Conservation of somatic tissue derived from collared peccaries (*Pecari tajacu* Linnaeus, 1758) using direct or solid-surface vitrification techniques

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Abstract Cryopreservation of somatic tissue can be applied in biodiversity conservation, especially for wild species as collared peccary. We aimed to evaluate the effect of vitrification techniques of ear tissue of collared peccary [direct vitrification in cryovials (DVC) or solid-surface vitrification (SSV)] on the layers of epidermis and dermis by conventional histology and cell ability during the in vitro culture. Thus, both the vitrification methods were able to maintain normal patterns of the epidermis as the cornea and granular layers, furthermore the intercellular space and dermal–epidermal junction of the spinous layer when compared to fresh control. Nevertheless, DVC and SSV percentage of normality decreased in the morphological integrity of cytoplasm (37.5 and 25.0%) of spinous layer, respectively, as compared to the fresh fragments (100%, $p < 0.05$). Moreover, other differences between the fresh control

(100%) and DVC tissues were verified in the intra-epidermal cleavage of the spinous (37.5%) and basal (37.5%) layers. In general, DVC and SSV techniques were efficient for the recovery of the somatic cells according to most of the evaluated parameters for the in vitro culture ($p > 0.05$). In addition, only at time of 72 h (D3), in the growth curve, DVC fragments showed a reduced cell concentration than fresh control. In conclusion, SSV was found to be a more efficient method for vitrifying collared peccary skin tissue when compared to DVC. These results are relevant for the tissue cryopreservation from collared peccary and could also be useful for mammals with phylogenetic relationships.

Keywords Wild animals · Cryopreservation · Somatic cell · In vitro culture · Dermis · Epidermis

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Introduction

The vulnerability to extinction of several species caused by unnatural factors is a growing concern in the context of animal biodiversity. Besides captive breeding of wild animals with commercial purposes is an alternative for ranchers that have limited productive conditions for breeding domestic animals, it is also characterized as a strategy for some species conservation (Santos et al. 2009). In this context, the development of conservation strategies have been required for collared peccaries, especially in the

Caatinga and Atlantic Forest biomes, where they are endangered (Desbiez et al. 2012).

In general, collared peccaries are very attractive for captive breeding due to the high commercial value of its meat and leather in the international market, which results in a great interest to commercial management, particularly in the multiplication of the individuals with valuable genetic characteristics (Nogueira and Nogueira-Filho 2011). Moreover, due to phylogenetic proximity, collared peccaries can be used as an experimental model for closely related species classified as vulnerable, *Tayassu peccary* Link, 1795, or under risk of extinction, *Catagonus wagneri* Rusconi, 1930 (IUCN 2016).

Thus, the cryopreservation of somatic tissue has been studied in order to support the biodiversity conservation. The establishment of tissue banks to obtain skin cells has been indicated as a practical approach for the preservation of species (León-Quinto et al. 2009; Cetinkaya and Arat 2011). It is associated with the somatic cell nuclear transfer (cloning) that is highlighted for the possibility of providing restoration of endangered/threatened species (Li et al. 2009; Folch et al. 2009).

Several factors as the sampling of a larger group of individuals as well as the independence of gender or age highlight the use of skin samples for the cryobank formation (León-Quinto et al. 2011). Moreover, this tissue can be quite easily obtained by using a simple and low-cost methodology (Silvestre et al. 2002).

Different cryopreservation techniques, such as slow and rapid freezing and vitrification can be employed for tissue preservation. In general, these techniques vary on several aspects as the method, the type and concentration of cryoprotectants, the temperature variations, and others (Carvalho et al. 2011). The major difficulty found in the use of slow and rapid freezing is the formation of intracellular ice crystals, which can provoke irreversible cell damage after warming. In this context, the vitrification appears as an alternative to prevent the formation of ice crystals. This method consists in establishing a vitreous stage in biological systems, which prevents water crystallization and allows cell survival (Carvalho et al. 2013).

