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Effect of cryoprotectant type and concentration on the vitrification of collared peccary (*Pecari tajacu*) ovarian tissue

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ABSTRACT

The aim of the present study was to establish a protocol for solid surface vitrification of peccary ovarian tissue by using different cryoprotectants. Ovarian pairs from five adult females were fragmented and two fragments (fresh control group) were immediately subjected to morphological evaluation using classical histology, transmission electron microscopy, and viability analysis using fluorescent probes. The remaining fragments ($n = 18$) were vitrified using a solid surface method with different concentrations (3 or 6 M) of ethylene glycol (EG), dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF). After 2 weeks, samples were re-warmed and evaluated. A decrease in the percentage of morphologically normal preantral follicles (PFs) was verified for all the groups in comparison to the fresh control (92.0 ± 2.8%); however, if only the primordial follicles are considered, the most effective preservation ($P < 0.05$) was achieved with the use of EG at 3 M (74.2 ± 7.3%) or DMSO at 6 M (75.0 ± 4.2%). Ultrastructural analysis indicated there were well-preserved PFs in all the groups evaluated, having well-defined membranes, a few vacuoles, and organelles that were uniformly distributed throughout the cytoplasm, mainly round and elongated mitochondria in close association with lipid droplets. Viability was preserved ($P < 0.05$) with the use of EG at 3 (97%) or 6 (97%) M, DMSO at 3 (100%), and DMF at 6 (97%) M. Solid surface vitrification, therefore, is an effective method for conservation of peccary female germplasm, especially with the use of EG at 3 M, which was highly effective for preservation of both the morphology and viability of PFs.

1. Introduction

Due to its importance for the maintenance of ecosystems, the collared peccary (*Pecari tajacu* Linnaeus, *1758*), a type of wild pig, has been a focus of the scientific community during the last decade (Silva et al., 2017). Although it is currently classified as a species of least concern with regard to endangerment, its population is decreasing and it is extinct in some regions due to over-hunting for its

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meat and excessive destruction of its natural habitats (Gongora et al., 2013). In this context, strategies for gamete rescue and preservation are of great importance for species preservation so as to facilitate the formation of biobanks and use in captive breeding programs (Silva et al., 2017). Recently, many efforts for the development of protocols for the storage of peccary sperm have been described (Souza et al., 2016; Bezerra et al., 2018). Strategies for the preservation of female genetic material, however, are not available to date.

Ovarian tissue preservation allows the storage of a large amount of female genetic samples, as the ovary represents a large pool of oocytes enclosed in preantral follicles (PFs) (Santos et al., 2010). The tissue vitrification method has gained popularity for this purpose because of its contribution to the establishment of biobanking in various wild species such as the dromedary camel (Madboly et al., 2017) and the red-rumped agouti (Praxedes et al., 2018), as well as the domestic pig (Gabriel et al., 2017). The use of vitrification procedures has been reported to allow for avoiding some of the damage caused when there is use of conventional freezing protocols, such as the risk of ice crystal formation (Saragusty and Arav, 2011). The successful preservation of ovarian tissue using solid-surface vitrification (SSV) was first achieved in domestic goats (Santos et al., 2007), and there were similar results for domestic cats (Brito et al., 2018). It is considered a low-cost and reasonably easy method to utilize that can be performed in field conditions with no need for special equipment, making it a good alternative for use in various settings often encountered with wildlife species, including after sudden animal death (Amorim et al., 2011).

For cell survival during vitrification, the use of cryoprotectants (CPA) is indispensable. It, however, is necessary to establish appropriate concentrations for these reagents, because the metabolites resulting from CPA degradation might have toxic effects (Fahy, 2010). Of the CPAs, the alcohols dimethyl sulfoxide (DMSO) and ethylene glycol (EG) have been extensively used for ovarian tissue preservation in various species, such as swine (Gabriel et al., 2017) and felines (Brito et al., 2018). For vitrification of cattle oocytes (Siqueira-Pyles et al., 2004) and sheep embryos (Dos Santos-Neto et al., 2017), the use of dimethyl formamide (DMF) has been proposed as an alternative to avoid the toxicity of conventional CPAs and, therefore, is an option for the preservation of ovarian tissue.

