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Morphological evaluation of adult domestic cat testicular biopsy after vitrification

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Research Article

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Summary

Testicular biopsies (9 mm^3) from domestic cats ($n = 10$) submitted to orchiectomy were submitted to equilibrium vitrification in the presence of ethylene glycol (EG) alone or combined with dimethylsulfoxide (DMSO) as intracellular cryoprotectants, and sucrose or trehalose as extracellular cryoprotectants. The samples were vitrified with 40% EG or 20% EG + 20% DMSO, plus 0.1 M or 0.5 M of sucrose or trehalose. The study was divided into Step 1 and Step 2. In Step 1, intratubular cells (spermatogonia, spermatids, spermatocytes, and Sertoli cells) were quantified and classified as intact or degenerated (pyknotic and/or vacuolated cells). Cryodamage of seminiferous cords was determined by spermatogonia and Sertoli cell scoring of nuclei alterations, tubular basement membrane detachment, epithelium shrinkage, and tubular measures (total area, epithelium area, larger and smaller diameter, and height of the epithelium). In Step 2, Hoechst 33342 stain and propidium iodide (PI) fluorescent stain were used to assess the cell viability of the four best experimental groups in Step 1. The effect of treatments on all analyses was accessed using analysis of variance (ANOVA), and Fisher's post hoc test at $P < 0.05$ significance was considered. In Step 1, the mean percentage of spermatogonia and Sertoli cells morphological integrity did not show a difference when using both sugars at different concentrations, but their morphology was more affected when DMSO was used. EG use associated with 0.1 M of sucrose or trehalose positively affected spermatocyte and spermatid morphology, respectively. The larger diameter and epithelium height of seminiferous tubules were increased using DMSO plus 0.5 M sucrose and DMSO plus 0.1 M trehalose. The changes in spermatogonial/Sertoli nucleoli visualization were best scored in the EG groups, while the nuclei condensation was lower with sucrose. The basement membrane was satisfactorily preserved with 0.1 M sucrose. In Step 2, the percentage of cell viability was higher when EG plus 0.1 M sucrose was used. Therefore, DMSO's negative effect on the vitrification of testicular biopsies of adult domestic cats was evident. The EG plus 0.1 M of sucrose or trehalose associations are the most suitable CPAs to preserve the testicular histology structure of adult domestic cats in vitrification.

Introduction

Improving reproductive techniques to preserve genetic material from endangered wild cats is needed due to the number of feline species on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Endangered Species. Worldwide, there are 38 endangered feline species, and only seven species are not in danger of extinction (International Union for Conservation of Nature and Natural Resources, 2020). The most obvious example is the domestic cat (*Felis catus*). The phylogenetic proximity between domestic and wild felids, allows their use as an experimental model for the development of procedures to preserve reproductive cells (Sowińska, 2021). In reproductively ageing males, cryopreservation of testis biopsies appears to be the only alternative to preserve the genetic potential of animals that unexpectedly die (Comizzoli and Wildt, 2013). The mature testicular tissue allows access to all stages of the sperm cycle, which would be *in vitro* cultured or grafted by xenotransplant to produce viable cells for assisted reproduction in the future (Abrishami *et al*., 2010; Comizzoli, 2015).

Testicular biopsy equilibrium vitrification is a potential technique for cryopreservation due to its low cost and easy operability (Amorim *et al*., 2011). This technique has already been described in humans (Dumont *et al*., 2016), rats (Radaelli *et al*., 2017), fish (Higaki *et al*., 2017), pigs (Abrishami *et al*., 2010), collared peccary (da Silva *et al*., 2019) and in prepubertal cats, solid-surface vitrification with 20% dimethylsulfoxide (DMSO), 20% glycerol (GLY) and 24% ethylene glycol (EG) was evaluated (Lima *et al*., 2017a; Lima *et al*., 2018). However, the method needs to be optimized for mature cats.

EG and DMSO are the most commonly used intracellular cryoprotectants in equilibrium vitrification. They are small molecules that enter the cell and bind with water molecules, limiting the amount of intracellular and extracellular water, which protects intracellular organelles (Prentice and Anzar, 2010). DMSO, as the only permeable cryoprotectant, can be toxic at high concentrations (Gurtovenko and Anwar, 2007). Conversely, EG reduces the time of the tissue permeabilization process and is a good alternative for the vitrification of human adult testicular biopsy (Unni *et al.*, 2012) and epididymal sperm of adult domestic cats (Lima *et al.*, 2021). Therefore, the association of both CPAs can be an alternative to increase total penetration and reduce the harmful effects of vitrification (Baert *et al.*, 2012; Poels *et al.*, 2012).

