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ANIMAL SCIENCE

Comparative effect of cryoprotectant combinations on the conservation of somatic cells derived from jaguar, *Panthera onca*, towards the formation of biologic banks

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Abstract: Due to the reduction of the jaguar population, the formation of somatic cell cryobanks represents an interesting tool for its conservation. Nevertheless, the success of these cryobanks depends on the cryoprotectants used in cryopreservation. We evaluated the effects of the intracellular cryoprotectants (10% dimethyl sulfoxide, DMSO; 10% ethylene glycol, EG) in the absence or presence of an extracellular cryoprotectant (0.2 M sucrose, SUC) on the morphology, confluence, viability, and metabolism of somatic cells derived from five jaguars belonging to Brazilian zoos. The morphology was presented in a descriptive manner, while the confluence, viability and metabolic activity were presented as means and compared using statistical tests. Non-cryopreserved cells were used as control and compared to frozen/thawed cells using cryoprotectants. No difference was observed for the morphology and confluence among non-cryopreserved and cryopreserved cells, regardless of the cryoprotectants. Only cryopreserved cells in EG (45.8%±12.9) had a reduction in their viability when compared to non-cryopreserved cells (97.8%±1.1). Only cryopreserved cells in DMSO with SUC (76.0%±2.7) or absence of SUC (77.0%±3.7) maintained their metabolic activity after thawing, when compared to non-cryopreserved cells (100.0%±6.7). Therefore, combinations of DMSO in the absence and presence of SUC were efficient in the cryopreservation of somatic cells of jaguars.

Key words: Biological resource banks, cryopreservation, *Panthera* genus, slow freezing, wild felids.

INTRODUCTION

The *Panthera* genus consists of the felids represented by lions, tigers, leopards, and jaguars, which the latter are the only ones distributed on the American continent (Morato et al. 2016). Although being the largest felid in the Americas and widely distributed (Morato et al. 2014), the jaguar has been suffering a decline in its quantitative population, which has been estimated at about 55% of its initial population since the last century (Jędrzejewski et al. 2018). Consequently, this species is considered globally as a species almost threatened with extinction (Quigley et al. 2017), with subpopulations of Latin America being identified as threatened or critically endangered (Morato et al. 2018).

Considering this scenario, conservation strategies for jaguars are necessary, including the use of artificial insemination (Goeritz et al. 2012), embryo transfer (Pope et al. 2012), *in vitro* maturation and intracytoplasmic sperm injection (Fernandez-Gonzalez et al. 2015), cloning by nuclear somatic cell transfer (Moulavi et al. 2017) and studies on nuclear

reprogramming mechanisms (Verma et al. 2013). For the latest purposes, a crucial step is the formation of somatic cell cryobanks, whose success depends on the proper choice of the cryopreservation technique and cryoprotectants employed. In general, slow freezing has been the cryopreservation technique routinely employed for somatic cells of some wild felids, as *Panthera trigris altaica* (Song et al. 2007), *Panthera tigris tigris* (Guan et al. 2010), and *Panthera onca* (Mestre-Citrinovitz et al. 2016). This technique consists in the controlled temperature reduction using systems, such as programmable freezer (Guan et al. 2010) and Mr. Frosty (Mr. Frosty™ Freezing Container, León-Quinto et al. 2014) and its efficiency has been measured according to the lower amount of cryoinjury generated during freezing and thawing.

In order to reduce cell injury, the proper use of intracellular and extracellular cryoprotectants plays a significant role in the cryobank formation. In somatic cells, both dimethyl sulfoxide (DMSO) and ethylene glycol (EG) have been used as intracellular cryoprotectants and its protection's ability depends of species, and cell type (Li et al. 2006). In general, the low molecular weight of EG (62.1 g/mol) and DMSO (78.0 g/mol) ensures the high permeability in the cell; nevertheless, such permeability may cause osmotic stress (Schneider & Mazur 1984), provoking cell injury and, therefore, the use of cryoprotectant must be optimized for cell in study. Moreover, to minimize the effects of toxicity from intracellular cryoprotectants, extracellular cryoprotectants have been employed, such as sucrose (León-Quinto et al. 2014). This molecule acts by increasing the osmolality of the extracellular medium, promoting the outflow of water from the intracellular environment, reducing the injuries during cryopreservation, and resulting in a positive effect on cellular functionality (Gurruchaga et al. 2019).