Consequently, different tissue vitrification techniques have been studied over the years (Al-Aghbari and Menino 2002; Silvestre et al. 2004; Carvalho et al. 2011). Vitrification can be usually conducted through different methods, from which the direct vitrification

in cryovials (DVC) and the solid-surface vitrification (SSV) are highlighted (Carvalho et al. 2011). In the latter system, the sample is superimposed on a cubic hollow metal surface partially submerged in liquid nitrogen causing vitrification followed by its storage in cryovials. This technique consists of a low cost and high feasibility system already used for ovary tissue preservation (Aerts et al. 2008; Carvalho et al. 2011). Thus, the choice of an appropriate vitrification method consists of evaluating the tissue of interest. Currently, there is no information on the efficiency of SSV on the conservation of somatic tissue.

Therefore, the aim of this study was to evaluate the effect of vitrification techniques of ear tissue of collared peccary, DVC and SSV, on the layers of epidermis and dermis by conventional histology and cell ability during in vitro culture.

Materials and methods

All experimental protocols and animal care were approved by the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92), in compliance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-1). The reagents, media and solutions used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA), Gibco-BRL (Carlsbad, CA, USA) and Labimpex (São Paulo, Brazil).

Animals

Eight animals, four males and four females, obtained from the Centre for Wild Animals Multiplication (CEMAS/UFERSA, No. 1478912) were used. The individuals were clinically healthy and presented a proximate age (7–12 months old).

Skin biopsy and experimental design

Since the usual management of the collared peccaries include their identification by ear sections, these skin fragments (1–2 cm²) were recovered and used for the experiment. Skin samples were transported to the laboratory in phosphate buffer saline (PBS) supplemented with 2% (v/v) antibiotic–antimycotic solution (penicillin G, streptomycin and amphotericin B) at

37 °C for 30 min. At the laboratory, the tissue fragments were washed in 70% ethanol and Dulbecco modified Eagle medium (DMEM) supplemented with 2.2 g/L (w/v) sodium bicarbonate and 2% (v/v) antibiotic–antimycotic solution.

Subsequently, the 24 fragments derived from each individual were equally distributed into three treatments. The first treatment consisted in the fresh control; in the second one, samples were submitted to the direct vitrification in cryovials (DVC); and in the third one, to solid-surface vitrification (SSV). In each treatment, the fragments were equally distributed for histological and in vitro culture analysis. After 2 weeks, fragments derived from DVC and SSV were warmed and fresh and vitrified fragments were evaluated for morphological aspects of the epidermis and dermis layers by conventional histology. Moreover, samples were destined to primary tissue culture and subcultures for up to third passages of up to 35 days. Cells derived from cultured tissues were analyzed for morphology, adhesion, subconfluence, proliferative activity through cell growth curve and determination of population doubling time (PDT) and viability by trypan blue, as described in the following.

Somatic tissue vitrification

Skin fragments ($3.0 \times 3.0 \times 1.0 \text{ mm} = 9.0 \text{ mm}^3$) were randomly allocated for each treatment. A vitrification solution (VS) constituted of DMEM supplemented with 20% (v/v, 3.58 M) ethylene glycol (EG), 20% (v/v, 2.82 M) dimethyl sulfoxide (DMSO), 0.25 M (w/v) sucrose and 10% (v/v) fetal bovine serum (FBS) was used for both vitrification techniques adopted.

Direct vitrification in cryovials

The direct vitrification in cryovials (DVC) technique was conducted according to a methodology previously described for porcine somatic tissues (Silvestre et al. 2003). Briefly, fragments were immediately transferred for cryovials containing 2.0 mL VS and kept for 15 s at room temperature. Then, the cryovials were stored in liquid nitrogen.

Solid-surface vitrification

For the solid-surface vitrification (SSV) procedures, we adopted a methodology previously described for

caprine ovarian tissues (Carvalho et al. 2011, 2013). Briefly, two fragments were exposed to 1.8 mL VS for 5 min; tissues were then dried on absorbent paper. Subsequently, the fragments were individually placed on a metal cubic surface partially immersed in liquid nitrogen, transferred to cryovials and stored in liquid nitrogen.