As extracellular cryoprotectants, sucrose and trehalose are two isomers of a non-reducing disaccharide that can balance the extracellular and intracellular osmotic pressure during vitrification and devitrification, playing a role in cell membrane stabilization (Pan *et al.*, 2017). Studies indicate they can interact directly with membrane lipids and proteins, changing their phase transition behaviour and hydration status (Crowe *et al.*, 1988). Therefore, sugars optimize the vitreous transition and increase cell viability (Tian *et al.*, 2015). Trehalose preserves cell viability and reduces oxidative stress by vitrification in bovine calf testicular tissue (Zhang *et al.*, 2015), and sucrose was successfully used in the vitrification of testicular tissue in prepubertal cats, maintaining the proliferative potential of cells (Lima *et al.*, 2017b).

In the present study, we evaluated the effect of EG (40%) alone, associated with DMSO (20% EG + 20% DMSO), combined or not with sucrose (0.1 M or 0.5 M) or trehalose (0.1 M or 0.5 M) on the vitrification of parenchymal testicular tissue in adult domestic cats.

Materials and methods

Chemicals

Unless stated otherwise, all chemicals and media used in this study were purchased from Sigma Chemical Company 43 (St. Louis, MO, USA).

Tissue collection

Ten mixed-breed adult cats ($n = 10$), aged 1–5 years and weighing 2.5–4 kg, were sterilized by elective orchiectomy in the Veterinary Hospital School, as requested and authorized by their owners. Before the surgical procedure, a clinical examination was performed to detect the animal's health status, confirmed by a complete blood count and biochemical profile, and the physical examination of the reproductive tract. The evaluation of each male considered testicle consistency, shape, mobility, symmetry, and biometrics (height, length, width, and volume). The cats were submitted to an open bilateral orchiectomy, after which the tests were transported to the laboratory within 30 min in saline solution (0.9% NaCl) at 20°C.

Experimental design

The testicles of five males were divided longitudinally into 18 fragments of 9 mm^3. The testicular pieces were allocated to the control or vitrified groups in duplicate. As a control, two samples of each animal were immediately fixed in Davidson fixative for 24 h for histological analysis. The remaining fragments were exposed for 3 min at 20°C to the different equilibrium solutions prepared in phenol red-free RPMI-1640 as the base medium (Brito *et al.*, 2016), as follows: 20% EG or 10% EG + 10% DMSO, either combined with sucrose (0.1 or 0.5 M) or trehalose (0.1 or 0.5 M); two fragments per treatment. Subsequently, the tissues were exposed for 2 min at 20°C to the different vitrification solutions, as follows: 40% EG or 20% EG + 20% DMSO, either combined with sucrose (0.1 or 0.5 M) or trehalose (0.1 or 0.5 M); two fragments per treatment. Vitrification was performed using the solid-surface method, as previously described (Santos *et al.*, 2007), and tissues were stored in liquid nitrogen (−196°C). After 1 week of storage, the fragments were exposed to room temperature (~25°C) for 30 s, followed by 1 min in the water bath (37°C) and three subsequent washing steps of 3, 5, and 7 min in RPMI-1640 medium added with decreasing concentrations of sucrose or trehalose, according to the vitrification solution, i.e. 0.05, 0.025, and 0 M for groups vitrified with 0.1 M, and 0.25, 0.125, and 0 for groups vitrified with 0.5 M (Figure 1). After warming, all the fragments were immediately fixed in Davidson fixative and processed for routine histological analysis. The morphological analysis included germ and Sertoli cells integrity, spermatogonia/Sertoli cells nuclei, tubular epithelium score scale, and tubular component morphometry (Figure 2). For this, images were captured with a digital camera (DS-Ri2, Nikon Corporation, Tokyo, Japan) coupled to a light microscope (Eclipse Ni-U, Nikon Corporation, Tokyo, Japan), and measurements were performed with the aid of Nis Elements AR software (4.50.00 64 bit, version 1991–2015, Nikon Corporation, Tokyo, Japan). In total, 30 tubules were evaluated per experimental group.

In experiment 2, the testes of five males were divided longitudinally into 10 fragments of 9 mm^3, which were randomly divided in Duplo into control or vitrified groups. Two samples from each animal were submitted to mechanical dissociation of testicular cells as a control. In brief, the two fragments were presented to successive cuts into a Petri dish and recovered by suspension in 1 ml of RPMI medium. The solution was transferred to a Falcon tube, and 20 aliquots were pipetted. After that, a cell strainer (40 µm) was used to recover the isolated cells. The samples were centrifuged at 1000 rpm for 5 min, and 150 µl of the cell suspension was incubated with 2.5 µl of Hoechst-33342 stain (0.5 µg/ml) and 1 µl propidium iodide (PI) (2 µg/ml) for 30 min in a dark room (Lima *et al.*, 2017b). After incubation, 10 µl of each sample was evaluated, and 200 cells were counted using an epifluorescence microscope (Eclipse Ni-U, Nikon Corporation, Tokyo, Japan) at ×400 magnification. The emitted fluorescence signals of Hoechst stain and PI were collected at 380/535 and 555/645 nm, respectively. Cells were classified as non-viable if stained positively with PI. The remaining fragments were exposed for 3 min at 20°C to the different equilibrium solutions prepared in phenol red-free RPMI 1640 as the base medium (Brito *et al.*, 2016), as follows: 20% EG either combined with sucrose (0.1 or 0.5 M) or trehalose (0.1 or 0.5 M); two fragments per treatment. Subsequently, the tissues were exposed for 2 min at 20°C to the different vitrification solutions: 40% EG combined with sucrose (0.1 or 0.5 M) or trehalose (0.1 or 0.5 M); two fragments per treatment. Vitrification and thawing were performed as described in experiment 1. After warming, all the fragments were immediately submitted to the same procedure as the control fragments, and cell viability was assessed.