Therefore, this present study designed to evaluate the effects of the intracellular cryoprotectants (DMSO or EG) in the absence or presence of an extracellular cryoprotectant (SUC) on the morphology, confluence, viability, and metabolic activity of somatic cells derived from jaguars, aiming the formation of biological banks for the species.

MATERIALS AND METHODS

Unless otherwise noted, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco-BRL (Carlsbad, CA, USA). The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was purchased from Grainer (Miami, FL, USA).

Bioethics and animals

The experimental protocols were performed with the approval of the Animal Ethics Committee of the Universidade Federal Rural do Semi-Árido (CEUA/UFERSA, no. 23091.0011507/2017-61), in compliance with the Instituto Chico Mendes para Conservação da Biodiversidade (ICMBio, no. 57460-1).

Two skin samples (1–2 cm^2) of each individual were obtained from five jaguars belonging to the São Francisco do Canindé Zoo (Canindé, CE), Zoo and Parque Dois Irmãos (Recife, PE), Parque Zoobotânico Arruda Câmara (João Pessoa, PB), and Ecopoint (Fortaleza, CE). The animals had estimated age ranging from 7–16 years, being four males and one female. After administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, New York, USA) and mechanical containment (Araujo et al. 2018), peripheral skin samples from the ear were collected and transported to the laboratory in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% solution of 10,000 units/

mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin at 4°C for 8 h.

Recovery of somatic cells and experimental design

In the laboratory, the tissue fragments were trichotomized, washed in 70% ethanol and in DMEM containing 10% FBS and 2% solution of 10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin. Successively, the skin was fragmented in 9.0 mm^3 (3 × 3 × 1 mm) and explants were cultured by primary and secondary systems for recovery of somatic cells, according to Praxedes et al. (2017, 2019). All fragments from each animal were processed and cultured separately. All explants were incubated at 38.5°C, 5% CO_2, and 95% air in DMEM with 10% FBS, 2% solution of 10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin. The culture medium was changed every 24 h and the cells were harvested until they reached 70% subconfluency. Finally, cells were subcultured into other dishes until reach a concentration of 1.0 x 10^5 cells/mL to be cryopreserved (Praxedes et al. 2019).

Then, somatic cells were divided among non-cryopreserved (control) and cryopreserved groups using a concentration of 1.0 x 10^5 cells per 1.0 mL for each treatment. The cryopreservation solution was prepared using DMEM with 10% FBS and supplemented with ethylene glycol (EG), dimethyl sulfoxide (DMSO) without or with sucrose (SUC; 0.2 M), as follows: DMSO (10% DMSO), DMSO-SUC (10% DMSO and 0.2 M SUC), EG (10% EG), EG-SUC (10% EG and 0.2 M SUC).

Cryopreservation of somatic cells

Somatic cells were randomly allocated for each group, and cryopreserved using slow freezing, according to Mestre-Citrinovitz et al. (2016) with modifications. Initially, cells were suspended in 350 µL of DMEM containing only 10% FBS in cryovials. Then, 350 µL of DMEM containing DMSO or EG were added to the initial cell suspension. For the SUC-containing groups the 300 µL to reach the 1.0 mL volume were added of DMEM containing SUC. For the groups without SUC, 300 µL DMEM were added to reach the 1.0 mL volume. Finally, cell suspension in cryovials were maintained at 4°C for 10 min, and transferred to a -80°C freezer in Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, NY, USA) for 12 h using a cooling rate of 1°C/min. Subsequently, all cryovials were stored in liquid nitrogen (-196°C).

After two weeks, the cryovials were maintained for 1 min at 25°C and immersed in a water bath at 37°C for 4 min, according to León-Quinto et al. (2014). For removal of cryoprotectants, cell suspensions derived from groups contained SUC were added in DMEM plus 10% FBS and 0.2 M SUC and maintained at 4°C for 15 min, followed by centrifugation at 1300*g* for 10 min. Subsequently, the supernatant was removed; and cellular content was suspended in DMEM with 10% FBS, maintained at 25°C for 15 min, followed by another centrifugation at 1300*g* for 10 min. Finally, cell suspensions derived from groups without SUC were centrifuged twice as previously described using DMEM plus 10% FBS. After first and second medium addition, cells were maintained at 4°C for 15 min and 25°C for 15 min, respectively.