The aim of the present study, therefore, was to establish the most suitable cryoprotectant for the vitrification of collared peccary ovarian tissues utilizing the SSV method. There was comparison of different concentrations (3 or 6 M) of conventional CPAs such as DMSO and EG, and assessment of DMF as an alternative CPA for ovarian tissue preservation.

2. Materials and methods

All experimental protocols were approved by the Ethical Committee in Animal Research from UFERSA (Process nº 23091.000254/11-88). The study was authorized by the Chico Mendes Institute for Biodiversity (SISBio no. 37329). All procedures were supervised and managed by a veterinarian. Unless stated otherwise, the chemicals and media used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Animals

The female collared peccaries belonged to the Centre of Multiplication of Wild Animals from UFERSA (Mossoró, RN, Brazil; 5°10'S, 37°10'W), where the climate is semi-arid, with an average annual temperature of 27 °C. The animals were maintained in captivity and fed fruits and a balanced ration produced at UFERSA. Animals had access to feed and water ad libitum. As a means of animal population control, a scheduled peccary slaughter is conducted every year and the animal tissues are then used in experiments. For the present study, ovaries from five sexually mature females were used.

2.2. Preparation of ovaries

The ovarian pairs ($n = 5$) were removed aseptically immediately after slaughtering. The ovaries were rinsed once with 70% ethanol for 10 s and twice in sterile phosphate-buffered saline (PBS). In the laboratory, the ovarian cortex from each pair was sliced into 21 pieces (14 fragments of 3 mm × 3 mm × 1 mm and 7 fragments of 1 mm × 1 mm × 1 mm).Of these tissue segments, one piece was fixed for routine histological analysis; another piece was subjected to follicular isolation followed by viability analysis, and a third one was fixed for ultra-structural analysis. The remaining 18 ovarian cortical fragments were subjected to SSV (Santos et al., 2007), applying one of the six vitrification solutions as subsequently described in this manuscript.

2.3. Vitrification

The SSV method, as well as the protocol for incubating tissue in vitrification solutions for 5 min at room temperature, the dilution regimen to remove the cryoprotectants after warming, and the time and temperature of exposure, were chosen based on methods previously described (Santos et al., 2007). In brief, vitrification solutions were prepared immediately prior to use. The vitrification solutions were composed of an intracellular cryoprotectant (dimethyl sulfoxide – DMSO; ethylene glycol – EG; or dimethylformamide – DMF) at final concentrations of 3 or 6 M in the solution. These cryoprotectants were added to minimum essential medium (MEM) supplemented with sucrose (0.25 M) and 10% fetal calf serum (FCS). The ovarian fragments ($n = 18$) were placed for 5 min in one of the six vitrification solutions and placed on the surface of a metal cube partially immersed in liquid nitrogen (LN_2). The vitrified fragments were transferred into cryovials, using nitrogen-cooled forceps, and stored in the liquid phase of a liquid nitrogen tank. Vitrified ovarian fragments were maintained in cryostorage for 2 weeks. For warming, cryovials were exposed to room temperature

(~25 °C) for 1 min, and fragments separately subjected to cryoprotectant removal. Fragments were immersed in MEM (code M8052) which contained amino acids, inorganic salts, vitamins and pyruvate, supplemented with 10% FCS (MEM^+), at 37 °C, as follows: (i) MEM^+ + 0.5 M sucrose (5 min), (ii) MEM^+ + 0.25 M sucrose (5 min), and (iii) MEM^+ (5 min). After removal of cryoprotectant, one ovarian fragment per treatment was fixed for histological analysis and another one for ultra-structural analysis. Due to the small number of follicles recovered for viability analysis, one pool of follicles per treatment was used to obtain the percentage of viable PFs.