Warming

After 2 weeks of cryostorage for both vitrification techniques, the cryovials were maintained for 1 min at room temperature and then immersed in a water bath at 37 °C. For removal of cryoprotectants, fragments were washed three times for 5 min in DMEM plus 10% (v/v) FBS supplemented with sucrose at decreasing concentrations (firstly 0.50 M, then 0.25 M, and finally without sucrose).

Histological analysis

Fragments derived from both control and vitrified groups were fixed in Carnoy solution for 12 h, sectioned at 7 μm , stained with hematoxylin-eosin and evaluated by using a light microscope (Olympus CX 31 RBSFA, Tokyo, Japan). The morphological parameters of normality, cell identification and presence of granulation and vascularization in the dermis and epidermis layers were assessed (Ge et al. 2010). For each parameter, three slides per animal were assessed, being eight individuals evaluated per treatment, resulting in a total of 24 slides per treatment. These slides were evaluated over the entire area. The normal pattern was established taking as basis the initial evaluation of the pattern verified in the fresh control treatment. Therefore, the variations found in vitrified groups were considered as not normal when compared to the control group and they were quantified.

For the epidermis evaluation (Ge et al. 2010 adapted), the aspects of cornea, granular, spinous (intercellular space and cytoplasm, presence of intra-epidermal cleavage, presence of separation of the dermis–epidermis junction, presence of vacuoles or halos in nucleus) and basal layers were analyzed. Such aspects were determined as normal when the cornea layer presented little detachment; when the granular layer was unicellular; when the spinous layer presented a discreet intercellular space as well as the

cell's cytoplasm did not show perinuclear halos, and there was no intra-epidermal cleavage. Moreover, the integrity of the dermis–epidermis junction was determined when there was no separation between the two layers and the cell's nucleus presented no vacuoles or halos. Finally, the basal layer was considered as normal when imperceptible. In the dermis, the dermal collagen was evaluated, being considered as normal when there were no spaces nor lysis among fibers, that showed an evident nucleus.

Cell culture, morphological assessment and viability

Fresh and vitrified ear fragments were cultured in DMEM supplemented with 2.2 g/L (w/v) sodium bicarbonate solution, 2% (v/v) antibiotic–antimycotic solution and 10% (v/v) FBS at 38.5 °C, 5% CO₂ in air and 95% relative humidity. The total culture medium was daily substituted. Petri dishes (35 mm) were used for the primary culture. The cells were harvested when they reached 70% subconfluence and were subcultured into petri dishes of 100 mm for 35 days. 70% subconfluence was defined when 70% of the petri dishes showed somatic cells (Silvestre et al. 2003).

The assessment of the morphological characteristics was performed on the cells from the first passage in terms of cell adherence and confluency during culture using an inverted microscope (Nikon TS100, Tokyo, Japan). The following parameters were evaluated: morphology, number of attached explants, number of explants with subconfluence, days of all attached explants, number of explants that grow to subconfluence, days of subconfluence for explants, and total time for subconfluence (Silvestre et al. 2003).

The cellular viability was assessed by the use of a trypan blue staining, and the test was conducted in duplicate. Briefly, cells were suspended in culture medium and a cell aliquot was stained with 0.4% (w/v) trypan blue (in PBS, ratio 1:40) and counted in a Neubauer chamber. The cells were considered viable cells when they were not stained, i.e., cells without a ruptured membrane; the non-viable cells became blue because of the penetration of the dye (Strober 2001).

Proliferative activity

Proliferative activity was quantified according to the elaboration of the growth curve and determination of

PDT. Briefly, cells were seeded in four-well dishes at 1.0×10^4 cell/mL on the second passage. Cells from two wells were counted at 24 h intervals for up to 96 h of culture. After each interval, the mean cell counts were recorded, and the cell growth curve was generated (Costa et al. 2005). Finally, PDT was estimated according to Roth (2006), through the formula:

$$\text{PDT} = T \ln 2 / \ln(X_e/X_b),$$

PDT is the publication doubling time of the culture (in h); T is the incubation time; X_b is the number of cells at the beginning of the time incubation; X_e is the number of cells at the end of the incubation time; ln is Napierian logarithm.