Evaluation of cell morphology and confluence

Cells in culture were analyzed daily for cell morphology and confluence. Thus, the culture plates were arranged under an inverted microscope (Nikon TS100, Tokyo, Honshu, Japan) for observation of the morphology, analyzing the size, appearance, shape, and adhesion patterns. Moreover, the confluence was performed at the same time under the same circumstances described, but for this method, after observing

the entire surface of the plate, a value was adopted, resulting in a confluence rate ranging from 0–100%, according to number of cells that covered the perimeter of interest (Praxedes et al. 2017).

Evaluation of cell viability

The viability was determined by the trypan blue exclusion assay according to León-Quinto et al. (2014) in non-cryopreserved, cryopreserved and cryopreserved/cultured cells. A total of 200-300 cells were counted for each group. Briefly, thawed cells or cultured after five days of thawing were centrifuged and suspended in culture medium; a cell aliquot (20 µL) was stained with 0.4% trypan blue (in phosphate buffer saline) in the ratio 1:4 and counted in a Neubauer chamber resulting in a viability rate according to the formula:

Viability rate = (alive cells/total counted cells) * 100.

Evaluation of metabolic activity

Non-cryopreserved and cryopreserved cells were seeded into 12-well dishes at a concentration of 5.0×10^4 cells/mL and cultured at 38.5°C in a humid atmosphere of 5% CO_2 according to Mosmann (1983) with adaptations. After 5 days, 1.0 mL of MTT (5 mg/mL in DMEM) was added to each well for 3 h in the same conditions. After incubation, the MTT solution was removed, 1.0 mL of DMSO was added to each dish, and the dishes were incubated for 5 min under light stirring to solubilize the formazan crystals. Finally, the absorbance of the samples was measured using a spectrophotometer (Shimadzu® UV-mini-1240, Kioto, Kioto, Japan), using an absorbance wavelength of 595 nm (León-Quinto et al. 2014).

Statistical analysis

All data were expressed as mean ± standard error (one animal/one replicate) and analyzed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively. When they did not show normal distribution, the data were transformed into arcsine and analyzed by ANOVA followed by Tukey test. Significance was considered with $p < 0.05$.

RESULTS

Morphology and confluence of cryopreserved cells

The peripheral ear skin samples of five jaguars resulted in 20 explants that were placed in primary culture and subcultures up to the fourth passage to reach a required concentration of fibroblast-like cells for cryopreservation. During the primary culture (Figure 1a) and all subcultures before cryopreservation (Figure 1b), subcultures after thawing with more than 70% confluence (Figure 1c), and cells with a fusiform aspect with cytoplasmic extensions, abundant cytoplasm, and a central oval nucleus (Figure 1d) were observed in all groups.

Moreover, all groups maintained the confluence patterns during the analysis, and no confluence reduction was observed. After trypsinization, in approximately 72 h, the cells reached a confluence above 45%, reaching over 59% in 96 h of culture. These means were similar ($p > 0.05$) before freezing (68.0% ± 0.4) and after thawing [DMSO (65.0% ± 0.1), DMSO-SUC (60.0% ± 0.1), EG (59.0% ± 0.5) and EG-SUC (61.0% ± 0.1)] and observed after with 96 h of culture.

Viability of cryopreserved cells

For viability rate after thawing cells (Table I), DMSO, DMSO-SUC and EG-SUC groups-maintained values similar to control ($p > 0.05$), and only EG was different from non-cryopreserved group ($p = 0.028$). Moreover, the