2.4. Histological analysis

After fixation in Carnoy solution, ovarian fragments (3 mm × 3 mm × 1 mm) were dehydrated in ethanol, clarified with xylene, and embedded in paraffin wax. Serial sections (7 µm) of ovarian tissue were cut, and every fifth section was mounted on glass slides and stained with hematoxylin-eosin. All sections were examined using a light microscope (Carl Zeiss Optical Inc., Chester, USA) at a magnification of 100 × . The PFs were classified as primordial, primary, and secondary as previously described in a study with collared peccaries (Lima et al., 2013). For each treatment, at least 30 follicles were counted per female. To avoid counting a follicle more than once, PFs were counted only in the sections where the oocyte nucleus was observed. Follicular quality was evaluated based on the morphological integrity of the oocyte, the granulosa cells, and the basement membrane. Briefly, PFs were classified as (i) histologically/morphologically normal when the follicles contained an intact oocyte and granulosa cells, (ii) degenerated grade 1 when the oocyte nucleus had become pyknotic, and (iii) degenerated grade 2 when the oocyte was shrunken and its nucleus pyknotic and when granulosa cells were sometimes detached from the basement membrane and were of an enlarged volume.

2.5. Assessment of oocyte and granulosa cell viability

Fresh and vitrified ovarian fragments (3 mm × 3 mm × 1 mm) were subjected to follicular isolation using a mechanical procedure previously described by Lucci et al. (1999). Briefly, the ovarian cortex was cut into small fragments with a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 75 µm. Samples were then placed in MEM supplemented with 3 mg/mL bovine serum albumin (BSA) at room temperature (25 °C) and then pipetted 100 times with a Pasteur pipette to release the PFs. The suspension was filtered through a 200-µm nylon-mesh filters, followed by the isolation procedure within 10 min. The PFs smaller than 200 µm were collected using a dissecting stereomicroscope (SZ-STS, Olympus, Tokyo, Japan) and transferred to HEPES-buffered M-199 + 1% bovine serum albumin (holding medium). Isolated follicles were incubated in the holding medium for 15 min at 37 °C in a mixture of 4 µM calcein AM and 2 µM ethidium homodimer-1 (Molecular Probes Europe, Leiden, The Netherlands) to detect esterase enzyme activity and to assess membrane integrity and to enable the counting of nuclei, respectively. After being labeled, stained follicles were washed three times in PBS, mounted on a glass microscope slide, and were subsequently examined using an epifluorescence microscope (BH2-RFCA microscope, Olympus, Tokyo, Japan) equipped with a digital camera. The emitted fluorescent signals of calcein and ethidium homodimer were collected at 488 nm, and 568 nm, respectively. Oocytes and granulosa cells were classified as viable if the ooplasm was stained positively with calcein-AM. Thirty PFs were analyzed in each treatment.

2.6. Transmission electron microscopy

For more precise evaluation of the follicular morphology, an ultrastructural analysis was conducted on fresh control and vitrified/warmed ovarian fragments using a protocol described by Lima et al. (2013). Tissue fragments with a maximum dimension of 1 mm^3 were subsequently fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h. After fixation and five washes, specimens were post-fixed in 1% osmium tetroxide and immersed in 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h. Subsequently, the samples were dehydrated through a gradient of acetone solutions (31%–100%) and the tissues were embedded in Spurr. Semi-thin sections (3 µm) were stained with toluidine blue. The ultra-thin sections (60–70 nm) were contrasted with uranyl acetate and lead citrate, and examined using a Jeol JEM 100C (Carl Zeiss Microscopy, Jena, Germany) transmission electron microscope. Qualitative assessment of the ovarian tissue ultrastructure included evaluations of shape and dimensions of the oocyte nucleus, dispersion of the nucleus chromatin structure, presence of an intact oolemma, quality of the organelles, cytoplasmic vacuolization, and attachment to the surrounding granulosa cells.