Statistical analyses

Eight repetitions were conducted for the experiments (one animal/one repetition). Histological results were expressed as percentage and in vitro culture results as mean $\pm$ standard deviation. The PDT assay and viability were performed in duplicate for each animal of each treatment. *In vitro* culture values were verified for normality by the Shapiro–Wilk test and homoscedasticity by Levene's test. Since data regarding the trypan blue test did not show a normal distribution, they were arcsine transformed. Comparison among treatments (control vs DVC vs SSV) on the histological values were conducted using Fisher's exact test. For the values derived from the in vitro culture, values were subjected to the variance analysis (ANOVA) followed by paired and unpaired *t* test. All data were evaluated using the StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Differences among treatments were considered significant when $p < 0.05$.

Results

Morphological features in fresh (control) or vitrified (DVC and SSV) somatic tissue are shown in Fig. 1. The fresh control fragments presented no degenerated aspects (100%, Tables 1, 2) for any of the histological parameters evaluated.

Both vitrification procedures (DVC and SSV) were able to maintain normal patterns of the epidermis as the cornea and granular layers, besides of the intercellular space and dermal–epidermal junction of the spinous layer when compared to the fresh control

Fig. 1 Histological analysis of ear tissue of collared peccaries in fresh control (**a**, **a'**, **a''**), DVC technique (**b**, **b'**, **b''**) and SSV technique (**c**, **c'**, **c''**). **a–c** represent an overview of the skin layers; **a'–c'** represent layers of the epidermis; **a''–c''** represent

only the dermis. Layer cornea (LC), granular layer (GL), spinous layer (SL), basal layer (BL) and collagen dermal (CD). (*thick arrow*) spacing of the collagen fibers and (*thin arrow*) presence of nuclei

(Table 1). Nevertheless, for histological analysis, DVC and SSV caused a significant decrease on the morphological integrity of the cytoplasm of the spinous layer cells as compared to the fresh fragments (Table 1). There were also evident differences between DVC and fresh tissues with regard to the intra-epidermal cleavage of the spinous layer and basal layer. In the dermis, both techniques caused

damages related to spacing between the fibers, lysis of the fibers and nuclei absence in the dermal collagen (Table 2) when compared to fresh control fragments.

In relation to in vitro culture (Fig. 2), both vitrification techniques (DVC and SSV) were adequate for the recovery of the somatic cells, indicating that vitrification is a suitable method for somatic tissue cryopreservation (Table 3). However, differences

Table 1 Histological evaluation of normal epidermis of ear skin tissue derived collared peccaries after vitrification techniques

	Cornea	Granular	Spinous				Basal	
			Intercellular space	Cytoplasm	Intra-epidermal cleavage	Dermal-epidermal junction	Nucleus	
Fresh	8/8 (100) ^a	8/8 (100) ^a	8/8 (100) ^a	8/8 (100) ^b	8/8 (100) ^a	8/8 (100) ^a	8/8 (100) ^b	8/8 (100) ^a
DVC	4/8 (50.0) ^a	6/8 (75.0) ^a	7/8 (87.5) ^a	3/8 (37.5) ^a	3/8 (37.5) ^b	6/8 (75.0) ^a	6/8 (75.0) ^b	3/8 (37.5) ^b
SSV	4/8 (50.0) ^a	6/8 (75.0) ^a	5/8 (62.5) ^a	2/8 (25.0) ^a	4/8 (50.0) ^{ab}	5/8 (62.5) ^a	3/8 (37.5) ^a	4/8 (50) ^{ab}

Values shown normal epidermis (%). Fresh control

DVC technique direct vitrification in cryovials, *SSV technique* solid surface vitrification

^{a,b} Different letters show differences statistically significant in the same column ($p < 0.05$) when compared to fresh control and between groups

Table 2 Histological evaluation of normal dermis of ear skin tissue derived collared peccaries after vitrification techniques

	Spacing the fibers	Lysis of the fibers	Disappearance of nuclei
Fresh	8/8 (100) ^b	8/8 (100) ^b	8/8 (100) ^b
DVC	1/8 (12.5) ^a	2/8 (25.0) ^a	1/8 (12.5) ^a
SSV	1/8 (12.5) ^a	3/8 (37.5) ^a	1/8 (12.5) ^a