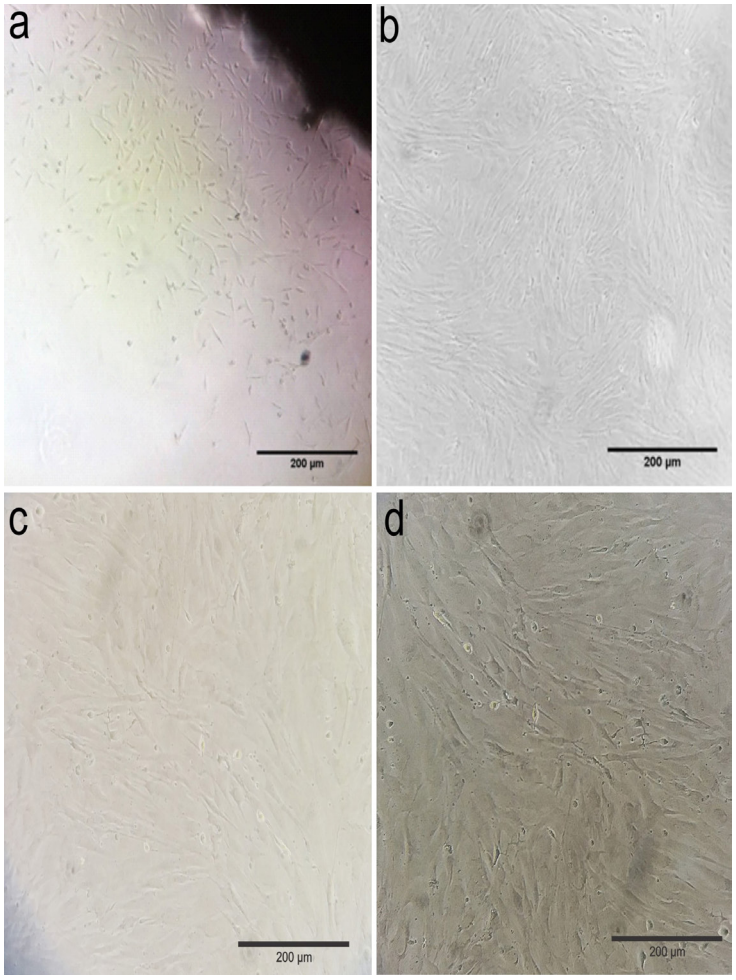

Figure 1. Outgrowth of primary fibroblast-like cells from jaguar skin explants demonstrating morphology and adhesion of somatic cells. (a) Cells during the primary culture. (b) Cell subculture before cryopreservation. (c) Cell subculture after thawing with approximately 70% confluence and (d) Cells with fusiform aspect similar to fibroblast cells.

viability after *in vitro* culture of all groups, DMSO (95.7% ± 1.3), DMSO-SUC (98.6% ± 0.3), EG (95.2% ± 3.5) and EG-SUC (96.5% ± 1.1) showed similar results to the control (97.8% ± 1.1 $p < 0.05$). In fact, after *in vitro* culture there was an increase in the viability rates in contrast to samples after thawing, regardless the cryoprotectants, and with a lower variance among the groups.

Metabolic activity of cryopreserved cells

The mean optical density values of the non-cryopreserved cells were considered 100% for comparison with the other groups. The values obtained from the reading of the groups were transformed into percentage by dividing by the mean value obtained with the non-cryopreserved group. For metabolic activity (Figure 2), only DMSO (77.0% ± 3.7) and DMSO-SUC (76.0% ± 2.7) groups were similar to control (100.0% ± 6.7, $p < 0.05$), statistically differing from the EG (74.0% ± 4.9) and EG-SUC (75.0% ± 5.2).

DISCUSSION

Aiming to establish somatic cell cryobanks for endangered wild mammals, such as jaguars, cryopreservation is an essential step to be optimized, especially in the types of cryoprotectants used (León-Quinto et al. 2014, Praxedes et al. 2018). The implementation of an ideal cryoprotectant solution allows the reduction of cellular cryoinjuries, resulting in the maintenance of the cellular morphology and confluence, besides of the viability and

Table I. Evaluation of different cryopreservation solutions on viability of somatic cells derived from jaguar.

Groups	Viability in % (mean ± S.E.)
Non-cryopreserved cells	97.8 ± 1.3[a]
Cells cryopreserved with	
DMSO	53.7 ± 9.8[a]
DMSO-SUC	58.6 ± 15.6[a]
EG	45.8 ± 12.9[b]
EG-SUC	52.4 ± 14.9[a]

In the column, different letters indicate significant difference ($p < 0.05$). S.E.: standard error.

metabolic activity after the freezing and thawing stages. In this study, we observed the positive effect of 10% DMSO with 10% FBS in absence or presence of 0.2 M sucrose on the conservation of jaguar-cryopreserved cells. Additionally, 10% DMSO has already been used in slow freezing in fibroblasts of different wild felids, such as Siberian tiger (*Panthera tigris altaica*), obtaining viability rates above 80% (Song et al. 2007).