2.7. Statistical analysis

Results were expressed in terms of mean and standard deviation and evaluated using GraphPad Prism Windows 6.04 software (GraphPad Software, La Jolla, California, USA). The normality of distribution and homogeneity of variance were evaluated using the Kolmogorov–Smirnov and Bartlett tests, respectively. Comparisons among experimental groups with regards to morphology were performed using one-way analysis of variance (ANOVA) followed by Fisher LSD test. For viability analysis, follicles were taken as a pool and evaluated by Fisher exact probability test. Differences were considered significant when $P < 0.05$.

Table 1
Percentage (± SEM; normal/total) of morphologically normal ovarian preantral follicles from collared peccaries (*Pecari tajacu*) of the fresh control group and after solid surface vitrification (SSV) using 3 or 6 M of dimethylsulfoxide (DMSO), ethylene glycol (EG) and dimethylformamide (DMF) (n = 30 follicles per treatment x 5 females).

Follicle category	Control	EG		DMSO		DMF	
		3M	6M	3M	6M	3M	6M
Primordial	91.2 ± 3.2[a] (196/214)	74.2 ± 7.3[a] (137/182)	72.2 ± 4.6[b] (167/229)	71.6 ± 5.2[b] (132/185)	75.0 ± 4.2[a] (148/194)	73.2 ± 5.2[b] (143/196)	72.0 ± 2.9[b] (97/132)
Primary	95.8 ± 2.7[a] (35/37)	85.6 ± 7.4[b] (33/40)	63.0 ± 15.6[b] (14/23)	80.6 ± 14.0[b] (22/30)	80.2 ± 5.8[b] (23/29)	75.3 ± 8.8[b] (19/26)	80.3 ± 10.2[b] (10/13)
Secondary*	100.0 ± 0.0 (6/6)	83.3 ± 16.7 (3/4)	91.5 ± 8.5 (6/7)	90.0 ± 10.0 (10/11)	62.5 ± 21.7 (11/16)	100.0 ± 0.0 (3/3)	100 ± 0.0 (3/3)
Total	92.2 ± 2.8[a] (237/257)	76.0 ± 6.5[b] (173/226)	72.0 ± 5.3[b] (187/259)	72.8 ± 6.2[b] (164/228)	75.8 ± 2.7 [b] (182/239)	73.2 ± 4.1[b] (165/225)	73.3 ± 2.5[b] (110/148)

[a,b,c] Lowercase superscript letters indicate differences among columns ($P < 0.05$).
* Due to the small number of follicles for this category, it was not possible to perform statistical analysis.

3. Results

3.1. Histological analysis

A total of 1582 PFs were evaluated (˜45 PFs per ovarian fragment). After rewarming, there was a decrease in the total percentage of morphologically normal PFs for all treatment groups (Table 1). When considering primordial follicles alone, however, the most effective preservation of morphology ($P < 0.05$) was achieved with the use of EG at 3 M (74.2 ± 7.3%) or DMSO at 6 M (75.0 ± 4.2%). In Fig. 1 there are photomicrographs of the PFs of peccaries before and after vitrification.

3.2. Viability analysis

Viability was examined in a total of 210 PFs (evaluating oocytes and granulosa cells) using a fluorescent labeling technique (approximately 30 follicles per treatment; Fig. 3). The percentage of viable oocytes was less ($P < 0.05$) only when ovarian tissue was vitrified with use of 6 M DMSO or 3 M DMF (Table 2).

3.3. Ultrastructural features

Results from ultrastructural analysis indicated PFs from fresh control tissues had well-defined membranes, a few vacuoles, and organelles that were uniformly distributed throughout the cytoplasm. Round and elongated mitochondria were observed in close association with lipid droplets, which were the most obvious organelles (Fig. 2A). For all the groups evaluated, vitrified ovarian tissue results indicated that there were well-preserved PFs with characteristics similar to those observed for the fresh control tissues, with no differences when different cryoprotectants were used. In all treatment groups, the oocyte nucleus was well-defined by the nuclear envelope and granulosa cells had a normal appearance (Fig. 2B). A large quantity of vacuoles and swollen mitochondria were, however, observed (Fig. 2C). These vacuoles were different from lipid droplets.