Values shown normal dermal collagen (%). Different letters show differences statistically significant in the same column ($p < 0.05$) when compared to fresh control and between groups

DVC technique direct vitrification in cryovials, *SSV technique* solid surface vitrification

were observed between the fresh and vitrified groups for the day of all attached explants and the day of all explants with cell grow (Table 3). One fresh fragment showed contamination in the early days of primary culture, and it was later removed from the experiment. In general, epithelial-like and fibroblast-like cells migrated from tissue fragments at 6.6, 10.7 and 9.2 days after explanting for fresh control for DVC and SSV groups, respectively. Over time, cells continued to proliferate and were subcultured when they reached 70% confluence from 17.5 to 18.0 days for all groups. All cultured cells were morphologically consistent with fibroblast with fusiform aspect and a central oval nucleus. Moreover, no difference was observed among all groups for viability and PDT assays ($p > 0.05$, Table 3), with daily cell duplication averaging approximately 13.5, 16.6 and 16.6 h for the fresh, DVC and SSV fragments, respectively.

The growth curves (Fig. 3) for the fresh control and the experimental groups presented a typical sigmoid form, which consist of the lag phase and the exponential phase and absence of the stationary phase, because the culture had duration of only few days. The samples of the fresh control group remained in log

phase for only 48 h. The DVC samples already showed a lag phase until 48 h, and then the exponential phase. Finally, cells submitted to the SSV treatment showed the lag phase until 48 h, followed by the exponential phase. Moreover, only the DVC group differed from the fresh control at 72 h (or D3) with a lower cell concentration ($p < 0.05$).

Discussion

In this study, we did not observe difference between fresh control and vitrified (DVC and SSV) groups with regard to the analysis of viability and PDT assay. This statement evidences that the vitrification process, regardless of the method used, does not affect the ability of the collared peccaries' ear tissue and cells during in vitro culture up to 35 days. Thus, both vitrification methods were suitable for the maximum conservation of the morphology of the tissues derived from skin biopsies as well as for the conservation of the cell ability during in vitro culture. These factors highlight the possibility of obtaining somatic cells from the vitrified ear tissue of peccaries. Probably, the

Fig. 2 In vitro culture of ear tissue of collared peccary demonstrating morphology, adhesion, and proliferation of somatic cells in fresh control groups (a–c), DVC technique

(a'–c') and SSV technique: (a''–c''); a, a', a'' primary culture; b, b', b'' early detachment and cell growth; c, c', c'' secondary culture and subconfluence

damage caused by the vitrification affected only the characteristics of tissue fragments attached on the substrate, not damaging the recovered somatic cells. This can be explained as the explants formed by several layers of tissue and cell types act in protection of cells located internally, allowing their preservation and further culture (Abazari et al. 2013). Therefore,

the most appropriate vitrification method was chosen according to the characteristics of the tissue cell type.

Through histological analysis, in general, DVC treatment provoked evident damages on the tissue in comparison to the fresh control group. This could be a consequence of tissues from the DVC group which were subjected to longer exposure to cryoprotectants

Table 3 Establishment of primary cell culture and subculture cell of ear skin samples from derived collared peccaries after vitrification techniques and cells cultured in vitro for 35 days

Technique	No. of samples			No. of attached samples			Viability $\pm$ SD (%)	PDT $\pm$ SD (h)
	Initial	Attached (%)	Day all attached explants $\pm$ SD	Grow to subconfluence (%)	Day all cell grow explants $\pm$ SD	Subconfluence total time (days) $\pm$ SD		
Fresh	32	31 (96.9) ^a	1.3 $\pm$ 0.5 ^b	31 (96.9) ^a	6.6 $\pm$ 1.6 ^b	18.0 $\pm$ 0.6 ^a	91.0 $\pm$ 6.0 ^a	54.0 $\pm$ 8.3 ^a
DVC	32	32 (100) ^a	6.0 $\pm$ 4.3 ^a	32 (100) ^a	10.7 $\pm$ 3.2 ^a	17.5 $\pm$ 0.6 ^a	89.0 $\pm$ 2.0 ^a	66.1 $\pm$ 92.8 ^a
SSV	32	32 (100) ^a	4.1 $\pm$ 1.7 ^a	32 (100) ^a	9.2 $\pm$ 2.1 ^a	18.3 $\pm$ 1.2 ^a	86.0 $\pm$ 4.5 ^a	66.3 $\pm$ 44.4 ^a

