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Effect of cryopreservation on sperm DNA fragmentation and apoptosis rates in the testicular tissue of domestic cats

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ABSTRACT

The objectives of the present study were to evaluate the damage caused by cryopreservation on sperm DNA and estimate the percentage of cell apoptosis in tissue after thawing. Testicles of cats were sectioned into of 0.3 cm^3 and 0.5 cm^3 fragments and evaluated for DNA damage using acridine orange and semi-quantitatively through histo-morphological and immunohistochemical methods (caspase-3). Other fragments were placed in cryotubes with diluent containing either 3% glycerol or 3% propanediol, and were cryopreserved. Evaluation using acridine orange indicated there was a difference with use of propanediol and glycerol on DNA damage in 0.5 cm^3fragments, with the latter being more effective than the former for cryopreservation. Results from histomorphological evaluations indicated there was a greater cell integrity among germ cells that were not cryopreserved, based on criteria assessed (detachment of cells from basal membrane, retraction of seminiferous tubule epithelium, visibility of the spermatogonia nucleoli and nuclear spermatogonia condensation), for both sizes of fragments. The values for these variables decreased after cryopreservation, with there being no differences as a result of size of fragment stored or between cryoprotectants used ($P > 0.05$). The staining for caspase-3 differed for the cytoplasm, nuclei and germ cells. Assessment of these staining patterns indicated the fresh fragments had an amount of cell damage and there was a similar amount of damage detected in cryopreserved fragments. This finding indicated that there was considerable efficacy in preserving the tissue fragments with use of the freezing protocols that were evaluated in this study.

1. Introduction

Conservation of cryopreserved genetic material, either as gametes or as embryos, is a resource that has been investigated for a long time in relation to techniques of assisted reproduction, with a goal being the preservation of species (Pukazhenti et al., 2006; Buarpung et al., 2013). Testicles are an important source of usable genetic material, even in cases in which the male has the oligozoospermia or obstructive azoospermia conditions, or has undergone therapeutic orchiectomy, or even if the testicles are

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obtained postmortem (Wyns et al., 2008; Buarpung et al., 2012). Results of many studies in recent decades indicate sperm obtained from testicular tissue can be cryopreserved and later used by applying techniques such as intracytoplasmic sperm injection (ICSI), thus resulting in development of embryos and birth of viable offspring (Marks et al., 1994; Schlatt et al., 2002; Shinohara et al., 2002; Hori et al., 2004; Jahnukainen et al., 2007; Milazzo et al., 2008; Abrishami et al., 2010; Milazzo et al., 2010; Wu et al., 2011; Hori et al., 2011; Thuwanut et al., 2013; Buarpung et al., 2013).

Many animal species, including wild felids, are threatened with extinction. The domestic cat is an important species for developing studies and defining techniques that might come to be used in non-domesticated felids (Luvoni, 2006; Tharasanit et al., 2012). With use of the domestic cat, important results have been obtained by using testicular germ cells obtained from cryopreserved tissues (Comizzoli et al., 2006; Buarpung et al., 2012). The birth of healthy kittens has been the most notable result demonstrating maintenance of the capacity of oocytes to be fertilized after ICSI using this material (Tharasanit et al., 2012). The successful cases of embryonic development using these technologies have been few, and this may have been due to issues relating to the cryopreservation of either testicular sperm or embryos.

These results highlight the need for novel research to increase the effectiveness of cryopreservation techniques and for determination of the utility in feline species. Among the factors that require more attention are the most suitable cryoprotectants for cat testicular tissue. This knowledge is needed to ensure cell viability and prevent morphological damage caused by thermal and osmotic conditions (Watson, 2000).

Regarding the efficiency of the cryoprotectant, another important factor for ensuring good-quality tissue post-thawing is the size of the cryopreserved fragment (Crabbé et al., 1999) because each cryoprotectant has a different extent of penetration into the tissue, largely depending on its density and molecular weight (Abrishami et al., 2010). Results from a recent evaluation indicated glycerol had the most desirable efficacy for fragment penetration of 0.5 cm^3 (Macente et al., 2017). The assessments that were conducted (histomorphological analysis and thiobarbituric acid reactive substance test), however, need to be extended with use of immunohistochemical analysis.

The objective of the present study was to evaluate the damage to DNA and estimate the apoptosis rates in testicular fragments obtained from domestic cats. Values for these variables were compared between type of cryoprotectant used (3% propanediol and 3% glycerol) and the size of the testicular fragment (0.3 cm^3 and 0.5 cm^3).

2. Material and methods

The procedures used in this study were approved by the Institutional Ethics Committee on Animal Use of the Universidade Estadual Paulista (UNESP), Jaboticabal campus (Process nº 013583/14).