4. Discussion

As an initial attempt for the development of a biobank for collared peccary female germplasm, the results from the present study indicate the SSV method can be used to preserve morphologically normal (> 70%) and viable (> 80%) PFs. These findings will facilitate the development of storage and conservation protocols for sourcing female gametes from this species in which the

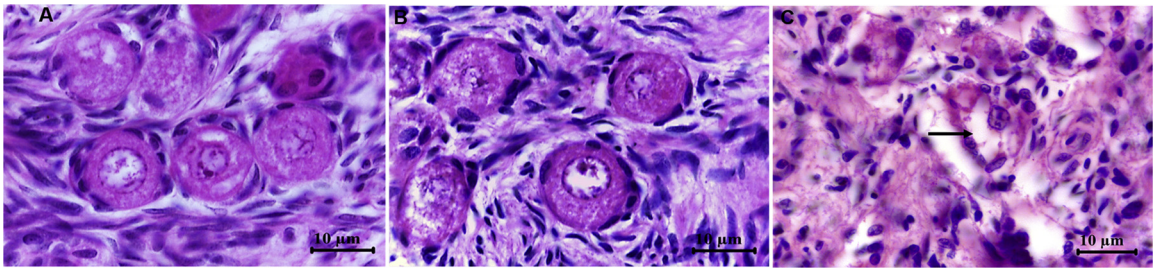

Fig. 1. Photomicrographs of fresh and vitrified ovarian sections from *Pecari tajacu*; Morphologically normal preantral follicles before (A) and after vitrification (B); Degenerated vitrified follicles (C) with oocyte cytoplasm retraction (arrows) and disorganization of granulosa cell layers.

Table 2
Viability of collared peccary (*Pecari tajacu*) ovarian preantral follicles (PFs) from fresh the control group and PFs vitrified using ethylene glycol (EG), dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF) at 3 or 6 M.

Treatments	Percentage (%)	Viable/Total
Fresh control group	97.0	29/30
EG 3 M	97.0 [ab]	29/30
EG 6 M	97.0 [ab]	29/30
DMSO 3 M	100.0 [a]	30/30
DMSO 6 M	87.0 [bc]	26/30
DMF 3 M	80.0 [c*]	24/30
DMF 6 M	97.0 [ab]	29/30

[a,b,c]Lowercase superscript letters indicate differences among treatments; *Asterisk indicates difference from the fresh control group ($P < 0.05$).

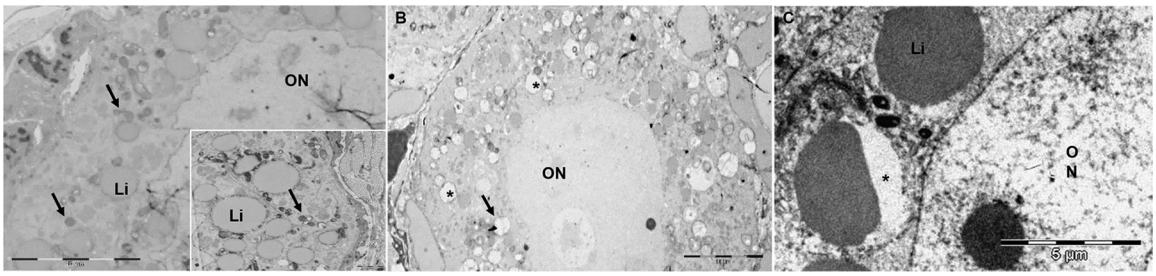

Fig. 2. Electron micrographs of *Pecari tajacu* ovarian follicles before (A) and after vitrification (B and C); (A) oocyte nuclei (ON), granulosa cells (GC), lipid droplets (Li) and round mitochondria (arrows) were the most abundant organelles; (B) Some vacuoles (*) and swollen mitochondria (arrows) were observed after vitrification process; (C) A few alterations (*) were observed in lipid droplets (Li) distributed in the oocyte cytoplasm (OC).

