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Brazilian green propolis doesn't have a beneficial effect on the cryopreservation of domestic cat epididymal spermatozoa

A própolis verde brasileira não tem efeito benéfico na criopreservação de espermatozóides epididimários de gato doméstico

El propóleo verde brasileño no tiene un efecto beneficioso sobre la criopreservación de espermatozoides epididimarios de gatos domésticos

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Abstract

This study evaluated the effect of different concentrations of Brazilian green propolis on the cryopreservation of epididymal sperm from domestic cats. Spermatozoa were collected from cauda epididymis by slicing technique in a TRIS extender, preheated to 37ºC. Then, the sperm were evaluated and divided randomly between the control group (without adding propolis in the extender) and the treatment groups (adding different concentrations of propolis extract in the extender): P1 (0.1 mg/mL), P2 (0.3 mg/mL) e P3 (0.6 mg/mL). Subsequently, sperm were cryopreserved. The evaluated parameters were sperm kinetics by the Computer Assisted Semen Analysis, vigor, viability, membrane functionality, chromatin condensation and morphology. The parameters were measured before and after cryopreservation. Propolis didn't show toxicity to the sperm in any concentrations, with no changes observed in the

motility pattern. There was no significant influence of propolis on motility, vigor, percentage of viable spermatozoa, chromatin quality and other kinetic parameters in the cryopreserved samples. In sperm morphology, it was demonstrated a reduction in the percentage of normal cells in P1 and P2 groups in relation to the fresh sample, control and P3 group. In addition, an interesting effect from the propolis groups was observed on possible growth inhibition of microorganisms in the contaminated samples. Propolis didn't provide superiority to the sperm parameters evaluated after thawing, except for the values of plasma membrane functionality, which were better. The propolis is an interesting component to be incorporated in the cryoprotectant medium due to its non-toxicity and the potential inhibition of microbial growth.
Keywords: Feline; Reproductive biotechiniques; Spermogram; CASA; Green propolis.

Resumo

Este estudo avaliou o efeito de diferentes concentrações da própolis verde brasileira na criopreservação dos espermatozoides epididimários de gatos domésticos. Os espermatozóides foram coletados da cauda do epidídimo pela técnica de fatiamento, em extensor TRIS, pré-aquecido a 37ºC. Em seguida, os espermatozoides foram avaliados e divididos aleatoriamente entre o grupo controle (sem adição de própolis no diluente) e os grupos de tratamento (adição de diferentes concentrações de extrato de própolis no diluente): P1 (0,1 mg/mL), P2 (0,3 mg/mL) e P3 (0,6 mg/mL). Posteriormente, os espermatozoides foram criopreservados. Os parâmetros avaliados foram: cinética espermática pela Análise de Sêmen Assistida por Computador, vigor, viabilidade, funcionalidade da membrana, condensação da cromatina e morfologia. Os parâmetros foram medidos antes e pós-criopreservação. A própolis não apresentou toxicidade aos espermatozoides em nenhuma concentração, não sendo observada alteração no padrão de motilidade. Não houve influência significativa da própolis na motilidade, vigor, porcentagem de espermatozóides viáveis, qualidade da cromatina e outros parâmetros cinéticos nas amostras criopreservadas. Na morfologia espermática, foi demonstrada redução no percentual de células normais nos grupos P1 e P2 em relação à amostra fresca, controle e grupo P3. Além disso, foi observado um efeito interessante dos grupos de própolis sobre a possível inibição do crescimento de microrganismos nas amostras contaminadas. A própolis não apresentou superioridade aos parâmetros espermáticos avaliados pós-descongelação, exceto para os valores de funcionalidade da membrana plasmática, que foram melhores. A própolis é um componente interessante de ser incorporado ao meio crioprotetor devido à sua não toxicidade e potencial inibidor do crescimento microbiano.
Palavras-chave: Felinos; Biotecnologias reprodutivas; Espermograma; CASA; Propolis verde.

