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Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants

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ABSTRACT

This study aimed to evaluate different vitrification methods using distinct cryoprotectants (CPAs) for the preservation of collared peccary ovarian preantral follicles (PFs). Ovarian pairs from six females were fragmented and three fragments (fresh control group) were immediately evaluated for morphology, viability, cell proliferation capacity (assessed by quantifying the number of argyrophilic nucleolus organizer regions – NORs), and apoptosis (by the identification of activated caspase-3 expression). The remaining 18 fragments were vitrified using the solid surface vitrification (SSV) method or the ovarian tissue cryosystem (OTC) with 3 M ethylene glycol (EG), 3 M dimethylsulfoxide (DMSO), or a combination of the two (1.5 M EG/1.5 M DMSO). After two weeks, samples were rewarmed and evaluated as described previously. The OTC with any of the CPAs provided a similar conservation of morphologically normal PFs as the fresh control group (75.6 ± 8.6%); however, the SSV was only efficient with DMSO alone (63.9 ± 7.6%). Regarding the viability or cell proliferation, all tested groups provided post rewarming values similar to those observed for the fresh control group, 84.0 ± 2.9% viable cells with 2.0 ± 0.2 NORs. Related to apoptosis analysis, only the OTC with EG (46.7%) and the SSV method with EG (43.4%) or the combination of EG and DMSO (33.4%) provided similar values to those found for the fresh control group (36.7%). Our findings indicate the utilization of a closed system, the OTC, with 3 M EG as the CPA for the vitrification of collared peccary ovarian tissue.

1. Introduction

Even if it is globally considered a stable population, poaching and deforestation are decreasing the population of the collared peccary (*Pecari tajacu*), with this species already extinct in some biomes [22]. Due to its ecological importance as seed dispersers [35] and as part of the food chain for great carnivores [17], there is a great need for the development of conservation strategies for this species. Besides the conservation of the collared peccary in its natural habitats or even under captivity, the formation of biobanks arises as an alternative to safeguard its valuable biological material [42].

Numerous attempts regarding the establishment of protocols for male germplasm conservation have been conducted [33,43]. For the female, however, a recent study proposed that the use of the solid surface vitrification (SSV) method using 3 M ethylene glycol (EG) as the cryoprotectant (CPA) provided effective conservation of the morphology and viability of peccary ovarian preantral follicles (PFs) [31]. Thus, detailed analysis of PFs is required because these are controversial findings indicating that vitrification may result in the emergence of apoptosis [26,40] or not [2].

Despite being used for the preservation of the female germplasm of domestic [13] and wild [37,38] species, SSV is considered an open

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system that allows contact between the sample and nitrogen vapors, which can exposure tissues to cryogenic resistant pathogens [24]. The ovarian tissue cryosystem (OTC) is an alternative closed system that has been proven to efficiently preserve ovarian tissue derived from goats [20], sheep [5], and cats [11].

Regardless of the vitrification method used, high concentrations of CPAs and cooling rates are required, which can cause fatal cell damage and osmotic stress [45]. To reverse this problem, a combination of two different types of CPAs has been proposed as being less toxic and more effective than their isolated use, as demonstrated by the use of a combination of dimethyl sulfoxide (DMSO) and EG for ovary preservation in swine [34], which is a domestic species that is closely related to the peccary [16].

The aim of the present study was to compare different vitrification methods (SSV and OTC) associated with the use of different CPAs (EG and DMSO), alone or in combination, on the morphology, viability, cell proliferation, and expression of markers for apoptosis in peccary ovarian PFs.

2. Material and methods

The Ethics Committee of Universidade Federal Rural do Semi-Árido (UFERSA) approved the experimental protocols as well as the animal care procedures used in the present study (Process no. 23091.006525/2016–82). The study was authorized by the Chico Mendes Institute for Biodiversity (SISBio Process no. 37329). All chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Animals and the collection of ovaries

Ovaries were obtained from six mature females, aged 2 years old and weighing 22 kg, from the Center for Multiplication of Wild Animals from UFERSA (Mossoró, RN, Brazil; 5°10′ S, 37°10′ W). The females were subjected to the programmed slaughter that is conducted every year for population control at the Center, with the carcasses destined for various experiments. After euthanasia of the animals, the ovaries (n = 12) were washed twice in 70% alcohol and Tissue Culture Medium- (TCM) Hepes, and were then transported to the laboratory in the same medium.