population of PFs was previously estimated as 33,273.5 ± 5 789.9 PFs per ovary (Lima et al., 2013). The results from the present study are promising because for the closely related domestic pig, results from previous studies indicate that PFs vitrified *in situ* have a sustained developmental capacity when subjected to xenografting procedures (Moniruzzaman et al., 2009). In addition, Lima et al. (2018) recently reported that peccary ovarian tissue can be successfully cultured *in vitro* in a medium supplemented with follicle stimulant hormone (FSH). Hence, the consideration of results obtained from experiments where there was *in vitro* culturing and vitrification techniques utilized indicates there could be development of techniques for having viable PFs to be used in other reproductive strategies to conserve collared peccaries. These results can possibly be extrapolated to other endangered tayassuids such as the white-lipped (*Tayassu pecari*) and the Chacoan (*Catagonus wagneri*) peccaries.

The promising results of vitrification are attributed to its effectiveness in avoiding the detrimental effects of freezing and, consequently, mitigating or decreasing intracellular ice formation. In this process, when the glass state prevails the cryoprotectant solution is exceedingly viscous and all chemical reactions cease to occur that require molecular diffusion, leading to metabolic inactivity and stability of the structures being stored(Sakai and Engelmann, 2007). This viscosity is achieved by using concentrated cryoprotective solutions, which result in supercooling to very low temperatures and solidification into a stable glass state, without crystallization (Mukaida and Oka, 2012). Specifically with respect to the vitrification method used in the present study, SSV is an open system that allows the preservation of a large percentage of follicles in small solution volumes, with there being a rapid cooling rate and, thus, a reduced probability of cryoinjury (Santos et al., 2007).

Because the vitrification method requires a concentrated cryoprotectant solution, a great concern in cryobiology is the potentially lethal mechanical stresses that cells are exposed when stored in solutions with high osmolarity (Cox et al., 2012). In this regard, many studies have been performed to evaluate the effect of different CPAs and concentrations and association with other substances, such as non-permeating protectants, in an attempt to diminish the toxicity of cryoprotectants (Moniruzzaman et al., 2009; Villamil et al., 2011). In peccaries, the CPAs used for tissue vitrification in the present study, functioned similarly with respect to the preservation of morphologically normal and viable PFs. The EG and DMSO are among the permeable cryoprotectants suggested to be the most adequate CPAs for ovarian tissue cryopreservation in various species, including domestic swine. In this species, Borges et al. (2009) reported that there was a more desirable ovarian tissue preservation when using conventional cryopreservation, in DMSO or EG than with use of propanediol (PROH) or glycerol (GLY). These findings were attributed to these being effective cryoprotectants as a result of the lesser molecular weight of these compounds (EG: 62.02 g/mol; DMSO: 78 g/mol), which permits more rapid penetration than with other agents (PROH: 76.10 g/mol; GLY: 92.10 g/mol).

It is important to note that although the use of CPAs is necessary for the success of gamete preservation, the metabolites derived from gamete metabolism of CPAs might be toxic, making this a limiting factor in utilization of CPAs for cryopreservation (Fahy, 2010). Thus, the use of minimal amounts of CPAs that are efficacious is suggested to diminish the toxicity potential. In this regard, the

