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ÉRIKA ALMEIDA PRAXEDES

DESCRIÇÃO HISTOLÓGICA, CULTIVO *IN VITRO* DE FIBROBLASTOS E CRIOPRESERVAÇÃO DA PELE DO PAVILHÃO AURICULAR DE ONÇA-PINTADA, *Panthera onca* (LINNAEUS, 1758)

MOSSORÓ–RN
2019

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Mestre em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Orientadora: Profa. Dra. Alexsandra Fernandes Pereira

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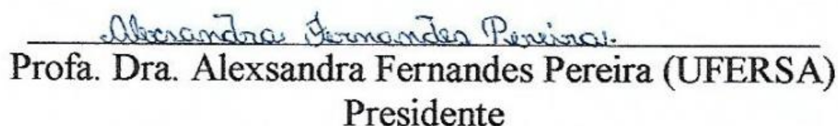

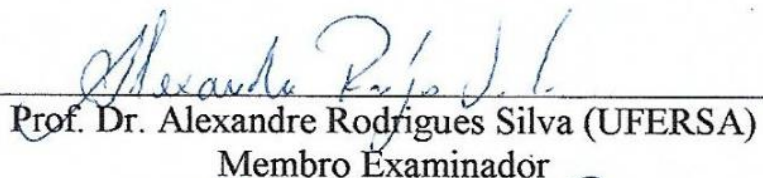

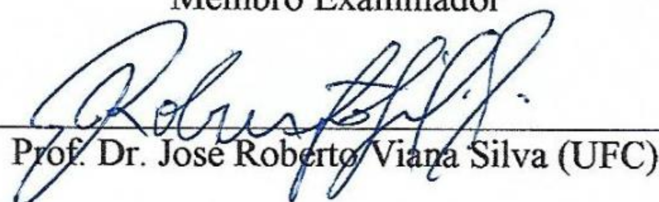

DADOS CURRICULARES DA AUTORA

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“Assim como os pássaros, precisamos aprender a superar os desafios que nos são apresentados, para alçarmos voos mais altos.”

(Dirk Wolter)

DESCRIÇÃO HISTOLÓGICA, CULTIVO *IN VITRO* DE FIBROBLASTOS E CRIOPRESERVAÇÃO DA PELE DO PAVILHÃO AURICULAR DE ONÇA-PINTADA, *Panthera onca* (LINNAEUS, 1758)

PRAXEDES, Érika Almeida. DESCRIÇÃO HISTOLÓGICA, CULTIVO *IN VITRO* DE FIBROBLASTOS E CRIOPRESERVAÇÃO DA PELE DO PAVILHÃO AURICULAR DE ONÇA-PINTADA, *Panthera onca* (LINNAEUS, 1758). 2019. Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2019.

RESUMO: A onça-pintada é um carnívoro de elevada importância ecológica para a biodiversidade mundial. Sua atual condição de vulnerabilidade a extinção requer estratégias de conservação, como a criopreservação de tecidos somáticos. Contudo, o emprego de técnicas de criopreservação depende do conhecimento das características histológicas e celulares dos tecidos em estudo. Portanto, os objetivos foram descrever histologicamente e por cultivo *in vitro* a pele do pavilhão auricular apical (**Etapa 1**) e comparar três técnicas de criopreservação [congelação lenta (CL), vitrificação direta em criotubos (VDC) e vitrificação em superfície sólida (VSS)] sobre a conservação dessas amostras de onça-pintada (**Etapa 2**). Para tanto, fragmentos foram recuperados de cinco animais oriundos de zoológicos do Brasil. Na primeira etapa, amostras de apenas dois animais, sendo um de pelagem amarela e outro de pelagem preta, foram avaliadas quanto à espessura da pele, quantificação e distribuição das células, percentual de matriz colágena, atividade proliferativa e viabilidade dos tecidos após cultivo. Para a segunda etapa, fragmentos foram criopreservados por CL, VDC ou VSS, e comparados com fragmentos não criopreservados (controle) quanto à espessura da pele, número de células, percentual de matriz colágena, e atividade proliferativa tecidual. Além disso, células resultantes dos fragmentos cultivados foram avaliadas quanto à morfologia, aderência, confluência, viabilidade, atividade proliferativa e metabólica. Assim, na primeira etapa, o estudo histomorfométrico mostrou uma espessura da pele total de 273,2 µm e 274,6 µm para onça pelagem amarela e preta, respectivamente. Além disso, melanócitos e fibroblastos para onça amarela foram de 9,3 e 23,0 e para onça preta foram de 11,3 e 26,8, respectivamente. Um percentual de matriz colágena de 67,0% e 49,0% foi observado para onça de pelagem amarela e preta, respectivamente. Adicionalmente, ambos os animais apresentaram uma atividade proliferativa celular variando de 1,20–1,30 e todos os fragmentos foram hábeis para promover o desprendimento celular, atingindo a subconfluência entre 10 a 15 dias. Na segunda etapa, todos os fragmentos criopreservados, independente da técnica empregada, mostraram uma redução na espessura da derme e da pele ($P < 0,05$). Embora uma matriz colágena similar ao grupo controle tenha sido observada somente para os fragmentos derivados dos grupos CL e VSS, todas as técnicas mantiveram o número de fibroblastos ($P > 0,05$). Além disso, VDC e VSS mantiveram a atividade proliferativa dos tecidos após o aquecimento. Após o cultivo, somente CL e VSS foram eficientes para a recuperação de células somáticas, de acordo com a maioria dos parâmetros avaliados. Em conclusão, a pele do pavilhão auricular de onça-pintada amarela e preta possui algumas variações em relação a outros mamíferos, quanto à espessura, densidade de matriz colágena, e número de melanócitos e fibroblastos. Contudo, o padrão de crescimento celular foi similar a outros felídeos silvestres. Além disso, a VSS foi a técnica mais eficiente para a criopreservação de pele de onça-pintada, quando comparada a VDC e CL. Estes resultados irão contribuir para a formação criobancos nesta espécie, direcionando a criopreservação adequada de amostras somáticas para aplicações em medicina regenerativa e tecnologias de reprodução assistida.

Palavras-chave: Bancos de recursos biológicos, criopreservação tecidual, células somáticas.

HISTOLOGICAL DESCRIPTION, FIBROBLAST *IN VITRO* CULTURE AND CRIOPRESERVATION OF JAGUAR, *Panthera onca* (LINNAEUS, 1758) EAR SKIN

PRAXEDES, Érika Almeida. HISTOLOGICAL DESCRIPTION, FIBROBLAST *IN VITRO* CULTURE AND CRIOPRESERVATION OF JAGUAR, *Panthera onca* (LINNAEUS, 1758) EAR SKIN. 2019. Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2019.

ABSTRACT: The jaguar is a carnivore of high ecological importance for the world's biodiversity. Its current condition of vulnerability to extinction requires conservation strategies, such as cryopreservation of somatic tissues. Nevertheless, the use of cryopreservation techniques depends on the knowledge of the histological and cellular characteristics of the tissues under study. Therefore, the aims were described by histological techniques and by *in vitro* culture the apical ear skin (**Step 1**) and to compare three cryopreservation techniques [slow freezing (SF), direct vitrification in cryotubes (DVC) and solid-surface vitrification (SSV)] on the conservation of these jaguar samples (**Step 2**). Thus, fragments were recovered derived from five animals from zoos of Brazil. In the first step, samples of only two animals, one with yellow and one black pelage, were evaluated for skin thickness, cell quantification and distribution, percentage of collagen matrix, proliferative activity and tissue viability after culture. For the second step, fragments were cryopreserved by SF, DVC or SSV, and compared to non-cryopreserved fragments (control) for skin thickness, number of cells, percentage of collagen matrix, and tissue proliferative activity. Moreover, cells resulting from the cultured fragments were evaluated for morphology, adhesion, confluence, viability, proliferative and metabolic activity. Thus, in the first stage, the histomorphometric study showed a total skin thickness of 273.2 μm and 274.6 μm for jaguars of yellow and black pelage, respectively. Likewise, melanocytes and fibroblasts for yellow jaguar were 9.3 e 23.0 and to black jaguar were of 11.3 e 26.8, respectively. A percentage of collagen matrix of 67.0% e 49.0% was observed for jaguars of yellow and black pelage, respectively. Additionally, both animals had a cell proliferative activity ranging from 1.20–1.30 and all the fragments were able to promote cell detachment, reaching the subconfluence between 10 and 15 days. In the second step, all the cryopreserved fragments, regardless of the technique employed, showed a reduction in the thickness of the dermis and skin ($P < 0.05$). Although a collagen matrix similar to the control group was observed only for the fragments derived from the SF and SSV groups, all techniques maintained the number of fibroblasts ($P > 0.05$). Additionally, DVC and SSV maintained tissue proliferative activity after warming. After culture, only SF and SSV were efficient for the recovery of somatic cells, according to most of the evaluated parameters. In conclusion, the apical ear skin of the yellow and black jaguar has some variations relative to other mammals, regarding thickness, collagen matrix density, and number of melanocytes and fibroblasts. Nevertheless, the pattern of cell growth was similar to other wild felids. Moreover, SSV was the most efficient technique for jaguar skin cryopreservation when compared to DVC and SF. These results will contribute to the formation of crybanks of this species, directing the adequate cryopreservation of somatic samples for applications in regenerative medicine and assisted reproduction technologies.

Keywords: Biological resources banks, tissue cryopreservation, somatic cells.

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LISTA DE SÍMBOLOS E SIGLAS

± Mais ou menos

< Menor

°C Graus celsius

% Percentual

AgNOR Região organizadora nucleolar marcada com sais de prata

BJ Black Jaguar

BK Basal layer

cm^2 Centímetro quadrado

CL Congelação lenta (*SF: slow freezing*)

CL Corneum Layer

CENAP Centro Nacional de Pesquisa e Conservação de Mamíferos Carnívoros

CEUA Comitê de Ética no Uso de Animais

CS Cryopreservation solution

CO_2 Dióxido de carbono

DE Dermis

DMSO Dimetilsulfóxido

DMEM Dulbecco Modification of Minimum Essential Medium

DNA Ácido desoxirribonucleico

EP Epidermis

FBS Fetal Bovine Serum

FSH Follicle stimulating hormone

FGF Fibroblast Growth Factor

GT Gomory Trichrome

h Hora

HE Hematoxilina-eosina

HF Hair Follicle

ICMBio Instituto Chico Mendes de Biodiversidade

iPS Induced Pluripotent Stem Cells

IUCN International Union for Conservation of Nature

LDH Lactate dehydrogenase

LH Luteinizing Hormone

LIF	Leukaemia Inhibitory Factor
M	Molar
MCR1	Receptor de Melanocortina 1
MDH	Malic dehydrogenase
µm	Micrômetro
μm^2	Micrômetro quadrado
mL	Mililitro
mm	Milímetro
mm^3	Milímetro cúbico
MTT	3-(4,5-dimetylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
N°	Número
NI	Non-informed
Ng/mL	Nanograma por mililitro
PDT	Population Double Time
RGB	Red, Green, and Blue format
NORs	Regiões Organizadoras Nucleolares
PBS	Solução tampão fosfato
SEG	Sebaceus Gland
SG	Sweat Gland
SL	Spinosum Layer
SUC	Sucrose
TNCS	Transferência Nuclear de Células Somáticas
U/mL	Unidade por mililitro
Vs.	*Versus*
VDC	Vitrificação direta em criotubos (*DVC: direct vitrification in cryovials*)
VSS	Vitrificação em superfície sólida (*SSV: solid-surface vitrification*)
YJ	Yellow Jaguar

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CAPITULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

As onças-pintadas são mamíferos emblemáticos do continente sul americano, sendo as únicas representantes do gênero *Panthera* e o maior felídeo das Américas (ZARRATE-CHARRY et al., 2010). Devido principalmente as ações antrópicas, como a destruição e a fragmentação de habitat além da caça ilegal, a sua população foi reduzida em 54% do seu quantitativo original (PAVIOLO et al., 2016). Assim, internacionalmente, a espécie é classificada como quase ameaçada (QUIGLEY et al., 2017), e nacionalmente, como vulnerável a extinção (MORATO et al., 2013).

Em se tratando de seus aspectos ecológicos, esses animais desempenham um importante papel no funcionamento dos ecossistemas, atuando na predação de um grande número de presas (ARROYO-ARCE et al., 2018). Além disso, as onças-pintadas requerem ambientes saudáveis e extensos para sua sobrevivência, sendo por essas razões consideradas indicadores de qualidade ambiental e espécie guarda-chuva na proteção de outras (RODRÍGUEZ-SOTO et al., 2013). Ainda, em virtude de sua beleza, a onça-pintada é uma espécie bandeira em projetos de conservação (VILAS BOAS; DIAS, 2010). Já do ponto de vista econômico, o desenvolvimento do ecoturismo por meio da exposição da espécie em parques ecológicos promove ações de educação ambiental e crescimento econômico local (TORTATO et al., 2017). Dessa forma, tendo em vista a importância desses animais e seu atual estado de vulnerabilidade a extinção, faz-se necessário o desenvolvimento de estratégias que promovam a sua conservação.

Nesse cenário, os bancos de recursos somáticos têm sido implementados para várias espécies, visando resguardar genótipos raros, permitindo a exploração do potencial de amostras biológicas (GOLACHOWSKI et al., 2018). Quando comparados aos bancos de gametas e embriões, os bancos de amostras somáticas, especialmente derivados da pele, têm sido considerados vantajosos em virtude de alguns fatores, como: (i) maior acessibilidade das regiões de colheita dos tecidos, (ii) maior variedade de tipos celulares recuperados a partir de diferentes tecidos, (iii) obtenção de tecidos independente do gênero e da idade do animal, (iv) obtenção de um grande número de células a partir de um único fragmento tecidual, e (v) aplicação em técnicas de reprodução assistida e medicina regenerativa por meio da indução de células à pluripotência (PRAXEDES et al., 2018). Associado a todos esses fatores e em

virtude da redução da variabilidade genética de onça-pintada, a conservação da genética de um único indivíduo torna-se essencial para a manutenção da biodiversidade (SRBEK-ARAUJO et al., 2018).

O uso de bancos de recursos somáticos para a conservação da fauna já tem sido observado para alguns felídeos silvestres (GOLACHOWSKI et al., 2018). Dentre esses bancos podem ser citados àqueles desenvolvidos para a conservação do lince-ibérico (*Lynx pardinus*) na Espanha (LEÓN-QUINTO et al., 2009), do gato marmorado (*Pardofelis marmorata*) e do gato-de-cabeça-chata (*Prionailurus planiceps*) na Tailândia (THONGPHAKDEE et al., 2010). Em 2016, Mestre-Citrinovitz et al. implantaram no Zoológico de Buenos Aires, um biobanco contendo 45 diferentes espécies ameaçadas, incluindo as onças-pintadas. Esse único estudo realizado até a presente data apresenta apenas uma descrição da colheita, criopreservação e cultivo *in vitro* dos tecidos somáticos de alguns indivíduos mantidos no território argentino, sendo necessária a otimização dos protocolos de criopreservação de tecidos, visando à conservação de onças-pintadas.

Nesse sentido, para atingir tais objetivos, faz-se necessário identificar inicialmente a arquitetura da região que se pretende obter as amostras somáticas (PEREIRA et al., 2018). No que se refere à pele, órgão mais empregado para a formação de bancos somáticos, sua composição e estrutura variam entre espécies, especialmente quanto aos parâmetros histomorfométricos, como espessura da pele, número e distribuição de células, e densidade de matriz proteica (HOSSAIN et al., 2016). Portanto, elucidar parâmetros morfológicos da pele pode direcionar protocolos de criopreservação, tornando-se uma ferramenta valiosa para formação de bancos de recursos somáticos.

Além disso, a escolha da técnica de criopreservação que promova menores danos ao tecido deve ser estabelecida para a espécie em questão. Mestre-Citrinovitz et al. (2016) relataram apenas o uso da congelação lenta na criopreservação da pele de onças-pintadas, sendo esta técnica responsável por um maior quantitativo de cristais de gelo, aspecto deletério para os tecidos criopreservados. Assim, avaliar os métodos de vitrificação, (BORGES et al., 2017), como a vitrificação direta em criotubos e vitrificação em superfície sólida, poderá ser útil para a conservação de amostras somáticas, uma vez que a vitrificação possui um custo reduzido, praticidade e reduzida formação de cristais de gelo, quando comparada à congelação lenta.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. ASPECTOS FILOGENÉTICOS DA ONÇA-PINTADA

Os primeiros estudos relacionados a taxonomia da família Felidae reportam a ocorrência de 28 espécies de felídeos (KLEIMAN; GEIST, 2004). Esses estudos utilizavam principalmente as avaliações morfológicas de fósseis, os quais por terem poucos exemplares, geravam informações escassas e ambíguas (CLAVIJO; RAMÍREZ, 2009). Dessa forma, com a possibilidade de uso de técnicas moleculares e maior acurácia nas análises, esses números foram elevados para 38 espécies (**Figura 1**) (JOHNSON et al., 2006).

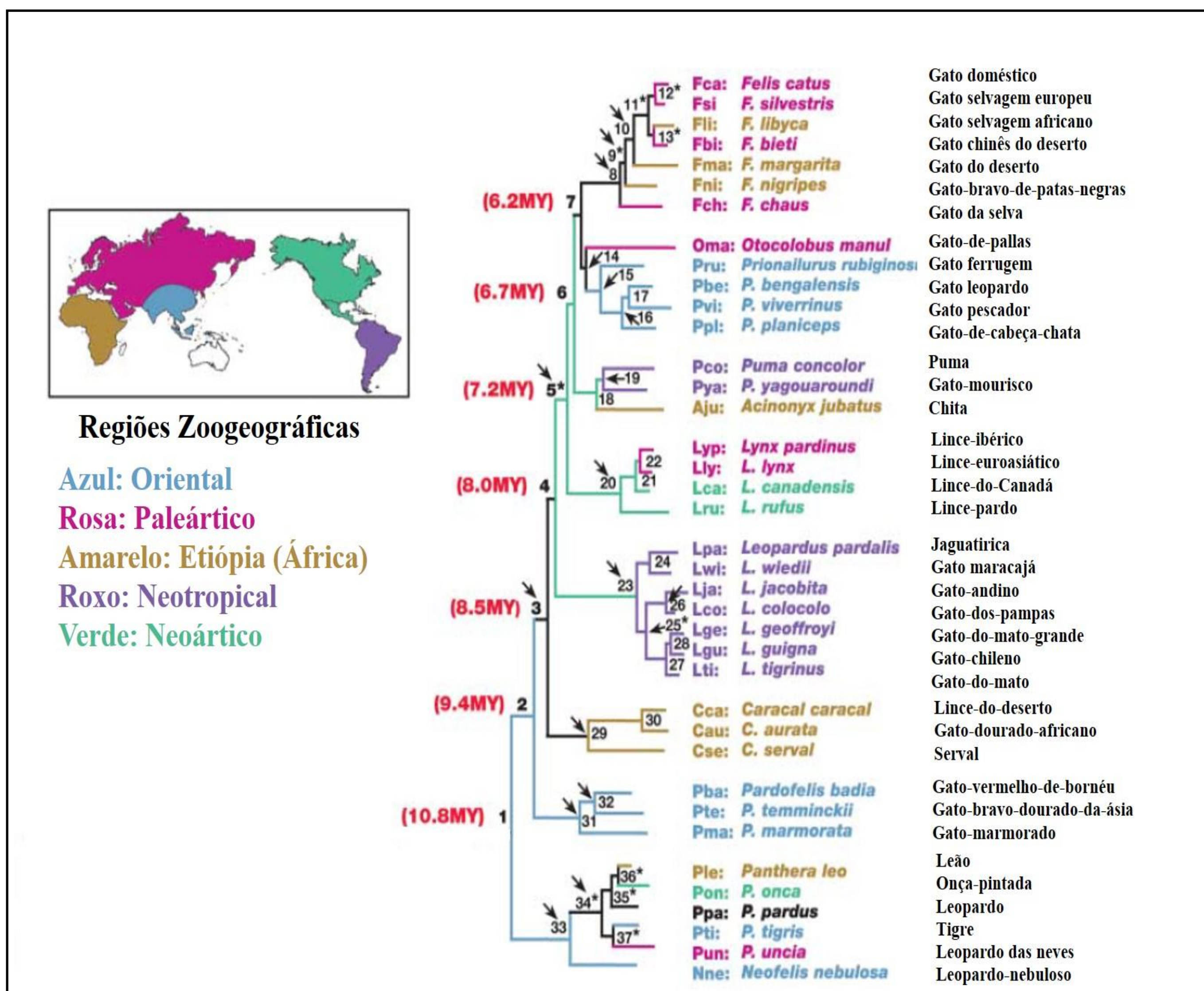


Figura 1. Relações filogenéticas entre 38 espécies de felídeos e suas regiões zoogeográficas históricas. Fonte: Johnson et al. (2006) com modificações.

Atualmente, a família Felidae é representada por duas subfamílias, 14 gêneros e 40 espécies dentre as quais se incluem a *Panthera onca*, conhecida popularmente como onça-pintada, jaguar ou jaguaretê, e considerada o maior felídeo do continente americano (CASO et al., 2008; CLAVIJO; RAMÍREZ, 2009). Essa espécie, pertencendo ao gênero *Panthera* é agrupada na subfamília Pantherinae juntamente com os gêneros *Uncia* e *Neofelis* (HEMMER, 1981). Os demais felídeos estão agrupados na subfamília Felinae e compreendem os gêneros *Caracal*, *Catopuma*, *Felis*, *Leopardus*, *Leptailurus*, *Lynx*, *Pardofelis*, *Profelis*, *Prionailurus*, *Puma* e *Acinonyx* (WOZENCRAFT, 2005).