2.1. Testicle collection, cryopreservation and thawing

Testicles were obtained from 31 domestic cats (crossbred, 1 to 5 y of age) submitted for elective orchiectomy at the Centro de Esterilização de Cães e Gatos of the Faculdade de Ciências Agrárias e Veterinárias, UNESP, Jaboticabal campus, Brazil. The testicles were transferred into a 50 ml falcon tube containing NaCl 0.9% and the tube was placed inside an isothermal box (18 °C) and transported to the laboratory within 2 h. Testicular tissue was separated from the blood vessels, tunica albuginea and epididymides, washed three times in saline solution supplemented with streptomycin (100 μg/mL) plus penicillin (100 UI/mL) and sectioned in 0.3 cm^3 and 0.5 cm^3 sized pieces (measured with a digital pachymeter).

One fragment of each size (0.3 and 0.5 cm^3) was immediately evaluated for sperm DNA fragmentation (acridine orange) and two other fragments were fixed in Bouin solution to be further semi-quantitatively evaluated using histomorphology and immunohistochemistry (cleaved caspase-3). Details of these procedures are described subsequently in this manuscript.

The remaining 0.3 and 0.5 cm^3 fragments were placed in cryotubes with 1 mL egg yolk Tris Equex STM extender containing 3% of glycerol or 3% propanediol for 10 min at room temperature. These fragments were subsequently maintained at 4 °C for 10 min and were positioned horizontally on a rack 4 cm above the liquid nitrogen vapor (−90 °C) for 30 min, before being plunged into liquid nitrogen (−196 °C) for cooling and storage.

For thawing, the samples were exposed to air for 10 s and submerged in 37 °C water bath for 30 s. The testicular tissues were placed in TRIS extender medium at 37 °C for 5 min. Frozen-thawed fragments were also evaluated for sperm DNA fragmentation, histomorphological alterations and assessment of cleaved caspase-3.

2.2. Sperm DNA fragmentation

The evaluation of sperm DNA fragmentation in the tissues was conducted using fluorescence microscopy, utilizing the previously described protocol of Thuwanut et al. (2008). Briefly, the DNA of sperm was extracted using longitudinal and transverse slicing of the tissue fragments immersed in PBS solution at 37 °C. The solution was subsequently filtered using a 40 μm nylon mesh (BD Sterile Cell Strainer®) to isolate the sperm cells. This recovered solution was used in sperm evaluation: a small (10 μl) drop of the recovered solution was smeared onto a microscope slide, air-dried and fixed with a solution of glacial acetic acid and methanol (1:3; Carnoy's solution) overnight at room temperature. The smear was then stained using 1% acridine orange at pH 2.5 (10 mL of acridine orange at 10 mg/mL, 40 mL of 0.1 M citric acid solution, and 2.5 mL of 0.3 M Na_2HPO_4 solution) for 5 min, washed with distilled water and a cover slip was applied. The percentage of sperm cells with DNA fragmentation was determined by evaluating 200 sperm cells using a fluorescent microscope (with excitation at 450–490 nm) in 40x magnification. Sperm cells with normal, intact double-stranded DNA emitted green fluorescence and those with denatured, single-stranded DNA had red, orange or yellow fluorescence.

2.3. Histomorphological evaluation

The fresh and frozen-thawed testicular fragments were fixed in Bouin solution for 6 to 10 h at 48 °C, dehydrated in 70% etanol and subsequently embedded in paraffin wax. The samples were sectioned, stained with hematoxylin and eosin (HE) dye and semi-quantitatively evaluated for integrity and structural alterations using a light microscope. Five randomized areas of the sectioned tissue were classified based on Milazzo et al. (2008) with modifications: (i) detachment of cells from the basement membrane was scored as 0 if absent, 1 < 75% and 2 > 75% of the circumference, (ii) gap formation and shrinkage were scored as 0 if absent, as 1 if slight and as 2 if more obvious; (iii) distinction between Sertoli cells and spermatogonia nuclei was scored as 0 if easy, 1 if difficult and 2 if impossible, (iv) observation of nucleoli was scored as 0 if easy (visible in 40% of cells) and scored as 1 if indistinguishable; (v) pyknotic nuclei was scored as 0 if absent, as 1 if < 40% and as 2 if > 40% were pyknotic. The final evaluation of the epithelia of the seminiferous tubules and for the nuclei of spermatogonia and Sertoli cells were combined by evaluation of the scores from five randomized areas. The data obtained were statistically analysed and the results were reported as frequencies of each score.