Statistical analysis

The data are expressed as mean ± standard deviation (SD) and analyzed by the Statview 5.0 program (SAS Institute Inc., Cary, NC, USA). All data were submitted to the Kolmogorov–Smirnov test

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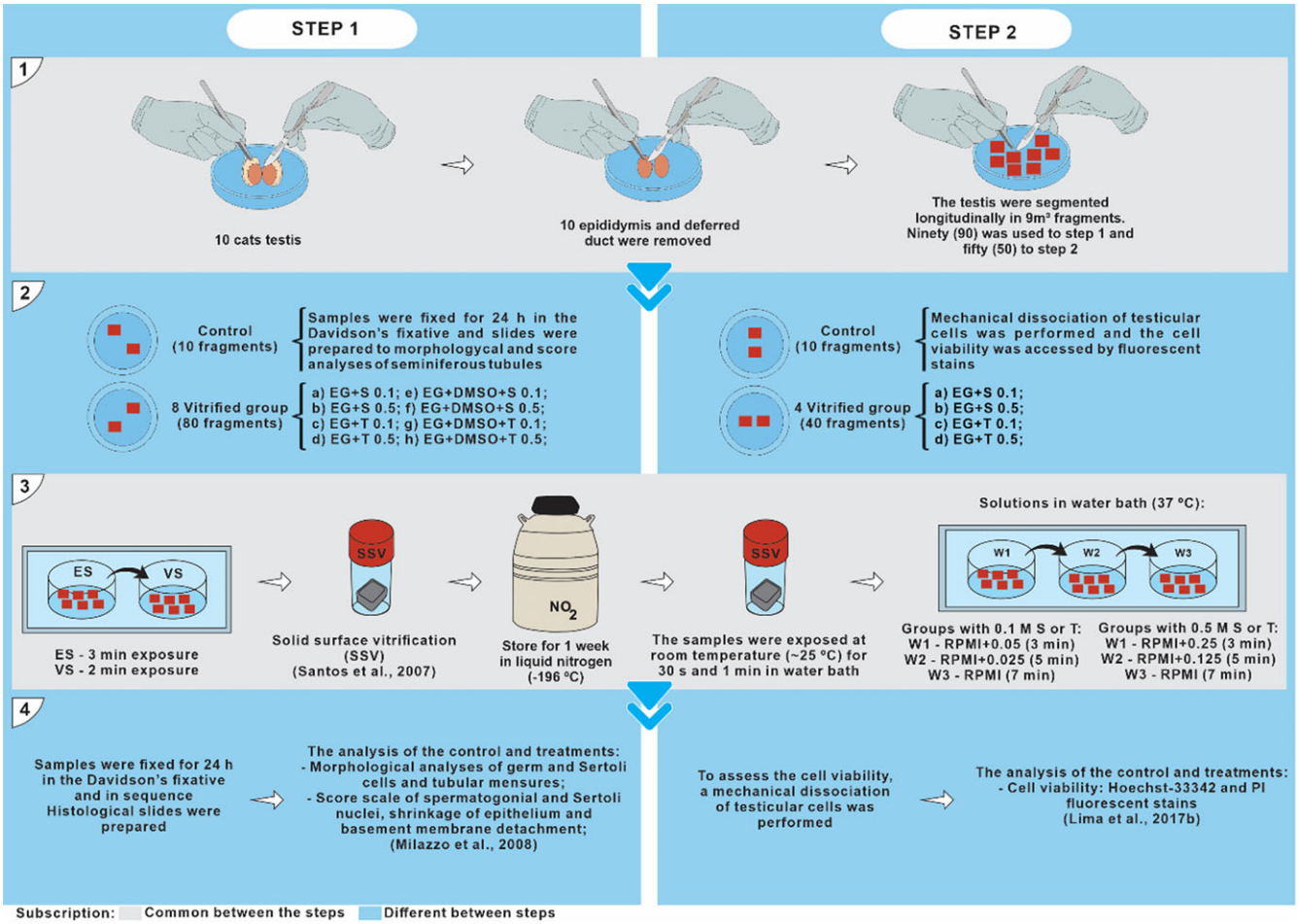

Figure 1. Experimental design. Performance of testis fragment recovery tests for Steps 1 and 2 (1). Separation of experimental groups and control (2). Exposure of equilibrium solution (ES) and vitrification solution (VS), vitrification process and exposure to warming bath with warming solutions (3). Testis fragments after warming processing and analysis (4).