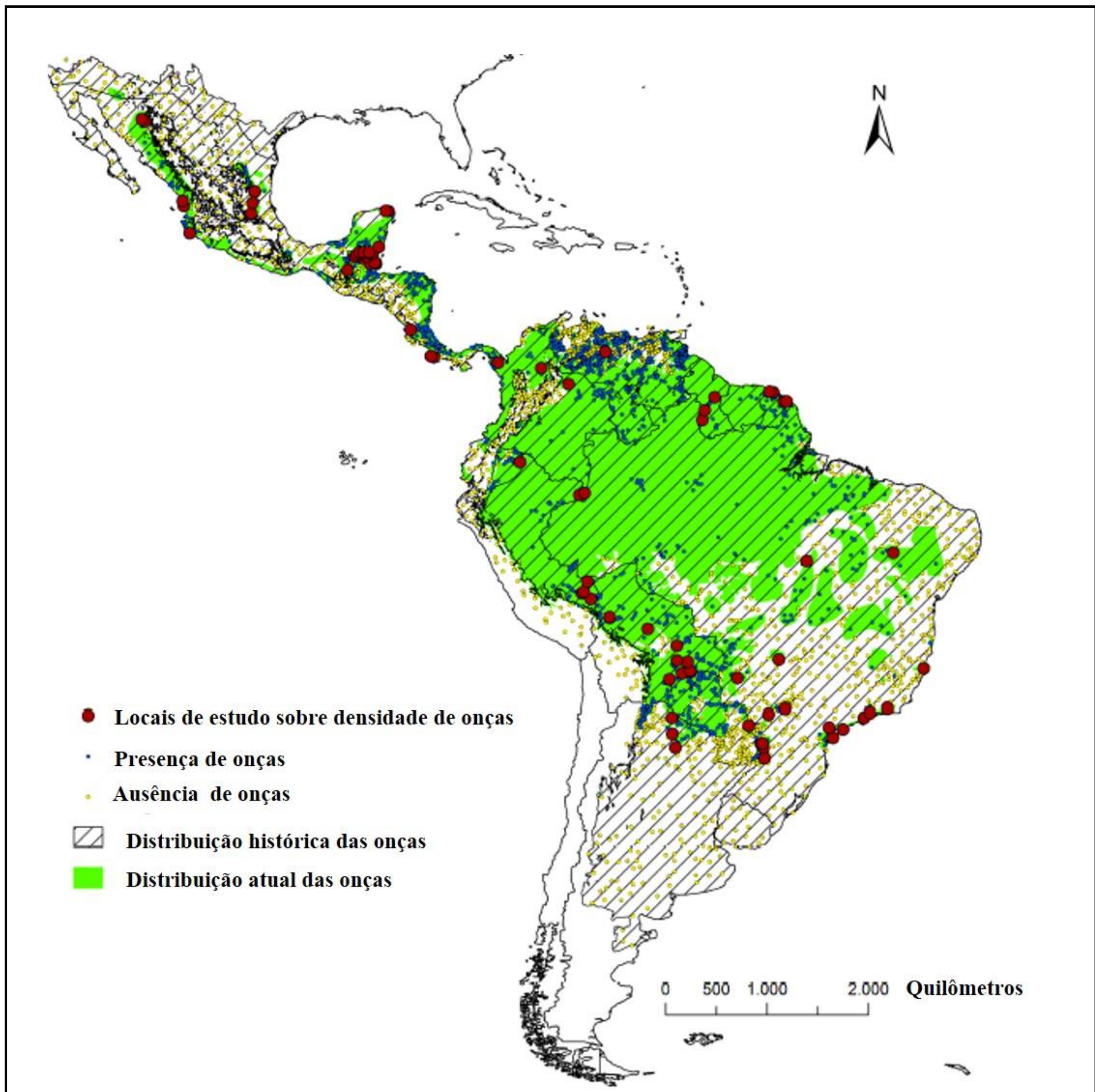
Um estudo realizado em 2001 utilizando DNA mitocondrial evidenciou a existência de quatro grupos filogeográficos de onça-pintada que se encontram distribuídos em quatro distintas regiões, México e Guatemala, Sul da América Central, ao Norte e ao Sul da América do Sul (EIZIRIK et al., 2001). Para tanto, foram avaliadas amostras sanguíneas de 44 onças-pintadas de diferentes regiões geográficas, como México, Guatemala, Nicarágua, Venezuela, Costa Rica, Panamá, Brasil, Peru, Guiana Francesa, Bolívia e Paraguai. Nesse estudo, os autores observaram padrões evolutivos gerais para a espécie e evidenciaram a ausência de subdivisões acentuadas.

Além disso, duas pelagens (HAAG et al., 2009) podem ser observadas na onça-pintada, a coloração amarelada e a coloração completamente preta (**Figura 2**).

Figura 2. Diferentes padrões de pelagem observados em onça pintada **(A)** e melânica **(B)**, Fontes: ECOPOINT Parque Ecológico (Fortaleza, CE) e Parque Zoobotânico Arruda Câmara (João Pessoa, PB), respectivamente.

113 Ambas possuem rosetas pretas distribuídas em toda extensão corporal; contudo, a pelagem
114 escura das onças melânicas dificulta sua visualização (NÚÑEZ; JIMÉNEZ, 2009). Dessa
115 forma, apesar de poucos esclarecimentos sobre as bases moleculares e adaptativas do
116 melanismo em onças-pintadas, sabe-se que esse padrão de pelagem é resultado de uma
117 deleção no gene do receptor de melocortina-1 (MCR1) com padrão de herança dominante
118 (EIZIRIK et al., 2003). Ainda, essa coloração negra pode conferir certas vantagens
119 adaptativas em circunstâncias ecológicas, sugerindo que em florestas mais densas e ambientes
120 mais úmidos são favoráveis a indivíduos melânicos (SÁENZ-BOLAÑOS et al., 2015).
121
122 ## 2.2. IMPORTÂNCIA DA ONÇA-PINTADA PARA O ECOSSISTEMA GLOBAL
123
124 Em termos ecológicos, as onças-pintadas possuem um papel significativo na
125 estruturação e funcionamento dos ecossistemas por meio de seu papel na predação (SILVER
126 et al., 2004). Sua posição no topo de teias alimentares os faz reguladores de cerca de 90
127 diferentes presas (CHIARELLO et al., 2008). Dessa forma, para sua sobrevivência em um
128 determinado ambiente é necessário que haja disponibilidade de extensos territórios e que estes
129 tenham abundância de presas, sendo por isso animais considerados indicadores de qualidade
130 ambiental (ABREU et al., 2004).
131 Além disso, a preservação de habitats visando à conservação das onças-pintadas
132 beneficia o amparo a outras espécies presentes no mesmo ambiente, sendo consideradas
133 espécies guarda-chuva de proteção dessas espécies (RODRÍGUEZ-SOTO et al., 2013).
134 Adicionalmente, a beleza e a força desse animal o elege muitas vezes como símbolos de
135 projetos sociais, recebendo a denominação de espécie bandeira (VILAS BOAS; DIAS, 2010).
136 Associado à sua importância ecológica, a onça-pintada também possui relevância
137 econômica, especialmente pelas atividades de ecoturismo mais intensas no Pantanal brasileiro
138 (TORTATO et al., 2017). Essas atividades permitem gerar renda e empregos para moradores
139 locais, estimulando um maior conhecimento da cultura regional e incentivando programas de
140 conservação ambiental (NASSAR et al., 2013).
141 Atualmente, o declínio de populações de onças-pintadas tem despertado grande
142 preocupação. Entre as principais causas para este cenário podem ser citadas principalmente a
143 caça, a destruição e a fragmentação de habitat decorrente da expansão agrícola
144 (HOOGESTEIJN; HOOGESTEIJN, 2011). Inicialmente, a ocorrência da espécie abrangia
145 territórios que compreendiam deste os Estados Unidos, América Central e América do Sul.

146 Contudo, em algumas regiões como El Salvador e Uruguai já não podem ser encontrados
147 esses indivíduos (CASO et al., 2008) (**Figura 3**). Além disso, em locais que ainda é possível
148 verificar a presença de onças-pintadas ocorreu uma grande redução em número de indivíduos
149 (VALDEZ et al., 2015). Internacionalmente (QUIGLEY et al., 2017), na escala de avaliação
150 da União Internacional para a Conservação da Natureza (IUCN), a espécie se encontra
151 classificada como quase ameaçada de extinção.
152



153
154 **Figura 3.** Distribuição geográfica histórica e atual da onça-pintada nas Américas e locais de
155 estudo de sua densidade. Fonte: Jędrzejewski et al. (2018) com modificações.
156

157 O Brasil concentra a maior variedade de habitats com presença de onça-pintada
158 (**Figura 4**). Esse animal ainda pode ser encontrado em quase todos os biomas, exceto o
159 Pampas, havendo variações quanto à escala de risco de extinção entre as diferentes regiões
160 (MORATO et al., 2013). Em geral, no país, a espécie se classifica como vulnerável à
161 extinção, sendo as áreas mais afetadas em termos de redução populacional, a Caatinga e a
162 Mata Atlântica, classificando-os como criticamente em perigo (MORATO et al., 2013).
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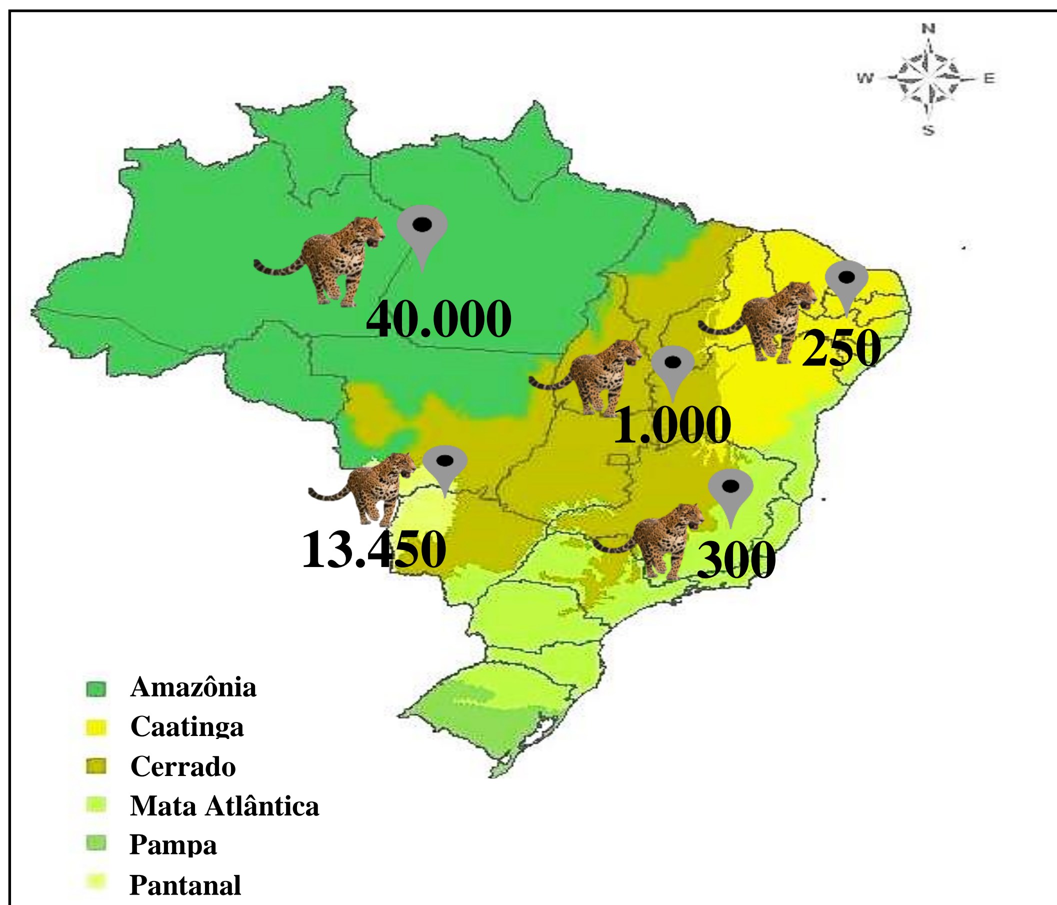

182
183 **Figura 4.** Cenário da conservação de onças-pintadas no Brasil segundo o Centro Nacional de
184 Pesquisa e Conservação de Mamíferos Carnívoros (CENAP) do ICMBio, evidenciando a
185 distribuição de aproximadamente 55 mil animais remanescentes nos biomas brasileiros.
186 Fonte: ICMBio (2018) com modificações.
187
188
189

190 2.2. ESTRATÉGIAS DE CONSERVAÇÃO APLICADAS À ONÇA-PINTADA

191

192 Diferentes estratégias têm sido adotadas visando à manutenção da máxima
193 biodiversidade existente, especialmente de espécies que se encontram em diferentes níveis de
194 ameaça (ANDRABI; MAXWELL, 2007). Estas podem ser realizadas de maneira *in situ*,
195 quando são desenvolvidas ferramentas que auxiliam na sobrevivência dos animais no
196 ecossistema (SANDERSON et al., 2002), bem como de maneira *ex situ,* a qual é subdividida
197 em *in vivo* ou *in vitro*. Assim, enquanto o *in vivo* consiste no transporte de animais para
198 reservas ou zoológicos (RUEDA-ZOZAYA et al., 2016)*,* o *in vitro* se caracteriza pelo
199 transporte e armazenamento de amostras biológicas na forma de criobancos (MESTRE-
200 CITRINOVITZ et al., 2016).

201

202 **2.2.1. Conservação *in situ* e *ex situ in vivo***

203

204 Diante do preocupante declínio que populações de onças-pintadas vêm sofrendo no
205 decorrer dos últimos anos, estratégias de conservação visam inicialmente promover a
206 manutenção de populações mínimas viáveis no ecossistema ou de modo *in situ* (NORA;
207 FRANCO, 2017). Essa estratégia é realizada por meio da proteção de habitats e do manejo
208 das populações na natureza visando à garantia mínima de variabilidade genética, demográfica
209 e ecológica (SANDERSON et al., 2002).
210 Assim, com esse intuito, estudos buscam esclarecer a densidade populacional e
211 distribuição geográfica de onças-pintadas nos diferentes biomas (TORRE et al., 2018;
212 JĘDRZEJEWSKI et al., 2018), bem como conhecer os padrões de comportamento e
213 alimentação da espécie em seu habitat (CONDE et al., 2010; ARROYO-ARCE et al., 2018).
214 Além disso, analisar a variabilidade genética faz-se importante para predição dos níveis de
215 conectividade entre as populações remanescentes (SRBEK-ARAUJO et al., 2018). Dessa
216 forma, para realizações de tais avaliações ferramentas não invasivas são empregadas,
217 utilizando armadilhas fotográficas (CASANOVA; BERNARDO, 2017) e radiotelemetria com
218 GPS (SOISALO; CAVALCANTI, 2006).
219 Além da manutenção desses animais em seu habitat natural, existem também
220 estratégias realizadas de maneira *ex situ in vivo* por meio do transporte desses animais para
221 reservas ou zoológicos (CAMPOS et al., 2005; RUEDA-ZOZAYA et al., 2016). Nesses
222 ambientes, os animais são úteis do ponto de vista educativo (GODINEZ et al., 2013), bem

223 como possibilitam a realização de estudos para compreensão de mecanismos fisiológicos e
224 reprodutivos (GONZALEZ et al., 2017). Finalmente, esses animais podem ser empregados
225 como reservatório genético, bem como uma fonte de indivíduos para reintroduções e reforço
226 demográfico e genético de populações existentes (RUEDA-ZOZAYA et al., 2016). Para
227 onças-pintadas, no Brasil, alguns zoológicos mantêm em geral de 1 a 3 animais em seus
228 recintos, e realizam trabalhos que vão desde promoção do bem-estar animal através de
229 enriquecimento ambiental, quanto avaliação de parâmetros reprodutivos em parceria com
230 laboratórios de pesquisa (JOÃO PESSOA, 2019; ZOOLÓGICO DE SÃO PAULO, 2019).
231 Em geral, programas de conservação para onças-pintadas em países sul-americanos
232 vêm sendo instituídos (SANDERSON et al., 2002; MORATO et al., 2013). No Brasil,
233 organizações governamentais lançaram o plano de ação para conservação da onça-pintada
234 durante 2011 a 2016, atingindo 41% das 46 metas estabelecidas, envolvendo ações nas
235 temáticas de comunicação e educação, políticas públicas, pesquisa, perda e fragmentação de
236 habitat, caça e conflitos, para conservação *in situ* da espécie (MORATO et al., 2013; ICMBio,
237 2018).
238 Além disso, organizações não governamentais desenvolvem ações para conservação
239 da espécie. Entre eles podem ser citados o Instituto Onça-Pintada que visa promover a
240 conservação *in situ* da espécie nos biomas brasileiros e o Projeto Onçafari que busca
241 promover o desenvolvimento sócio econômico das regiões em que está inserida por meio da
242 habituação de onças-pintadas a presença humana e desenvolvimento do ecoturismo no
243 Pantanal (INSTITUTO ONÇA PINTADA, 2019; ONÇAFARI, 2019). Em ambas as situações
244 também são realizadas pesquisas voltadas para o monitoramento, manejo, reintrodução de
245 animais na natureza e estudos genéticos. Finalmente, a mobilização nacional para conservação
246 da espécie resultou na criação da portaria no. 08/2018, pelo ministério do meio ambiente,
247 determinando o dia 29 de novembro como dia Nacional da onça-pintada (ICMBio, 2018).
248 Contudo, essas estratégias *in situ* e *ex situ in vivo* possuem algumas limitações.
249 Primeiramente, para conservação *in situ* são requeridos extensos territórios e monitoramento
250 destes, bem como conscientização e contribuição da população (SILVA et al., 2008;
251 ZARRATE-CHARRY et al., 2010). Além disso, a captura de onças-pintadas de vida livre é
252 de difícil realização em virtude do difícil acesso aos locais, grande dimensão territorial, bem
253 como comportamento noturno do animal e baixas densidades remanescentes (SILVER et al.,
254 2004). Para conservação *ex situ in vivo*, as onças-pintadas apresentam baixo potencial
255 reprodutivo em condições de cativeiros, devido às condições de acondicionamento, nutrição e

estresse nesses ambientes (MORATO et al., 2004; PAZ et al., 2006; GUTIÉRREZ-GONZÁLEZ et al., 2015). Portanto, em virtude desses obstáculos, o estabelecimento de criobancos é uma prioridade para a onça-pintada.

2.2.2. Conservação *ex situ in vitro*

Em conjunto com as estratégias *in situ* e *ex situ in vivo* citadas anteriormente, as estratégias *ex situ in vitro* por meio da criopreservação de amostras biológicas, como gametas, embriões, células e tecidos somáticos pode garantir a conservação do material genético de espécies existentes naquele momento (PAZ et al., 2007; MESTRE-CITRINOVITZ et al., 2016). Posteriormente, essas amostras podem ser empregadas em técnicas de reprodução assistida, como a inseminação artificial, fecundação *in vitro*, produção *in vivo* de embriões e clonagem por transferência nuclear de células somáticas (SILVA et al., 2016). Tais técnicas podem auxiliar na conservação e multiplicação de indivíduos, possibilitando estudos biológicos básicos e transporte de germoplasma entre populações *in situ* e *ex situ* (MORATO; BARNABE, 2001; SILVA et al., 2016).

Assim, a compreensão dos parâmetros reprodutivos da espécie faz-se imprescindível, tanto para manutenção de populações mantidas em seu ambiente natural, quanto para a obtenção de sucesso reprodutivo por meio da aplicação de técnicas de reprodução assistida em espécies mantidas em cativeiros (GONZALEZ et al., 2017). Para fêmeas, o monitoramento do ciclo estral evidenciou a ocorrência de múltiplos estros ao longo do ano, com duração de 22 a 65 dias, (BARNES et al., 2016). Além disso, a gestação dura em torno de 91 a 110 dias, com nascimento de 1 a 4 crias (MORATO et al., 2013; BARNES et al., 2016).

Quanto aos parâmetros reprodutivos referentes aos machos, Azevedo et al. (2006) realizaram a avaliação da morfometria testicular em onças mantidas em cativeiro, revelando um diâmetro de 257 µm e uma espessura de 90,3 µm para o epitélio seminífero, correlacionando-os com a massa corporal dos animais. Além disso, as onças-pintadas apresentam atividade androgênica durante todo o ano, com picos de produção nas estações chuvosas (MORATO et al., 2004). Finalmente, quanto às avaliações espermáticas, Paz et al. (2003) por meio de esfregaços obtidos a partir de citologia aspirativa por agulha fina em testículo de onças-pintadas, realizaram a avaliação da atividade espermatogênica e identificação de células germinativas, apresentando 4,4% de espermatogônias, 6,6% de espermatócitos primários, 0,7% de espermatócitos secundários, 14,3% de espermátides iniciais, 29,7% de espermátides finais , 39,4% de espermatozoides e 16,4% de células de

289 Sertoli. Adicionalmente, Costa et al. (2008) descreveram a produção de 16, 9 x 10^6
290 espermatozoides por grama de testis por dia, apresentando um ciclo espermatogênico com
291 oito estádios, baseando-se na avaliação morfológica do acrossoma.
292
293 *2.2.2.1. Obtenção e manipulação de gametas masculinos e femininos*
294
295 A obtenção de espermatozoides em onças-pintadas, por se tratar de um animal
296 silvestre de grande porte, é realizada por meio da eletroejaculação, visando fornecer uma
297 maior segurança a equipe (SWANSON et al., 1996; MORATO et al., 2001). Contudo, Araujo
298 et al. (2017) realizaram pela primeira vez a colheita seminal por meio da cateterização uretral
299 após indução farmacológica com uso de medetomidina. Esses autores relataram este método
300 como prático, efetivo e seguro.
301 Além disso, visando estabelecer parâmetros reprodutivos de referência para a espécie,
302 diferenças qualitativas seminais entre animais de cativeiro e de vida livre já foram relatadas,
303 demonstrando uma maior qualidade de espermatozoides provenientes de animais livres
304 (MORATO et al., 2001; ARAUJO et al., 2017). Essas divergências são representadas
305 principalmente por um maior percentual de espermatozoides com morfologia normal para
306 animais de vida livre (73%) além de um maior vigor espermático (4,1), quando comparado a
307 animais de cativeiro com (50% e 2,8) respectivamente (MORATO et al., 2001).
308 Quanto aos protocolos de criopreservação, Swanson et al. (1996) e Morato; Barnabé.
309 (2001) criopreservaram espermatozoides de onças-pintadas com solução constituída por
310 lactose, glicerol e gema de ovo, obtendo baixas taxas de motilidade, em torno de 30% após a
311 descongelação. Além disso, visando avaliar a funcionalidade espermática após a
312 descongelação, Paz et al. (2007) avaliaram a capacidade de ligação e penetração *in vitro* de
313 espermatozoides de onça-pintadas em oócitos sem zona pelúcida heterólogos de hamster.
314 Nesse estudo, foram encontradas baixas taxas de penetração (15,4%) além de anormalidades
315 espermáticas (72,4%), resultando numa baixa qualidade após criopreservação.
316 Adicionalmente, Silva et al. (2017) avaliaram a qualidade seminal de onças-pintadas, durante
317 uma curva de refrigeração, comparando o uso dos diluidores Tris e ACP-117c. Ambos os
318 diluidores apresentaram influência positiva na motilidade, vigor e integridade de membrana
319 espermática após o resfriamento.
320 No que se refere à obtenção de gametas femininos, estudos em onças-pintadas ainda
321 são escassos. Morato et al. (2001) realizaram um estudo pioneiro de superestimulação com

322 FSH e LH e posterior recuperação oocitária por laparoscopia. Contudo, os autores relataram
323 apenas resultados preliminares para quatro fêmeas, havendo recuperação de 28 oócitos em
324 diferentes graus de qualidade. Os autores reportaram ainda que não foi possível produzir
325 embriões por fecundação *in vitro* em virtude da baixa qualidade seminal e tempo inadequado
326 utilizado para recuperação oocitária.