2.2. Experimental design

The pairs of ovaries (n = 6) were divided into 21 fragments (3 mm × 3 mm × 1 mm). For the fresh control group, one fragment was immediately fixed in Carnoy's solution and underwent histological procedures for morphological evaluation; a second fragment was fixed in paraformaldehyde for subsequent immunohistochemical analysis to identify activated caspase-3 expression and assessment of cell proliferation; and a third fragment was subjected to the isolation protocol to evaluate the viability of the follicles. The other 18 fragments were vitrified and subsequently analyzed.

2.3. Ovarian tissue vitrification

For SSV [31], a vitrification solution composed of minimal essential medium (MEM) supplemented with sucrose (0.25 M), 10% fetal bovine serum (FBS), and CPAs was used. As experimental groups, DMSO and EG were individually tested at 3 M concentration, as well as in combination (DMSO 1.5 M and EG 1.5 M). The ovarian fragments were exposed to vitrification solution for 5 min and placed on a metal cube surface partially immersed in liquid nitrogen (LN_2) for vitrification. Once vitrified, the samples were transferred to cryovials for storage in LN_2 (at −196 °C).

The vitrification using the OTC (Fig. 1) followed the same procedure as that described for the SSV method; however, the entire process of exposure to the CPAs was conducted inside the OTC and then the solution was removed, the device closed, and the sample transferred to the LN_2 [14].

For both methods, the fragments remained stored for two weeks before being rewarmed at room temperature (25 °C) for 1 min, and immersed in a water bath at 37 °C for 5 s. CPAs were removed by three consecutive washes of 5 min in MEM supplemented with 10% FBS and decreasing concentrations of sucrose (0.50 M, 0.25 M, and 0.0 M sucrose) [31]. After the procedure, the fragments were analyzed.

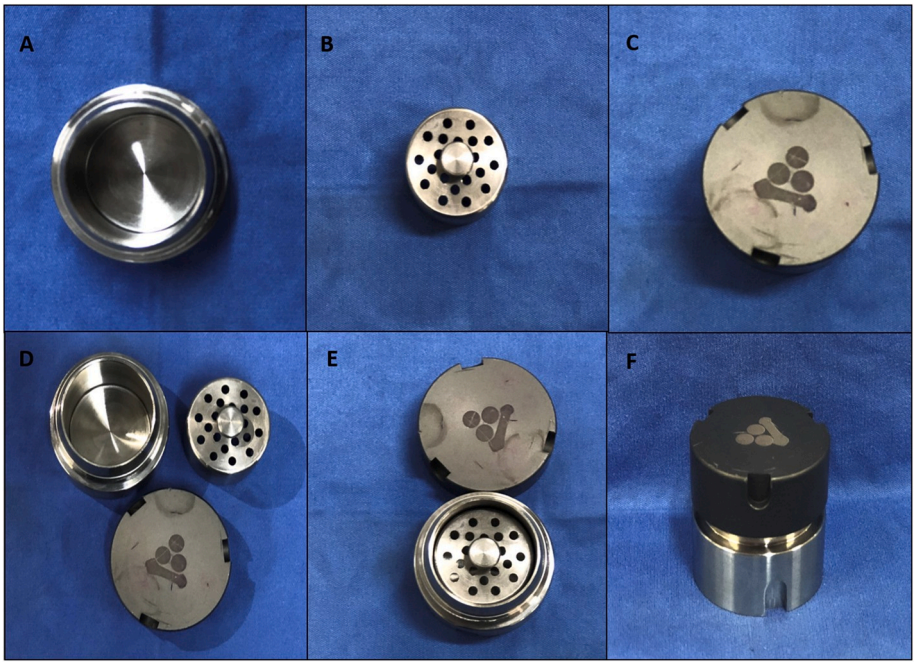

Fig. 1. Detailed view of the Ovarian Tissue Cryosystem – OTC. The container basis (A); the insert presenting perforations in the upper portion to facilitate placement and removal of solutions (B); the cover (C); view of the three different parts of the OTC (D); the cover and the basis containing the insert (E); the complete closed system (F).