2.4. Immunohistochemical evaluation

The immunohistochemical evaluation was conducted to evaluate the extent of apoptosis of intra-tubular nuclei (DNA fragmentation) using a horseradish peroxidase based (HRP) system.

Sections for use in histological analyses were initially set aside. The sections for immunohistochemical analysis, of thickness 4 μm, were then dewaxed and rehydrated. The antigen extraction was then conducted using a 10 mM citrate solution (pH 6), in a pressure cooker (Pascal®, Dako, Carpinteria, CA, EUA) for 30 min. The samples were subsequently cooled in a water bath containing ice for 20 min and were washed with deionized water. The samples then underwent a stage of blocking of exogenous peroxidase (Envision™ Flex Peroxidase-Blocking reagent, Dako, Carpinteria, CA, USA), followed by blocking of proteins (ScyTek Laboratories, Logan, UT, USA) at 27 °C for 30 min. The primary rabbit monoclonal cleaved anti-caspase 3 (Cell signalling, Massachusetts, EUA) primary antibody was then diluted at 1:300, and incubated overnight. With the next step, the samples were washed with a Tris buffer solution (pH 7.4), and a polymer system was applied as a secondary antibody (Envision, Dako, Carpinteria, CA, USA) and incubated again at 27 °C for 1 h in a dark chamber. The slides were then washed again with the Tris buffer solution and the reaction occurred with a diaminobenzidine chromogen substrate (DAB, Dako, Carpinteria, CA, USA) for 5 min. The counterstaining was performed using Harris hematoxylin for 1 min.

The positive control was performing using a normal feline lymph node and the negative control with a peptide blocking assay, using a Cleaved Caspase-3 Blocking Peptide (Asp175, Cell signaling, Massachusetts, EUA) according to the manufacturer recommendations.

The slides were evaluated using an optical microscope (Leica DM 2500) with 20x and 40x objective. The extent of the caspase-3 reaction in the samples was semi-quantified using a modified version of what was described by Walker (2006), with differences regarding the cytoplasm, nucleus and spermatozoid staining. Each of these, presence of caspase-3 in cytoplasm, nucleus and spermatozoid was classified as: 0 = negative (no caspase-3 stain), 1 = < 10% stained cells, 2 = 10–50% stained cells or 3 = > 50% stained cells.

2.5. Statistical analysis

Statistical analyses were conducted using the Statistical Analysis Systems software package (Version 9.4; SAS Institute Inc., 2014, NC, USA). The Proc-freq technique was used in the semi-quantitative analyses to obtain frequencies. These frequencies were analysed using an one-way ANOVA using the SAS GLM procedure. Probabilities and associations of characteristics were assessed through Fisher's exact test. Statistical differences for repeated measurements were evaluated using an one-way analysis of variance (ANOVA), followed by the Duncan post-test, considering the normality of the data distribution verified by the Kolmogorov-Smirnov test. The *post-hoc* Student-Newmann-Keuls test was used to assess the values for the fresh and cryopreserved samples of spermatozoids that were recovered and for the testicular tissue. The probability was considered to be significant with a $P < 0.05$.

3. Results

For the percentage of DNA damage to sperm cells that were recovered from testicular tissues stained with acridine orange, there was no significant difference between fresh fragments with either of the sizes evaluated (22% and 30% for 0.3 cm^3 and 0.5 cm^3, respectively), or between the samples that were cryopreserved with glycerol (27% for 0.3 cm^3 and 31% for 0.5 cm^3) or with propanediol (35% for 0.3 cm^3 and 47% for 0.5 cm^3). There was a difference detected for DNA damage with use of propanediol (47%) and glycerol (31%) when the tissue fragment size was 0.5 cm^3 with glycerol preserved fragments having lesser damage (Fig. 1).

There were the most desirable results in the evaluation on the integrity of seminiferous tubule epithelial coatings in the fresh samples, regarding both detachment of basal membrane cells (16.3% of the samples without alterations) and retraction of epithelium (17.39% for 0.3 cm^3 and 16.30% for 0.5 cm^3; Fig. 2). Regarding the cryopreserved samples, there were considerable alterations only for the size of 0.5 cm^3. This fragment size had the greatest frequency (13.04%) of Grade 1, with the presence of membrane detachment affecting as much as 75% of the tubular circumference and with most samples having a slight retraction, using either