327 Portanto, percebe-se que estudos visando à conservação *ex situ in vitro* usando
328 gametas sofrem com alguns obstáculos, principalmente quanto à captura de animais de vida
329 livre, o conhecimento de parâmetros fisiológicos reprodutivos e os protocolos de manipulação
330 para gametas (MORATO; BARNABE, 2001; ARAUJO et al., 2017). Assim,
331 concomitantemente, explorar o potencial de armazenamento de amostras somáticas é uma
332 ferramenta promissora e que tem gerado avanços em diversas espécies de felídeos silvestres.
333

334 *2.2.2.2. Obtenção e manipulação de amostras somáticas*

335

336 Além da manipulação, processamento e criopreservação de gametas, a utilização de
337 amostras somáticas, principalmente em espécies de felídeos com eminente ameaça de
338 extinção, tem sido descrita como uma promissora ferramenta para fins de conservação
339 (PRAXEDES et al., 2018). A aplicação desse recurso biológico possui como vantagens a
340 possibilidade de recuperação de amostras em ambos os gêneros (THONGPHAKDEE et al.,
341 2010; WITTAYARAT et al., 2013), em fetos ou animais adultos (KITIYANANT et al., 2003;
342 HASHEM et al., 2007) vivos ou após a morte (VERMA et al., 2012; MORO et al., 2015) e a
343 partir de diferentes regiões e tecidos corporais, possibilitando assim a obtenção de uma ampla
344 amostragem biológica (MESTRE-CITRINOVITZ et al., 2016).

345 Além disso, a colheita a partir de regiões pouco invasivas, como o pavilhão auricular,
346 pode ser realizada durante a marcação de animais, possibilitando a recuperação de uma
347 grande quantidade de células a partir de um único fragmento (PRAXEDES et al., 2018).
348 Posteriormente, essas amostras podem ser aplicadas em biotécnicas como a clonagem por
349 transferência nuclear de células somáticas, estudos de indução de células a pluripotência e
350 geração de gametas (VERMA et al., 2012; YELISETTI et al., 2016).

351 Nesse sentido, para onças-pintadas, Mestre-Citrinovitz et al. (2016) realizaram um
352 relato técnico descrevendo alguns aspectos práticos envolvidos no estabelecimento de bancos
353 somáticos para conservação da espécie. Assim, os autores descrevem a obtenção de amostras
354 de pele, cartilagem e músculo, abordando condições de transporte a 5ºC em solução salina

acrescida de gentamicina, processamento, enfatizando a assepsia de todo material utilizado, criopreservação utilizando congelação lenta e aquecimento desses tecidos. Posteriormente, os tecidos foram cultivados *in vitro* na ausência e presença de digestão com colagenase. Dessa forma, a partir das etapas descritas no trabalho foi possível armazenar em um biobanco do Zoológico de Buenos Aires amostras de 45 diferentes espécies autóctones ameaçadas, incluindo as onças-pintadas. O número exato de amostras somáticas criopreservadas de onças-pintadas não foi informado.

Finalmente, reforça-se que qualquer órgão pode ser empregado na colheita de amostras somáticas; contudo, a pele tem sido considerada o órgão amplamente empregado para essa finalidade. Entre as razões para esse cenário consistem na pele ser um órgão rico em tipos celulares de diferentes tecidos, poder ser realizada a recuperação de tecidos de maneira menos invasiva e as células obtidas serem adequadas para a reprogramação nuclear, etapa essencial para a clonagem por transferência nuclear de células somáticas, produção de células pluripotentes e obtenção de gametas (PRAXEDES et al., 2018).

388 # 3. JUSTIFICATIVA

389

390 Nos últimos anos, a redução da população de onças-pintadas tem despertado a busca
391 por estratégias de conservação visando à manutenção da espécie para o ecossistema global.
392 Nesse sentido, inúmeras ferramentas podem ser empregadas, como a formação de bancos de
393 recursos somáticos. Esses bancos são importantes em virtude da possibilidade dessas células
394 serem empregadas na clonagem por transferência nuclear de células somáticas, produção de
395 células pluripotentes e obtenção de gametas. Todas essas finalidades são fundamentais,
396 especialmente em espécies de reduzido quantitativo populacional, como a onça-pintada.

397 Em se tratando de colheita de amostras somáticas de onças-pintadas, a pele
398 especialmente do pavilhão auricular consiste num órgão adequado para a recuperação de
399 células somáticas, uma vez que permite uma colheita menos invasiva e obtenção de amostras
400 eficientes para a reprogramação celular, etapa fundamental em todas as etapas anteriormente
401 citadas. Para garantir uma formação eficiente de bancos de recursos somáticos, alguns passos
402 tornam-se essenciais quanto à escolha dos métodos de criopreservação de tecidos somáticos
403 derivados da pele.

404 Inicialmente, as amostras somáticas devem ser caracterizadas quanto aos seus aspectos
405 histológicos, ou seja, parâmetros morfométricos e características celulares devem ser
406 identificados para a definição adequada dos protocolos de criopreservação e cultivo *in vitro*
407 dos fragmentos teciduais. Posteriormente, o estabelecimento da técnica de criopreservação
408 tecidual mais eficiente necessita ser definido, visando à obtenção de um protocolo que garanta
409 a manutenção de uma maior viabilidade dos tecidos após o aquecimento. Até a presente data,
410 apenas um estudo foi realizado quanto à criopreservação de tecidos somáticos derivados da
411 pele de onças-pintadas. O referido estudo abordou de forma descritiva a congelação lenta
412 como técnica de conservação desses tecidos; contudo, a busca por métodos mais práticos, de
413 baixo custo e que garantam uma maior eficiência, reduzindo os danos nos tecidos após o
414 aquecimento o que é imprescindível para o sucesso dos bancos de recursos somáticos.
415 Portanto, esta proposta pretendeu contribuir de forma significativa nas pesquisas relacionadas
416 à conservação das onças-pintadas, especialmente no desenvolvimento de bancos de recursos
417 somáticos da espécie.

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4. HIPÓTESES CIENTÍFICAS

I – A pele do pavilhão auricular, tanto de onças de pelagem amarela quanto de pelagem preta, apresenta padrões histológicos referentes constituição celular epidermal e espessura de camadas, que o diferenciam de espécies mamíferas domésticas e silvestres.

II – A vitrificação em superfície sólida é mais adequada para a criopreservação da pele do pavilhão auricular de onças-pintadas, quando comparada a congelação lenta e vitrificação direta em criotubos, proporcionando a manutenção da viabilidade dos tecidos após o aquecimento.

454 # 5. OBJETIVOS

455

456 ## 5.1. OBJETIVO GERAL

457

458 Estudar características histológicas da pele do pavilhão auricular de onças-pintadas, e
459 descrever métodos eficientes de criopreservação tecidual.

460

461 ## 5.2. OBJETIVOS ESPECÍFICOS

462

463 - Descrever a estrutura, composição e capacidade de recuperação celular após cultivo *in vitro*
464 da pele do pavilhão auricular de onças de pelagem amarela e preta, visando com esse
465 conhecimento aprimorar os protocolos de conservação tecidual;

466

467 - Comparar três técnicas de criopreservação (congelação lenta *vs.* vitrificação direta em
468 criotubos *vs.* vitrificação em superfície sólida) na conservação pele do pavilhão auricular de
469 onças-pintadas.

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Use of somatic cell banks in the conservation of wild felids

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ABSTRACT

The conservation of biological resources is an interesting strategy for the maintenance of biodiversity, especially for wild felids who are constantly threatened with extinction. For this purpose, cryopreservation techniques have been used for the long-term storage of gametes, embryos, gonadal tissues, and somatic cells and tissues. The establishment of these banks has been suggested as a practical approach to the preservation of species and, when done in tandem with assisted reproductive techniques, could provide the means for reproducing endangered species. Somatic cell banks have been shown remarkable for the conservation of genetic material of felids; by merely obtaining skin samples, it is possible to sample a large group of individuals without being limited by factors such as gender or age. Thus, techniques for somatic tissue recovery, cryopreservation, and *in vitro* culture of different wild felids have been developed, resulting in a viable method for the conservation of species. One of the most notable conservation programs for wild felines using somatic samples was the one carried out for the Iberian lynx, the most endangered feline in the world. Other wild felids have also been studied in other continents, such as the jaguar in South America. This review aims to present the technical progress achieved in the conservation of somatic cells and tissues in different wild felids, as well address the progress that has been achieved in a few species.

KEYWORDS: Biological bank, conservation tools, cryopreservation, cloning.

1. INTRODUCTION

Biological resource banks are considered sources of gametes, embryos, gonadal tissues, and somatic tissue and cells after collection, processing, and cryopreservation for long periods (León-Quinto et al., 2009); their use has been significant to the conservation and reproduction of domestic and wild mammals (Comizzoli, Mermillod, & Mauget, 2000). Though most banks store semen and embryos, the interest in somatic cell banks has increased over the years, especially with the rapid loss of a large number of species (Gubili et al., 2017; León-Quinto, Simón, Sánchez, & Soria, 2011).

According to the International Union for Conservation of Nature (IUCN), 38 of the 40 species of the Felidae family are included in the list of endangered animals classified among the different risk categories, from least concern to endangered (IUCN, 2018). The main causes of decline in the feline population are related to human action; these include factors such as agricultural expansion, which leads to the fragmentation of habitats, and hunting (Yin, Lee, Jin, Kim, & Kong, 2006). The importance of these animals is remarkable, and the need for conservation is emphasized by the large number of species threatened with extinction. Thus, conservation strategies such as somatic tissue banks have also been proposed for wild felids (León-Quinto et al., 2009).

Cryopreservation of somatic tissues is advantageous and important; it offers the possibility of a collection of a large number of individuals, allowing for the storage of many fragments and maximizing the preservation of a particular genetic population (León-Quinto, Simón, Cadenas, Martínez, & Sern, 2014). Cryopreserved biological material can be used for basic biological, genetic, toxicological, and epidemiological research; it may also be used for assisted reproductive techniques, as well as cloning by somatic cell nuclear transfer (SCNT) for genetic rescue (Mogollón-Waltero, Mello, & Burla-Días, 2014).

In view of this perspective, this review will present the technical progress achieved in the conservation of somatic cells and tissues in different wild felids.

2. SOURCES OF SOMATIC TISSUES

In wild felids, somatic cells can be obtained by biopsies (Verma, Holland, Temple-Smith & Verma, 2012) or necropsy (Moulavi et al., 2017) from various tissues, including skin from the

908 ear (Praxedes et al., 2017), inner groin area (Mestre-Citrinovitz, Sestelo, Ceballos, Barañao
909 & Saragueta, 2016), and abdomen (Wittayarat et al., 2013), as well as muscle (Thongphakdee
910 et al., 2006), oral mucosa, bone marrow, spinal cord, and intestines (León-Quinto et al., 2009)
911 of both genders (Thongphakdee et al., 2010; Wittayarat et al., 2013) and different ages
912 (Hashem, Bhandari, Kang, & Lee, 2007). In general, most studies have used skin tissues,
913 especially from the ear, due to its ease in acquisition during the routine management of
914 animals (Guan et al., 2010; Liu, Guo, Liu, Guan, & Ma, 2010).
915
916 Moreover, tissue collection is performed in individuals that were previously anesthetized
917 (Mestre-Citrinovitz et al., 2016). In jaguars (*Panthera onca*), dexmedetomidine hydrochloride
918 is a good sedative (Praxedes et al., 2017). The region to be biopsied is sanitized and tissue
919 samples are harvested using scalpel blades (Moulavi et al., 2017). After collection, tissue
920 fragments are transported in phosphate-buffered saline (PBS) (Veraguas, Gallegos, Castro &
921 Rodriguez-Alvarez, 2017) or Dulbecco Modified Eagle Medium (DMEM) (Moulavi et al.,
922 2017) containing antibacterial and antifungal agents (Wittayarat et al., 2013). Additionally,
923 the temperature during transport is maintained at 4°C to avoid dehydration and to preserve the
924 viability of the tissue (Mestre-Citrinovitz et al., 2016). Another important parameter is the
925 duration of transport, understanding the time between sample collection and processing in the
926 laboratory is imperative; as such, there are reports of transport duration ranging from 1
927 (Yelisetti, Komjeti, Katari, Sisinthy & Brahmasani, 2016) to 24 h (León-Quinto et al., 2014).
928
929 In the case of animals that are found dead, parameters such as the duration of time it takes to
930 obtain viable fibroblasts from the *post-mortem* tissue sample, as well as the temperature that
931 the body was maintained at can influence the occurrence of cell death and bacterial
932 contamination in the sample (Silvestre, Saeed, Cervera, Escribá & García-Ximéxez, 2003).
933 Some studies dealing with wild felids have discussed these parameters. Moulavi et al. (2017)
934 observed that embryos can be obtained by cloning using skin cells derived from an Asiatic
935 cheetah (*Acinonyx jubatus*) maintained at −20°C for 10 days without using cryoprotectants. In
936 marbled cat (*Pardofelis marmorata*), skin samples collected up to 24 h *post-mortem* and
937 preserved at −4°C in nutritive medium resulted in viable fibroblasts upon *in vitro* culture
938 (Wittayarat et al., 2013).
939

940 In the laboratory, tissues are washed in culture medium supplemented with antibiotics,
941 buffers, and protein sources, and then fragmented into variable sizes [1.0 mm^3 for Siberian
942 tiger (*Panthera tigris altaica*) (Liu et al., 2010), 8.0 mm^3 for marbled cat (Wittayarat et al.,
943 2013), and 9.0 mm^3 for jaguar (Praxedes et al., 2017)]. After fragmentation, tissues may be
944 cryopreserved and used for the establishment of *in vitro* culture systems.
945

946 **3. CRYOPRESERVATION TECHNIQUES OF SOMATIC CELLS AND TISSUES**

947 In wild felids, slow freezing (Mestre-Citrinovitz et al., 2016) is a technique routinely used for
948 the cryopreservation of somatic cells and tissues, especially those derived from skin. In
949 general, slow freezing uses low concentrations of cryoprotectants and is done through gradual
950 temperature reduction in a controlled manner. Although not performed in wild felids,
951 vitrification has also been a prominent method in tissue cryopreservation and has been used in
952 somatic tissues of some mammals (Borges et al., 2017; Caputcu, Akkoc, Cetinkaya, & Arat,
953 2013). This technique is performed through rapid temperature reduction and with high
954 concentrations of cryoprotectants.
955

956 For cells derived from the skin of wild felids, slow freezing is the method most commonly
957 employed, as observed in the cryopreservation of jaguar (Mestre-Citrinovitz et al., 2016) and
958 sand cat tissues (*Felis Margarita*) (Gómez et al., 2008). In general, 10% dimethylsulfoxide
959 (DMSO) is the highest concentration of intracellular cryoprotectant used, as observed in the
960 cryopreservation of Bengal tiger (*Panthera tigris tigris*) (Guan et al., 2010) and African wild
961 cat tissues (*Felis silvestris libica*) (Gómez et al., 2004). Moreover, DMSO is used in
962 combination with an extracellular cryoprotectant, such as 0.1 or 0.2 M sucrose, which protects
963 the cell membrane by binding to phospholipid groups and reducing osmotic shock by
964 controlling the entry of water into the cell (León-Quinto et al., 2011). Additionally, fibroblasts
965 derived from skin can be cryopreserved with 10% DMSO and 10% fetal bovine serum (FBS),
966 as observed in the cryopreservation of marbled cat (Imsoonthornruksa, Sangmalee, Srirattana,
967 Parnpai & Ketudat-Cairns, 2012) and leopard cat tissues (*Prionailurus bengalensis*) (Yin et
968 al., 2006).
969

970 For cryopreserved tissues of wild felids, Mestre-Citrinovitz et al. (2016) described procedures
971 to obtain somatic tissues derived from the skin of the ear of a jaguar. In this study, the authors
972 presented the aseptic conditions, as well as the materials and steps necessary for carrying out

tissue cryopreservation by slow freezing. Additionally, León-Quinto et al. (2011) evaluated the effects of different concentrations of DMSO (5%, 7.5%, 10%, 12.5%, or 15%) alone or in combination with sucrose (0.1 or 0.2 M) during slow freezing in skin tissues of Iberian lynx (*Lynx pardinus*) and concluded that 10% DMSO either with or without 0.2 M sucrose was adequate for tissue cryopreservation.

4. *IN VITRO* CULTURE OF SOMATIC CELLS

In wild felids, the protocols used for the isolation of cells derived from tissues have been based on studies dealing with domestic felids (Kitiyanant, Sakhun & Pavasuthipaisit, 2003). In jaguar, somatic tissues from skin, muscle, and cartilage were treated with collagenase (Mestre-Citrinovitz et al., 2016) for to accelerate the time required during *in vitro* culture procedures. Nevertheless, most studies are performed without enzyme treatment (Liu et al., 2010; Thongphakdee et al., 2010) and fragments are cultured immediately in DMEM with antibiotics and either 10% or 20% FBS at 38°C and 5% CO_2 (Liu et al., 2010; Praxedes et al., 2017).

During *in vitro* culture, different analyses can be performed for cell establishment and characterization (Song, Hua, Song & Zhang, 2007), as well as for evaluating the cryopreservation effect on cell recovery (Guan et al., 2010). In general, morphology is an important qualitative parameter analyzed in conjunction with membrane integrity using trypan blue (Guan et al., 2010; Praxedes et al., 2017). Additionally, the quality of the primary culture can be quantified by evaluating the fixation/adherence efficiency of the explants, as well as the detachment and cell growth around the explants (Liu et al., 2010). In Bengal tiger, the viability of fibroblasts after cryopreservation was assayed using trypan blue and 95.7% of the cells were determined to be viable (Guan et al., 2010). In jaguar, Mestre-Citrinovitz et al. (2016) observed the time of cell detachment and growth around the explants to be between 10 and 14 days.

Moreover, Praxedes et al. (2017) assayed mitochondrial dehydrogenase activity (MTT; brometo de 3- (4,5-dimetiltiazol-2-il) -2,5 difeniltetrazólio) after 5 and 7 days of culture of fibroblasts derived from the skin of the ear of jaguar and presented values of 100% viable cell functionality. Additionally, León-Quinto et al. (2011) performed MTT assay to evaluate cell viability after thawing and presenting mean values around 85%.