2.4. Histological analysis

After fixation in Carnoy's solution for 12 h, the ovaries from the control and vitrified groups were dehydrated in a graded series of ethanol (70%, 80%, 95%, and 100%), clarified with xylene, embedded in paraffin wax, and serially sectioned at 7 μm. Every 5th section was mounted on a glass slide that was stained with hematoxylin–eosin and evaluated using a light microscope at 400× magnification. The developmental stages of the PFs were classified as primordial, primary, or secondary [41].

The PFs were also identified and classified by a light microscope (400×) based on their integrity. Follicular morphology was evaluated based on the integrity of the oocyte, granulosa cells, and basement membrane. PFs were classified and counted as morphologically normal when they contained an oocyte with a regular shape and uniform cytoplasm, and organized layers of granulosa cells; or were classified as degenerated, when the oocyte exhibited a pyknotic nucleus and/or ooplasma shrinkage, and occasionally granulosa cell layers became disorganized, detached from the basement membrane, and/or included enlarged cells. The PFs were analyzed only in the sections where the oocyte nucleus was and 30 follicles were counted per treatment [41].

2.5. Viability analysis

Fresh and vitrified fragments were subjected to the mechanical procedure for follicular isolation [12]. The fragments were placed in a Petri dish and submitted to dissociation with a scalpel blade. After agitation, the suspension was filtered through a 500 μm nylon mesh filter and posteriorly it was centrifuged at 40 g for 10 min and evaluated with Trypan blue (0.4%). After 5 min, the samples were evaluated under an inverted microscope and PFs were classified as viable when the oocyte and <10% of granulosa cells remained unstained or as non-viable when the oocyte and/or >10% of granulosa cells were stained.

2.6. Assessment of cell proliferation capacity

The proliferation rate of follicular cells in all treatments was evaluated by quantifying the number of argyrophilic nucleolus organizer regions (NORs) as previously reported for the species [30]. The ovarian tissue fragments were fixed in 4% paraformaldehyde and subject to conventional histological processing. The blocks were then sectioned at 5 μm and the sections were immersed in 1% potassium metabisulfite (3 min). The slides were impregnated with silver nitrate (2:1 colloidal solution) in a darkroom (30 min) and then placed in a solution of sodium metabisulfite (3 min) and sodium thiosulphate (3 min) to allow impregnation of the silver nitrate. To quantify the NORs, 30 developing follicles were visualized under a light microscope (1000×) and NORs of all nuclei of visible granulosa cells were counted.

2.7. Immunochemistry for activated caspase-3

Immunohistochemistry was performed according to the methodology described by Barberino et al. [7], with some modifications. Ovarian fragments from the fresh and vitrified groups were fixed in paraformaldehyde. After 12 h of fixation, the ovarian tissue was dehydrated with increasing concentrations of ethanol, clarified in xylene, and embedded in paraffin. Sections (3 μm thick) from each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted on Star-Frost glass slides (Knittel, Braunschweig, Germany). The slides were incubated in citrate buffer (Dinamica) at 95 °C in a decloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with H_2O_2 (EasyPath) for 10 min. Nonspecific binding sites were blocked using 10% normal goat serum (EasyPath). Subsequently, the sections were incubated in a humidified chamber for 55 min at room temperature with polyclonal rabbit anti-activated caspase-3 (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, the sections were incubated for 20 min with Easylink One polymer (EasyPath). Protein localization was determined with diaminobenzidine (EasyPath) and the sections were counterstained with hematoxylin (Vetec, São Paulo, Brazil) for 1 min. Negative controls (reaction control) were included that did not contain the rabbit polyclonal anti-activated caspase-3. Sections were examined using an optical microscope. The PFs containing positively stained cells (granule cells/oocyte) for activated caspase-3 were counted manually; however, only those follicles presenting a visible oocyte nucleus were analyzed [18].