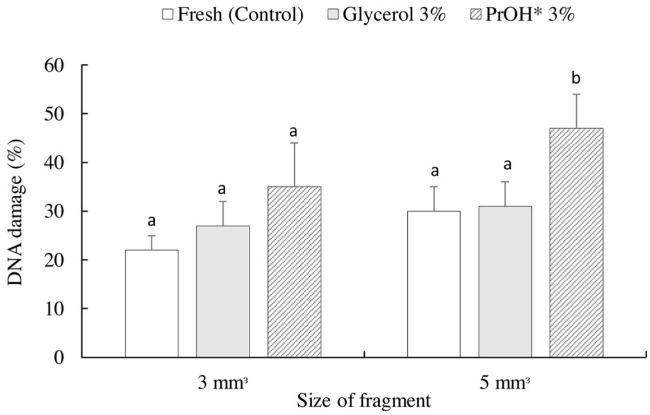

Fig. 1. Representative graphic of percentage on sperm DNA damage, recovered from testicular tissue of domestic cats, fragmented in two sizes, before and after cryopreservation, using the acridine orange stain. Different lowercase letters in the columns indicate significant difference in the Student-Newman-Keuls test ($P < 0.05$).

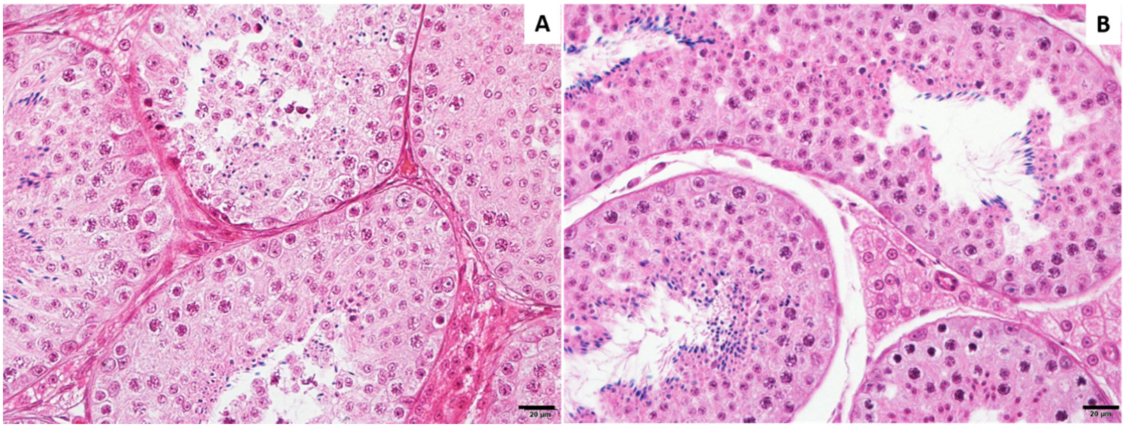

Fig. 2. Photomicrography of histological assessment of fresh integrate testicular tissue obtained from domestic cat, sectioned in 0.3 cm^3 (A) and 0.5 cm^3 (B). Note the quality of seminiferous tubular cells, with absence of detachment of basement membrane and pyknotic nucleus. Hematoxylin and eosin dye. Scale bars represents 20 µm.

glycerol or propanediol (Table 1; Figs. 3 and 4).

Regarding the evaluation of the integrity of spermatogonium nuclei, there was a difference in the visibility of the nucleoli between the fresh and cryopreserved samples, for both tissue sizes and both cryoprotectants. Most fresh samples did not have alterations (17.39% for 0.3 cm^3 and 0.5 cm^3; Fig. 2). Most of all cryopreserved samples did not have a distinguishable nucleolus (Table 2). Regarding the detection of nuclear condensation of the spermatogonia, most of the fresh samples were classified as Grade 1 (< 40%

Table 1
Histomorphology of the testicular tissue regarding frequencies of detachment of basal membrane cells and retraction of seminiferous tubule epithelium.

Size of fragment	Group	Detachment			Retraction		
		0 Absent	1 < 75%	2 < 75%	0 Absent	1 Slight	2 Obvious
0.3 cm^3	Fresh (control)	$16.30^{A,a}$	$1.09^{B,c}$	$0.00^{C,d}$	$17.39^{A,a}$	$0.00^{B,f}$	$0.00^{B,e}$
	Glycerol 3%	$3.26^{C,c}$	$8.70^{A,b}$	$4.35^{B,a}$	$3.26^{B,d}$	$6.52^{A,d}$	$6.52^{A,a}$
	PrOH* 3%	$6.52^{B,b}$	$8.70^{A,b}$	$1.09^{C,c}$	$7.61^{A,c}$	$7.61^{A,c}$	$1.09^{B,d}$
0.5 cm^3	Fresh (control)	$16.30^{A,a}$	$1.09^{B,c}$	$0.00^{C,d}$	$16.30^{A,b}$	$1.09^{B,e}$	$0.00^{C,e}$
	Glycerol 3%	$1.09^{C,e}$	$13.04^{A,a}$	$2.17^{B,b}$	$1.09^{C,e}$	$11.96^{A,a}$	$3.26^{B,c}$
	PrOH* 3%	$2.17^{B,d}$	$13.04^{A,a}$	$1.09^{C,c}$	$1.09^{C,e}$	$10.87^{A,b}$	$4.35^{B,b}$

Capital letters in the same line and lowercase letters in the same column indicate differences detected using the Student-Newman-Keuls test ($P < 0.05$).