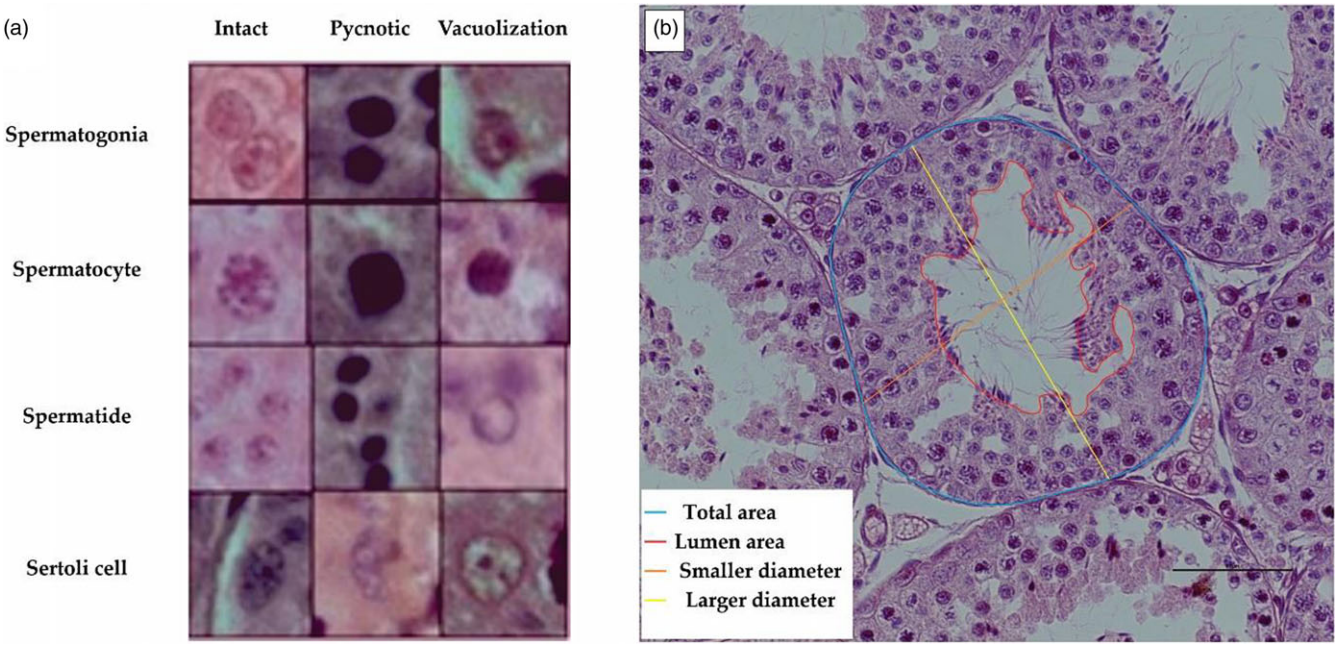

Figure 2. Germ and Sertoli cells are classified as intact, pyknotic or vacuolization (A). The blue line is the measure of total area, red line is the lumen area, larger and smaller diameters are represented for the yellow and orange lines, respectively (B). Scale bar represents 50 µm.

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Table 1. Percentage of intact germ and Sertoli cells before and after cat testicular parenchyma vitrification

Treatments	Spermatogonia (%)	Spermatocytes (%)	Spermatids (%)	Sertoli cells (%)
C	89 ± 1.7[a]	92.4 ± 1.6[a]	93.4 ± 1.9[a]	85.8 ± 7.2[a]
EF + 0.1 M S	52.6 ± 6.7[b]	29.6 ± 7.1[b]	30.6 ± 3.8[b,c]	58.2 ± 11.5[b,c]
EF + 0.5 M S	52.6 ± 5[b]	17.8 ± 8.2[c]	21 ± 1.7[c]	58.2 ± 10.9[b,c]
EF + 0.1 M T	58.8 ± 4.3[b]	28.6 ± 3.2[b]	33.6 ± 8.3[b]	66.6 ± 4[b]
EF + 0.5 M T	53.8 ± 5.9[b]	23.2 ± 8.4[b,c]	29 ± 7[b,c,d]	61.4 ± .7[b]
EF + DMSO + 0.1 M S	38 ± 6.9[c]	19.6 ± 8.9[c]	19 ± 7.5[d]	50.6 ± 6.5[c]
EF + DMSO + 0.5 M S	35.6 ± 3.3[c,d]	23.8 ± 3.2[b,c]	25.4 ± 8[b,c,d]	37.2 ± 8.3[e]
EF + DMSO + 0.1 M T	31.2 ± 5.2[c,d]	23 ± 10.3[b,c]	33.4 ± 5.5[b]	48.6 ± 10[c,d]
EF + DMSO + 0.5 M T	29.8 ± 71[d]	25.2 ± 8.2[b,c]	28.2 ± 6.3[b,c,d]	39.4 ± 7.5[d,e]

[a–d]Different superscripts indicate significant differences within the same row ($P < 0.05$).
C: Control; DMSO: dimethylsulfoxide; EG: ethylene glycol; S: sucrose; T: trehalose.

and underwent a logarithmic transformation. The effect of vitrification on the tubular component measures, percentage of typical germ and Sertoli cells, score scale, and cell viability was assessed by analysis of variance (ANOVA), and differences were located with Fisher's protected least significant difference (PLSD) post hoc test. A P-value < 0.05 was considered statistically significant.