2.8. Statistical analysis

Data were expressed as means and standard error of means, and were subjected to Smirnov–Kolmogorov and Bartlett tests to confirm normal distribution and homogeneity of variance, respectively. For morphology and cell proliferation assessments, comparisons between groups (fresh control versus vitrified by SSV or OTC using different CPAs) were undertaken by analysis of variance followed by the Fisher's least significance difference (LSD) test. For the analysis of viability and activated caspase-3 expression, data were taken as a pool and analyzed by dispersion of frequency with Fisher's exact probability test. Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. Histological analysis

A total of 1260 PFs were analyzed (~180 per treatment). Overall, 75.6% morphologically normal PFs were found in the fresh control group. After vitrification, the use of the OTC with any of the CPAs revealed morphologically normal PFs similar to those observed for the fresh control group (75.6 ± 8.6%); however, the SSV method was only efficient when used with DMSO alone (63.9 ± 7.6%), and there was an evident decrease ($P < 0.05$) in the morphologically normal PFs after the use of EG alone or DMSO and EG combined (Table 1).

From the histological analysis, it was found that the peccary PF had a spherical oocyte, with the nuclei occupying most of the cytoplasm and being positioned centrally or eccentrically in the oocyte. The granulosa cells were well organized around the oocyte, forming concentric layers. These features of morphologically normal PFs were found both in the fresh control (Fig. 2A) and the vitrified (Fig. 2B and C) groups, regardless of the method and CPA used. After the vitrification process, the main changes observed were the retraction of the oocyte, detachment of the granulosa cells from the basement membrane, and pyknotic oocyte nuclei in all treated groups (Fig. 2D).

Table 1
Values (mean ± S.E.M.) for normal morphology, viability, and cell proliferation in collared peccary preantral follicles subjected or not (fresh control) to vitrification by the solid surface method (SSV) or using the ovarian tissue cryosystem (OTC) with the cryoprotectants ethylene glycol (EG) or dimethylsulfoxide (DMSO), alone or in combination (EG + DMSO); n = 6 individuals.

Treatments		Morphology (%)	Viability (%)	Cell proliferation capacity (Number of Nucleolus Organizing Regions)
Fresh control		75.6 ± 8.6^{a}	84.0 ± 2.9	2.0 ± 0.2
SSV	EG	52.8 ± 5.9^{b}	79.0 ± 5.3	2.1 ± 0.2
	DMSO	63.9 ± 7.6^{ab}	82.0 ± 2.9	1.9 ± 0.1
	EG + DMSO	54.5 ± 10.4^{b}	79.3 ± 3.2	2.0 ± 0.2
OTC	EG	67.8 ± 6.8^{ab}	79.0 ± 4.3	2.0 ± 0.2
	DMSO	58.3 ± 8.7^{ab}	78.0 ± 2.1	2.0 ± 0.1
	EG + DMSO	64.5 ± 7.7^{ab}	77.0 ± 1.0	2.0 ± 0.1

[a,b] Superscript lowercase letters indicate significant differences among treatments ($P < 0.05$).