1006 Another important parameter is the population doubling time (PDT) and plotting the growth
1007 curve (León-Quinto et al., 2014). In Siberian tiger, fibroblasts showed a PDT of 24 h and cell
1008 growth was observed to yield a typical "S" curve (Liu et al., 2010). In another study
1009 conducted by Guan et al. (2010) using Bengal tiger fibroblasts, the PDT was determined to be
1010 28 h. Yelisetti et al. (2016) used fibroblasts derived from ear fragments of leopard (*Panthera*
1011 *pardus*), lion (*Panthera leo*), and tiger (*Panthera tigres*) collected *post-mortem* and measured
1012 the PDT to be 26.7, 27.2, and 34.7 h, respectively, attributing the differences to variations in
1013 culture conditions, passage number, and age of the animal.
1014
1015 Song et al. (2007) also evaluated the effect of prolonged culture (up to 8th passage) on
1016 Siberian tiger fibroblasts by chromosomal quantification and showed that, under prolonged
1017 culture conditions, the cells had normal chromosome numbers (2n: 38). Furthermore, in
1018 Siberian tiger (STF34) and Bengal tiger (BTF22) cell lines, Guan et al. (2010) and Liu et al.
1019 (2010) performed assays to detect microorganisms, as well as the isoenzyme patterns of
1020 lactate dehydrogenase (LDH) and malic dehydrogenase (MDH). In both works, the results
1021 were negative for contamination with bacteria, fungi, and yeasts. However, there were
1022 significant differences in the isoenzyme patterns of LDH and MDH were found between the
1023 two species and the other cell lines analyzed. These results indicate that there was no cross-
1024 contamination in the STF34 and BTF22 cell lines with the different cell lines simultaneously
1025 established in the laboratory.
1026
1027 **5. SOMATIC CELL BANKS IN CONSERVATION PROGRAMS IN ZOOS**
1028 The establishment and use of biobanks of wildlife has been central to the development of
1029 basic scientific research and is indispensable to the long-term storage of somatic cells
1030 (Comizzoli, 2017). Many studies that aim to conserve somatic tissues of wild felids are
1031 carried out in cooperation with zoos (Thongphakdee et al., 2006; Hashem et al., 2007; Verma
1032 et al., 2012; Wittayarat et al., 2013; Yelisetti et al., 2016). Studies are being developed to
1033 preserve somatic cells of endangered wild felids from various continents (Table 1).
1034
1035 León-Quinto et al. (2009) used somatic tissues from different regions of the body (muscle,
1036 oral mucosa, bone marrow, spinal cord, and intestines) of Iberian lynx for the maintenance of
1037 somatic tissues of 69 individuals, significantly greater in number than that collected for
1038 gonadal tissues (seven males and six females). Another work carried out by Mestre-

1039 Citrinovitz et al. (2016) described the collection, isolation, and culture of somatic tissues from
1040 jaguar by the Biobank at Buenos Aires Zoo. The Biobank has a collection of 570 samples
1041 from 45 autochthonous and endangered species, including jaguar. The fibroblasts generated
1042 were a part of 6 700 samples, including tissues such as muscle, ovarian, testicular, blood,
1043 fibroblast, sperm, hair and fluids, and cells from 450 individuals of 87 different species.
1044
1045 Furthermore, Thongphakdee et al. (2010) used somatic cells from epithelial and muscular
1046 tissues of male and female marbled cats (*Pardofelis marmorata*) and flat-headed cats
1047 (*Prionailurus planiceps*) in SCNT. The authors reported that the genomes of both species
1048 have been preserved since 2003 in the Genome Resource Bank, which was jointly developed
1049 by the Zoological Park Organization under the Royal Patronage of H.M. the King of
1050 Thailand.
1051
1052 Additionally, as a potential use of somatic cells kept in these cryobanks, cloning by
1053 interspecies SCNT (iSCNT) can be used to restore or expand threatened populations (Loi,
1054 Modlinski & Ptak, 2011). In this sense, Gómez et al. (2004) reported the first birth of a wild
1055 cat by iSCNT using cloned embryos produced by the fusion of fibroblast nuclei of African
1056 wild cat with domestic cat cytoplasts. Subsequently, the same group (Gómez et al., 2008)
1057 reported the birth of another offspring, in this case between different species, using sand cat as
1058 a nucleus donor and domestic cat as a cytoplast donor. In addition, an intergeneric nucleus
1059 transfer was performed using the fibroblasts of flat-headed cats and the cytoplasts of domestic
1060 cats, resulting in blastocysts (9%).
1061
1062 Finally, the advances in the area of induced pluripotency have also been achieved for wild
1063 felids. Induced pluripotent stem (iPS) cells can provide a source of pluripotent cells for use in
1064 wildlife conservation by cryopreservation of genetic resources, nuclear transfer using
1065 reprogrammed donor cells, and directed differentiation of gametes (Verma et al., 2012); this is
1066 notable especially with endangered wild felids, which are frequently difficult to breed both in
1067 captivity and natural conditions (Pope, 2000). Thus, Verma et al. (2012) derived and
1068 characterized iPS cells from snow leopard (*Panthera uncia*) ear fibroblasts from animals in
1069 Mogo Zoo (Australia) and used retroviral vectors to examine their differentiation potential.
1070 They observed that the NANOG gene was crucial in the reprogramming combination for the
1071 derivation of iPS lines in this felid.

1072 **6. FINAL CONSIDERATIONS**

1073 *In vitro* techniques have been shown to be useful and promising for the preservation of
1074 somatic samples from wild felids, helping in maximizing the genetic conservation of a
1075 population. The large number of felines threatened worldwide arouses interest and calls for
1076 the development of conservation strategies aiming to protect still existing biodiversity. The
1077 establishment of somatic cell banks has been described as an advantageous strategy to address
1078 the problem with wild felids that have been reported to be threatened in different continents.
1079 The technical variables involved in cryopreservation processes are constantly improving and
1080 previous works with species such as the Iberian lynx show the potential of the technique.
1081 Further studies should be developed that contribute to the conservation efforts for a wide
1082 range of feline species establishment of optimal cryopreservation conditions, and applications
1083 in interspecific cloning procedures.

1084

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1199 **TABLE 1**. Use of somatic cells in the conservation of wild felids.

Species	World location*	Level of threat*	Sample source	*In vitro* culture conditions	Cryopreservation conditions	Authors
African wild cat *(Felis silvestris libica)*	Europe, Africa and Asia	Endangered	Skin	DMEM supplemented with 10% FBS and antibiotics at 38°C, 5% CO_2 for 7–10 days of primary culture	10% DMSO and 10% FBS	Gómez et al. (2003, 2004)
Siberian tiger (*Panthera tigres*)	Asia	Endangered	Skin	DMEM supplemented with 10% FBS at 37°C, 5% CO_2 for 6–8 days of primary culture	10% DMSO	Song et al. (2007)
Iberian Lynx (*Lynx pardinus*)	Europe	Endangered	Skin, muscle, oral mucosa, bone marrow, spinal cord and intestines	DMEM supplemented with 15% FBS, 1000 U/mL of leukaemia inhibitory factor (LIF) and antibiotics at 37°C, 5% CO_2	NI	León-Quinto et al. (2009)
Iberian Lynx (*Lynx pardinus*)	Europe	Endangered	Skin	DMEM with 10%, 15% or 20% FBS, 5–10 ng/mL epidermal growth factor (EGF), 5–10 ng/mL fibroblast growth factor (FGF) and antibiotics at 37°C, 5% CO_2	5–15% DMSO and 0.1–0.2 M sucrose	León-Quinto et al. (2011)
Leopard (*Panthera uncia)*	Central Asia	Endangered	Skin	DMEM supplemented with 10% FBS and antibiotics at 38.5°C, 6% CO_2 for 7 days of primary culture	DMSO 10% and 90% FBS	Verma et al. (2012)
Asian golden cat (*Pardofelis temminckii*)	Southeast Asia	Near threatened	Skin	DMEM supplemented with 20% FBS and antibiotics at 37°C, 5% CO_2	NI	Wittayarat et al. (2013)

Marbled cat (*Pardofelis marmorata*)						
Cheetah (*Ancinonyx jabutus*)	Africa and Southwest Asia	Vulnerable	Skin	DMEM supplemented with 10% FBS and antibiotics at 39°C, 5% CO_2	10% DMSO and 10% FBS	Moro et al. (2015)
Jaguar (*Panthera onca*)	America	Near threatened	Skin, muscle and cartilage	DMEM supplemented with 10% FBS and antibiotics at 37°C, 5% CO_2 for 10–14 days of primary culture	10% DMSO	Mestre-Citrinovitz et al. (2016)
Asian cheetah (*Ancinonyx jabutus vanticus*)	Africa and Southwest Asia	Vulnerable	Skin	DMEM supplemented with 10% FBS and antibiotics at 38°C, 5% CO_2	10% DMSO and 50% FBS	Moulavi et al. (2017)

1200 * Species world location and levels of threat according to IUCN (2018). NI: non-informed.

CAPÍTULO 3 – ASPECTOS HISTOLÓGICOS QUANTITATIVOS E DESCRITIVOS DA PELE AURICULAR DE ONÇA-PINTADA, *Panthera onca* LINNAEUS, 1758) COMO FERRAMENTA PARA CONSERVAÇÃO

Artigo Experimental N° 01: Quantitative and descriptive histological aspects of jaguar (*Panthera onca* Linnaeus, 1758) ear skin as a tool for conservation

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Quantitative and descriptive histological aspects of jaguar (*Panthera onca* Linnaeus, 1758) ear skin as a tool for conservation

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Running title: Histomorphology of jaguar ear skin.

ABSTRACT

Skin of mammals vulnerable to extinction, such as the jaguar, is used as a source of material in conservation strategies. The composition of skin is not uniform among species and the ability to distinguish similarities in skin morphology in animal groups is fundamental in the application of skin tissue for use in biobanks. The aim of our study was to evaluate the structure, composition, and capacity for culture of ear skin from the yellow and black jaguar. Both qualitative and quantitative methods were used, focusing on skin thickness, cell quantification and distribution, collagen density, proliferative activity, and viability. Histomorphometrical study of the skin showed a total thickness of 273.2 µm and 274.6 µm for yellow and black jaguar, respectively. Melanocytes and

1263 fibroblasts for yellow jaguar were 9.7 and 23.0 and for black jaguar were 11.3 and 26.8,
1264 respectively. A collagen density of 67.0% and 49.0% was observed for yellow and
1265 black jaguar, respectively. Both animals presented a proliferative activity varying
1266 between 1.20–1.30. All tissues were able to promote cellular detachment, reaching
1267 subconfluence in 10–15 days. This kind of information from histomorphometrical
1268 features and cell cultures can be essential for a more targeted application of
1269 cryopreservation in this species.
1270

1271 **KEYWORDS:** Conservation strategies, histomorphometry, *in vitro* culture, wild felid,
1272 zoo.
1273

1274 **1 INTRODUCTION**

1275 Understanding the basic morphological aspects of skin from different species is a
1276 valuable tool in the application of skin in biological resource banks aiming at
1277 biodiversity conservation (Hossain et al., 2016; Pereira, Borges, Praxedes, & Silva,
1278 2018). Thus, the ability to identify the similarities and differences in skin morphology
1279 of different animal groups is fundamental for tissue cryopreservation protocols for the
1280 species of interest (Borges et al., 2017a). Attention to such tissue banks has increased in
1281 recent years (Tunstall et al., 2018), especially as skin provides a greater recovery of the
1282 genetic diversity of the population regardless of gender and age of the individual from
1283 which tissue is harvested (León-Quinto et al., 2009).
1284

1285 The skin is a complex organ playing an important role in thermoregulation and
1286 functioning as a mechanical and immunological barrier (Turner, Pezzone, & Badylak,
1287 2015). The structural architecture of skin is not uniform across different species (Souza,
1288 Fighera, Kommers, & Barros, 2009) and it may vary according to histomorphometric
1289 parameters that include thickness of layers, quantitative estimation of collagen, and
1290 appendages such as sweat glands, sebaceous glands, and hair follicles. All these
1291 histological aspects play an important role in the recovery of cells after the culture of
1292 cryopreserved tissues (Pereira et al., 2018).
1293

In jaguar, the largest, unique feline of the *Panthera* genus of the Americas (Zarrate-Charry, Trujillo, Balaguera-Reina, González-Maya, & Trujillo, 2009), quantitative histological aspects of the skin may be different from those of other animal groups. In this species, some animals have a yellow pelage with black spots or rosettes all over the body, and are called yellow jaguars, while others have a melanic pelage which makes it almost impossible to distinguish the rosettes and are called black jaguars (Núñez, & Jiménez, 2009). Eizirik et al. (2003) have shown that melanism in felines is the result of at least four genetic mutations that occurred independently of each other, with melanism in jaguars being a dominant feature caused by mutation in the melanocortin receptor 1 (*MCR1*) gene which regulates the synthesis of melanin. In recent years, both jaguars have suffered a large decrease in their population sizes and are considered as near threatened on the IUCN Red List (Quigley et al., 2017). Moreover, the black variant of the jaguar is rare, being found only in South America, including the states of Brazil, Peru, Guyana, Ecuador (Meyer, 1994), and Central America, including the Costa Rica (Núñez, & Jiménez, 2009).

Given the ecological and scientific importance of the species (Morato, Ferraz, Paula, & Campos, 2016), conservation tools such as skin cryopreservation become urgently needed strategies in conservation efforts due to the reduction in population sizes. The only work published thus far on such approaches for jaguars is the study conducted by Mestre-Citrinovitz Sestelo, Ceballos, Baranao, & Saragueta (2016). Cryopreserved tissues can be used in cell reprogramming studies for regenerative medicine (Verma, Holland, Temple-Smith, & Verma, 2012) and in combination with cloning by somatic cell nuclear transfer for multiplication of species (Moulavi et al., 2017). Thus, the morphological study of the skin and its description between jaguars with skin variations represents a first step in the establishment of ideal tissue conservation protocols.

The aim of this study was to describe the structure, composition, and capacity for *in vitro* culture of skin from black and yellow jaguars belonging to zoos for application in the creation of biological resource banks for this species.

2 MATERIALS AND METHODS

1326 All methodology was conducted in accordance with the Animal Use Ethics Committee
1327 of the Federal Rural University of the Semi-Arid (CEUA/UFERSA, no.
1328 23091.0011507/2017-61), and the Chico Mendes Institute for Biodiversity Conservation
1329 (ICMBio, no. 57460-1). The chemicals, reagents and media used were obtained from
1330 Sigma-Aldrich (St. Louis, USA), Gibco-BRL (Carlsbad, USA) and Labimpex (São
1331 Paulo, Brazil).

1332

1333 **2.1 Animals and ear skin collection**

1334 Skin samples (1–2 cm^2) were obtained from one male yellow jaguar (10 years of age)
1335 belonging to the São Francisco do Canindé Zoo (Canindé, CE, Brazil), and one male
1336 black jaguar (7 years of age) from the Zoo and Park Dois Irmãos (Recife, PE, Brazil).
1337 After an anesthetic procedure with 0.08 mg/kg IM dexmedetomidine hydrochloride
1338 (Dexdormitor ®, Zoetis, Campinas, Brazil) and mechanical containment (Araujo et al.,
1339 2017), peripheral skin samples from the ear were collected using pliers, washed with
1340 70% alcohol, and transported to the laboratory in Dulbecco modified Eagle medium
1341 (DMEM) supplemented with 10% fetal bovine serum (FBS) 2% penicillin,
1342 streptomycin and amphotericin solution at 4 °C for 8 h.

1343

1344 In the laboratory, the skin fragments (9.0 mm^3) were processed for four types of
1345 analysis: histological analysis using staining with a) hematoxylin-eosin, b) Gomori
1346 trichrome, c) silver salts, and d) capacity for *in vitro* culture of skin. Four skin
1347 fragments were prepared for each type of analysis, totaling sixteen fragments per
1348 animal.

1349

1350 **2.2 Morphometric analysis, cell quantification and distribution**

1351 The samples were fixed in 4% paraformaldehyde and processed for embedding in
1352 paraffin as described by Queiroz Neta et al. (2018) for morphometric analysis, cell
1353 quantification, and distribution analysis. Sections of 5.0 µm thickness were stained with
1354 hematoxylin-eosin. The histological analysis and morphometry were performed using
1355 ImageJ software software (US National Institutes of Health, Bethesda, Rockville, USA)
1356 at 400× magnification. The following parameters were examined: thickness of the
1357 epidermis, dermis, total skin, and epidermal layers (in µm); and number of epidermal

1358 cells, fibroblasts, and melanocytes. Twenty images per animal were acquired for this
1359 analysis.
1360

1361 **2.3 Assessment of collagen density**

1362 To assess the collagen fiber distribution, sections of 5.0 µm thickness were stained with
1363 Gomori trichrome, according to Borges et al. (2017a). The histological analysis was
1364 done using ImageJ software. Ten images at 400× magnification per animal were
1365 acquired and an area with presence of collagen fibers in the superficial dermis was
1366 selected in each image. The amount of fibers present in each area was evaluated and
1367 recorded applying the Threshold Color software plug-in and the 32-bit Red, Green, and
1368 Blue (RGB) format conversion. The following parameters were examined: image area
1369 (μm^2), collagen area (μm^2), and percentage of collagen (density) calculated as total area
1370 with collagen fibers divided by the total area of the analyzed section (Morais et al.,
1371 2017).
1372

1373 **2.4 Evaluation of proliferative activity**

1374 To evaluate the proliferative activity, argyrophilic nucleolar organizer region (AgNOR)
1375 staining was performed. The silver-staining solution was prepared with 1 part of a
1376 solution of 2% gelatin in 1% aqueous formic acid and 2 parts of 50% aqueous silver
1377 nitrate solution. The slides were exposed in a dark room for 30 min. Subsequently, the
1378 slides were washed in 5% thiosulfate solution for 10 min. The AgNOR dots were
1379 counted in 100 randomly selected nuclei on each slide using ImageJ software at 1000×
1380 magnification. AgNOR number/cell and AgNOR area/cell were quantified, with
1381 AgNOR number/cell counted as the average number of silver-labeled nucleoli within
1382 the 100 nuclei counted (Yang et al., 2013).
1383

1384 **2.5 Evaluation of capacity for *in vitro* culture of skin**

1385 To evaluate the culture capacity of skin during *in vitro* culture, skin fragments were
1386 cultured *in vitro* in DMEM supplemented with 10% FBS, 2% penicillin, streptomycin
1387 and amphotericin solution, at 38.5 °C under controlled atmosphere with 5% CO_2 and
1388 95% air, as described by Mestre-Citrinovitz et al. (2016). The culture medium was
1389 changed every 24 h. Daily assessment of the cell culture from the onset until the

1390 subconfluence stage was performed under an inverted microscope (Nikon TS100,
1391 Tokyo, Japan) and the following parameters were evaluated: morphology, number of
1392 attached explants, number of explants with subconfluence, day of all attached explants,
1393 number of explants grown to subconfluence, day of subconfluence explants,
1394 subconfluence total time, and total duration of primary culture.
1395

1396 **2.6 Data analysis**

1397 Data was expressed as mean ± standard error and analyzed descriptively. All
1398 histological aspects such as skin thickness, cell distribution, collagen density,
1399 proliferative activity, and ability of culture were counted from ear skin samples of both
1400 yellow and black jaguar.
1401

1402 **3 RESULTS**

1403 **3.1 Morphometric analysis, cell quantification and distribution**

1404 Morphological features in yellow and black jaguar ear skin are shown in Figure 1 a-b.
1405 The epidermis of both animals showed three evident layers: the basal and spinosum as
1406 viable epidermis and the corneum layer as non-viable epidermis (Figure 1a'-b'). The
1407 basal layer comprised a single layer of cells with a cuboid shape. The spinosum layer
1408 was generally seen with only one layer of cells and the corneum layer as a layer of dead,
1409 flattened cells without a nucleus.
1410

1411 In the dermal layer, the distinction between superficial and deep dermis was not very
1412 evident. Additionally, the adnexa of the skin, such as sebaceous and sweat glands and
1413 hair follicles, were observed (Figure 1a''-b''). The sebaceous glands were seen
1414 associated with hair follicles, usually several glands per follicle, while the sweat glands
1415 did not associate with follicles and were arranged as simple spiral tubular structures.
1416

1417 The histomorphometrical study of the skin revealed a total thickness of 273.2 ± 16.4 µm
1418 and 274.6 ± 9.2 µm for yellow and black jaguar, respectively (Figure 2a-b). The
1419 epidermal and dermal thickness for yellow jaguar were 29.3 ± 2.3 µm and 243.9 ± 17.0
1420 µm, respectively, while those for black jaguar were 32.6 ± 3.6 µm and 242.0 ± 8.4 µm,
1421 respectively (Figure 2 a-b). The spinosum layer of epidermis was the thickest layer in

1422 both jaguars (yellow: 14.0 ± 1.7 µm and black: 14.2 ± 1.3 µm). All the other layers
1423 together totaled a thickness varying between 15.3 µm to 18.4 µm (Figure 2a-b).
1424
1425 Cell quantification values for melanocytes and fibroblasts of yellow jaguar, 9.7 and 23.0
1426 and for black jaguar were 11.3 and 26.8, respectively (Table 1). Epidermal cells showed
1427 values ranging from 38.7 ± 2.5 to 47.4 ± 4.6. Epidermal cells were distributed in the
1428 spinosum as a single layer and melanocytes were observed only in the basal layer.
1429
1430 **3.2 Assessment of collagen density**
1431 The results of the analysis of the profile of collagen fibers present in the yellow and
1432 black jaguar ear dermis are shown in Figure 3 a-b. A thickening pattern of fibers in the
1433 deeper layers appears in blue. Percentage collagen density in skin was 65.0 ± 0.0% for
1434 yellow jaguar and 49.0 ± 0.0% for black jaguar (Figure 3c).
1435
1436 **3.3 Evaluation of proliferative activity**
1437 Cell proliferative activity was measured using by evaluating the number and area of
1438 nucleolar argyrophilic cells in fibroblasts present in the superficial dermis (Figure 4a-b).
1439 Proliferative activity of fibroblasts varied between 1.2 ± 0.2 and 1.3 ± 0.2 NOR/cell for
1440 yellow and black jaguar, respectively (Figure 4c). Furthermore, AgNOR area/cell was
1441 of 0.8 ± 0.4 μm^2 and 1.9 ± 0.8 μm^2 for yellow and black jaguar, respectively (Figure
1442 4c).
1443
1444 **3.4 Evaluation of capacity for *in vitro* culture of skin**
1445 All explants cultured from ear skin of both jaguars showed adherence between the first
1446 and second day of *in vitro* culture (Figure 5a-b). Cell growth around the explants
1447 occurred in 9–10 days and cellular subconfluence in 10–15 days. The duration of the
1448 total culture was of 30 days (Table 2). With regard to the morphological characteristics
1449 of the cells in culture, cells with a fusiform shape and oval central nucleus were
1450 observed (Figure 5a-b).
1451
1452
1453

4 DISCUSSION

In this study, we evaluated the structure, the composition, and capacity for *in vitro* culture of ear skin from the yellow and black jaguar, recognizing the similarities and differences in the skin morphology in the jaguars and their pelages. We described the histological aspects of ear skin of yellow and black jaguar, as this information is essential for a more targeted application of cryopreservation. The formation of skin banks is of growing interest in conserving genetic samples of wild felids (Guan et al., 2010; León-Quinto et al., 2009), especially jaguars (Mestre-Citrinovitz et al., 2016), aimed at ensuring the storage of valuable genetic material (Praxedes et al., 2018).