Resumen

Este estudio evaluó el efecto de diferentes concentraciones de propóleo verde brasileño en la criopreservación de esperma epididimario de gatos domésticos. Los espermatozoides se recolectaron de la cola del epidídimo mediante la técnica de corte, en un extensor TRIS, precalentado a 37ºC. Luego, los espermatozoides fueron evaluados y divididos aleatoriamente entre el grupo de control (sin agregar propóleo en el diluyente) y grupos de tratamiento (adición de diferentes concentraciones de extracto de propóleo en el diluyente): P1 (0,1 mg/mL), P2 (0,3 mg/mL) y P3 (0,6 mg/mL). Posteriormente, los espermatozoides fueron criopreservados. Los parámetros evaluados fueron: cinética espermática por análisis de semen asistido por computadora, vigor, viabilidad, funcionalidad de membrana, condensación de cromatina y morfología. Los parámetros se midieron antes y después de la criopreservación. El propóleo no mostró toxicidad para los espermatozoides a ninguna concentración, no se observó ningún cambio no estándar en la motilidad. No hubo influencia significativa del propóleo sobre la motilidad, vigor, porcentaje de espermatozoides viables, calidad de cromatina y otros parámetros cinéticos en muestras criopreservadas. En la morfología de los espermatozoides, se demostró una reducción del porcentaje de células normales en los grupos P1 y P2 en relación con la muestra fresca, el control y el grupo P3. Además, se observó un interesante efecto de los grupos propóleos sobre la posible inhibición del crecimiento de microorganismos en las muestras contaminadas. El propóleo no mostró superioridad a los parámetros espermáticos evaluados después de la descongelación, excepto por los valores de funcionalidad de la membrana plasmática, que fueron mejores. El propóleo es un componente interesante para ser incorporado al medio crioprotector por su no toxicidad y potencial para inhibir el crecimiento microbiano.
Palabras chave: Felino; Biotecnologías reproductivas; Espermograma; CASA; Propóleo verde.

1. Introduction

Cryopreservation is a biotechnology that conserves cells in a quiescent state at temperatures below the freezing point of water, preserving their composition and viability indefinitely (Sieme *et al.*, 2016). The application of this technology to the sperm cells of domestic animals, pets or animals with great zootechnical value, makes it possible to preserve their genetic material and possibly its application in other reproductive biotechniques (Martins & Justino, 2015; Kunkitti *et al*., 2016). For wild animals, this biotechnology is valuable to maintain the genetic variability, based on the formation of biobanks (Silva *et al.*, 2019). In felines, this justification is quite plausible since all species, with the exception of the domestic cat, are threatened or at risk of extinction

(IUCN, 2020).

Obtaining feline sperm for cryopreservation can be done by different techniques, such as electroejaculation, use of artificial vagina or even recovery of epididymal sperm. This last technique has some advantages over the others, such as the possibility of recovering sperm from post-mortem animals, high cell concentration after harvest, and elimination of animal training stage (Lima & Silva., 2017; Jelinkova *et al.*, 2018).

Despite its importance, the cryopreservation of sperm presents the setback of cellular stress by causing harmful effects to the morphofunctional features of spermatozoa due to temperature variations, the cryoinjuries generated by the formation of ice crystals, and cellular toxicity caused by the use of cryoprotectants in the medium (Sieme *et al.*, 2016).

In this context, there is a growing prospect of substances of natural origin that can provide protection to the sperm. One of these substances is propolis, which due to its biological properties, presents itself as a raw material with great potential for preserving the quality of sperm cells subjected to freezing, and is therefore intensively investigated for its antimicrobial and antioxidant activity. In addition, several studies seek to understand propolis' mechanisms of action on the morphology of reproductive organs and on the production of sperm cells, as well as its protective action on sperm cells structure (Capucho *et al.*, 2012; Cedikova *et al.*, 2014; Ögretmen *et al.*, 2014). Therefore, the aim of this study was to evaluate the effect of Brazilian green propolis extract in different concentrations on the epididymal sperm parameters after cryopreservation in domestic cat.

2. Methodology

This research had an exploratory and quantitative nature (Pereira *et al.*, 2018). All procedures performed in this study was approved by UFERSA Ethics Committee for Animal Use (CEUA) under protocol number 23091.001390/2018-11.

2.1 Propolis extract preparation

Green propolis was collected from Nazareno city (Minas Gerais, Brazil), with its predominant botanical origin being *Baccharis dracunculifolia*. An alcoholic extract was prepared based on the methodology adapted from Park *et al.* (1998), starting by grinding raw propolis, followed by the extraction in grain alcohol. The whole process was carried out in a thermostatic water bath, under manual agitation and constant heating at 60 ºC for 2 h. Once the solubilized extract was obtained, three dilutions were prepared based on the propolis mass/volume ratio of TRIS until reaching concentrations of 0.1 (P1), 0.3 (P2) and 0.6 mg/ml (P3).