Results

All males showed testicles with regular consistency, a round shape, free mobility in the scrotum, and normal symmetry.

After vitrification, the percentage of all intact morphological germ and Sertoli cells was statistically different from the control group (Table 1). In the sequence, spermatogonial cells were best preserved in the EG groups when compared with the treatments with DMSO, independent of disaccharides and their concentrations tested ($P < 0.0001$; Table 1). In the second cell type analyzed, spermatocytes were more preserved morphologically in vitrification, with EG associated with smaller sucrose ($P = 0.0075$) and trehalose ($P = 0.0092$) concentrations (Figure 3). In contrast, the highest sucrose concentration plus EG ($P = 0.0092$). Its small concentration plus DMSO ($P = 0.0305$) showed the most deleterious effect (Table 1).

In its turn, spermatid integrity had better results, associating 0.1 M of trehalose with EG ($P = 0.0087$) or EG + DMSO ($P = 0.0031$). In addition, the higher concentration of sucrose plus EG showed a harmful effect on them ($P = 0.0414$; Figure 3). At last, Sertoli cells were susceptible to the DMSO vitrification solutions. In addition, EF + trehalose (0.1 and 0.5 M) solutions resulted in better preservation (Table 3).

The means of total tubule area and area of seminiferous epithelium did not differ from the control group. Nevertheless, the highest concentration of sucrose in the DMSO-associated groups has a discrete influence in increasing these measures (Table 2). For the larger diameter of the seminiferous tubules, using EF + DMSO with 0.5 M sucrose showed a high increase in this measure ($P = 0.0112$; Table 2). Smaller diameters have no statistical difference among the groups (Table 2). To seminiferous epithelium height, the DMSO associated with 0.1 M trehalose increases this measurement ($P = 0.0405$; Table 2).

The control group differed statistically from all experimental groups at scale score parameters ($P = 0.0001$) (Table 3). There was no difference between the treatments at the spermatogonia and Sertoli cell nuclei distinction score. For nucleoli visualization, both EG groups with 0.1 M or 0.5 M sucrose and 0.1 M trehalose were best scored when compared with others ($P = 0.0009$). These groups still performed at the nuclei condensation score ($P = 0.0073$) (Table 3). At basement membrane detachment and epithelium shrinkage, the EG plus 0.1 M group was best rated ($P = 0.046$; $P = 0.0006$).

The four EG experimental groups were submitted to Step 2. The control group differed from all treatments except for the EG plus 0.1 M sucrose group ($P = 0.225$). In addition, the percentage of viable cells (Hoechst +) in this group was increased when compared with the other groups ($P = 0.005$; Figure 4).

Discussion

All the associations of intracellular and extracellular cryoprotectants were able to somewhat preserve the morphological integrity of seminiferous tubules. Nevertheless, generally, the groups with DMSO were inferior in preserving germinative cell morphologies. In the groups with EG, the recovery of spermatogonia with viable morphology was higher than in the associated groups, in agreement with previously described results that 40% EG was the best result for immature mouse spermatogonial viability (Gouk *et al.*, 2011). These results could be due to EG's high penetrability and perfusion, which minimize the deleterious effects on testicular cells (Unni *et al.*, 2012).

The EF + DMSO association revealed that DMSO is deleterious to cells when used in high concentrations on adult cat testicular biopsies. Nevertheless, DMSO is an effective cryoprotectant associated with glycerol for immature cat testicular tissue when analyzed by similar spermatogonia/Sertoli cells and basement membrane scores (Lima *et al.*, 2017a). Therefore, EG showed better results for adult mouse testicular tissue (Unni *et al.*, 2012), which justified the most outstanding results of this study. The same protective effect of EG is observed on Sertoli cells, which represents an important tool to support spermatogonia development (Ning *et al.*, 2012).