* PrOH = propanediol.

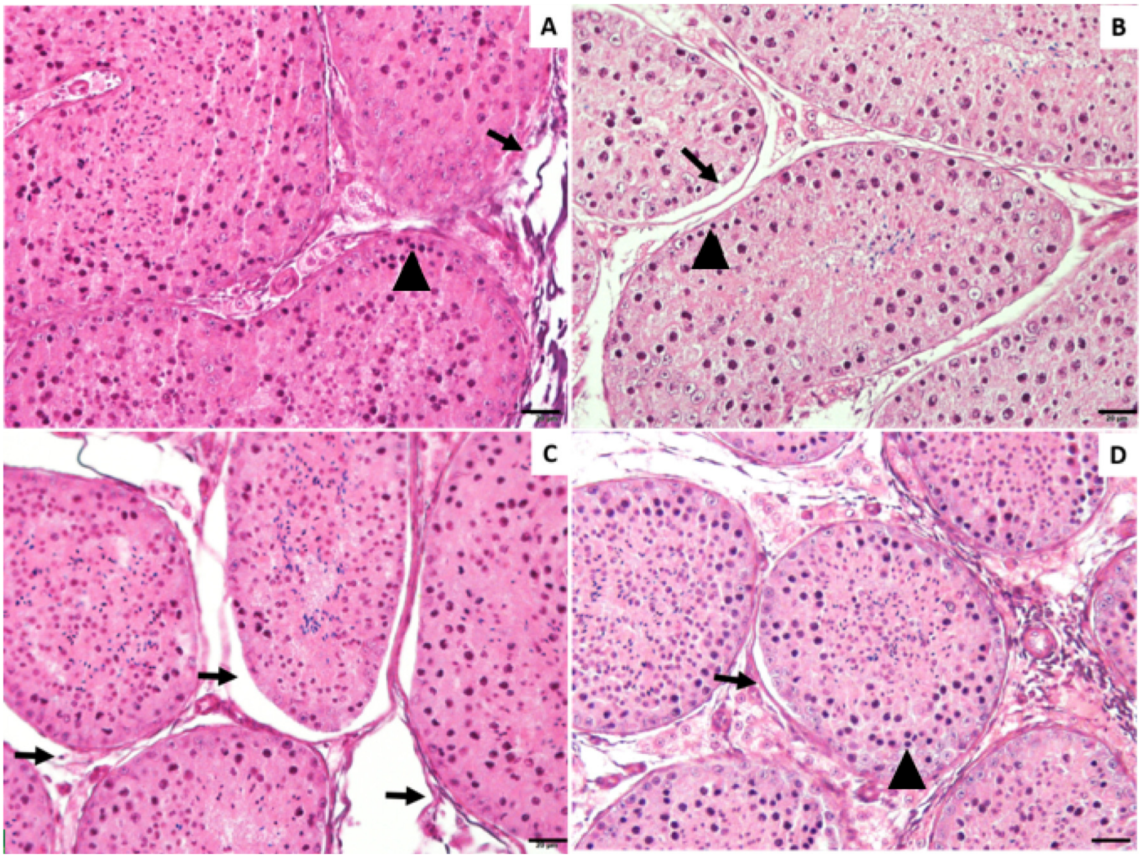

Fig. 3. Photomicrography of histological assessment of cryopreserved testicular tissue obtained from domestic cat. **A, B.** Fragment sectioned in 0.3 cm^3 cryopreserved with propanediol and glycerol, respectively. **C, D.** Fragment sectioned in 0.5 cm^3 cryopreserved with propanediol and glycerol, respectively. Presence of detachment of basement membrane (arrows) and pyknotic nucleus (arrowhead) in spermatogonia. Hematoxylin and eosin dye. Scale bars represents 20 μm.