In mammals, skin architecture is composed of an external epithelial portion, the epidermis, comprising the layers corneum, lucidum, granulosum, spinosum, and basal (Dal Monte et al., 2005). The deeper connective portion, the dermis, comprises collagen and elastic fibers along with arteries, veins, capillaries, lymphatic vessels, sensitive nerve fibers, and exocrine secretion glands (Dal Monte et al., 2005). Jaguar skin of both yellow and black pelages presented structural patterns of skin architecture similar to the general architecture in mammals, divided into epidermis and dermis.

Depending on the location on the body of the animal, the skin varies with regard to the thickness of the epidermis (Isola, Moraes, Rahal, & Machado, 2013) and density of hair (Meyer, Schwarz, & Neurand, 1978). Thus, in body regions that have thin skin, such as the ear region, there are usually 3 to 4 layers in the epidermis with the lucidum layer being absent and the granulosum layer absent or incomplete (Affolter, & Moore, 1994). Our data agrees with this characteristic and only three layers of epidermis were observed. Independent of jaguar pelage, the epidermal thickness varied only between 29.3 µm to 32.6 µm. The thickness of the epidermis in domestic cats is approximately 25.0 µm (Souza et al., 2009), which is a little less than indicated by our data for jaguars. In mammals the thickness of the epidermis varies between 10.0 and 45.0 µm (Meyer et al., 1978).

We did not observe any distinction between superficial and deep dermis, as is also seen in llamas (Atlee et al., 1997). We observed a dermal thickness of 243.9 µm for yellow

1486 jaguar and 242.0 µm for black jaguar. Thickness of both the epidermis and the dermis
1487 may influence the transport of substances (Grabau et al., 1995), such as cryoprotectants,
1488 used in cryopreservation protocols. Both values were lower than those found in the neck
1489 region of *Cuniculus paca* (3120.90 µm; Isola et al., 2013), and in the mid-thoracic
1490 region of rhesus monkey and pig (1457.2 µm and 3848.2 µm; Grabau, Dong, Mattie,
1491 Jepson, & McDougal, 1995). However, it was higher than the dermal thickness in the
1492 ear skin region of collared peccaries (*Pecari tajacu*, 222.6 µm, Borges et al., 2017a).
1493 Thus, these variations in skin thickness may be related to the different regions analyzed
1494 and species-specific factors (Grabau et al., 1995; Salehi, Lavvaf, & Farahvash, 2013).
1495
1496 Sebaceous and sudoriparous glands were observed as cutaneous appendages, as also
1497 hair follicles in both jaguars, similar to reports on cats (Souza et al., 2009). Furthermore,
1498 the sebaceous glands are in the region of the deep dermis, in line with the location
1499 described for the Egyptian water buffalo and humped camel (El-Shafey, Emam, &
1500 Kassab, 2017). The skin appendage structures generally vary as to their composition
1501 and distribution (Grabau et al., 1995). In general, sebaceous glands are associated with
1502 hair follicles and not related to sweat glands in mammals (Gartner, & Hiat, 2010), and
1503 the same is seen in the present study. These skin adnexal structures may vary in amount
1504 according to the region of skin studied and gender of the animal (Hossain et al., 2016).
1505
1506 The number of melanocytes showed only a small variation with values being between
1507 9.7–11.3 for the yellow and black jaguars. Functionally, melanocytes are responsible for
1508 the production of melanin pigment and a high pigmentation is not a sign of a large of
1509 melanocytes, but rather of the intense activity in the production of melanin (Khavkin, &
1510 Ellis, 2011). In black jaguars, this melanism is caused by a deletion in the *MC1R* gene
1511 (Eizirik et al., 2003). These findings are in accordance with our understanding of
1512 melanism in these animals. In cats, melanocytes represent 5–8% of the epidermal cell
1513 population, and are present in a proportion of 10–20 keratinocytes to one melanocyte
1514 (Affolter, & Moore, 1994; Souza et al., 2009). Additionally, the values found were
1515 similar to those seen for Yorkshire pigs, with an average number of 6–15 melanocytes
1516 (Navarro et al., 2001). However, these values differ from those found in collared
1517 peccaries, which have an average number of melanocytes ranging from 10–94 (Borges

1518 et al., 2017a). Finally, keratinocytes, Merkel, and Langerhans cells are also present as
1519 constituent cells of the epidermis (Souza et al., 2009).
1520
1521 In the present study, the percentage of collagen fibers varied between 49%–65% in the
1522 black and yellow jaguar. Some authors correlate the variation in the proportion of
1523 collagen fibers in the dermis with the age (Yang et al., 2017) and gender of the animal
1524 (Isola et al., 2013). This explanation for variation is not applicable to our study;
1525 however, a correlation was observed between the thickness of the dermis and the
1526 percentage and diameter of collagen fibers (Meyer, & Neurand, 1987). The dermis of
1527 the yellow jaguar was thicker than that of the black jaguar, and this aspect may have
1528 influenced the results obtained.
1529
1530 Fibroblasts present in the ear dermis of jaguars showed a proliferative activity of 1.2–
1531 1.3 number of NOR/cell in the present study. The NORs are segments of DNA
1532 responsible for the synthesis of ribosomal ribonucleic acids (rRNA), a main component
1533 of ribosomes, constituting the starting point for the synthesis of cellular proteins, and
1534 therefore associated with cellular proliferative activity (Godoy, Godoy, & Oehmichen,
1535 2000). Thus, the determination of the AgNOR number/cell and the AgNOR area/cell,
1536 can suggest rates of cellular proliferative activity (Heinisch, & Wozel, 1995). Preziosi,
1537 Sarli, & Marcato (2000) recorded 1.22 AgNORs in swine and the area in this species
1538 was approximately two times larger (4.39 µm), when compared to the values found in
1539 the present study. Additionally, the number of AgNOR found in collared peccaries was
1540 2.48 µm, demonstrating once again the presence of divergence even among wild species
1541 (Borges et al., 2017a).
1542
1543 The data of *in vitro* culture presented a very similar cell growth pattern for both jaguars,
1544 where cellular growth from the explants occurred in 9 and 10 days. This data was
1545 similar to that obtained by Mestre-Citrinovitz et al. (2016), who reported that cell
1546 growth occurred between 10 and 14 days for yellow jaguar. Furthermore, for other
1547 species of felines such as the Bengal tiger (*Panthera tigris tigris*) and Siberian tiger
1548 (*Panthera tigris altaica*), cell growth occurred after about 5–12 days of *in vitro* culture
1549 (Guan et al., 2010; Liu, Guo, Liu, Guan, & Ma, 2010). In addition, the cellular

1550 subconfluence was reached in 10–15 days, a shorter time than that required for the
1551 collared peccary at 18 days (Borges et al., 2017b). The cells presented a fusiform
1552 morphology with oval central nucleus similar to fibroblasts, as described in the yellow
1553 jaguar (Mestre-Citrinovitz et al., 2016).
1554

1555 **5 CONCLUSIONS**

1556 In conclusion, the ear skin from the yellow and black jaguar presented well-defined
1557 epidermal and dermal layers, with the presence of sebaceous glands, sweat glands, and
1558 hair follicles. The epidermal portion presented three layers (basal, spinosum, and
1559 corneum) similar to that of domestic cats, but different from that of other mammals.
1560 Some variations were observed in skin thickness, density of collagen fibers, and number
1561 of melanocytes and fibroblasts between jaguars and other mammals. The cell growth
1562 pattern was similar between yellow and black jaguar, resembling that of other wild
1563 felids but differing from that of some mammalian species. This information is essential
1564 for the development of cryopreservation protocols for jaguar ear skin and will contribute
1565 to the practical implementation of cryobanks for this species.
1566

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1573

1574 **CONFLICTS OF INTEREST**

1575 The authors declare that they have no conflict of interest.
1576

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1732 **TABLE 1** Mean values of different skin cells derived from the ear region from jaguar using hematoxylin-eosin staining.

Jaguar	**Epidermal cells**		**Melanocytes**		**Fibroblasts**	
	Mean ± S.E.	**Range**	**Mean ± S.E.**	**Range**	**Mean ± S.E.**	**Range**
Yellow	47.4 ± 4.6	34–82	9.7 ± 1.1	4–13	23.0 ± 2.9	12–37
Black	38.7 ± 2.5	24–47	11.3 ± 1.6	5–20	26.8 ± 2.7	18–43

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1741 **TABLE 2** Establishment of primary cultures of skin cells derived from yellow and black jaguar.

Jaguar	**No. samples**			**No. of attached samples**			**Duration of culture (days)**
	Initial	**Attached (%)**	**Day of all attached explants**	**Grow to subconfluence (%)**	**Day of all fragments with growth**	**Subconflunce day**	
Yellow	4	4 (100)	1	4 (100)	10	15	30
Black	4	4 (100)	1	4 (100)	9	10	30

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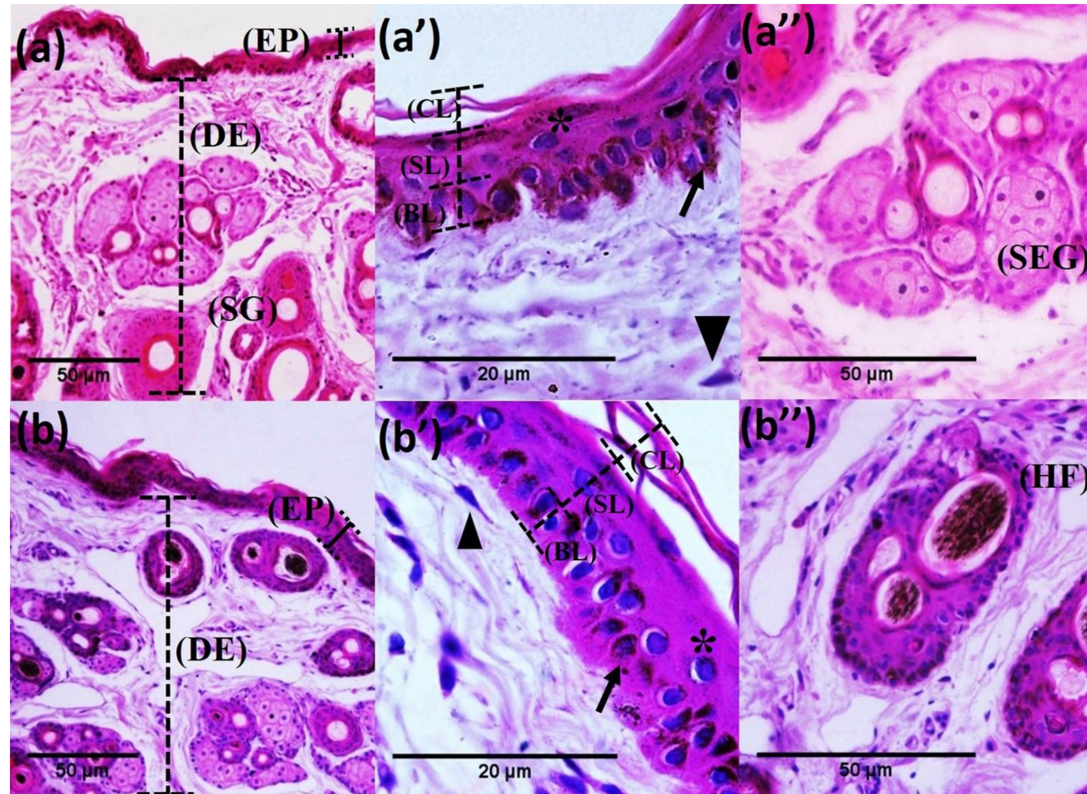
1748 **FIGURES**



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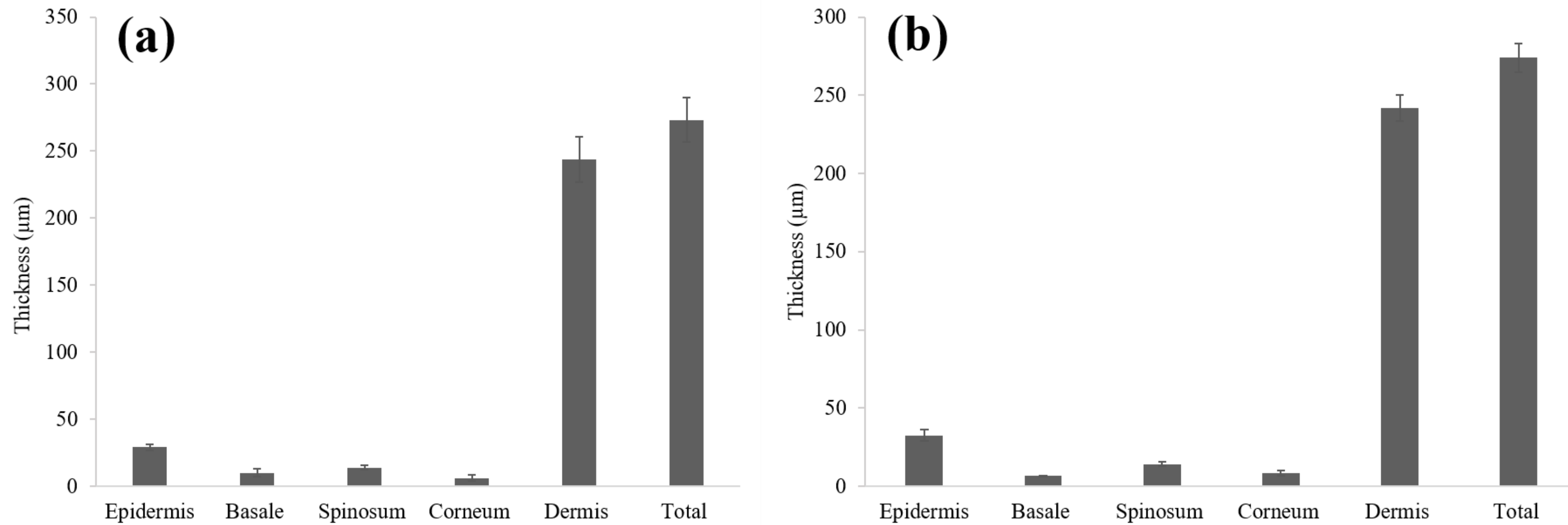
1750 **FIGURE 1** Histological analysis of ear skin of yellow jaguar **(a, a', a'')** and black jaguar **(b, b', b'')** using hematoxylin-eosin. a-b
1751 represent an overview of the skin layers; **a'-b'** represent layers of the epidermis; **a''-b''** represent only the dermis. Epidermis (EP),
1752 corneum layer (CL), spinosum layer (SL), basal layer (BL), dermis (DE), sweat gland (SG), sebaceous gland (SEG), and hair follicle (HF).
1753 Epidermal cell (*), melanocyte (arrow), and fibroblast (triangle).

1754



1755 **FIGURE. 2.** Measurement of the skin and epidermal layer sizes of the ear region from jaguar using hematoxylin-eosin staining. (a)
1756 Thickness of the epidermis and dermis and total of the ear skin derived from yellow and black jaguar. (b) Thickness of the epidermis layers
1757 of the ear skin derived from yellow and black jaguar. Bars represent standard error.

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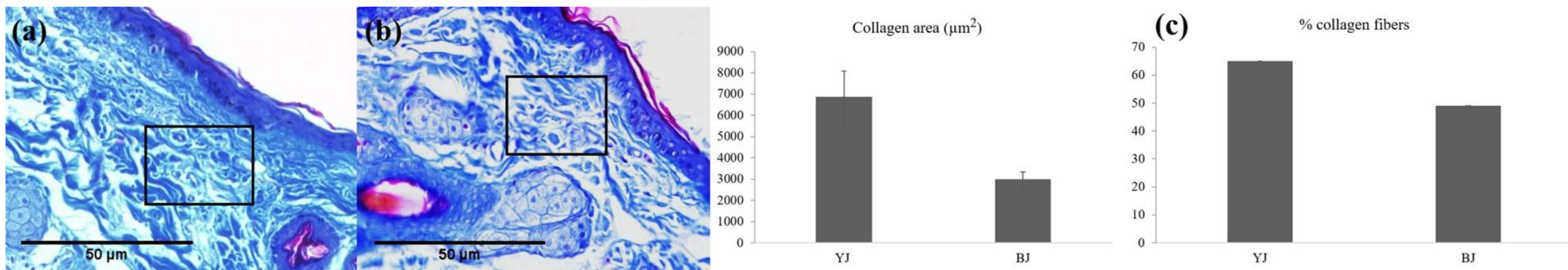

1759 **FIGURE 3** Evaluation of collagen density from ear skin jaguar using Gomori trichrome. (a) Staining of collagen fibers in the dermis from

1760 yellow jaguar (YJ). (b) Staining of collagen fibers in the dermis from black jaguar (BJ). (c) Collagen area and percentage of collagen in the

1761 dermis from yellow and black jaguar. Bars represent standard error.

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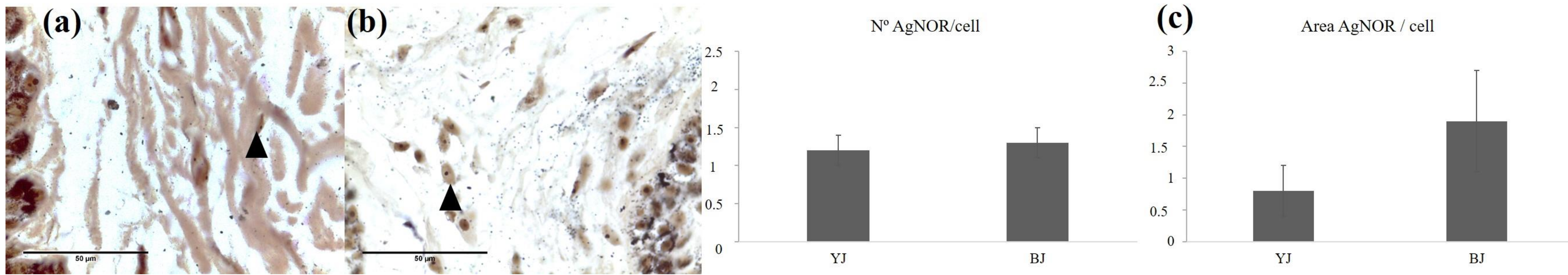

1767 **FIGURE 4** Proliferative activity of ear skin derived from jaguar. (a) Staining of AgNOR in the fibroblasts from yellow jaguar (YJ). (b)
1768 Staining of AgNOR in the fibroblasts from black jaguar (BJ). (c) Quantification of AgNOR number/cell and AgNOR area/cell. Triangles
1769 represent nuclei stained with AgNOR. Bars represent standard error.
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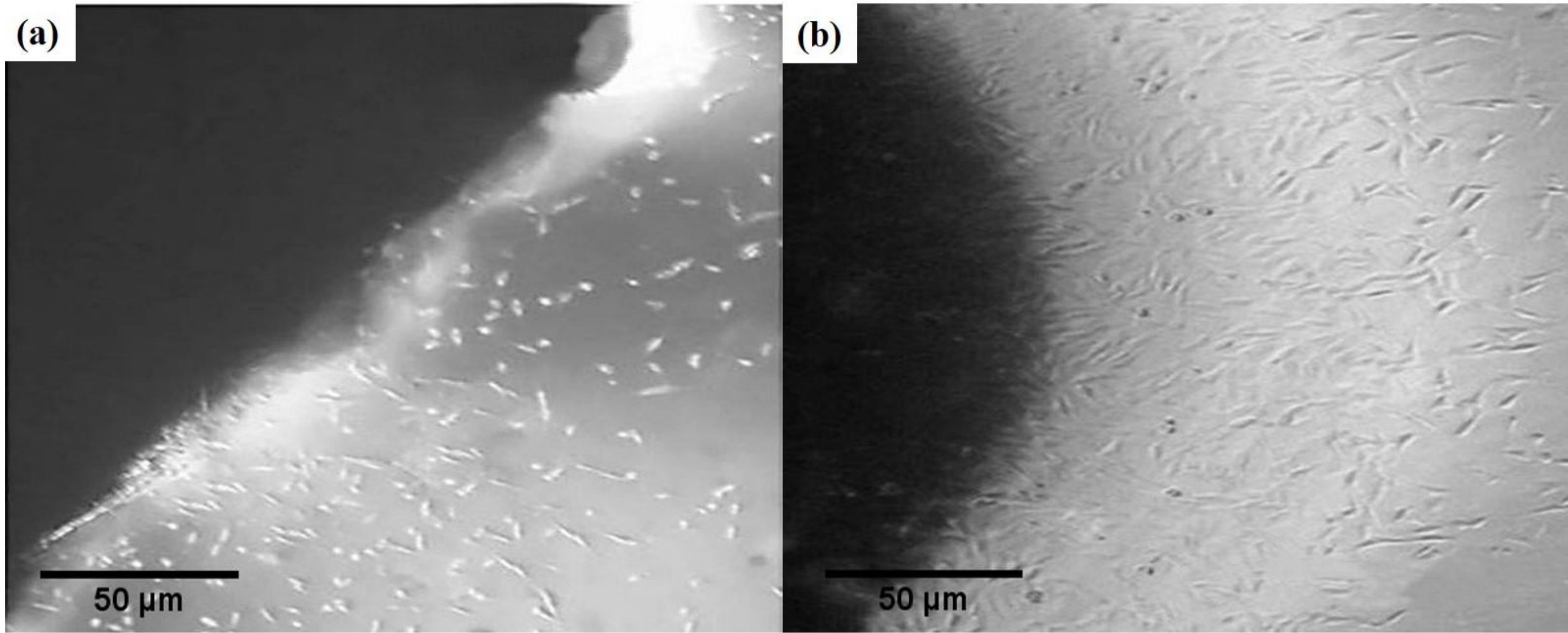

1772 **FIGURE 5** Primary of fibroblast-like cells from ear skin samples of jaguar. (a) Cells from ear skin from yellow jaguar. (b) Cells from ear
1773 skin from black jaguar growing from explants in nine and ten days.