Therefore, it was evident that high concentrations of DMSO induced pore formation in the phospholipid membrane (Gurtovenko and Anwar, 2007) and affected the epigenetic regulatory system by altering the methylation profile of DNA (Iwatani *et al.*, 2006). However, at low concentrations, DMSO induces membrane thinning and increases the fluidity of the

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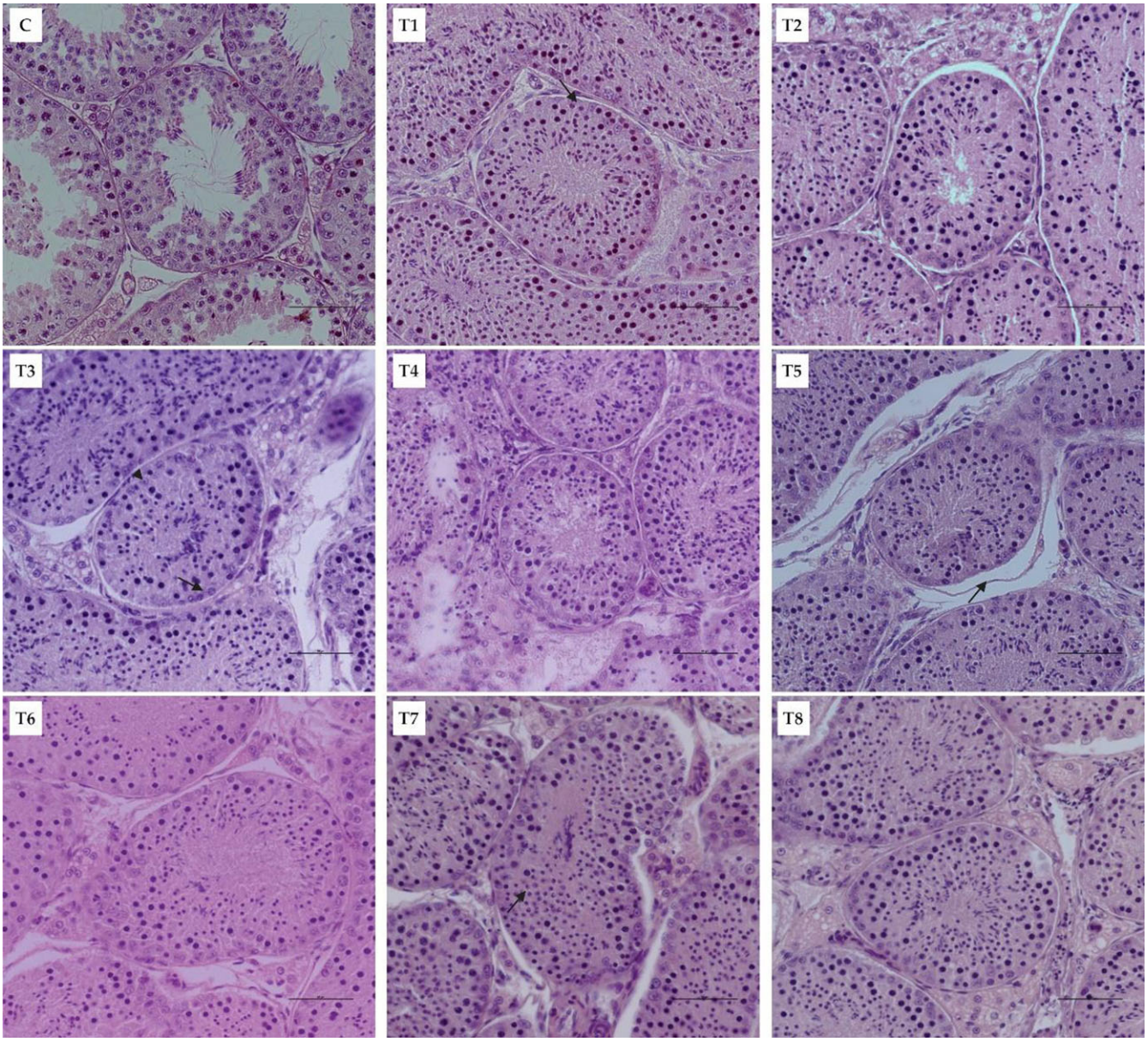

Figure 3. Histological sections of seminiferous tubules of domestic cat stained with haematoxylin and eosin (H&E). All analyses were made at ×400 magnification. Ethylene glycol (EG); dimethylsulfoxide (DMSO); sucrose (S), trehalose (T). Control group (C); EF + 0.1 M S (T1); EF + 0.5 M S (T2); EF + 0.1 M T (T3); EF + 0.5 M T (T4); EF + DMSO+0.1 M S (T5); EF + DMSO + 0.5 M S (T6); EF + DMSO + 0.1 M T (T7); EF + DMSO + 0.5 M T (T8). For Figure T1, the arrow shows normal spermatogonia after vitrification. For Figure T3, the arrow shows normal spermatocytes, and the arrowhead shows a normal Sertoli cell. For Figure T5, the arrow shows tubular basement membrane detachment. For Figure T7, the arrow shows normal spermatids. Scale bars represent 50 µm.

membrane's hydrophobic core (Gurtovenko and Anwar, 2007). This mechanism justifies the positive results of slow freezing (Curaba *et al.*, 2011; Pukazhenthi *et al.*, 2015), but the deleterious effect of using DMSO on the vitrification of mature testicular tissue (Hajiaghalou *et al.*, 2016; da Silva *et al.*, 2019) and sperm cells (Xu *et al.*, 2014). Probably, the concentration of 20% of DMSO in the vitrification solution was enough to induce membrane pores formation and increase the occurrence of membrane rupture, as evidenced by the epithelium score analyses of this study. Based on that, the absence of experimental groups containing only DMSO is explained by the substantial toxicity caused when used at high concentrations.