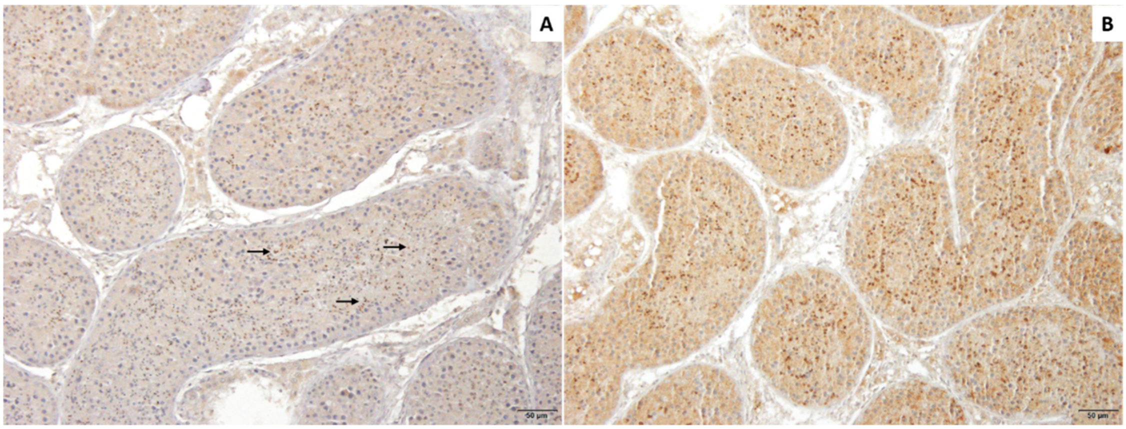

Fig. 4. Photomicrography of immunohistochemistry staining of cleaved caspase-3 in domestic cat testicular tissue. **A.** Fragment sectioned in 0.3 cm^3 cryopreserved with propanediol. Note the absence of nuclear staining in the epithelia and remarkable markings in spermatozoa (arrows). **B.** Fragment sectioned in 0.5 cm^3 cryopreserved with propanediol. Markings are intense in all structures (cytoplasm, nucleus and spermatozoa). Scale bars represents 50 μm.

cells with pyknotic nucleus). All the cryopreserved samples, however, had this alteration, and most of these cells were classified as Grade 2 (i.e., more than 40% of cells had a pyknotic nucleus; Table 2; Fig. 3).

The immunohistochemical analysis that was conducted to investigate whether there was cellular apoptosis using caspase-3 allowed for detection of differential staining between the cytoplasm and nuclei of seminiferous tubular cells, along with the staining of

Table 2
Evaluation of frequencies of histo-morphological characteristics of the nucleolus and nuclear condensation of the spermatogonia of testicular tissues of domestic cats.

Size of fragment	Group	Nucleolus visibility		Nuclear condensation		
		0 easy > 40%	1 Undistinguishable	0 Non-Pyknotic	1 < 40% Pyknotic	2 > 40% Pyknotic
0.3 cm^3	Fresh (control)	$17.39^{A,a}$	$0.00^{B,c}$	$4.35^{B,a}$	$11.96^{A,a}$	$1.09^{C,d}$
	Glycerol 3%	$0.00^{B,c}$	16.30A^{,a}	$0.00^{C,b}$	$1.09^{B,b}$	$15.22^{A,b}$
	PrOH* 3%	$2.17^{B,b}$	$14.13^{A,b}$	$0.00^{C,b}$	$1.09^{B,b}$	$15.22^{A,b}$
0.5 cm^3	Fresh (control)	$17.39^{A,a}$	$0.00^{B,c}$	$0.00^{C,b}$	$11.96^{A,a}$	$5.43^{B,c}$
	Glycerol 3%	$0.00^{B,c}$	$16.30^{A,a}$	$0.00^{B,b}$	$0.00^{B,c}$	$16.30^{A,a}$
	PrOH* 3%	$0.00^{B,c}$	$16.30^{A,a}$	$0.00^{B,b}$	$0.00^{B,c}$	$16.30^{A,a}$

Capital letters in the same line and lowercase letters in the same column indicate differences detected using the Student-Newman-Keuls test ($P < 0.05$).
* PrOH = propanediol.

spermatozoa heads (Fig. 4A), at different intensities. The most discerning cytoplasm assessments were obtained from fresh samples of 0.3 cm^3, of which 7.88% did not have any detectable staining. Most 0.5 cm^3 fresh samples (8.48%) had slight staining (Grade 1; Fig. 4B, Table 3). After cryopreservation, both fragment sizes had similar percentages of samples with caspase-3 staining. At least 10% of the cells observed in the microscopic fields evaluated (Table 3) were stained, with no particularly relevant finding for either cryoprotectant (Fig. 4).

