cubic.

Table 4.Plasma membrane, acrosome, morphology, and oxidative stress of goat semen frozen with different concentrations of EGCG

Parameters	Time	Concentrations of EGCG						Regression assay*		
								P>F		
		0µM	15µM	25µM	50µM	75µM	100µM	L	Q	C
PMi	0h	39.3±4.7[b]	44.5±6.8[ab]	41.0±5.5[ab]	43.5±8.2[ab]	43.3±4.2[ab]	51.3±8.3[a]	0.0117	ns	ns
	1h	37.6±6.6	34.8±10.0	36.1±9.9	31.5±13.0	31.6±7.2	33.0±5.0	ns	ns	ns
ACi	0h	44.8±7.0	45.1±8.1	47.0±10.5	46.0±6.6	47.8±4.9	49.7±7.6	ns	ns	ns
	1h	43.4±8.8	42.7±11.8	39.6±9.7	42.1±10.2	40.0±11.0	42.1±9.8	ns	ns	ns
nMOR	0h	86.6±5.3	84.5±6.3	84.5±6.8	84.8±6.4	87.5±1.8	84.4±6.9	ns	ns	ns
	1h	81.6±10.4	84.9±6.4	82.3±8.6	80.3±10.5	81.8±10.1	79.8±11.5	ns	ns	ns
-OE	0h	78.5±3.5	81.0±4.4	82.3±2.8	82.7±4.6	81.3±4.0	82.0±2.9	ns	ns	ns
	1h	74.0±2.9	76.0±4.6	78.7±4.7	78.0±3.9	78.8±2.2	77.2±2.9	ns	ns	ns

Different letters in the same line denote significant differences between groups (P<0.05). PMi: plasma membrane integrity (%); ACi: acrosome integrity (%); nMOR: normal morphology (%); −OS: negative oxidative stress (%). *L: linear; Q: quadratic; C: cubic.

Based on similar results, the use of EGCG at high concentrations is sometimes considered detrimental to sperm and linked to an increase in ROS production (De Amicis *et al*., 2012).Nevertheless, it is important to consider that the investigated catechins are inhibitors of F0F1-ATPase (Zheng and Ramirez, 2000) and cyclooxygenase (COX) (Kundu *et al*., 2003). F0F1-ATPase is an ATP synthase from mitochondria that represents one of the pathways to ATP synthesis (Zheng and Ramirez, 2000).Moreover, COX is a regulator of the prostaglandins production, which are modulators of sperm motility(Kennedy *et al*., 2003). In this way, catechins can be considered motility inhibitors by nature, in a dose-dependent response.

It is possible that the inhibitory effect of catechinson goat sperm kinematics occurred in a defensive manner. This hypothesis can be justified considering that the mitochondria are the main source of ROS in sperm (Koppers *et al*., 2008) and that their maximum activity occurs during the sperm capacitation (Ramio-Lluch *et al*., 2011). Moreover, COX is an enzyme linked to sperm capacitation and acrosome reaction (Joyce *et al*., 1987); events that can be induced prematurely during the cryopreservation process (Watson, 2000).

The treatment with (+)-catechin did not increase the percentage of cells with plasma membrane and acrosome integrity, normal morphology, or sperm without oxidative stress. However, the therapy with 100µM EGCG showed a powerful capacity to protect the plasma membrane integrity, immediately after thawing, evidencing a dose-dependent effect.No significant improvement was observed in the other parameters in Experiment 2; except for a tendency (P=0.07) of EGCG to maintain a greater number of cells without oxidative stress than the control group,2 h after semen thawing (dates not shown).Therefore, it is probable that the absence of plasma membrane integrity increase, after 1 h incubation, was due to the removal of the cryopreservation stress factor, and not to loss of EGCG activity.

In opposition to these results, Boonsorn *et al*. (2010) observed a protective effect of (+)-catechin(25, 50, 75, and 100 µM) on plasma membrane integrity and lipoperoxidation of cooled boar semen. Moreover, De Amicis *et al*. (2012) showed that the incubation of fresh human semen with EGCGatlow concentrations (2 and 20 µM) resulted in greater plasma membrane integrity than the control or a higher concentration (60 µM); although in the present study, this was observed with 100 µM EGCG. However, it is important to note that in the boar semen (Boonsorn *et al*., 2010), a chemically defined medium was used, and in the latter case (De Amicis *et al*., 2012), the semen samples were plasma free, diluted in a protein-free buffer, and maintained in a controlled atmosphere.

In the present study, although the seminal plasma had been removed, it was used with a milk-based extender with pH 6.8 and the atmosphere was not controlled. However, catechins have an affinity toproteins, such as milk proteins, with which they can bind and form stable complexes (Hassan *et al*., 2013). Moreover, catechin stability decreases with the increase in pH (pH 4 to 8), resulting in their degradation (Zhu *et al*., 1997) and acquisition of a negative charge that is repulsed by the cell (Martínez-Flórez *et al*., 2002). It is possible that catechins also suffer

oxidation when exposed to light and atmospheric oxygen (Nath *et al*., 2012). All these factors result in loss of catechin antioxidant activity (Nath *et al*., 2012; Hassan *et al*., 2013).

In addition, the decrease in somekinematic parameters has been associated with differences in extender density, viscosity, and number and size of particles (Tekin and Daşkin, 2016). This can justify the fact that, immediately post-thaw, the groups treated with the largest concentrations of polyphenols presented decreased VSL, VAP, and WOB in Experiment 1, without changes in other criteria, as well as decreased PM, LINand WOB in Experiment 2, associated with a higher PMi.

Despite reports about the dual role of EGCG (Martínez-Flórez *et al*., 2002; Weinreb *et al*., 2009), this flavonoid is considered the most powerful antioxidant among the catechins (Adela *et al*., 2010). Therefore, it is justifiable that the EGCG excelled over the (+)-catechin, despite the presence of some negative factors. In this context, there is a clear need for further studies on catechins in goat semen cryopreservation, taking into account the negative influences affecting this agent. Finding the appropriate conditions will enable the protective properties of catechins to bemaximized during goat semen cryopreservation.

CONCLUSION

Under the experimental conditions used, (+)-catechin or EGCG at higher concentrations (50 to 100µM) inhibits the sperm kinematics of frozen goat semen compared to low concentrations, in a transitory way over incubation time, and 100µM EGCG preserves the plasma membrane integrity of these gametes immediately after thawing.

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