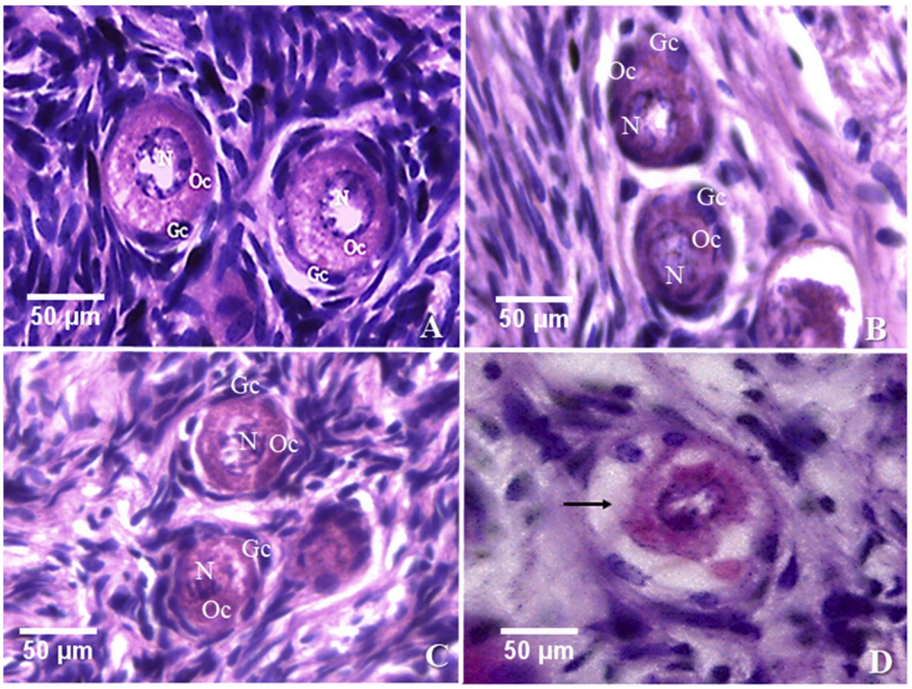

Fig. 2. Histological features of collared peccaries ovarian preantral follicles. Normal follicles from control group (A), after vitrification in SSV (B) and OTC (C) exhibited oocyte with homogenous cytoplasm (Oc), central nucleus (N) and intact and organized granulosa cells (GC). Degenerated follicle (D) showed cytoplasmic retraction (arrow) or disorganization of granulosa cells.

3.2. Assessment of viability and cell proliferation capacity

The percentage of viable PFs in the fresh control group was 84% (Table 1). After vitrification, all tested groups maintained a similar viability of peccary PFs to that of the control group, with values ranging from 77% to 82%. Concerning the cell proliferation capacity (Table 1), a similar amount of NOR was verified among the fresh control and vitrified groups, thus all interactions among vitrification methods and CPAs were able to preserve this PF characteristic.

3.3. Immunochemistry for activated caspase-3

None of the negative controls (Fig. 3A) marked with activated caspase-3 showed any immunoreactivity, whereas signal-positive activated caspase-3 was observed (Fig. 3 C, D, E, F, G, and H). The healthy (primordial, primary, and secondary) follicles were determined as negative activated caspase-3 for both the fresh control group (Fig. 3B) and the vitrified groups (Fig. 3 C, D, E, F, G, and H).

From a quantitative point of view, a total of 36.7% PFs were marked as activated caspase-3 in the fresh control group. After vitrification, only samples processed using the SSV method with EG (43.4%) or combined EG and DMSO (33.4%), and those vitrified using the OTC with EG