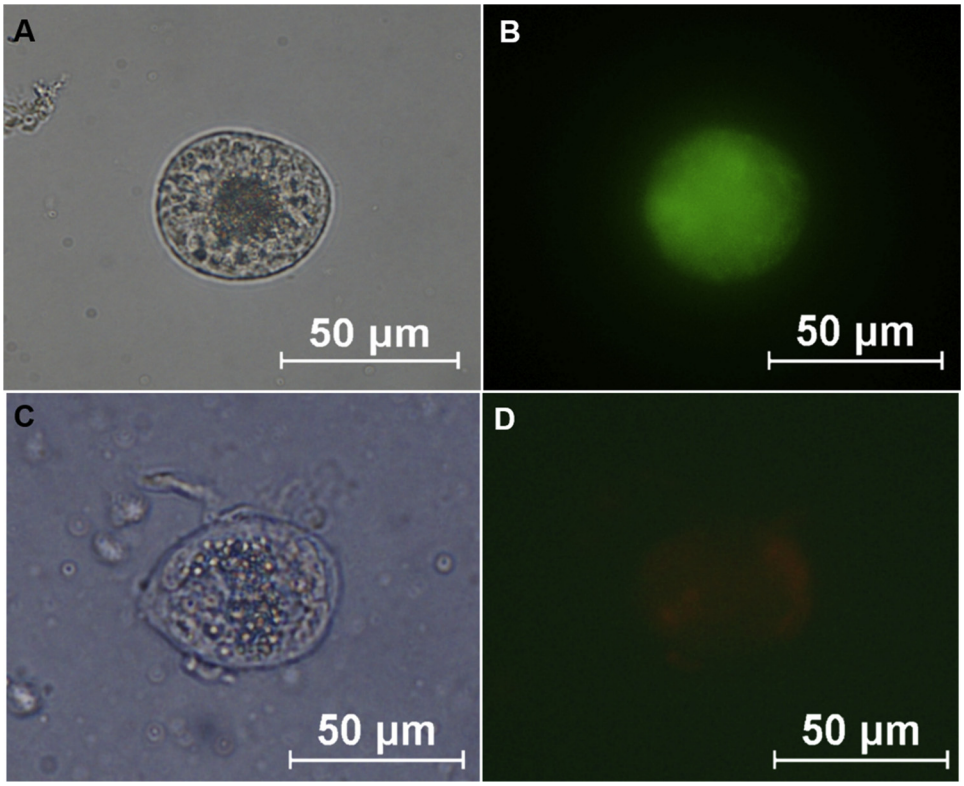

Fig. 3. Viability assessment of preantral follicles of collared peccaries using fluorescent probes; Viable preantral follicle from vitrified/warmed ovarian tissue using ethylene glycol (A and B) labeled with calcein-AM (green fluorescence); Nonviable preantral follicle from vitrified/warmed ovarian tissue (C and D) labeled with ethidium homodimer-1 (red fluorescence) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

preservation of peccary PF morphology and viability was achieved with the use of a small amounts of EG at a 3 M concentration. Although there were favorable cryopreservation effects when EG was used in the present and a previous study (Varago et al., 2014), it was reported that the glycolic acid resulting from EG metabolism leads to intracellular acidification. Depending on the quantity of the EG, the amount of this glycolic acid can exceed the buffering capacity of the cell, causing a decrease in pH and consequently resulting in cell acidosis (Carney et al., 1999).

The least concentration of DMSO (3 M) was adequate to promote the preservation of PF viability, while the largest amount (6 M) appeared to have deleterious effects, even though this amount resulted in acceptable PF morphological preservation. The increase in DMSO concentration could have promoted diffusion of intracellular fluids to the oocyte membrane, leading to excessive cell dehydration. The DMSO compound could induce thinning and expansion of the phospholipid bilayer and increase the fluidity of its hydrophobic core (Gurtovenko and Anwar, 2007). There were similar results when concentrations of greater than 3 M of DMSO were used for zebrafish oocyte cryopreservation. In this previous study, there was a lack of oocyte mitochondrial DNA replication, resulting in a decrease in intracellular ATP concentrations (Zampolla et al., 2009).

In the present study, there was the use of DMF as a CPA for ovarian tissue vitrification. Amides are permeant CPAs with a relatively lesser molecular weight (DMF: 73.09 g/mol) and viscosity (Lopes et al., 2009) than many other CPAs. The presence of the methyl (CH_3) radical in the DMF molecule increases its permeability through the cell membrane and improves the efficacy of its cryoprotective action (Bianchi et al., 2008). The use of DMF for cryopreservation procedures was previously reported to be effective for male (stallion - Oldenhof et al., 2017) and female (Siqueira-Pyles et al., 2004) gametes, as well as for embryo preservation (Varago et al., 2014). In peccaries, the use of DMF at the 6 M concentration provided adequate preservation of the morphology and viability of PFs, similar to that achieved with use of conventional CPAs. In contrast, the use of the least DMF concentration (3 M) led to a decrease in PF viability, suggesting that greater concentrations are required for maintenance of the viability of PFs. Based on these initial results, amides should be considered as an alternative for tissue vitrification, and further studies should be conducted for this purpose by assessing different concentrations or even associations among amides and other CPAs.