CAPÍTULO 4 – EFEITOS DE TÉCNICAS DE CRIOPRESERVAÇÃO SOBRE A CONSERVAÇÃO DA PELE AURICULAR – UMA ABORDAGEM ALTERNATIVA PARA CONSERVAÇÃO DE ONÇA-PINTADA, *Panthera onca* (LINNAEUS, 1758)

Artigo Experimental N° 02: Effects of cryopreservation techniques on the preservation of ear skin – An alternative approach to conservation of jaguar, *Panthera onca* (Linnaeus, 1758)

Periódico de submissão: Cryobiology.

Qualis (Medicina Veterinária): B1. Fator de Impacto: 2,050

Data de submissão: 19/01/2019.

1805 **Effects of cryopreservation techniques on the preservation of ear skin – An**
1806 **alternative approach to conservation of jaguar, *Panthera onca* (Linnaeus, 1758)**
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1828
1829 **Abstract**
1830 Currently, it has been observed that a considerable segment of the jaguar population is
1831 declining mainly because of hunting, and destruction and fragmentation of habitat.
1832 Given this scenario, efforts of the scientific community have been concentrated on the
1833 development of conservation strategies, such as the formation and use of somatic
1834 sample banks. We aimed to assess the effect of cryopreservation techniques of the ear
1835 skin of jaguar [slow freezing (SF) or direct vitrification in cryovials (DVC) or solid-
1836 surface vitrification (SSV)] on the morphological analysis and cell ability during the

culture. All cryopreserved fragments regardless of the technique used, showed a reduction in the dermis and total thickness of the skin. Although a collagen matrix similar to the control group (fresh) has been observed only for the fragments from SF and SSV groups, all cryopreserved techniques were able to maintain normal patterns of the fibroblasts. Moreover, DVC and SSV methods maintained the proliferative activity of the tissues even after warming. After the culture, SF and SSV techniques were efficient for the recovery of the somatic cells according to most of the evaluated parameters, especially with regard to the duration of culture and cell metabolic activity. In conclusion, SSV was found to be a more efficient technique for cryopreserving jaguar skin when compared to DVC and SF. These results are relevant for the formation of somatic resource banks of this species, directed at cryopreserving adequate samplings of different individuals and generations for future applications in regenerative medicine, and assisted reproductive technologies.

Keywords: *Panthera* genus; biological resource banks; vitrification; somatic cells.

1. Introduction

The jaguar, the third largest feline in the world and the largest in the Americas, is a carnivorous of high ecological and economic significance for global biodiversity [6,25]. In recent years, the population of jaguars has fallen sharply [24] mainly due to anthropogenic activities [33,35]. According to Jedrzejewski et al. [13], currently the world's jaguar population is estimated at 173,000 individuals, with Brazil, being the holder of half of the world's jaguar population with approximately 86,800, followed by Peru with as many as 22,200.

Professionals from different areas such as veterinarians, biologists, and zoologists have intensified their research aimed at the conservation of the jaguar population [13,22]. This multidisciplinary action aims to respond by immediate strategies of conservation of the biological material of this species, such as formation of biological resource banks [17]. Biological banks are defined as deposits of cryopreserved gametes, embryos, gonadal tissues, somatic cells, and tissues after recovery and processing [22].

Although gonadal samples and embryos are the first choice for biological banks, the interest in somatic samples, especially those derived from skin, has increased [22,30], particularly because these samples provide a greater recovery of the genetic diversity of the population caused by tissue harvest that is performed regardless of the gender and age of the animal [14]. Endangered wild felids are difficult to breed, and hence formation of somatic banks for obtaining pluripotent cells has aroused the interest of researchers from different sectors, such as regenerative medicine, assisted reproductive technologies, and development of biotechnologies [28,32,36].

Moreover, the application of suitable methods for the storage of skin at low temperatures aiming to implement cryobanks is one of the most important steps for obtaining cells of interest for nuclear reprogramming [19] and obtaining embryos by cloning [20]. A single work published so far has performed the cryopreservation of jaguar skin by slow freezing (SF) using living individuals kept in the Argentine territory [17]. However, this technique provokes the formation of intracellular ice crystals, which can incite irreversible cell damage after warming.

Vitrification appears as a substitute to reduce the formation of ice crystals [3]. Moreover, vitrification is preferred over SF due to faster execution procedure, not requiring expensive equipment, and being able to be carried out in the field [8]. Currently, there is no information regarding the efficiency of vitrification on the conservation of jaguar skin. The difference in the tissue morphology of the skin of different species does not allow the extrapolation of protocols to the species of interest that are developed in other species [4]. Likewise, vitrification can be usually conducted through different methods, such as direct vitrification in cryovials (DVC) and solid-surface vitrification (SSV), and the choice of appropriate vitrification method consists of evaluating in the tissues [3].

Therefore, the aim of this study was to evaluate the effects of cryopreservation techniques of the ear skin of jaguar (SF, DVC or SSV) on the morphological analysis and cell ability during the culture, and contribute to the conservation by appropriate storage of the skin of this species.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all the reagents, media and solutions were obtained from Sigma-Aldrich (St. Louis, MO, USA), Gibco-BRL (Carlsbad, CA, USA) and Labimpex (São Paulo, SP, Brazil).

2.2. Compliance with ethical standards and animals

The experimental protocols and animal handling procedures were performed with the approval of the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.0011507/2017-61), in compliance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 57460-1). A total of five jaguars, obtained from the zoos of the northeastern of Brazil, were used. Data on the main biological aspects, including age, gender, pelage and location of jaguars are presented in Table 1.

2.3. Skin collection and experimental design

For recovery of skin derived from peripheral ear, animals were previously anesthetized using 0.08 mg/kg of dexmedetomidine hydrochloride (Dexdormitor®, Zoetis, São Paulo, SP, Brazil) intramuscularly and monitored throughout the procedure [1]. Skin tissues of 1–2 cm^2 were recovered using pliers. After collection, skin samples were washed in 70% ethanol and transported to the laboratory in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% antibiotic–antimycotic solution at 4 °C for 3 to 8 h.

In the laboratory, the tissue fragments were trichotomized, washed in 70% ethanol and in DMEM containing 10% FBS and 2% antibiotic–antimycotic solution. Subsequently, the skin was fragmented in 9.0 mm^3 (3 x 3 x 1 mm) sections and distributed randomly in cryopreserved [slow freezing (SF) or direct vitrification in cryovials (DVC) or solid-surface vitrification (SSV)] and non-cryopreserved (fresh control) groups. Thus, fresh and cryopreserved/warmed fragments were evaluated for the morphological analysis with emphasis on epidermal and dermal thickness, cell and perinuclear halo

1933 quantification, collagen matrix, and tissue proliferative activity. For evaluation of
1934 morphological analysis by histology, 20 fragments were used from each group.
1935 Moreover, other samples were submitted to primary tissue culture and subcultures for
1936 up to 3 passages, being 20 fragments for each group. Cells were analyzed for
1937 morphology, adhesion, subconfluence, viability by trypan blue, metabolic activity by 3-
1938 (4.5-dimethylthiazole-2yl)-2.5-diphenyl tetrazoline bromide (MTT), and proliferative
1939 activity through cell growth curve and determination of population doubling time
1940 (PDT), as described in the following.
1941
1942 *2.4. Cryopreservation for skin conservation*
1943 A cryopreservation solution (CS) constituted of DMEM supplemented with 1.5 M
1944 dimethyl sulfoxide (DMSO), 0.25 M sucrose (SUC) and 10% FBS was used for all
1945 cryopreservation techniques, according to the combination of cryoprotectants employed
1946 for somatic samples of wild felid species [14,17].
1947
1948 *2.4.1. Slow freezing*
1949 The slow freezing (SF) technique was performed according to a methodology described
1950 for jaguar skin with modifications [17]. Briefly, fragments were inserted into cryovials
1951 containing 2.0 mL CS and transferred to a -80 °C freezer in Mr. Frosty system®
1952 (Thermo Scientific Nalgene, Rochester, NY, USA) for 12 h using a cooling rate of 1
1953 °C/min. Subsequently, all cryovials were stored in liquid nitrogen (-196 °C).
1954
1955 *2.4.2. Direct vitrification in cryovials*
1956 As there are no studies describing the vitrification methods in jaguar skin and other wild
1957 felids, the direct vitrification in cryovials (DVC) was performed according to the
1958 methodology described for skin of wild mammals [3,7]. Briefly, fragments were
1959 immediately transferred for cryovials containing 2.0 mL CS and kept for 15 s at 25 °C.
1960 Subsequently, all cryovials were stored in liquid nitrogen (-196 °C).
1961
1962 *2.4.3. Solid-surface vitrification*
1963 For the solid-surface vitrification (SSV) procedures, a methodology described for skin
1964 derived from wild mammals was implemented [7]. Briefly, fragments were exposed to

1965 1.8 mL of CS for 5 min, and then the excess solution was removed on absorbent paper.
1966 Subsequently, the fragments were placed individually on a metal surface partially
1967 immersed in liquid nitrogen (-196 °C), transferred to cryovials and stored in liquid
1968 nitrogen (-196 °C).
1969
1970 *2.4.4. Warming*
1971 After 2 weeks of storage in liquid nitrogen, all cryovials were maintained for 1 min at
1972 25 °C and immersed in a water bath at 37 °C. For removal of CS, fragments were
1973 washed three times for 5 min in DMEM plus 10% FBS supplemented with SUC at
1974 decreasing concentrations (0.50, 0.25 M and without SUC).
1975
1976 *2.5. Evaluation of the skin by histological analysis*
1977 For morphological evaluation using histological analysis, fragments derived from non-
1978 cryopreserved (fresh control) and cryopreserved (SF, DVC, and SSV) groups were fixed
1979 using 4% paraformaldehyde, processed for embedding in paraffin and sectioned at 5.0
1980 µm, according to Queiroz Neta et al. [23]. Subsequently, fragments of each group were
1981 stained with hematoxylin-eosin (HE), Gomori trichrome (GT) and argyrophilic
1982 nucleolar organizer region (AgNOR) for analysis of the morphometric aspects, collagen
1983 matrix, and tissue proliferative activity, respectively. Finally, images at 40x (HE and
1984 GT) and 100x (AgNOR) magnitude were obtained, using a light microscope (Leica
1985 DM500, Leica Microsystems, Wetzlar, HE, Germany) coupled with a camera (Leica
1986 ICC50 HD, Leica Microsystems, Wetzlar, HE, Germany).
1987
1988 For morphometric analysis, fragments stained with HE were evaluated for
1989 quantification of the thickness of total skin, epidermis and dermis in µm, number of
1990 perinuclear halos in the epidermis and dermal fibroblasts. For this analysis, 20
1991 images/animal were acquired for each group, totaling 100 images per group and
1992 assessed using Image J software (US National Institutes of Health, Bethesda, MA,
1993 USA).
1994
1995 For analysis of collagen matrix using GT, collagen fibers of dermis were quantified and
1996 presented in percentage. The slides were stained with Weigert's iron hematoxylin

1997 solution and trichrome solution. The percentage of collagen fibers was the result of the
1998 total area of collagen divided by the total area of the analyzed image, according to
1999 Morais et al. [18]. For this analysis, 10 images/animal were acquired for each group,
2000 totaling 50 images per group and evaluated using threshold color plug-in with the Image
2001 J software (US National Institutes of Health, Bethesda, MA, USA), employing 32-bit
2002 RGB format conversion.
2003
2004 For tissue proliferative activity analysis with AgNOR assay, dark spots marked by silver
2005 nitrate attached to nuclear proteins were counted according to the cell location [23].
2006 Briefly, the slides were exposed to silver nitrate solution and sodium metabisulfite
2007 solution. In each image, 100 nuclei of randomly selected stained fibroblasts were
2008 counted, and the AgNOR number/cell and AgNOR area/cell were quantified using
2009 Image Pro Plus software. For this analysis, 20 images/animal were acquired from each
2010 group, totaling 100 images per group.
2011
2012 *2.6. Evaluation of the skin by primary culture and subcultures*
2013 All fragments were cultured by primary and secondary systems in DMEM constituted
2014 with 10% FBS and 2% antibiotic–antimycotic solution at 38.5 °C, and 5% CO_2. The
2015 culture medium was changed every 24 h and the cells were harvested when they reached
2016 70% subconfluency and were subcultured into other dishes. The subconfluence of 70%
2017 was defined when 70% of the petri dishes presented somatic cells [27]. Subconfluent
2018 cells were trypsinized and passaged [17]. Cells were evaluated for their characteristics
2019 during primary culture and subcultures for morphology, viability, metabolic and
2020 proliferative activities.
2021
2022 *2.6.1. Assessment of tissue and cell morphology*
2023 The primary culture was evaluated using an inverted microscope (Nikon TS100, Tokyo,
2024 Japan). The following parameters were evaluated: morphology, number of attached
2025 explants, number of subconfluent explants, day on which all explants are attached, day
2026 at which explants reached subconfluence, total time required to attain subconfluence,
2027 and total culture duration, according to Queiroz Neta et al. [23].
2028

2029 *2.6.2. Analysis of viability and metabolic activity during subcultures*

2030 The cell viability was performed in the first passage of culture using trypan blue assay.
2031 All analysis was carried out in duplicate. Briefly, an aliquot of suspended cells was
2032 stained with 0.4% trypan blue (in PBS) in the ratio 1:1 and counted in a Neubauer
2033 chamber. The cells were considered viable when they are not stained owing to the
2034 ruptured membrane and the non-viable cells became blue because of the penetration of
2035 the dye. The percentage of viable cells was calculated by dividing the number of viable
2036 cells per total number of cells counted [3].

2037

2038 For evaluation of the metabolic activity, cells were submitted to MTT assay. Briefly,
2039 cells 5 x 10^4 cells/mL were cultured for 5 days in 5% CO_2 at 38.5 °C in DMEM
2040 constituted with 10% FBS and 2% antibiotic–antimycotic solution. After this period,
2041 cells were incubated with 5 mg/mL MTT solution for 3 h at 38.5 °C and 5% CO_2.
2042 Subsequently, DMSO was added as a solubilization solution for MTT and the readings
2043 were noted at 595 nm, according to Santos et al. [27].

2044

2045 *2.6.3. Study of proliferative activity*

2046 Proliferative activity of cells derived from non-cryopreserved and cryopreserved tissues
2047 was quantified according to the elaboration of the growth curve and determination of
2048 PDT. Cells (1 x 10^4 cells/mL) were plated in 24-well dishes and were trypsinized,
2049 counted and recorded at 24 to 168 h intervals. The average of the counts at regular
2050 intervals of 24 h was used for elaboration of the growth curve, and PDT was estimated
2051 [26], according to following equation:

2052

2053 PDT = T ln2/ln (Xe/Xb)

2054

2055 PDT is the time of the culture (in hours), T is the incubation time, Xb is the number of
2056 cells at the beginning of the time incubation, Xe is the number of cells at the end of the
2057 incubation time, and ln is Napierian logarithm.

2058

2059 *2.7. Statistical analysis*

Data from five jaguars was expressed as mean ± standard error (one animal/ repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. As data regarding the trypan blue test and metabolic activity did not show a normal distribution, they were arcsine transformed. Data of morphometric analysis were analyzed by ANOVA (multiple comparisons) followed by Tukey test. The results of AgNOR analysis, fibroblast and perinuclear halo numbers were already analyzed by Kruskal-Wallis and Dunn tests. All *in vitro* culture data was analyzed by ANOVA followed by unpaired t-test. Significance was set at $P < 0.05$.

3. Results

3.1. Evaluation of the skin by histological analysis

To determine the effects of cryopreservation techniques on the conservation of jaguar ear skin, we histologically analyzed 80 skin explants obtained from five jaguars. Initially, we evaluated the jaguar skin by hematoxylin-eosin stain and morphological features in non-cryopreserved (fresh control) or cryopreserved (SF, DVC, and SSV) skin were observed (Fig. 1a-1d). An evident distinction of the skin layers could be observed in all groups and demonstrated the presence of perinuclear halos in the epidermis and fibroblasts in the dermis. Moreover, all fragments cryopreserved, regardless of the techniques used, showed a reduction in the dermis and total thickness of the skin (Table 2). Nevertheless, only the thickness of epidermis from SF group was similar to the control group ($P > 0.05$). Likewise, all cryopreserved techniques were able to maintain normal patterns of the fibroblasts (Table 2). Additionally, only DVC group showed a number of perinuclear halos similar to non-cryopreserved group.

We further evaluated the jaguar skin by Gomori trichrome stain in non-cryopreserved (fresh control) or cryopreserved (SF, DVC, and SSV) groups as shown in Fig. 1a'-1d', and we observed a collagen matrix similar to the control group for fragments derived from SF and SSV groups (Table 2). Finally, DVC and SSV methods maintained the proliferative activity by AgNOR assay of the tissues after warming (Fig. 2). Both DVC and SSV showed an increase in AgNOR number per cell and AgNOR area per cell than the SF group ($P < 0.05$).

3.2. Evaluation of the skin by primary culture and subcultures

In relation to *in vitro* culture (Fig. 3), no difference was observed among non-cryopreserved and cryopreserved for the ability of tissue adherence and cellular confluence (Table 3). In general, epithelial-like and fibroblast-like cells migrated from tissue fragments of the fresh control, SF, DVC and SSV groups at 9.2, 8.4, 11.8 and 8.0 days respectively after explanting (Table 3 and Fig. 3a-3d). Nevertheless, SF and SSV techniques were efficient for the recovery of the somatic cells according to the day on which all attached explants with the cell grow and the total duration of culture (Table 3 and Fig. 3a'-3d'). Thus, a longer culture for the DVC group was a result of longer time required for cells growing from explants.

After the first passage, all groups maintained the quality presenting viability above 90% (Fig. 4a). Through analysis of metabolic activity with MTT, only SF and SSV groups were similar to the fresh group ($P < 0.05$) showing a negative effect of DVC cryopreservation on the metabolic activity of the cells (Fig. 4b).

The cell growth curve generated observes a typical "S" shape in all cryopreserved groups similar to the fresh group with a lag phase, exponential phase and steady phase (Fig. 5b). Cells derived from the DVC group had a lower metabolic activity, besides a change in the cell growth curve between 96 and 120 h. Furthermore, the population doubling time did not change among cryopreserved and fresh groups (Fig. 5a).

4. Discussion

Only few studies evaluated the potential of somatic tissue vitrification aiming at the conservation of wild mammals [3,4,7]. This technique is considered advantageous by the as it is less time-consuming, practical and an economic technology to perform in the field [8]. Thus, in the present study, we evaluated for the first time the potential of vitrification techniques on jaguar ear skin tissue cryopreservation. Consequently, a superior maintenance of the histological parameters was obtained with the vitrification techniques (DVC and SSV). Furthermore, during the *in vitro* culture, SSV and SF showed greater efficiency in cell recovery, with superior metabolic activity potential.

2124 Therefore, based on the data obtained, SSV showed to be most suitable technique for
2125 conservation of skin derived from jaguars.
2126
2127 Histological analysis revealed retraction of the dermal layer and total skin thickness in
2128 all cryopreserved groups when compared to the fresh group. Further, the epidermal
2129 thickness of only SF was similar to the fresh group. Thus, cryopreservation techniques
2130 in general may lead to a certain retraction and cell deformation due to the efflux of
2131 water from the interior of the cells to the extracellular medium [11]. Thereby, this efflux
2132 of water could justify the retraction occurred in the total and dermal thickness of the
2133 skin in all groups. Moreover, the vitrification techniques may have caused damage to
2134 the epidermis because this is the most superficial layer and thus is more exposed to
2135 damage as a result of the abrupt decrease in temperature [3].
2136
2137 The number of perinuclear halos showed a subtle increase in the SF and SSV groups.
2138 These halos are structures that signal the beginning of apoptosis, being formed by
2139 separation of the nucleus from the cytoplasm [2]. This increase was not observed in the
2140 DVC group, probably due to less time used in the vitrification procedures associated
2141 with the cryoprotectant solution present during all the stages of the DVC. Nevertheless,
2142 as the cells of interest for subsequent studies are fibroblasts found in dermis [20], the
2143 retraction of the epidermis and increase of halos caused by vitrification would not be a
2144 limiting factor.
2145
2146 Moreover, all cryopreservation techniques were able to promote the maintenance of the
2147 number of fibroblasts. Studies show that the use of a combination of cryoprotectants
2148 acting both intra and extracellularly can reduce the damage caused by cryopreservation
2149 processes, providing the maintenance of cell membrane integrity [3]. In the present
2150 study, DMSO was used; it is an intracellular cryoprotectant with low toxicity and high
2151 permeability in membranes [29]. Additionally, its combination with FBS and SUC aims
2152 to aid in cell survival and proliferation after warming [5], protection of cell membranes
2153 and reduction of osmotic shock [4]. Thus, the combination of cryoprotectants, DMSO,
2154 SUC and FBS may have aided in the cell maintenance, not causing damage to the
2155 fibroblasts present in the dermal region in either group.

2156

2157 The collagen matrix was maintained in the SF and SSV groups**.** During the
2158 cryopreservation and warming procedure, no redistribution or realignment of collagen
2159 fibers occurred in SF and SSV groups [21]. This phenomenon may have already
2160 occurred in the DVC group, resulting in a negative effect and associated with non-
2161 permeabilization of the cryoprotectant throughout the thick dermal layer. Moreover, the
2162 tissue proliferative activity evaluated by AgNOR showed a higher AgNOR number and
2163 AgNOR area in the DVC and SSV groups. Thus, it can be hypothesized that the slow
2164 decrease of temperature in the SF process can lead to the formation of intra cellular ice
2165 crystals, leading to ruptures and tissue damage [9,16] thereby negatively affecting the
2166 proliferative capacity of the tissues. Additionally, all procedures of vitrification may
2167 have allowed the maintenance of this proliferative activity of the tissues.