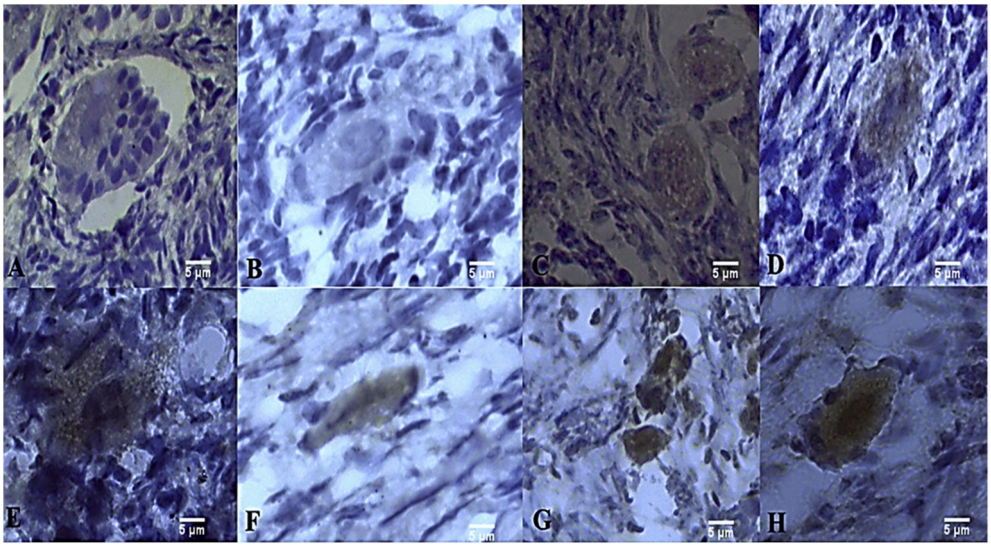

Fig. 3. Immunohistochemical analysis for the expression of activated caspase-3 in collared peccary ovarian preantral follicles. Negative control (A); Fresh control group (B); Groups vitrified through solid surface method using ethylene glycol (C), dimethyl sulfoxide (D) or its combination (E); Groups vitrified through the ovarian tissue cryosystem with ethylene glycol (F), dimethyl sulfoxide (G) or its combination (H).

(46.7%) provided similar values as those found for the fresh control group (Fig. 4).

4. Discussion

Biobank formation based on the female germplasm allows for the storage of many PFs for long periods and facilitates the transport of genetic material between different reproductive centers [6]. The present study represents an important step for the preservation of the collared peccary and related endangered species, such as the Chacoan peccary (*Catagonus wagneri)* [3], because it highlights the possibility of safeguarding female genetic material under different vitrification methods for an undetermined time.

The morphological examination of the peccary ovarian tissue showed the possibility of preserving more than 50% of the total PF population when the samples were vitrified using both the open and closed systems. The results found from the OTC were similar to those reported for goats (58.1%) [14]; however, they were higher than those verified for sheep (30.66%) [5] and cats (37%) [21]. During vitrification with the OTC, the entire procedure of exposure and removal of the cryoprotective agent is performed inside the device, making the technique more practical and faster than other vitrification methods. In addition, the fragments do not come into contact with LN_2, thus avoiding contamination of the samples [14]. In contrast, the SSV method can provide extremely fast rates of cooling due to heat exchange through direct contact with the LN_2 cooled surface [44], and is considered an inexpensive method [39].

In comparison to the fresh control group, the OTC provided an efficient preservation of the morphology of PFs when using any of the tested CPAs. EG and DMSO are the CPAs that have been suggested for use with OTC in previous studies for goats [14], sheep [5], and cats [11]. Conversely, SSV was only efficient at preserving the morphology of PFs with values similar to the fresh control group when DMSO was used alone. This CPA, which is also efficient for sheep [36], mice [28], and goat [15], interacts with membranes and crosses them rapidly via diffusion. Moreover, DMSO and its metabolites have low toxic potential [4].

According to Fauque et al. [19], the viability test using Trypan blue vital dye should be routinely used as a quality control method for the cryopreservation of ovarian tissue. The viability analysis in the present study revealed that both vitrification methods provided a high rate of viable follicles (ranging from 77% to 82%), regardless of the CPA used. Therefore, both methods did not cause rupturing of the cell membrane, as they used viscous and highly concentrated CPA solutions that presented enough permeation capacity into the ovarian tissue, reducing osmotic stress. This fact, associated with the abrupt drop in temperature, causes the direct passage of the media from a liquid to an amorphous state, without the formation of ice crystals, thus allowing for successful follicular conservation [39].

NORs are segments of DNA that transcribe ribosomal RNA that, in turn, is later translated into protein, forming ribosomes [10]. Therefore, NORs are directly related to cell proliferation, so the higher the cell proliferation activity, the greater the number of NORs observed [23]. In the collared peccary, the vitrified tissue presented the same proliferation capacity as that in the fresh tissue because the number of NORs among the treatments was similar, indicating that the proliferative capacity remained active even after the vitrification process. Similar results have been observed in donkeys [32], where the authors found 1.79 ± 1.4 NORs/cell in the fresh control group and 1.89 ± 0.7 NORs/cell in samples vitrified using the SSV method with a combination of EG and DMSO.