^{a,b} Different letters show differences statistically significant in the same column ($p < 0.05$) when compared to fresh control and between groups. Fresh control

DVC technique direct vitrification in cryovials, *SSV technique* solid surface vitrification, *PDT* population doubling time

Fig. 3 The growth curve of skin cells derived from somatic tissue of collared peccary in fresh control and vitrified fragments (DVC and SSV). *Differences statistically significant for the same time with the fresh control group ($p < 0.05$)

during warming than for the SSV method. In the SSV treatment, the samples are not in contact with a large amount of cryoprotectants which decreases toxicity due to exposure during drastic temperature changes (Carvalho et al., 2011). In fact, Saragusty and Arav (2011) reported the need of a minimum volume of cryoprotectant as one of the important factors to achieve the vitreous state.

In comparison to the fresh control group, both vitrification techniques (DVC and SSV) provoked morphological damages in the dermal collagen; however, they were equally efficient in preserving the epidermis parameters. These clear differences can be attributed to various skin layers and their sources; the epidermis has ectodermal origin whereas dermis has mesodermal origin, resulting in different cell types

that compose each of these layers. The epidermis is avascular and the dermis is vascularized. The epidermis has stronger characteristics for this layer and is an important skin barrier to protect the body, which is more resistant than the dermis (Debeer et al. 2013; Summerfield et al. 2015).

In relation to cryoprotectants used in this study (DMSO, EG and sucrose), DMSO is considered the most widely used agent for porcine skin cryobanks (Brockbank et al. 2010). Commonly, DMSO is used alone or in combination with other cryoprotectants, such as EG (Santos et al. 2007). However, EG is recommended as the most permeable cryoprotectant (Silvestre et al. 2002). Sucrose is used to avoid excessive swelling and osmotic shock, stabilizing cell membranes during the vitrification (Rodrigues et al. 2008). Nevertheless, despite the protection that cryoprotectants provide to the cells during the cryopreservation, there is a risk of cell damage, especially when used in high concentrations (León-Quinto et al. 2011). These concentrations may reduce cellular ionic strength, contraction and cell electrolyte leakage (Praus et al. 1980).

In swine somatic tissue, Silvestre et al. (2002, 2003), using a combination of the DVC technique and DMSO/EG (20, 20%), we observed that all vitrified fragments reached subconfluency and cells derived from these fragments reached confluency up to 92% during subculture. In addition, the use of SSV technique and the same cryoprotectant combination was already reported for porcine ovarian tissue cryopreservation (Moniruzzaman et al. 2009). Thus, cryoprotectant concentrations used in this study were adequate, since they did not affect the culture quality of the recovered cells.

For the *in vitro* culture, differences between fresh control and vitrified groups regarding the day of attached fragments and day of all explants with cell growth were observed. These results allow to affirm the vitrification method although it alters the normal growth pattern and not adversely affects both vitrification groups, it only delays the appearance of the cells. In addition, the normal characteristics of the cell do not vary according to the method. It is important to note that changes in cellular characteristics in culture can occur for long intervals between passages or extended maintenance of the cell confluence (Mehrabani et al. 2014). Further, biological characteristics, especially the genetic characteristics of cells, can be altered by *in vitro* culture conditions following many passages, with a minimum number of passages recommended for its conservation (Mehrabani and Manafi 2013).

Although all samples presented adhesion and cell confluence, the fragments of DVC group had more difficulty in reaching confluence, since various cellular debris appeared during this period. This observation can be justified with the fact that a large amount of cryoprotectants are still in contact with the tissue, even during warming, and the long time required for warming the samples. Prolonged exposure during warming can generate cell interactions/cryoprotectant which may damage their proliferative capacity (May and Wainwright 1985).