2.2 Experimental design

Seventeen healthy adult male cats, aged 1.5 to 4 years, underwent elective bilateral orchiectomy. The pair of testis-epididymis complex (CTE) was kept in 0.9% saline solution at 37 ºC, until processing, which occurred within 1 h.

The recovered samples were evaluated for sperm concentration, sperm kinetics, total motility, vigor, viability, membrane functional integrity, morphology and chromatin integrity. They were divided and subsequently randomized between the groups: control (n = 12), P1 (n = 12), P2 (n = 12) and P3 (n = 12). All frozen medium were supplemented with 20% egg yolk as cryoprotectant. For samples diluted in P1, P2 and P3 treatment groups, an immediate evaluation of motility, vigor and sperm kinetics was carried out in order to investigate the possible effect of propolis on fresh sperm.

The diluted sperm in the respective treatments were equilibrated at 5ºC for 1 h and cryopreserved in liquid nitrogen. Frozen samples were thawed after a week and evaluated.

2.3 Epididymal sperm collection, cryopreservation and thawing

The spermatozoa were recovered from cauda epididymis by slicing technique, in a Petri dish containing 1 mL of Tris buffer solution (Tris-hydroxymethyl-aminomethane plus fructose and citric acid monohydrate) at 37ºC (Barbosa *et al.*, 2020).

The recovered sperm were centrifuged at 500g for 6 min. Formed *pellet* was resuspended in TRIS and subsequently divided randomly into the groups. Given the non-toxic effect of the plant extract, the samples were cryopreserved according to the protocol described by Cocchia *et al.* (2009).

The cryopreservation consisted of subjecting the sample to equilibrium curve of 5°C for 60 min. Subsequently, to complete the treatment final volume, there were added a second part of the respective diluent volume containing 8% glycerol, for a final concentration of 4% of glycerol. The sperm sample (with a final concentration of 50 x 10^6 sptz/ml) was placed in 0.25 mL plastic straws, exposed to nitrogen vapors (6 cm distance from the nitrogen surface) for 20 minutes and stored in liquid nitrogen (Cocchia *et al.*, 2009).

For thawing, straws were removed from the canister, kept at room temperature (approximately 28ºC) for 5 sec and then immersed in a water bath at 37 ºC for 30 sec. The sperm were transferred to microtubes, preheated to 37ºC, and immediately evaluated (Cocchia *et al.*, 2009).

2.4 Sperm evaluation

The vigor (quality of progressive spermatozoa movement on a scale of 0 to 5) was evaluate using a drop of sperm sample placed on a preheated slide, under optical microscope (100x) (CBRA, 2013).

For morphology analysis, smears of sperm sample were stained with rose bengal dye and 200 cells per slide were randomly counted, between normal and abnormal, under optical microscopy (1000x) (VillaVerde *et al.*, 2008). Sperm viability was determined by analyzing a slide stained with bromo-phenol blue, under an optical microscope (400x), quantifying 100 cells per slide, among those considered viable (unstaining) and non-viable (blue staining) (Lima *et al.*, 2016).

The functionality of the sperm membrane was verified through the hypoosmotic swelling test (HOST) using a hypoosmotic solution (150 mOsm/L). A total of 100 cells were counted, and those with a coiled tail were considered to have a functional membrane (Lima *et al.*, 2016).

For the chromatin condensation analysis test, 10 µl of the sample was added on a slide, followed by smear and fixation, respectively. The slide was washed, dried and 5 µl of the toluidine blue dye (pH 4) was added, 500 sperm cells were counted under the microscope (400x). The state of chromatin condensation was evaluated based on the presence or absence of the blue color (Kamimura *et al.*, 2010).

To analyze the kinetic of movement, the samples were submitted to Computer Assisted Semen Analysis (CASA; IVOS 7.4G, Hamilton-Thorne Research, Beverly, MA, USA). The system was adjusted considering feline species values, set as follows: temperature of analysis: 37 ºC; Frame rate (Hz): 60; Minimum static contrast: 30; Average path velocity cut-off (VAP; µm/s): below 30; Straight-line velocity cut-off (VSL; µm/s): below 20; Progressive minimum VAP (µm/s): equal to or greater 70; Minimum straightness (%): 75 (Villaverde, 2007). The analyzed variables were total motility (TM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

2.5 Statistical analysis

The data were expressed as mean and standard error, analyzed using the statistical software *R* version 3.5.1 (*The R Foundation, Viena, Áustria*). The data were compared by Analysis of Variance (ANOVA) followed by Student-Newman-Keuls (SNK) test. The results were considered significant when $p < 0.05$.