The morphology observed after the cryopreservation process was similar to the fibroblasts-like cells, presenting a fusiform shape with cytoplasmic extensions, and a large oval nucleus, as well as rapid growth patterns, being these characteristics have already been reported in the literature for this cell type (Praxedes et al. 2017). The maintenance of the morphological characteristics is associated with a decrease of the ice crystals formation in the process by exposing the cells to the combination of intra- and extracellular cryoprotectants (León-Quinto et al. 2014).

The preservation of the cellular confluence in all groups was due to a stable growth pattern that can be observed in fibroblast-like cells of this species, such as Mestre-Citrinovitz et al. (2016) have already described. Moreover, the same authors denoted the importance of using cells for freezing up to the fourth passage, because after many subcultures the cells will have their level of growth diminished and will take longer to reach the desired confluence, besides dying by cellular senescence.

Viability assays showed a maintain cell membrane integrity, and this is an important parameter in the enhancement of slow freezing protocols, because the cells that have ruptured membranes are not viable for future applications for presenting initial apoptosis (Park et al. 2017). The presence of cryoprotectants has been shown to reduce the effects of osmotic pressure, becoming useful for cell maintenance. The technique employed also plays an important role in the maintenance of cell viability, where slow freezing is commonly associated with better results for wild felid somatic cells (Mestre-Citrinovitz et al. 2016).

Moreover, the addition of 10% EG in combination or not to 0.2 M SUC did not return promising results compared to the DMSO group. The better rates associated with groups containing DMSO are related to the fact that it is the most penetrating agent used to protect cells against cryoinjuries, because it has the ability to protect cells against freezing injury by reducing ice formation inside and outside the cells (León-Quinto et al. 2014). In general, the DMSO forms high-energy hydrogen bonds with water molecules, replacing them intracellularly (Weng et al. 2011). Briefly, DMSO and other intracellular cryoprotectants cross the cell membrane and forms hydrogen bonds with the water molecules present in the cytosol, so the freezing point of the water decreases, and there is a lower probability of formation of ice crystals inside the cell, as well as the integrity of the membrane is conserved (Cetinkaya et al. 2014). Moreover,

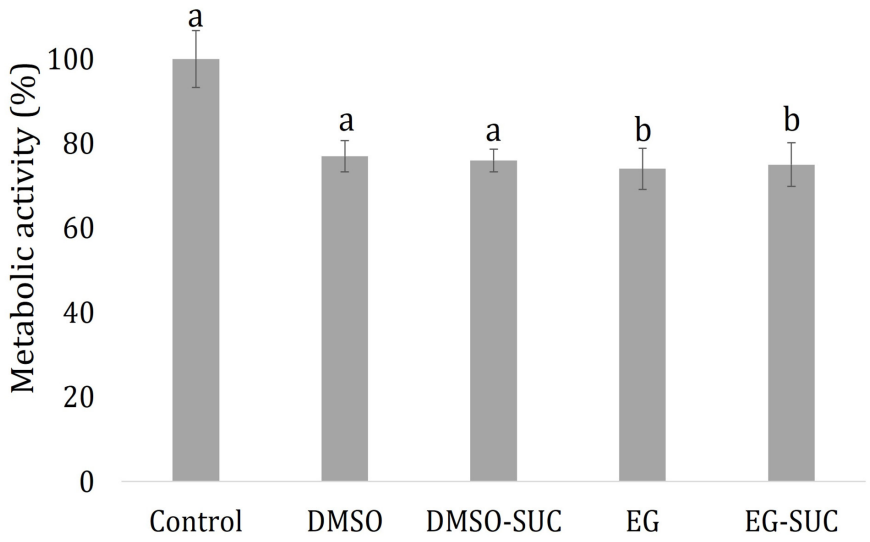

Figure 2. Evaluation of different cryopreservation solutions on the metabolic activity of somatic cells derived from jaguar. Letters in the column indicate statistical difference among the groups ($p < 0.05$). Bars represent standard error.

DMSO stabilizes cell proteins and displaces intracellular fluid to equilibrate intracellular and extracellular electrolyte concentrations (Liu et al. 2010), besides possessing a fast cellular penetration due to S-O (binding of sulfur with oxygen) grouping, in his composition (Castro et al. 2011). Thus, protein stabilization is mediated via hydrophobic interactions among DMSO and positively charged proteins, including cell membrane phospholipids (Anchordoguy et al. 1991). On the other hand, EG has a higher protein denaturant effect when samples are thawed, and this mechanism is due to a preferential binding of co-solvent with proteins at higher temperatures (Weng et al. 2018). Additionally, the metabolism of the EG generates potentially toxic byproducts, such as glycolic acid and oxalate, which promotes an accumulation of these compounds in the intracellular environment (McMartin & Wallace 2005) and these effects probably resulted in a lower viability rate in cryopreserved cells in the presence of EG alone.