Regardless of the cryoprotectant used in the present study, there were changes in ultrastructural features, such as the presence of swollen mitochondria and vacuoles in collared peccary vitrified PFs. Mitochondrial swelling is commonly described in cryopreserved oocytes (Ebrahimi et al., 2011) and embryos (Dalcin et al., 2013). In pigs, PFs preserved using the conventional cryopreservation method had swollen mitochondria and empty spaces in the oocyte cytoplasm (Borges et al., 2009); these void areas are hypothesized to represent endoplasmic reticulum swelling (Sobaniec-Lotowska and Lebensztejn, 2006). These characteristics result because of

changes in ionic balance caused by an altered plasma membrane permeability due to the osmotic effects related to CPAs (Borges et al., 2009). Thus, oocytes that continue to be viable after cryopreservation have less glutathione (GSH) content which leads to an increase in the reactive oxygen species (ROS) concentrations (Somfai et al., 2007). Mitochondria have important functions in oocyte energy production and regulation of ROS and Ca^{2+} concentrations during maturation and embryonic development (Baril et al., 2001). Although swollen mitochondria could affect cellular metabolism, this change may be reversible, as previously reported in oocytes (Borges et al., 2009) and embryos (Dalcin et al., 2013).

The promising results in the present study could also be attributed to the use of nonpermeable (sucrose) in combination with permeable cryoprotectants. It has been suggested that sugars can function to preserve the structural and functional integrity of membranes when there is a low water activity (Kuleshova et al., 1999). The use of sucrose for vitrification of swine ovarian tissues prevent the losses due to lack of viability of PFs after vitrification (Moniruzzaman et al., 2009). Similar effects were observed in peccaries in the present study where the percentage of morphologically normal pPFs was maintained after vitrification with the use of all CPAs. It is to recognize the surface area to volume ratio of PFs is high which is an excellent morphological characteristic for substance exchange. Besides being smaller than the developing follicles, the primordial follicles are less differentiated with fewer organelles, containing oocytes with no zona pellucida and cortical granules, and are less metabolically active (Campebell and Picton, 1999). All these characteristics contribute to the efficacy of the cryopreservation protocols (Oktay et al., 1998), especially for vitrification.

The aim of the present study was to focuson the use of efficacious vitrification/rewarming procedures for peccary ovarian tissues, an approach largely reported for in various mammals such as cattle (Celestino et al., 2008), goats (Carvalho et al., 2011), and dogs (Lopes et al., 2016). It, however, was recently postulated that the effect of cryoprotectants and vitrification on cell metabolism and function may not be evident in the relatively fresh tissue samples when evaluation occurs immediately after warming. Rather it was suggested that ovarian tissues should be cultured before analysis (Mouttham et al., 2015; Mouttham and Comizzoli, 2016).In this regard, future studies on peccary ovarian cryopreservation should be conducted to assess culturing of vitrified tissues before evaluation, mainly because an *in vitro* culture system was recently reported for this species (Lima et al., 2018).

5. Conclusions

In conclusion, the results of the present study indicate that solid surface vitrification is an effective method to conserve the female germplasm from *Pecari tajacu*, regardless of the CPA used (EG, DMSO, or DMF). The use of EG at a 3 M concentration, however, appears to be the most efficacious cryoprotectant concentration and compound for preservation from both a viability and morphological perspective of peccary PFs. In addition, the use of DMF appears to be a viable alternative as a cryoprotectant for mammalian ovarian tissue as a result of findings using the peccary in the present study. This is important information not only for the establishment of biobanks with material derived from peccaries, but also to apply the findings of the present study to other related threatened species.

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