3. Results

3.1 Fresh evaluation of the sperm sample diluted in medium containing propolis

The results of the evaluation of fresh diluted sperm containing propolis for the analysis of sperm kinetics and vigor are shown in Table 1. There was no difference ($p < 0.05$) in any of the control variation values in relation to P1, P2 and P3 treatment groups. These results allow us to infer that none of the concentrations tested was toxic to epididymal sperm, since there was no negative impact on the movement quality.

Table 1: Evaluation of sperm kinetics and vigor of the epididymal sperm diluted in medium containing propolis.

Parameters	Fresh	P1	P2	P3
Vigor (0-5)	3.17 ± 0.17	2.67 ± 0.42	3.00 ± 0.10	3,00 ± 0.45
MT (%)	78.33 ± 3.87	53.33 ± 8.52	58.67 ± 6.77	60.50 ± 6.90
VAP (µm/s)	64.27 ± 1.72	60.98 ± 3.34	60.05 ± 1.59	70.17 ± 4.69
VCL (µm/s)	128.68 ± 2.09	110.50 ± 7.07	113.90 ± 4.37	120.22 ± 3.08
VSL (µm/s)	45.48 ± 1.44	46.35 ± 3.28	44.42 ± 2.68	56.37 ± 4.77
LIN (%)	37.50 ± 1.45	43.50 ± 1.68	43.17 ± 3.11	46.83 ± 3.11
ALH (µm)	6.90 ± 0.50	7.43 ± 0.82	8.60 ± 0.48	8.80 ± 0.80
BCF (Hz)	20.15 ± 1.12	18.80 ± 0.92	20.52 ± 1.60	19.43 ± 0.82

P1: propolis concentration at 0.1 mg/mL; P2: propolis concentration at 0.3 mg/mL; P3: propolis concentration at 0.6 mg/mL; Control: No propolis added. MT: Total Motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity; LIN: Linearity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency. Source: Authors.

3.2 Evaluation of frozen-thawed sperm samples

The results of the sperm kinetics of the frozen-thawed samples in the groups with or without propolis are shown in Table 2. The reduction in total motility after cryopreservation was noted in all treatment groups ($p < 0.05$). However, there were no differences between the groups containing propolis and the control group ($p > 0.05$). The velocity parameters (VAP, VCL and VSL), LIN, ALH and BCF, showed no difference ($p > 0.05$) between the fresh and cryopreserved sample or between treatments in the same group.

Table 2: Evaluation of sperm kinetics and vigor of the epididymal sperm diluted and cryopreserved in medium containing propolis.

Parameters	Fresh	Frozen-thawed			
		Control	P1	P2	P3
MT (%)	78.33 ± 3.87[a]	18.83 ± 3.78[b]	14.22 ± 4.19[b]	14.45 ± 2.18[b]	16.00 ± 2.86[b]
VAP (µm/s)	64.27 ± 1.72	53.57 ± 4.39	64.23 ± 5.65	62.55 ± 15.37	67.32 ± 12.11
VCL (µm/s)	128.68 ± 2.09	89.33 ± 12.66	95.18 ± 15.62	90.57 ± 23.83	103.68 ± 18.50
VSL (µm/s)	45.48 ± 1.44	43.57 ± 3.53	53.33 ± 6.33	55.73 ± 14.51	49.92 ± 11.20
LIN (%)	37.50 ± 2.98	60.00 ± 5.37	62.17 ± 8.94	56.50 ± 12.36	48.83 ± 9.52
ALH (µm)	6.90 ± 0.50	6.83 ± 2.39	6.00 ± 1.73	6.22 ± 1.90	6.55 ± 2.15
BCF (Hz)	20.15 ± 1.12	14.75 ± 4.79	13.07 ± 3.17	10.23 ± 4.75	13.83 ± 4.78

[a, b, c] Different lowercase letters indicate differences between fresh and frozen-thawed sperm in treatments (Control, P1, P2 and P3; $p < 0.05$). P1: propolis concentration at 0.1 mg/mL; P2: propolis concentration at 0.3 mg/mL; P3: propolis concentration at 0.6 mg/mL; Control: No propolis added. MT: Total Motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity; LIN: Linearity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency. Source: Authors.