During the first passage, similar viability was observed among the treatments. Therefore, the tissue vitrification methods used in this study had no negative effect on cell culture, as also seen by Dariolli et al. (2013). They could show that after cryopreservation of the porcine adipose tissue it was possible to obtain mesenchymal stem cells. Cell viability is an information that significantly contributes to the optimization of vitrification methods (Carvalho et al. 2011), confirming that the skin can be conserved for long time without losing its viability, if stored at low temperatures (Praus et al. 1980; Hu et al. 2013).

With respect to the growth curve, the absence of a stationary phase in all groups was observed, showing the need of an observation period longer than 4 days of culture. These data also corroborate with Hu et al. (2013) who demonstrated that fibroblasts derived from cryopreserved goat ear tissue reached similar time of the lag phase (48 h) with 65.5 h of PDT. In porcine cells, a PDT ranging from 55h to 140 h was observed

(Hao et al. 2009). Moreover, at 72 h or on the third day (D3), only the DVC group showed a low cell number with respect to fresh fragments. It shows that the tissue required a long time to recover damages caused by the DVC technique for collared peccaries considering the long period of warming and exposure to cryoprotectants at room temperature. Thus, these samples in culture took a long time to adapt and initiated the process of cell detachment.

Regarding the fragment used, the tissue showed high survivability and maintained its structural integrity during the *in vitro* culture, showing that the size of the tissue samples (9.0 mm³) corresponded to the specifications of vitrification methods. This did not affect the samples following culture. In general, vitrification requires conditions to stop the chemical and biological activity of the cell for long periods of storage without affecting the cellular functionality after warming (Jomha et al. 2002). The vitrification has been very successful in terms of metabolic cell function and viability (Magalhães et al. 2012).

One episode of contamination of a fresh control fragment was observed during the experiment. As the contamination occurred only in the fresh control group, we ruled out any possibility of contamination source with liquid nitrogen. Thus, the methods chosen for vitrification have another advantage to not to expose the biological material to liquid nitrogen. In both methods, the samples were stored in cryovials, avoiding the risk of contamination during vitrification and storage (Bos-Mikich et al. 2012).

The differences observed between the DVC and SSV groups could also be attributed to the fact that samples are usually exposed to a large amount of cryoprotectants during warming in DVC, but not in SSV, which reduces the risk of toxicity generated by cryoprotectants. Thus, the results indicate that SSV method is more recommended for the cryopreservation of ear tissue derived from collared peccary when compared to the DVC. In this sense, the cryopreservation of collared peccary somatic tissue seems to be a simple alternative approach that allows the obtaining of viable cells. These cells are able to be used in various reproductive biotechnologies, as cloning (Saini et al. 2015), studies on cell differentiation and nuclear reprogramming (Hao et al. 2009), and monolayer formation to other cultures, especially in developing embryos (Shah et al. 2015). In addition, vitrification of somatic tissue is a relevant tool for

the conservation of germplasm and preservation of biodiversity (Song et al. 2007). We emphasize, however, that despite the SSV method providing an efficient preservation of ear skin cells derived from collared peccaries, there is a lack of information regarding the detailed characterization of peccaries epithelium, which could help us to establish trustworthy parameters for evaluating the SSV technique efficiency.

We clearly demonstrated the possibility of using the ear tissue vitrification as an expectation to obtain genetic material from few peccaries, as demonstrated for other wild species (Guan et al. 2010). In general, the somatic tissue is easily obtained, which supports the efficiency of vitrification. Thus, in regions of difficult access, a greater number of individuals can be collected, without restricting the age and gender, thus allowing an unlimited number of samples and the extrapolation of these cell characteristics for the entire population (León-Quinto et al. 2009). Therefore, somatic tissue samples can contribute to preservation programs (Caputcu et al. 2013; Silvestre et al. 2004). These skin cryobanks permit the maintenance of a maximal representation of biodiversity population, being an important tool in wildlife preservation programs (León-Quinto et al. 2014).

In conclusion, SSV treatment was the most appropriate method for the vitrification of somatic tissue of collared peccaries in comparison to DVC and the recovered viable somatic cells can be used in cloning. Thus, the SSV technique is suitable for cryopreservation of collared peccaries' ear tissue, aiming at the conservation of genetic material both of this species as of other phylogenetically closely related species.

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