Besides using a conventional approach to analyze the morphology, viability, and cell proliferation of the PFs, the present study is the first to evaluate the incidence of apoptosis in peccary ovarian tissue after vitrification. Caspases play a central role as an indicator of apoptosis in the intrinsic and extrinsic pathways [25], especially activated caspase-3, which is usually related to follicle atresia [27]. This parameter was of fundamental importance for establishing the differences in the efficiency of the tested treatments. Results from the present study showed that the OTC with EG, and the SSV method with EG or the combination of CPAs can efficiently preserve peccary PFs by providing similar expression values for activated caspase-3 similar to those observed for the fresh control group. However, the use of the SSV method provoked an increase in the morphological damage of peccary PFs. Therefore, the OTC is efficient at using EG as a CPA for both conserving the morphology and preventing apoptosis in peccary PFs.

Similar to that shown for the collared peccary, the effectiveness of EG at providing low rates of apoptosis induction after ovary vitrification has been reported for humans [2], murids [1,26], and equine [21]. EG has a lower molecular weight (62.07 g/mol) than that of DMSO (78.13 kDa), which facilitates its penetration into tissues and it also presents a lower toxicity compared to other CPAs, which may contribute to the preservation of PFs after vitrification [8]. Moreover, DMSO has a considerable toxicity effect on ovarian cell morphology [9,29]; however, the mechanism(s) by which the toxicity effects of CPAs trigger the activation of the apoptotic process in ovarian cells remains unclear [21].

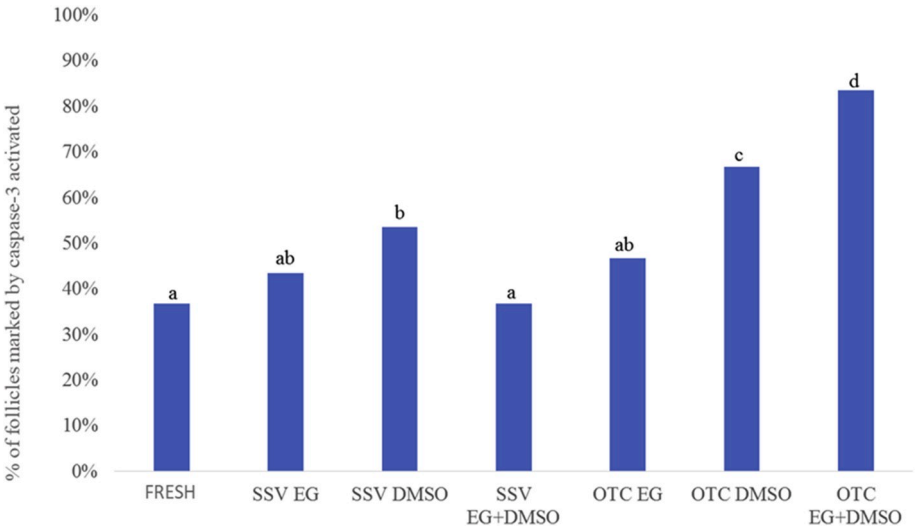

Fig. 4. Percentage of collared peccary preantral follicles marked for caspase-3 activated in the fresh control group and after vitrification through solid surface method or ovarian tissue cryosystem using different cryoprotective agents (EG, DMSO and EG + DMSO).

5. Conclusions

We demonstrated that the OTC, with 3 M EG as the CPA, is a good cryopreservation system for the vitrification of collared peccary ovarian tissue in terms of providing excellent cell viability and morphology, maintaining the cell proliferation capacity and preventing apoptosis. Moreover, this system offers the advantage of being a closed system, thus enabling the preservation of the species and the formation of a germplasm bank, which is an important tool for the conservation strategies of collared peccary and other endangered peccary species.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.10.193.

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