The intervals observed in the kinetics analysis by CASA were: 53.57 – 76.45 µm/s for VAP; 89.33 – 136.05 µm/s for VCL; and 43.57 – 58.78 µm/s for VSL. The LIN values varied between 37.50% and 62.17%, ALH between 6.00 µm and 8.80 µm and BCF between 10.23 Hz and 23.78 Hz (Table 2).

The Table 3 has the data regarding the vigor, morphology, viability, sperm membrane functionality and chromatin integrity proportions. The vigor parameter did not show difference between the treatment groups and the fresh sample ($p > 0.05$). The same was observed in the chromatin integrity parameter ($p > 0.05$), with little variation between data from 99.5% to 99.9%.

Table 3: Evaluation of vigor, morphology, viability, functionality (HOST) and chromatin integrity of epididymal spermatozoa diluted and cryopreserved in medium containing propolis.

Parameters	Fresh	Frozen-thawed			
		Control	P1	P2	P3
Vigor (0-5)	3.17 ± 0.17	2.17 ± 0.54	1.83 ± 0.40	2.00 ± 0.45	2.33 ± 0.42
Normal morfology (%)	59,00 ± 6.07^{ab}	61.00 ± 5,17^{a}	51.80 ± 3.96^{ab}	45.73 ± 3.23^{b}	62.71 ± 5.03^{a}
Viability (%)	59.50 ± 8.44^{a}	36.50 ± 7.05^{b}	38.00 ± 2.58^{b}	32.67 ± 2.84^{b}	40.33 ± 4.20^{b}
HOST (%)	70.00 ± 5.88^{a}	49.17 ±4.51^{b}	54.67± 6.34^{ab}	61.33± 3.98^{ab}	50.50 ± 7.28^{b}
Chromatin condensation (%)	99.70 ± 0.30	99.50 ± 0.17	99.87 ± 0.07	99.77 ± 0.08	99.90 ± 0.05

$^{a, b, c}$ Different lowercase letters indicate differences between fresh and frozen-thawed sperm in treatments (Control, P1, P2 and P3; $p < 0.05$). P1: propolis concentration at 0.1 mg/mL; P2: propolis concentration at 0.3 mg/mL; P3: propolis concentration at 0.6 mg/mL; Control: No propolis added. Source: Authors.

On morphology parameter (Table 3), P1 and P2 groups showed a reduction in the percentage of normal sperm after cryopreservation ($p < 0.05$); whereas, P3 did not differ from the control and the fresh sample ($p > 0.05$). The values recorded after morphological evaluation varied between 39.92% and 66.08% in different treatments.

The decrease in viability occurred in all treatments after cryopreservation ($p < 0.05$). However, they did not differ from each other ($p > 0.05$). For the plasma membrane functionality analysis, the control and P3 groups showed a significant reduction in the percentage of functional sperm, compared to the fresh sample ($p < 0.05$). While treatments P1 and P2 were statistically similar to the others ($p > 0.05$) (Table 3).

4. Discussion

This study showed for the first time the effect of the alcoholic extract of green propolis on the domestic cat's epididymal sperm. Propolis has several biological interest properties already reported and it was used in this study in order to reduce cell damage caused by free radicals produced by oxidative stress during the cryopreservation procedure (Cedikova *et al.*, 2014). To ensure that the real effect of propolis on sperm was tested, a first evaluation was performed before cryopreservation. This evaluation showed no adverse effects of propolis on the epididymal sperm in any of the concentrations tested. However, it is known that propolis can have a toxic effect on sperm, and this effect seems to be linked to the species studied and the concentration used. Propolis toxicity has already been reported on equine sperm cells when subjected to refrigeration (Santos, 2014) and goats after cryopreservation (Castilho *et al.*, 2009). On the other hand, when used in carp sperm (*Cyprinus carpio*) in concentrations of 0.8 and 1.0 mg/ml, it was responsible for the increase in motility and fertilization capacity of sperm cells (Ögretmen *et al.*, 2014).