The presence of caspase-3 staining in the nuclei of the tubular cells was observed in most samples. Between 10% and 50% of the cells were stained, both in fresh and in frozen samples, regardless of the tissue fragment size and cryoprotectant used (Table 4).

The sperm cells in the histological sections also were stained due to the presence of caspase-3, with the pattern of staining indicating as many as 50% of the spermatozoa were undergoing apoptosis (Table 5; Fig. 4A).

4. Discussion

Cryopreservation of testicular tissue is an important procedure for preservation of genetic material (Wyns et al., 2008; Buarpung et al., 2012). There are several applications of testicular tissue fragments: one can isolate spermatogonia and sperm cells that can be used in cell cultures or ICSI (Jahnukainen et al., 2007; Levron et al., 2009; Honaramooz, 2012) or the whole fragment can be transplanted in autologous or xenotransplantations (Shinohara et al., 2002; Jahnukainen et al., 2007).

For these fragments to be used after thawing, however, cryopreservation protocols must be used that allow for retention of the integrity of the DNA of seminiferous tubular cells and the structural quality of tissues (Thuwanut et al., 2013). In the present study, two cryoprotectants and different fragment sizes were evaluated regarding the effectiveness of the cryopreservation procedures for testicular tissues of domestic cats.

With regards to sperm DNA fragmentation, the use of the 0.5 cm^3 tissue fragment size resulted in most undesirable results (47% of the cells had DNA damage) when propanediol was used, and this differed from that when there was use of glycerol (31% of the cells showed DNA damage) when the same processing conditions were used. Regarding the findings for fragments measuring 0.3 cm^3, Chatdarong et al. (2016) evaluated cryopreservation of the testicular tissue of domestic cats in fragments of this size and using the

Table 3
Immunohistochemical evaluation of the seminiferous-tubule cell cytoplasm using caspase-3 staining differences for assessments of domestic cat testicular tissue.

Size	Group	Caspase-positive cytoplasm			
		0 Absent	1 < 10%	2 = 10 to 50%	3 > 50%
0.3 cm^3	Fresh (control)	$7.88^{A,a}$	$3.64^{B,c}$	$3.64^{B,c}$	$3.03^{C,d}$
	PrOH* 3%	$1.21^{C,d}$	$5.45^{A,b}$	$5.45^{A,a}$	$4.24^{B,c}$
	Glycerol 3%	$1.21^{D,d}$	$3.64^{C,c}$	$4.85^{B,b}$	$6.67^{A,a}$
0.5 cm^3	Fresh (control)	$5.45^{B,b}$	$8.48^{A,a}$	$2.42^{C,d}$	$2.42^{C,e}$
	PrOH* 3%	$2.42^{D,c}$	$3.03^{C,d}$	$3.64^{B,c}$	$6.67^{A,a}$
	Glycerol 3%	$1.21^{D,d}$	$1.82^{C,e}$	$5.45^{B,a}$	$6.06^{A,b}$

Different capital letters in the same line and lowercase letters in the same column indicate differences detected using the Student-Newman-Keuls test ($P < 0.05$).
* PrOH = propanediol.

Table 4
Immunohistochemical evaluation of the staining pattern for caspase-3 of the nucleus of seminiferous tubule cells of domestic cats.

Size	Group	Caspase-positive nucleus			
		0 absent	1 < 10%	2 = 10 to 50%	3 > 50%
$0.3\ cm^3$	Fresh (control)	$1.21^{C,a}$	$4.24^{B,b}$	$12.73^{A,b}$	$1.21^{C,d}$
	PrOH* 3%	$0.61^{D,b}$	$4.24^{B,b}$	$10.30^{A,c}$	$1.21^{C,d}$
	Glycerol 3%	$0.61^{D,b}$	$3.03^{B,b}$	$10.30^{A,c}$	$2.42^{C,b}$
$0.5\ cm^3$	Fresh (control)	$0.61^{C,b}$	$1.82^{B,d}$	$13.33^{A,a}$	$1.82^{B,c}$
	PrOH* 3%	$1.21^{D,a}$	$4.85^{B,a}$	$6.67^{A,e}$	$3.03^{C,a}$
	Glycerol 3%	$0.00^{D,c}$	$3.03^{B,c}$	$9.70^{A,d}$	$1.82^{C,c}$

Different capital letters in the same line and lowercase letters in the same column indicate differences as detected using the Student-Newman-Keuls test ($P < 0.05$).
* PrOH = propanediol.

Table 5
Immunohistochemical evaluation of spermatozoa cell staining using caspase-3 in the testicular tissue of domestic cats.