2168

2169 During *in vitro* culture, it was possible to observe that tissue adhesion capacity and cell
2170 growth were not affected using any of the cryopreservation techniques. Thus, the
2171 composition of the culture medium was an auxiliary factor in the maintenance of these
2172 parameters through the supply of substances that favor both tissue adhesion and cell
2173 growth [27]. In the present study, 10% FBS was added to the culture medium, similar to
2174 that employed in domestic cat [12] and cheetah [19], providing growth factors, proteins,
2175 vitamins, trace elements and hormones, essential for the growth and maintenance of
2176 cells [31].

2177

2178 The duration of culture and the cell explants growth day were similar between SSV and
2179 SF when compared to fresh group. These cells showed an increase in metabolic activity
2180 through the MTT assay; thus, it can be deduced that the metabolic activity of the cells
2181 was not affected by SSV and SF techniques. This may have occurred because
2182 cryopreservation has the advantage of exposing the tissue to the cryoprotectant solution
2183 with minimum warming time, resulting in lowering of the cells [7,8].

2184

2185 Cell viability after the first passage was greater than 90%, independent of the
2186 cryopreservation method used, indicating a high cellular viability, corroborating with
2187 the values found by León-Quinto et al. [14], after cryopreservation of skin from Iberian

lynx (*Lynx pardinus*). The PDT values have not undergone any changes with a maximum of 26 h similar to those found for other wild felids of *Panthera* genus, such as Bengal tiger (*Panthera tigris tigris*) at 28 h [10] and Siberian Tiger (*Panthera tigris altaica*) at 24 h [15].

The cell growth curve in the fresh, SF and SSV groups demonstrated a typical "S" shape representing the stages of cell growth, the lag or latency phase, exponential phase and steady phase [34], as presented in other wild felids [10,15]. In contrast, the DVC group presented a decrease in cell concentration at 96 and 120 h changing this curve, demonstrating that the DVC technique presented a lower efficiency in maintaining cell quality parameters during *in vitro* culture, probably because of cryoprotectant toxicity after warming [8].

5. Conclusions

In summary, it can be observed that with the three techniques used, it was possible to recover the cell after *in vitro* culture of the cryopreserved tissues. Nevertheless, the SSV was the most adequate for cryopreservation of jaguar ear skin tissue, based on the results found in both the histology and *in vitro* culture analysis. This technique is more advantageous in view of SF because it is more economical, quick to perform and can be carried out in the field. Thus, the construction of a biological reserve for this endangered species through storage of skin opens innovative possibilities for the storage of genes that would be lost. Therefore, this genetic reserve can be a promising alternative for cell isolation and their application for cloning aiming to multiplication, knowledge on nuclear reprogramming mechanisms, induction to pluripotency of the cell, and obtaining gametes in jaguar, as already observed in other endangered wild felid species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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2351 **Table 1** Details of the main biological aspects from jaguars used in this study.

Animal	Estimated age (years)	Pelage	Gender	Location
J1	16	Yellow	Female	Ecopoint, Fortaleza, CE, Brazil
J2	15	Yellow	Male	Ecopoint, Fortaleza, CE, Brazil
J3	10	Yellow	Male	Zoobotanical Park Arruda Câmara, João Pessoa, PB, Brazil
J4	10	Yellow	Male	São Francisco de Canindé Zoo, Canindé, CE, Brazil
J5	7	Black	Male	Zoo Dois irmãos, Recife, PE, Brazil

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Table 2 Thickness of the epidermis and dermis, collagen matrix, and mean number of fibroblasts and perinuclear halos of the ear skin derived from jaguars after cryopreservation using different techniques.

	Thickness (µm)			**Collagen fibers (%)**	**No. of perinuclear halos**		**No. of fibroblast**	
Group	**Epidermis ± S.E.**	**Dermis ± S.E.**	**Total ± S.E.**	**Area ± S.E.**	**Mean ± S.E.**	**Range**	**Mean ± S.E.**	**Range**
Fresh	31.0 ± 3.6^{a}	256.5 ± 13.7^{a}	287.3 ± 13.7^{a}	59.0 ± 0.0^{a}	14.2 ± 3.0^{a}	3–33	20.6 ± 3.2^{a}	8–44
SF	32.3 ± 4.4^{a}	233.6 ± 16.4^{b}	265.0 ± 15.9^{b}	57.0 ± 0.0^{a}	16.3 ± 3.1^{b}	3–40	18.8 ± 3.1^{a}	6–38
DVC	35.8 ± 4.9^{b}	235.9 ± 15.0^{b}	270.5 ± 14.8^{b}	56.0 ± 0.0^{b}	15.1 ± 3.0^{a}	1–41	18.9 ± 3.4^{a}	5–41
SSV	35.4 ± 5.2^{b}	227.5 ± 13.4^{b}	261.4 ± 14.4^{b}	57.0 ± 0.1^{a}	17.6 ± 3.9^{b}	0–46	21.7 ± 4.5^{a}	1–46

SF: Slow freezing, DVC: direct vitrification in cryovials. SSV: solid-surface vitrification. S.E.: standard error. Different letters show differences statistically significant in the same column ($P < 0.05$).

2365 **Table 3** Establishment of primary cultures and subcultures of skin cells derived from jaguars after cryopreservation using different techniques.

Group	No. of samples		No. of attached samples				
	Initial	Attached (%)	Day all attached explants ± S.E.	Grow to subconfluence (%)	Day all cell grow explants ± S.E.	Subconfluence total time (days) ± S.E.	Duration of culture (days) ± S.E.
Fresh	20	20 (100)[a]	2.0 ± 0.7[a]	20 (100)[a]	9.2 ± 1.2[a]	12.0 ± 0.9[a]	29.2 ± 0.3[a]
SF	20	20 (100)[a]	1.2 ± 0.3[a]	20 (100)[a]	8.4 ± 0.3[a]	9.0 ± 0.0[a]	30.6 ± 0.7[a]
DVC	20	19 (95)[a]	1.0 ± 0.0[a]	19 (95)[a]	11.8 ± 0.9[b]	11. 8 ± 0.9[a]	33.8 ± 0.9[b]
SSV	20	19 (95)[a]	1.0 ± 0.0[a]	19 (95)[a]	8.0 ± 0.3[a]	9.0 ± 0.0[a]	30.8 ± 0.8[a]

2366 SF: Slow freezing, DVC: direct vitrification in cryovials. SSV: solid-surface vitrification. S.E.: standard error. Different letters show differences

2367 statistically significant in the same column ($P < 0.05$).

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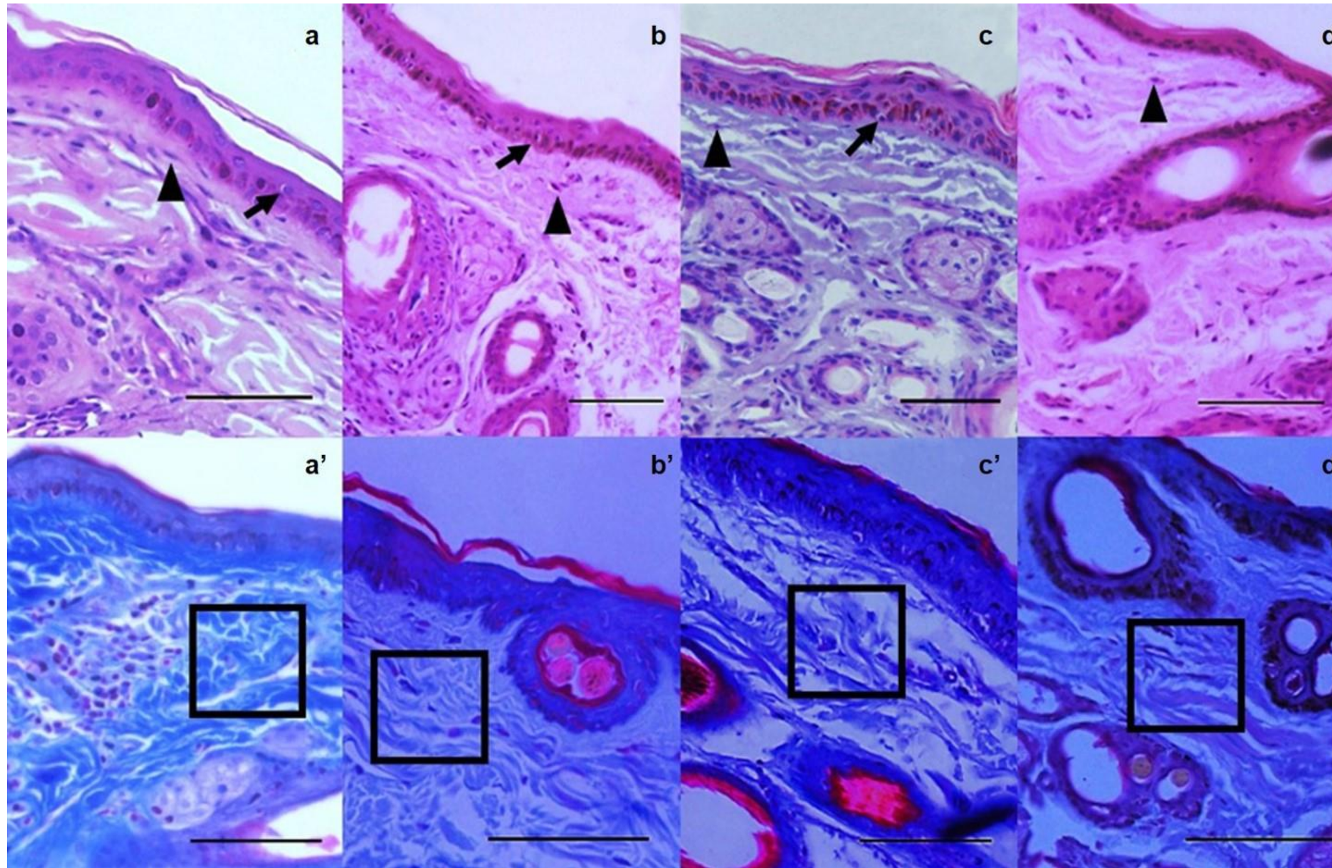
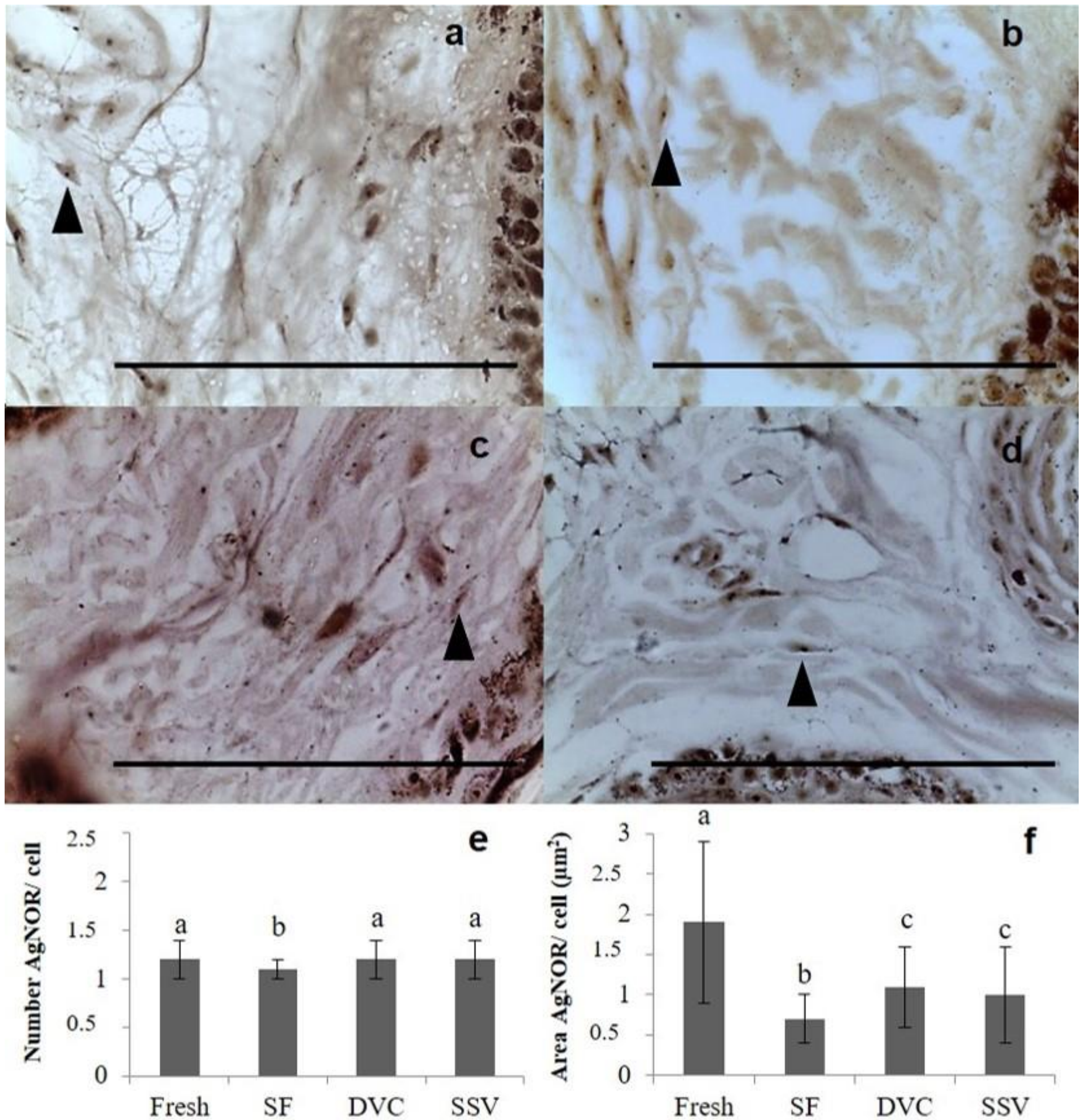


2373 **Fig. 1** Histological sections of non-cryopreserved and cryopreserved skin derived from jaguar using hematoxylin-eosin and Gomori trichrome. **a**
2374 and **a')** fresh skin (control); and cryopreserved tissues by **b** and **b')** slow freezing, **c** and **c')** direct vitrification in cryovials and **d** and **d')** solid-
2375 surface vitrification. Arrow indicates the presence of perinuclear halos in the epidermis; triangle indicates fibroblasts in the dermis. Square
2376 exemplifies dermal area of evaluation of collagen matrix. Scale bar: 50 µm. Magnification 40x.



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2378 **Fig. 2** Proliferative activity of non-cryopreserved and cryopreserved skin derived from
2379 jaguars submitted to different techniques. **a)** fresh skin (control); **b)** Slow freezing **c)**
2380 direct vitrification in cryovials, and **d)** solid-surface vitrification. **e)** Quantification of
2381 AgNOR number/cell. **f)** Quantification of AgNOR area/cell. SF: Slow freezing, DVC:
2382 direct vitrification in cryovials. SSV: solid-surface vitrification. Different letters show
2383 differences statistically significant among the groups (*P* < 0.05). Bars represent
2384 standard error. Triangle indicates AgNOR number. Scale bar: 50 µm. Magnification
2385 40x.

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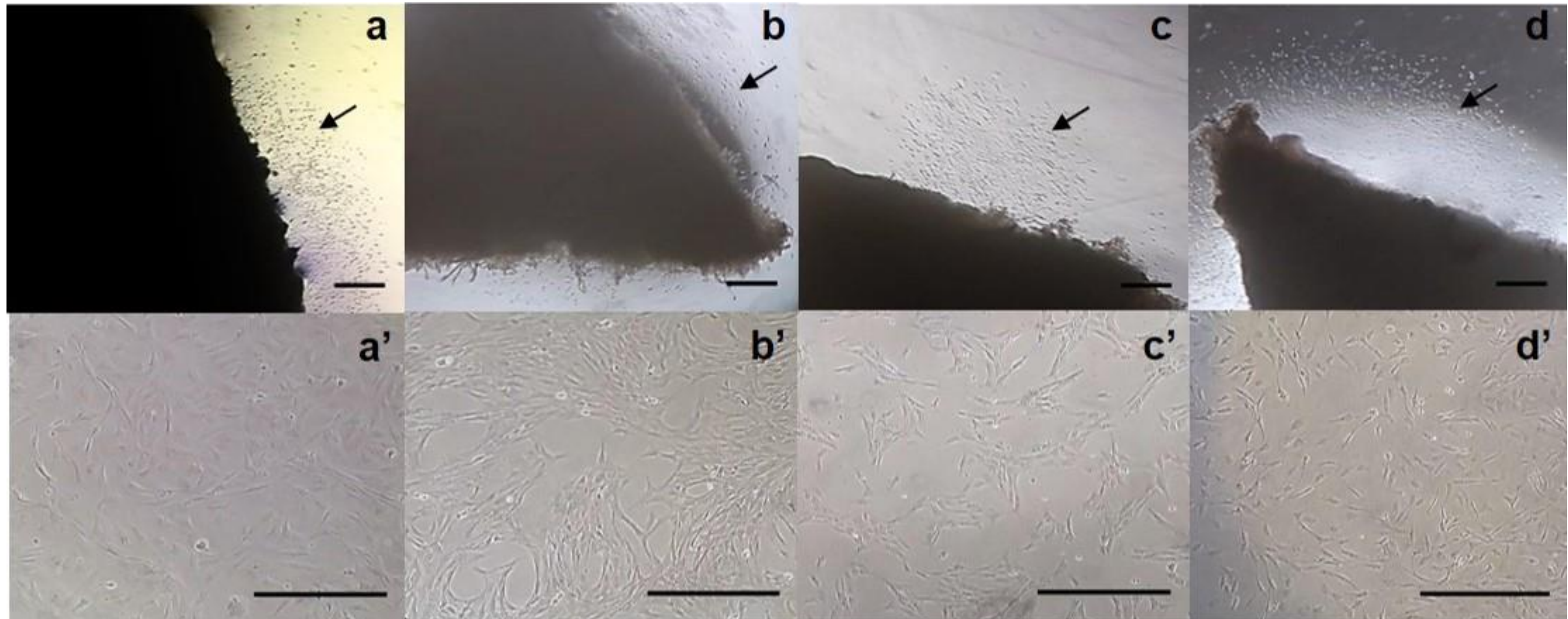

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2388 **Fig. 3** Primary and subcultures of fibroblast-like cells from ear skin samples of jaguars. **a** and **a')** Cells from non-cryopreserved tissues; and cell
2389 from cryopreserved by **b** and **b')** Slow freezing, **c** and **c')** direct vitrification in cryovials, **d** and **d')** solid-surface vitrification. Arrow indicates the
2390 beginning of cell detachment in primary cultures. Scale bar: 100 (a, b, c and d) and 50 µm (a', b', c' and d'). Magnification 4x (a, b, c and d) and
2391 10x (a', b', c' and d').

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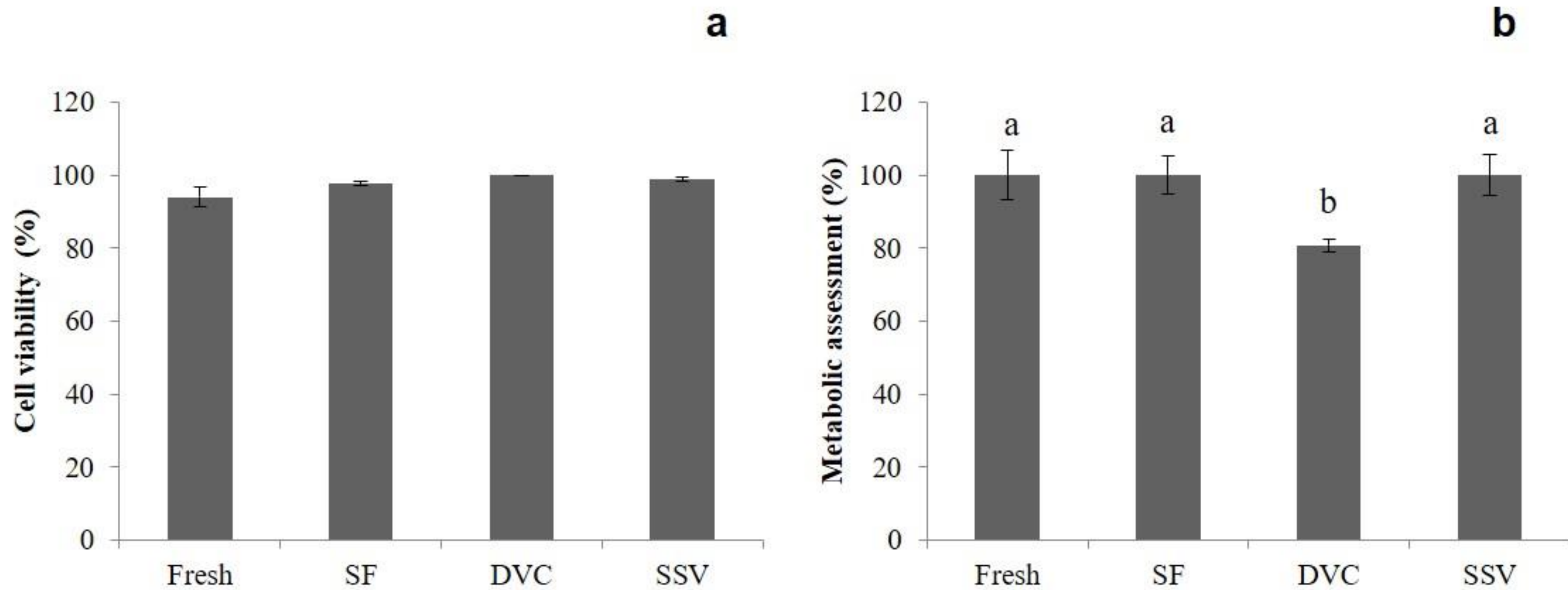

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2394 **Fig. 4** Viability and metabolic activity of the non-cryopreserved and cryopreserved skin cells of jaguars. **a)** Cell viability by trypan blue stain. **b)**
2395 Metabolic assessment by the MTT assay. SF: Slow freezing, DVC: direct vitrification in cryovials. SSV: solid-surface vitrification. Different
2396 letters show differences statistically significant among the groups ($P < 0.05$). Bars represent standard error.