Through the computerized analysis by the CASA, we obtained total motility values of 78.33 ± 9.37% (range 69 - 93%) in the fresh samples without adding propolis. This result shows the good initial quality of the sperm, which is extremely important for submission to freezing; and it is in accordance with reported results on epididymal sperm in the species (43.10% to 68.75%) (Kunkitti *et al.*, 2016; Lima *et al.*, 2016; Prochowska *et al.*, 2016). The addition of propolis also kept motility in all treatment groups (interval 30 – 82%), although some samples were below the cited value.

The other kinetic variables (VAP, VCL, VSL, LIN, ALH and BCF), in samples with or without propolis, resemble the values described in the literature for sperm from the epididymis: 47.15 µm/s, 86.55 µm/s, 33.75 µm/s, 39.65%, 3.94 µm and 5.96 Hz, respectively (Lima *et al.*, 2016; Prochowska *et al.*, 2016).

The quality of sperm movement is closely related to the source of sperm recovery. This difference is notable when comparing semen with epididymal sperm. Factors such as the contact of sperm cells with seminal fluids probably have an influence on these parameters. It is known, for example, that the content of seminal plasma is directly linked to male fertility and has several functions on sperm metabolism and the fertilization process such as the activation of sperm motility, which may explain the fact that seminal samples have greater motility than those recovered from the epididymis (Guasti *et al.*, 2012; Araújo, 2014) . Thus, it is believed that propolis in the evaluated concentrations has no significant effect on parameters related to sperm motility. However, the contact of feline sperm with seminal plasma may possibly result in increased motility.

Another theory that is linked to this result involves reactive oxygen species (ROS). Although these substances in high quantities cause damage at molecular (genetic material, lipids, and proteins) and functional (oxidative stress and decreased metabolism) levels, the presence of these compounds in low amounts is linked to the sperm capacitation process such as motility hyperactivation (De Lamirande *et al.*, 1997; 2008). The semen components are responsible for the production of superoxide anion and hydrogen peroxide, both related to this process (Maia & Bicudo, 2009). However, studies are still needed to make this comparison between epididymal sperm and semen in a specific and standardized way, in order to better understand the characteristics and factors that influence this difference.

Regarding the effect of propolis after cryopreservation, all concentrations used in the media reduced the total motility. The low sperm motility after thawing in treatments containing propolis (< 16.00 ± 2.85%) may be related to the cryopreservation process and not the composition of the medium or presence of the additive, since this reduction was also observed in the control group (18.83 ± 3.78%). However, Rizk *et al.* (2014) defend that the improvement of sperm motility by the addition of propolis depends on the composition of the extender. In addition, greater sensitivity of feline epididymal sperm to cryoinjuries was inferred from this result (Hermansson & Axnér, 2007). The literature shows sperm motility values after thawing from 15.5% to 40% for cat epididymal sperm (Macente *et al.*, 2012; Buranaamnuay, 2015; Kunkitti *et al.*, 2016; Prochowska *et al.*, 2016; Brusentsev *et al.*, 2018); and 52.8% - 70.6% for semen from the same species (Platz *et al.*, 1978; Villaverde, 2007; Chatdarong *et al.*, 2010).

Sperm morphology is extremely important for sperm fertilizing ability assessment (Arruda *et al.*, 2011). All values obtained in this study are within the limits that characterize sperm samples as normozoospermic for the domestic cat species (Axnér *et al.*, 2004); although, a reduction in the percentage of morphologically normal sperm has been observed in P1 and P2 groups. The morphological changes indicate incompatibility of the process and/or freezing medium to the needs of the cells (Santos *et al.*, 2015). It is possible that smaller amounts of propolis negatively affect sperm morphology, showing a positive correlation between the presence of antioxidants and morphology, since this substance reduces the oxidation rate, inhibiting the production and the harmful effects of free radicals on sperm structures (Silva *et al.*, 2013).

In addition, the composition of epididymal fluids may have inferred the response/interaction of the sperm cell with propolis, and consequent sperm morphology. Even with the reduction in the percentage of normal sperm, P1 and P2 groups remained within the limits reported for the cryopreserved sample (40% - 54.5%) (Cocchia *et al.*, 2009; Jiménez *et al.*, 2013;

Barbosa *et al.*, 2020).