The percentage of morphologically viable spermatocytes in domestic cats was better in the EG group associated with 0.1 M sucrose or trehalose. However, when associated with 0.5 M sucrose, the effect was deleterious, which could show that the protective effect of sucrose as a nonpermeable CPA was not cumulative, and perhaps in higher concentrations led to unequal dehydration of the cells (Sum *et al.*, 2003). Therefore, the present results were confirmed when the percentage of cell viability was higher at 0.1 M sucrose when compared with other concentrations of sugars, closer to some results that show that sucrose at 210 mmol is more efficient when compared with other smaller and higher concentrations in the vitrification of spermatogonia stem cells in pigs (Pan *et al.*, 2017). In

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Table 2. Tubular measurements and epithelium height before and after testicular tissue vitrification of adult tomcats

Groups	Tubular area (μ^2)	Seminiferous epithelium (μ^2)	Larger diameter (μ)	Smaller diameter (μ)	Epithelium height (μ)
C	25,170.6 + 5872	22,435.6 + 5715.1	200.4 + 19.6^{a,b}	154 + 24.2	59.8 + 11.1^{a,b}
EF + 0.1 M S	20,965.2 + 7 42.4	20,329 + 7573.8	180,2 + 36.5^{b}	139.4 + 29.4	64.9 + 16.5^{a,b}
EF + 0.5 M S	21,494.4 + 3722.1	19,737.4 + 3166.6	197.2 + 18.9^{a,b}	132.6 + 19.1	56.9 + 6.2^{b}
EF + 0.1 M T	23,914.2 + 5828.1	22,722.6 + 5632.4	207.8 + 33.7^{a,b}	134.8 + 18.6	65.6 + 13^{a,b}
EF + 0.5 M T	18,977 + 5106.3	18,381.8 + 5490	177.2 + 20.7^{b}	131.4 + 20.3	64.4 + 13.6^{a,b}
EF + DMSO + 0.1 M S	23,016.2 + 6645.4	22,187.8 + 6490.7	197.8 + 34.5^{a,b}	141.2 + 21.9	67.9 + 15.2^{a,b}
EF + DMSO + 0.5 M S	21,637 + 7520.2	20,860.3 + 7006.5	236.2 + 54.3^{a}	142.2 + 31.6	72.3 + 11.1^{a,b}
EF + DMSO + 0.1 M T	22,006.8 + 3599.4	21,326.6 + 3204.6	204.4 + 11.7^{a,b}	124 + 30.1	73.6 + 8.6^{a}
EF + DMSO + 0.5 M T	18,525.2 + 4687.9	17,643.5 + 4390.9	193 + 44.2^{b}	134.6 + 25.8	67 + 13.5^{ab}

^{a,b}Different superscripts indicate significant differences within the same column ($P < 0.05$).
C: Control group; DMSO: Dimethylsulfoxide; EG: Ethylene glycol; S: Sucrose; T: Trehalose.

Table 3. Score scale of cat testicular seminiferous tubules

	Control	EF + S0.1 M	EF + S0.5 M	EF + T0.1 M	EF + T0.5 M	EG/DMSO + S0.1 M	EG/DMSO + S0.5 M	EG/DMSO + T 0.1 M	EG/DMSO + T 0.5 M
Spermatogonia/Sertoli distinction	0.03 + 0.03^{a}	1.2 + 0.19^{b}	1.2 + 0.23^{b}	1.2 + 0.08^{b}	1.4 + 0.15^{b}	1.4 + 0.06^{b}	1.2 + 0.1^{b}	1.4 + 0.15^{b}	1.4 + 0.18^{b}
Nucleoli visualization	0^{a}	0.5 + 0.14^{b}	0.7 + 0.12^{b}	0.7 + 0.11^{b}	0.9 + 0.04^{c}	1 + 0.03^{c}	0.9 + 0.04^{c}	1 + 0.03^{c}	1 + 0^{c}
Nuclear condensation	0.2 + 0.08^{a}	0.8 + 0.2^{b}	0.7 + 0.16^{b}	0.9 + 0.06^{b,c}	1 + 0.07^{c}	1.2 + 0.1^{c}	1.1 + 0.07^{c}	1.2 + 0.16^{c}	1.1 + 0.07^{c}
Basal membrane detachment	0^{a}	1 + 0.4^{b}	1.5 + 0.3^{b,c}	2 + 0.2^{c}	2.2 + 0.2^{c}	1.9 + 0.3^{c}	2.2 + 0.2^{c}	1.9 + 0.3^{c}	2.2 + 0.2^{c}
Shrinkage of epithelium	0.3 + 0.1^{a}	1 + 0.3^{b}	2 + 0.3^{c}	2.2 + 0.2^{c}	2 + 0.3^{c}	2.5 + 0.1^{c}	2.3 + 0.1^{c}	2.5 + 0.3^{c}	2.5 + 0.2^{c}

$^{a–c}$Different superscripts indicate significant differences within the same row ($P < 0.05$).
DMSO: dimethylsulfoxide; EG: ethylene glycol; S: sucrose; T: trehalose.