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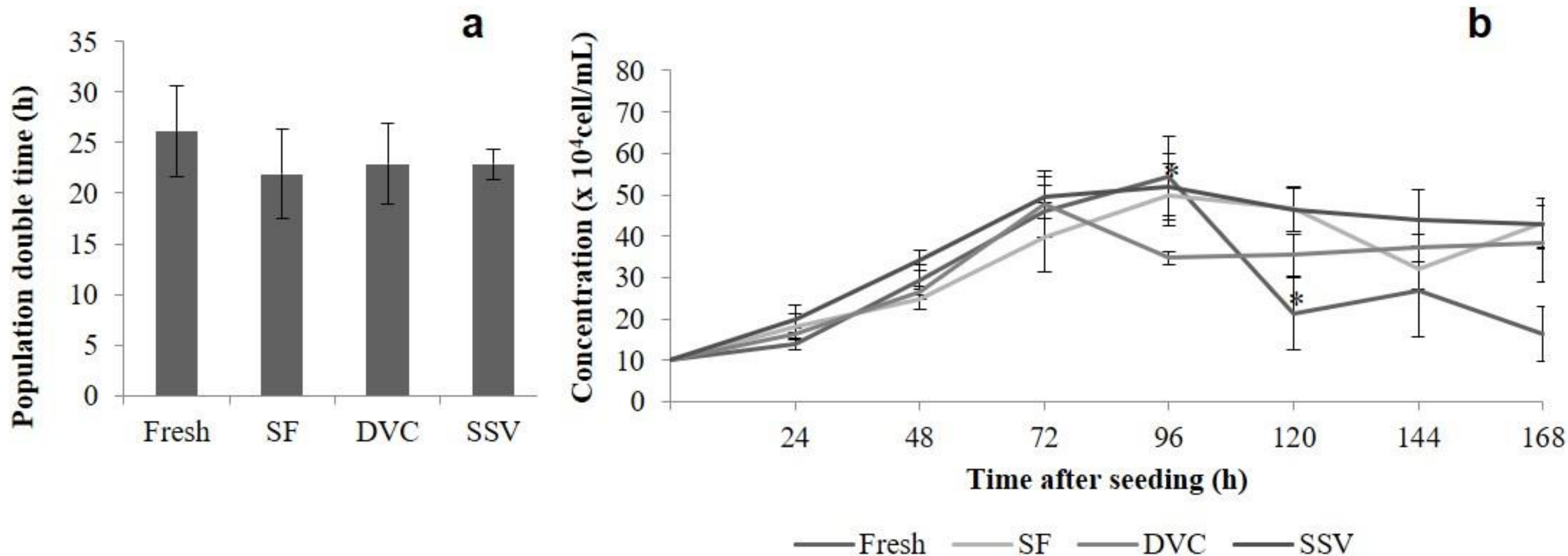

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2402 **Fig. 5** The population double time and growth curves of cells derived from of the non-cryopreserved and cryopreserved skin cells of jaguars. SF:
2403 Slow freezing, DVC: direct vitrification in cryovials. SSV: solid-surface vitrification. Each value represents mean of cells/mL in duplicate. Bars
2404 represent standard error. No difference was observed for PDT values among groups ($P > 0.05$). *: Differences statistically significant for same
2405 time with other groups ($P < 0.05$)

CONCLUSÕES GERAIS E PERSPECTIVAS

O presente trabalho descreveu pela primeira vez os parâmetros histológicos da pele do pavilhão auricular de onças de pelagem amarela e preta. Assim, a pele do pavilhão auricular de onça-pintada amarela e preta possui variações entre si principalmente em relação a espessura das camadas da pele, densidade de matriz colágena, e número de melanócitos e fibroblastos. Além disso, a camada epidermal de ambas as onças se mostrou mais espessa quando comparada a felinos domésticos, com uma espessura dermal inferior ao apresentado em outras regiões corporais de pacas e suínos. Contudo, o padrão de crescimento celular foi similar a outros felídeos silvestres, como o tigre de Bengala (*Panthera tigris tigris*) e o tigre siberiano (*Panthera tigris altaica*).

Além disso, a vitrificação em superfície sólida foi a técnica mais eficiente para a criopreservação da pele de onças-pintadas, quando comparada as técnicas de vitrificação direta em criotubos e congelação lenta, de acordo com os parâmetros histológicos e celulares. Adicionalmente, a vitrificação em superfície sólida foi mais vantajosa, quando comparada à congelação lenta, especialmente no que se refere à praticidade, rapidez e baixo custo de realização, sendo, portanto, interessante para uso a campo.

Finalmente, esses resultados irão contribuir para a formação de bancos de recursos somáticos desta espécie, direcionando a criopreservação adequada de amostras somáticas para aplicações em medicina regenerativa e tecnologias de reprodução assistida. Portanto, esse trabalho compreendeu a primeira etapa, visando o uso dessas amostras para as diferentes finalidades, desde os estudos voltados para a multiplicação de indivíduos a produção de células pluripotentes e obtenção de gametas a partir dessas células.

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2452 # ANEXOS

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ANEXO – A: COMPROVANTE DE SUBMISSÃO DO ARTIGO: QUANTITATIVE AND DESCRIPTIVE HISTOLOGICAL ASPECTS OF JAGUAR (*PANTHERA ONCA* LINNAEUS, 1758) EAR SKIN AS A TOOL FOR CONSERVATION À REVISTA ANATOMIA, HISTOLOGIA, EMBRYOLOGIA 19 – JAN – 2019

Submission Confirmation

Thank you for your submission

Submitted to	Anatomia, Histologia, Embryologia
Manuscript ID	AHE-01-19-OA-017
Title	Quantitative and descriptive histological aspects of jaguar (Panthera onca Linnaeus, 1758) ear skin as a tool for conservation
Authors	Praxedes, Érika Queiroz Neta, Luiza Borges, Alana Silva, Maria Bárbara Santos, Maria Valeria Ribeiro, Leandro Silva, Herlon Victor Pereira, Alexsandra
Date Submitted	19-Jan-2019

2484 ANEXO – B: COMPROVANTE DE SUBMISSÃO DO ARTIGO: EFFECTS OF
2485 CRYOPRESERVATION TECHNIQUES ON THE PRESERVATION OF EAR SKIN – AN
2486 ALTERNATIVE APPROACH TO CONSERVATION OF JAGUAR, *Panthera onca*
2487 (LINNAEUS, 1758) À CRYOBIOGY 19 – JAN – 2019

Ref: CRYO_2019_26
Title: Effects of cryopreservation techniques on the preservation of ear skin – An alternative approach to conservation of jaguar, Panthera onca (Linnaeus, 1758)
Journal: Cryobiology

Dear Dr. Fernandes Pereira,

Thank you for submitting your manuscript for consideration for publication in Cryobiology. Your submission was received in good order.

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Kind regards,

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APÊNDICES

2535 **APÊNDICE A**: Resumo científico apresentado no Encontro de Biotecnologia do Nordeste –
2536 RENORBIO, realizado em Natal, RN, de 8 a 11 de agosto de 2017.

2537 **CARACTERIZAÇÃO HISTOLÓGICA DE TECIDO SOMÁTICO DE ONÇA-**
2538 **PINTADA (*Panthera onca*): RESULTADOS PRELIMINARES**

2539 Érika Almeida Praxedes[1]; Luiza Bento de Queiroz Neta[1]; Maria Bárbara Silva[1]; Francilane
2540 Nascimento Costa[1]; Cibelle Anne dos Santos Costa[1]; Alana Azevedo Borges[1]; Maria Valéria
2541 de Oliveira Santos[1]; Herlon Victor Rodrigues Silva[2]; Leandro Rodrigues Ribeiro[2]; Alexandre
2542 Rodrigues Silva[1]; Moacir Franco de Oliveira[1]; Alexsandra Fernandes Pereira[1]

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2545 O conhecimento sobre o perfil histológico de mamíferos silvestres, principalmente àqueles em
2546 risco de extinção, é fundamental para o desenvolvimento de estratégias biotecnológicas de
2547 conservação. Nesse sentido, o uso do tecido somático como fonte biológica tem sido
2548 proposto, especialmente para a onça-pintada. Essa espécie, por requerer ambientes saudáveis
2549 e com abundância de presas, é útil como indicadora de qualidade ambiental em diferentes
2550 áreas rurais. Portanto, o objetivo do presente trabalho foi caracterizar a região tegumentar
2551 auricular periférica da onça-pintada. Para tanto, biópsias de pele da região auricular periférica
2552 foram recuperadas a partir de dois machos com idade de 10 e 15 anos, anestesiados com 0,08
2553 mg/kg de cloridrato de dexmedetomidina e provenientes de zoológicos localizados no
2554 Nordeste do Brasil. As amostras foram transportadas por 3 a 5 h em meio essencial mínimo
2555 modificado por Dulbecco (DMEM) suplementado com 2,2 g/L de bicarbonato de sódio, 10%
2556 de soro fetal bovino e 2% de solução de antibiótico-antimicótico, a 4°C. No laboratório,
2557 fragmentos (9,0 mm³) foram fixados em paraformaldeído tamponado em solução fosfato,
2558 desidratados por etanol e diafanizados em xilol. Em seguida, os mesmos foram inclusos em
2559 parafina, seccionados em cortes de 5,0 µm e corados com hematoxilina-eosina para
2560 quantificação de halos e fibroblastos, bem como a mensuração da proporção volumétrica da
2561 derme e epiderme. Todos os dados foram expressos como média ± desvio padrão. Assim,
2562 tamanhos de 9,1 ± 2,7 µm e 63,7 ± 5,1 µm foram observados para epiderme e derme, com
2563 uma proporção volumétrica de 12,6% ± 3,9 e 87,3% ± 3,9, respectivamente. Além disso, na
2564 epiderme foram obtidos valores de 10,5 ± 4,4 para halos perinucleares e a derme apresentou
2565 15,1 ± 4,3 de fibroblastos. Adicionalmente, foi observada uma fina espessura da epiderme
2566 apresentando em maior parte apenas uma camada de células. Em toda a derme foi identificada
2567 a presença de glândulas sebáceas, sudoríparas e folículos pilosos, sendo a localização das
2568 glândulas sebáceas tanto laterais quanto inferiores aos folículos pilosos. Em conclusão, de
2569 maneira preliminar, o sistema tegumentar auricular periférico de onça-pintada apresentou uma
2570 epiderme notoriamente delgada e derme com número reduzido de fibroblastos presentes.
2571 Essas informações auxiliarão no estabelecimento de protocolos de criopreservação tecidual,
2572 visando à aplicação em biotecnologias avançadas de conservação.
2573

2574 **Suporte financeiro:** CNPq e CAPES.
2575 **Palavras-chave:** Conservação animal, felinos silvestres, histologia clássica.

2576 **APÊNDICE B**: Resumo científico apresentado no Annual Meeting of the Brazilian Embryo
2577 Technology Society (SBTE), realizado Cabo de Santo Agostinho, PE, de 17 a 19 de agosto de
2578 2017.

2579 **ISOLATION AND *IN VITRO* CULTURE OF SOMATIC CELLS DERIVED FROM**
2580 **JAGUAR (*Panthera onca*) EAR TISSUE**

2581 E.A. Praxedes[1]; L.B. Queiroz Neta[1]; H.V.R. Silva[2]; L.R. Ribeiro[2]; A.A. Borges[1]; M.B. Silva[1];
2582 M.V.O. Santos[1]; A.R. Silva[1]; A.F. Pereira[1]*

2583 [1]Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró-RN, Brasil. [2]Universidade
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2585 The establishment of *in vitro* culture of cells derived from skin fragments has been proposed
2586 as a useful technique for the conservation of endangered species, especially when associated
2587 with nuclear transfer (cloning). Among the species vulnerable to extinction, the jaguar
2588 (Panthera onca) requires strategies for the conservation of its genetic diversity. Therefore, the
2589 aim of the present work was to describe the *in vitro* culture of somatic cells derived from *P.*
2590 *onca* skin, using morphological analysis, trypan blue cell viability assay and metabolic
2591 activity by the 3-(4,5- Dimethylthiazol-2yl) -2,5-diphenyl tetrizoline bromide) or MTT. Thus,
2592 skin biopsy derived from peripheral ear region were recovered using surgical scissors from
2593 two males with age of 10 and 15 years, anesthetized and from zoos located in northeastern
2594 region of Brazil. Samples were transported for 3 to 5 h in minimal essential medium modified
2595 by Dulbecco (DMEM) supplemented with 2.2 g/L sodium bicarbonate, 10% fetal bovine
2596 serum and 2% penicillin and streptomycin solution, pH 7.4 at 4°C. In the laboratory,
2597 fragments (9.0 mm^3) were cultured under controlled atmosphere (38.5°C, 5% CO2) and
2598 evaluated every 24 h. After the cells reached 70% confluency, the first cell subculture was
2599 performed to obtain the desired concentration (5.0 x 104 cells/mL) for the MTT assay.
2600 Additionally, a pool of trypsinized cells was evaluated for viability with trypan blue. All data
2601 were expressed as mean ± standard deviation. Thus, the total culture time was 29 days for the
2602 samples derived from both individuals and from the eight fragments submitted to the culture
2603 (four fragments per animal) all presented adherence on the first day with cell growth around
2604 the explants from the ninth day (9.0 ± 2.8). Cells reached 70% confluence forming monolayer
2605 on day 12.5 ± 2.1. In general, from the morphological analysis, all cells had fusiform
2606 morphology with a centralized oval nucleus, showing to be similar to fibroblasts. As for the
2607 trypan blue test, a viability of 99.6% ± 0.6 was obtained. In the MTT assay, the percentage of
2608 100% ± 14.9 was obtained in D5 and in the D7 100% ± 18.1 of metabolic activity. In
2609 conclusion, skin biopsy derived from jaguar peripheral ear region allowed the isolation of
2610 viable cells similar to fibroblasts and with high metabolic functionality, providing a valuable
2611 source for the somatic cell nuclear transfer, aiming at the genetic conservation of this species.
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APÊNDICE C: Resumo científico apresentado no Congresso Norte e Nordeste de Reprodução Animal (CONERA), realizado em Belém, PA, de 10 a 12 de setembro de 2018.

Conservação de células somáticas derivadas de onça-pintada (*Panthera onca* Linnaeus, 1758) usando diferentes combinações de crioprotetores

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A onça-pintada, terceiro maior felino do mundo e o maior do continente americano, é um exemplar de mamífero carnívoro de elevada importância ecológica e econômica. No Brasil, esta espécie encontra-se classificada como vulnerável à extinção e estratégias de criopreservação representam ferramentas interessantes para a conservação de seu material genético. Dentre as estratégias aplicáveis, tem-se a criopreservação de células somáticas, a qual o seu sucesso depende da escolha adequada dos crioprotetores. Portanto, o objetivo foi avaliar a eficiência de diferentes combinações de crioprotetores sobre a conservação de células somáticas de onça-pintada. Para tanto, tecidos recuperados (1–2 cm^2) de biópsias de pele da região auricular periférica foram coletados de cinco onças-pintadas anestesiadas e mantidas em zoológicos da região nordeste do Brasil. Após a colheita, fragmentos (9,0 mm^3) foram cultivados em meio essencial mínimo modificado por Dulbecco (DMEM) suplementado com 10% de soro fetal bovino (SFB) e 2% de solução de antibióticos (38,5°C, 5% de CO_2) e avaliados a cada 24 h. Após as células atingirem 70% de confluência, células foram subcultivadas para obtenção da concentração desejada (1,0 x 10^5 células/mL). Para a criopreservação, células foram submetidas à congelação lenta em meio contendo DMEM acrescido de 10% de SFB e o crioprotetor, de acordo com os grupos: 10% de dimetilsulfóxido [DMSO], 10% de DMSO e 0,25 M de sacarose [DMSO-SAC], 10% de etilenoglicol [EG], e 10% de EG e 0,25 M de sacarose [EG-SAC]. Após duas semanas, amostras foram descongeladas e submetidas à análise de viabilidade usando o ensaio de azul de tripan imediatamente após a descongelação e após sete dias de cultivo. Células não submetidas à criopreservação e cultivadas foram consideradas como grupo controle. Todos os dados foram expressos como média ± erro padrão e comparações foram realizadas usando ANOVA seguido de teste de Tukey ($P < 0,05$). Após cinco repetições (um animal/uma repetição), uma taxa de viabilidade celular de 97,8% ± 2,7 foi obtida antes da congelação lenta. Após análise com azul de tripan imediatamente após a descongelação, células derivadas do grupo DMSO (53,7% ± 24,1), DMSO-SAC (58,6% ± 38,2), e EG-SAC (52,5% ± 36,6) apresentaram taxas de viabilidade similares ao controle ($P > 0,05$). Além disso, células congeladas em EG tiveram a viabilidade reduzida logo após a descongelação (45,8% ± 31,6). Sete dias de cultivo após a descongelação, todas as células apresentaram similares taxas de viabilidade (DMSO: 95,7% ± 3,3; DMSO-SAC: 98,6% ± 0,7, EG: 95,2% ± 8,5; EG-SAC: 96,5% ± 2,6), as quais foram superiores as taxas observadas imediatamente após a descongelação. Assim, a adição da sacarose à solução de criopreservação melhorou as taxas de viabilidade celular, sendo os valores acentuando quando em associação com EG. Isso ocorreu provavelmente devido a influência positiva da sacarose como crioprotetor extracelular de controle do equilíbrio osmótico durante os ciclos da criopreservação. Em conclusão, tanto o DMSO quanto sua associação com sacarose e o EG em combinação com a sacarose podem ser empregados na congelação de células somáticas derivadas de onça-pintada. Adicionalmente, o cultivo *in vitro* melhorou a viabilidade de células somáticas descongeladas

2660 **APÊNDICE D:** Resumo científico apresentado no Seminário de Iniciação Científica da
2661 UFERSA (SEMIC), realizado em Mossoró, RN, de 27 a 29 de novembro de 2018.

2662 **Viabilidade de células somáticas de onça-pintada (*Panthera onca* Linnaeus, 1758) após**
2663 **criopreservação usando diferentes crioprotetores intracelulares**

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2666 O estabelecimento de bancos de células somáticas a partir de fragmentos de pele tem sido
2667 proposto como uma ferramenta interessante para a conservação de espécies ameaçadas de
2668 extinção, como a onça-pintada. Nesse sentido, faz-se necessário estabelecer os protocolos de
2669 criopreservação de células somáticas, como a escolha dos crioprotetores, visando à formação
2670 adequada dos criobancos. Portanto, o objetivo foi avaliar a viabilidade de células somáticas
2671 derivadas de onças-pintadas após a criopreservação usando diferentes crioprotetores
2672 intracelulares. Para tanto, biópsias de pele da região auricular periférica foram recuperadas de
2673 quatro machos e uma fêmea, anestesiados e provenientes de zoológicos localizados nos
2674 estados do Ceará, Paraíba e Pernambuco. Todos os procedimentos foram aprovados pelo
2675 Comitê de Ética de Uso de Animais (CEUA/UFERSA, no. 23091.0011507/2017-61) e
2676 Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, no. 57460-1). Após a
2677 colheita dos fragmentos de pele, amostras foram transportadas por 3 a 7 h em meio essencial
2678 mínimo modificado por Dulbecco (DMEM) suplementado com 10% de soro fetal bovino
2679 (SFB) e 2% de solução de antibióticos e antimicóticos, a 4°C. No laboratório, fragmentos (9,0
2680 mm^3) foram cultivados em condições e atmosfera controladas (38,5°C, 5% de CO_2) e
2681 avaliados a cada 24 h quanto ao desprendimento de células. Após as células atingirem 70% de
2682 confluência, foi realizado o primeiro subcultivo celular para obtenção da concentração
2683 desejada ($1,0 \times 10^5$ células/mL). Para a criopreservação, células foram submetidas à
2684 congelação lenta usando o sistema Mr. Frosty (taxa de resfriamento de 1°C min^{-1}) em freezer
2685 -80ºC, e em meio contendo DMEM acrescido de 10% de SFB e o crioprotetor intracelular, de
2686 acordo com os grupos: i) 10% de dimetilsulfóxido (DMSO), ii) 10% de etilenoglicol (EG) e
2687 iii) associação de 10% de DMSO e 10% de EG. Após duas semanas, amostras foram
2688 descongeladas a 35°C, lavadas e centrifugadas para remoção dos crioprotetores.
2689 Posteriormente, as células foram submetidas ao cultivo *in vitro* nas mesmas condições
2690 anteriormente citadas e analisadas quanto à viabilidade usando o ensaio de azul de tripan,
2691 antes, imediatamente após a descongelação e após sete dias de cultivo. Todos os dados foram
2692 expressos como média ± erro padrão e analisados por ANOVA e Tukey ($P < 0,05$). Após
2693 cinco repetições (1 animal/1 repetição), uma taxa média de viabilidade celular de 97,8% ± 2,7
2694 foi obtida antes da congelação. Após análise com azul de tripan imediatamente após a
2695 descongelação, células derivadas do grupo DMSO (53,7% ± 24,1) e DMSO-EG (71,2% ±
2696 18,3) apresentaram maiores taxas de viabilidade quando comparadas às células do grupo EG
2697 (45,8% ± 31,6). Além disso, após sete dias de cultivo, todas as células descongeladas
2698 apresentaram similares taxas de viabilidade DMSO: 95,7% ± 3,3; EG: 95,2% ± 8,5; DMSO-
2699 EG: 95,5% ± 4,5), as quais foram superiores as taxas observadas imediatamente após a
2700 descongelação. Em conclusão, tanto o DMSO quanto sua associação com EG podem ser
2701 empregados na criopreservação de células somáticas derivadas de onça-pintada.
2702 Adicionalmente, o cultivo por sete dias melhorou a viabilidade de células somáticas após a
2703 descongelação.
2704 **Palavras-chave:** Ensaio de exclusão. Bancos somáticos. Congelação lenta. Felídeos
2705 silvestres.