Although the DMSO without SUC has been effective, authors report a reduction in negative effects during the process when extracellular cryoprotectants are used, leading to an improving in the viability rates when these substances are present (León-Quinto et al. 2014). Among the most used, SUC is highlighted because it acts dehydrating the cell, avoiding excessive turgidity and osmotic shocks (Erdag et al. 2002). During the dehydration and the rehydration in the cryopreservation, SUC interact with the plasma membrane phospholipids, and reorganize the membrane, increasing its fluidity and inducing a depression in the membrane phase transition temperature of dry lipids and forming a glass drying (Khalili et al. 2009). Thus, these sugars probably play a key role in preventing deleterious alteration to membranes during reduced-water states (Elliott et al. 2017). The results found corroborate with León-Quinto et al. (2011, 2014), which promoted slow freezing in Iberian lynx (*Lynx pardinus*) fibroblasts using a cryoprotectant solution composed of DMEM, FBS, 10% DMSO and 0.2 M SUC. Those authors obtained positive results with viability rates above 80% after cryopreservation, emphasizing the efficiency of the use of sucrose, to promote the osmotic maintenance of the medium and to perform a synergistic protection with the intracellular cryoprotectant, obtaining better results in their use in combination. In addition, EG-SUC group also obtained higher viability indices in comparison to the EG group, which corroborates with the already reported

information on the cellular protection capacity when an extracellular cryoprotectant is used in the cryopreservation solution (Léon-Quinto et al. 2014).

Another constituent present in the freezing solution was the FBS. This compound acts as an extracellular cryoprotectant ensuring the protection of cellular proteins and stabilization of the osmotic pressure, reducing the damage caused by the stages of slow freezing (Verdanova et al. 2014). Different studies have reported the use of 10% FBS with 10% DMSO for cryopreservation of cells derived from the skin of different felids, such as marbled cat, *Pardofelis marmorata* (Thongphakdee et al. 2006), leopard, *Prionailurus bengalensis* (Yin et al. 2006), and jaguar (Mestre-Citrinovitz et al. 2016).

Moreover, the viability rates presented in this study, after *in vitro* culture, corroborated with Barnes et al. (2003) and Subramanian et al. (2018), which states that cultures sourced from cryopreserved cells and their non-cryopreserved counterparts yield similar performance in growth, viability, and productivity, because cell population after established in the culture can recovered with similar rates to the non-cryopreserved group. Also, the MTT assay revealed a metabolic activity of over 70%. These data corroborate with those found by León-Quinto et al. (2011) where after cryopreserving Iberian lynx fibroblasts with 10% DMSO and performing the MTT assay found metabolic activity above 80%. For this test, the absorbance is directly proportional to the number of cells metabolically active, and therefore, it was necessary to promote cell growth to reach the ideal parameters with a homogeneous cell population for the analysis of the metabolic activity assay. Additionally, this cell growth was based on similar species of wild felines, such as leopard (*Panthera pardus*), tiger (*Panthera tigres*) and lion (*Panthera leo*) where the exponential growth phase of them are reached within 5–7 days on the growth curve (Yelisetti et al. 2016).

In summary, combinations of DMSO in the absence and presence of SUC were efficient in the cryopreservation of somatic cells of jaguars, reducing the damage caused by the formation of ice crystals and allowing future uses with optimal viability rates. Therefore, the results generated represent a breakthrough for the establishment of somatic genetic banks for jaguars.

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L.R.M. Oliveira and A. F. Pereira designed the experimental protocols. L.R.M. Oliveira, E.A. Praxedes, M.B. Silva. L.R. Rodrigues and H.V.S. Rodrigues carried out the experiments. A.F. Pereira performed statistical analysis, interpretation of data, and drafted the manuscript. L.R.M. Oliveira and A.F. Pereira participated in a substantial contribution to projetc conception, interpretation of data, and revising it critically for important intellectual content. All authors have read, critically revised, and approved the final version of the manuscript.