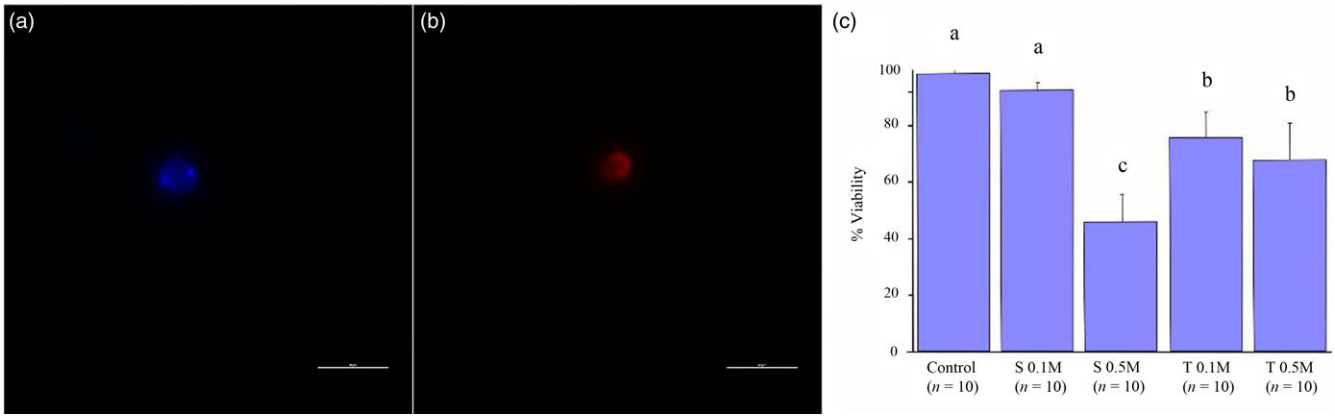

Figure 4. Cell viability accessed by Hoechst-33342 stain and propidium iodide (PI) fluorescence dyes for EG groups at Step 2. (A) Tubular isolated cell emitting fluorescence by Hoechst-33342 stain. (B) Cat testicular cell emitting propidium iodide (PI) stain. (C) Graphic representation of isolated cell viability percentage before and after vitrification process. The columns show the mean and standard deviation of the mean of each experimental group. Sucrose (S); Trehalose (T). Scale bars represents = 50 µm. $^{a-c}$Differences indicate significant differences among treatments ($P < 0.05$).

addition, the vitrification of the cat epididymal tail shows great preservation of sperm integrity with EG and 0.1 M sucrose vitrification (Lima *et al.*, 2021). Unfortunately, the studies on concentrations of sugars in cat testicular biopsies for vitrification are limited, which shows the importance of this research.

Concerning spermatids, the indifference between the use of EG or EG + DMSO with trehalose maybe because this disaccharide has the flexibility to expand and contract its glucose rings and adjust to the proper dimensions between the lipid headgroups, minimizing the effects of the intracellular cryoprotectant (Sum *et al.*, 2003). The sucrose density is highest in the middle of the aqueous region of the membrane. In contrast, trehalose exhibits a uniform distribution throughout the aqueous region (Sum *et al.*, 2003).

The height of the germinal epithelium of adult domestic cats in reproductive age can vary from approximately 55 µm (Hoshino *et al.*, 2002) 67–78 µm (Diagone *et al.*, 2012), reaching up to 81 µm (França and Godinho, 2003). The association of DMSO with 0.1 M trehalose increases the testicular epithelial height after vitrification, which may be related to the alteration in cell-to-cell junctions and swelling caused by DMSO on testicular tissue (da Silva *et al.*, 2019).

For the larger tubular diameter, although the group with EG + DMSO and trehalose at 0.5 M has shown higher values, these are within the usual range for domestic cat tubular measurements (Hoshino *et al.*, 2002; França and Godinho, 2003). The tubular area, epithelium area, and smaller diameter also agree with normospermic domestic cats (Neubauer *et al.*, 2004). Therefore, vitrification does not significantly influence the occurrence of tubular retraction due to cold.

In conclusion, Although all cryoprotectant associations were effective to some degree in preserving the morphological parameters of domestic cat testicular tissue and germ and Sertoli cell integrity. The DMSO-associated groups showed more damage when compared with the EG-associated groups. In addition, the 0.1 M concentration of sucrose was more efficient at recovering the most significant percentage of viable cells after vitrification.

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Competing interests. All the authors have no conflict of interest.

Ethical approval. The Research Ethics Committee of the Federal University of Pará approved the study (protocol number: 2890040516).

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