Size	Group	Caspase-positive spermatozoa			
		0 absent	1 < 10%	2 = 10 to 50%	3 > 50%
$0.3\ cm^3$	Fresh (control)	$5.49^{B,b}$	$8.54^{A,b}$	$3.05^{C,e}$	$0.61^{D,a}$
	PrOH* 3%	$0.00^{D,d}$	$9.15^{A,a}$	$6.10^{B,b}$	$0.61^{C,a}$
	Glycerol 3%	$1.22^{C,c}$	$9.15^{A,a}$	$5.49^{B,c}$	$0.61^{D,a}$
$0.5\ cm^3$	Fresh (control)	$6.10^{B,a}$	$9.15^{A,a}$	$4.27^{C,d}$	$0.00^{D,b}$
	PrOH* 3%	$1.22^{C,c}$	$5.49^{B,d}$	$7.93^{A,a}$	$0.61^{D,a}$
	Glycerol 3%	$1.22^{C,c}$	$6.71^{B,c}$	$7.32^{A,a}$	$0.00^{D,b}$

Different capital letters in the same line and lowercase letters in the same column indicate differences as detected using the Student-Newman-Keuls test ($P < 0.05$).
* PrOH = propanediol.

same diluent, but in a different concentration (5%) and with use of the two-step freezing technique and the cryoprotectants, ethylene glycol and DMSO. In this previous study, the protocol that was used did not affect the integrity of the DNA because the integrity in these samples was not different from the DNA integrity in fresh samples (Chatdarong et al., 2016). Similarly, glycerol was more effective than propanediol for cryopreservation of fragments measuring $0.5\ cm^3$, which can be explained by the difference in the rate of penetration of these two cryoprotectants into the tissue, considering the difference in the size and molecular weight of these two compounds (glycerol is larger than propanediol; Macente et al., 2017).

The results from the histomorphological evaluation in the present study indicated that in fresh samples the coating epithelial tissue was preserved while this integrity of this tissue was moderately damaged after cryopreservation. There were similar findings in the present study for the nuclear evaluation of spermatogonia, in which the fresh samples had greater tissue integrity than the cryopreserved samples. These differences in fresh and cryopreserved tissues were expected because the process of cryopreservation induces cell damage and decreases cell integrity, regardless of the technique used (Ball and Vo, 2001).

The immunohistochemical results from the present study indicate there was a marked damage to all tissue fragments. Caspase-3 is a protein that functions in the cascade of reactions of cell apoptosis. The detection of caspase-3 in the cytoplasm indicates the cell has begun the process of degradation, although the occurrence of cell death is determined by the type of nuclear staining (Earnshaw et al., 1999). Caspase-3 was detected in the cytoplasm of cells in most samples in the present study, regardless of tissue fragment size or type of cryoprotectant used, thus indicating that there was cell apoptosis at the time of initiation of processing of the fresh samples. The positive staining for nuclear caspase-3 indicates there was a protective action of the cryoprotectants because the samples in which cryoprotectants were used had the same extent of apoptosis as the fresh tissue samples.

The evaluation of samples with immunohistochemical analysis was more precise for determining the deleterious effects caused by cryopreservation than was basic histological evaluation (Negoescu et al., 1996). Unlike what was observed in a previous study (Macente et al., 2017) using immunohistochemistry, there were not any differences between the sizes of the fragments evaluated, and the cryoprotectants used for effective preservation of the integrity of the cat testicular tissues.

Based on the present results, the use of propanediol and glycerol at a 3% concentration in a diluent and stabilization at 4 °C for 10 min were both effective procedures for maintaining the integrity of seminiferous tubule cells after thawing, in fragments measuring 0.3 and $0.5\ cm^3$. It, however, would be useful to complement the present study with use of ICSI, using either gametes recovered from cryopreserved tissues or tubular seminiferous cells grown in vitro, or fragments in xenotransplants. These techniques might provide greater predictive efficacy for the accuracy of these findings.

5. Conclusion

In conclusion, the results from the present study indicate both glycerol and propanediol at a concentration of 3% provided protection against damage caused by cryopreservation for both sizes of fragments evaluated in the present study. This protection was evident in as many as 50% of testicular tissue cells. There, however, were differences in the efficacy of the cryoprotectants regarding protection capacity depending on the type of the cell within the tissue. Furthermore, testicular tissue can be damaged in a short period of time, which highlights the importance of transporting the material immediately after orchidectomy and being efficient in the processing of the samples.

Author contributions

BIM and MA have designed the study, and participated in the acquisition, interpretation of data and drafting of the manuscript. GHT, CFMM, MRT, CEFA, BPS, PHLB, EST, ROV have participated in the acquisition and interpretation of data. All authors have read and approved the final version of the manuscript.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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