The preservation of the integrity and functionality of the plasma membrane is fundamental for the process of acquisition of hyperactivated motility and subsequent fertilization, for the mechanisms of interaction with the proteins of the pellucid zone, acrosome reaction and oocyte penetration (Gadella et al., 2001). In this study, the decline in post-thaw viability and functionality results from the physical-osmotic changes undergone by the sperm cells and the formation/dissolution of ice crystals during freezing-thawing, which can cause structural changes in the membrane, generate ruptures, or loss of osmoregulation capacity (Figueroa *et al.*, 2016). Changes in the plasma membrane also result from the lipid peroxidation, due to the overproduction of ROS in the cryopreservation process, and the interaction of these with the membrane biomolecules, especially fatty acids (Angrimani *et al.*, 2018).

The viability data did not show superiority for the samples with propolis. This result is in accordance with El-Harairy *et al.* (2018), which they reported the effect of propolis only on the increase in motility, with no improvement in the viability and integrity of biological membranes. In contrast, the best response of P1 and P2 groups to hiposmotic test, especially this latter, refers us to the choice of intermediate concentrations of propolis to inhibit ROS on biological membranes and, consequently, improve the quality of epidymal sperm. The antioxidant mechanisms of propolis involve the elimination of ROS by flavonoids, phenethyl caffeic acid and other phenolic compounds present; inhibition of xanthine oxidase activity and activation of antioxidant enzymes superoxide dismutase and catalase (El-Seadawy *et al.*, 2017; Kasiotis *et al.*, 2017; Baykalir *et al.*, 2018).

The integrity of the chromatin was maintained after thawing in all treatments. Previous studies report the excellent stability and cryoresistance of feline sperm chromatin, not distancing from the values observed in this study (Manee-In *et al.*, 2014; Kunkitti *et al.*, 2016; Barbosa *et al.*, 2020). Sperm chromatin is a cluster of DNA, RNA and proteins that are located inside the nucleus of the sperm. The degree of compaction of these molecules is important for the transmission of the genetic information to offspring. The matured sperm have compacted chromatin; however, genetic, environmental and physiological factors can lead to changes in the pattern of compaction and fragmentation of chromatin, making reproduction and/or application of biotechniques unfeasible (Courtens & Loir, 1981).

Finally, the chromatin analysis supports the hypothesis that propolis is non-toxic to domestic feline epididymal spermatozoa. Although, specific techniques for DNA integrity analysis should be performed in the future to ratify the results found in this research.

Among the 12 samples analyzed, 3 of them presented non-spermatic cells with progressive and spiral movement, which had bacillary morphology (DATA NOT PUBLISHED). Occasionally, these cells tended to group into clusters with more than 3 cell units, generating morphology similar to streptobacillus microorganisms. It is believed to be contamination from egg yolk, a product which may contain bacteria strains of *Salmonella* (Oliveira & Silva, 2000) and *Pseudomonas genus* (Mendes *et al.*, 2014). It is worth mentioning that the same egg yolk was used in the preparation of the cryoprotectant medium of the control group and the treatment groups containing propolis. However, only the control groups showed the presence of bacillary microorganisms. Thus, we believe that all treatments containing propolis had effects in reducing cell multiplication or elimination of these organisms since the treatments were added with propolis from the beginning of the preparation of the media and there are reports of the antimicrobial effect of propolis in the literature (Cedikova *et al.*, 2014).

5. Conclusion

Epididymal sperm were able to maintain good motility rates when subjected to different concentrations of green propolis extract, at room temperature. However, none of these concentrations provided superiority to the sperm parameters evaluated

after the cryopreservation process, except for the hypoxic test, in which there was a slight improvement in the percentage of functional cells.

Even though, the propolis are an interesting component to be added in the cryoprotective medium, since it is non-toxic to the sperm cell and potentially acts to inhibit the growth of contaminating microorganisms. And its antioxidant effect should be further investigated in sperm from the epididymis.

We believe that our contribution will instigate further studies regarding the use of propolis as an addictive substance to conventional thinners, aiming at the complete elucidation of the mechanism of this substance on the sperm cell. There is also the prospect of developing a product of plant origin with commercial application in the area of reproductive biotechnology. Finally, it was demonstrated the need for studies aimed at the conservation of feline gametes; we hope, therefore, the development of better protocols for gamete cryopreservation, especially aimed at those of epididymal origin, since they are more cryosensitive.

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