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GABRIELA PEREIRA DE OLIVEIRA LIRA

# CRIOPRESERVAÇÃO DE TECIDO SOMÁTICO E OBTENÇÃO DE LINHAGENS FIBROBLÁSTICAS DERIVADAS DO PAVILHÃO AURICULAR DE ONÇA-PARDA, *Puma concolor* (LINNAEUS, 1771)

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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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GABRIELA PEREIRA DE OLIVEIRA LIRA

# CARACTERIZAÇÃO CELULAR E AVALIAÇÃO DOS DANOS CAUSADOS PELA CRIOPRESERVAÇÃO EM AMOSTRAS SOMÁTICAS DERIVADAS DO PAVILHÃO AURICULAR DE ONÇA-PARDA, *Puma concolor* (LINNAEUS, 1771)

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## DADOS CURRICULARES DA AUTORA

**GABRIELA PEREIRA DE OLIVEIRA LIRA** – Nascida em Mossoró, RN, no dia 7 de março de 1996, mulher, mãe, filha de Neide Pereira da Silva e Efraim de Oliveira Lira. Graduou-se em Biotecnologia pela Universidade Federal Rural do Semi-Árido (UFERSA). Fez a sua trajetória como discente de iniciação científica e estagiou no Laboratório de Biotecnologia Animal (LBA/UFERSA), participando de Programas de Iniciação Científica (Programa Institucional de Bolsas de Iniciação Científica, 2016–2017 e 2018–2019), aprendendo e participando em diferentes experimentos relacionados à produção *in vitro* de embriões, criopreservação, histologia e cultivo *in vitro* de células somáticas em mamíferos silvestres, sob a orientação da Profa. Dra. Alexsandra Fernandes Pereira. Em março de 2019, iniciou as atividades de mestrado pelo Programa de Pós-Graduação em Ciência Animal (PPGCA/UFERSA), recebendo bolsa de auxílio financeiro pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e desenvolvendo seus experimentos no LBA/UFERSA, com muito orgulho.

.

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*Dedico.*

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*“Juntando novas pedras*

*E construindo novos poemas.*

*Recria tua vida, sempre, sempre, sempre.*

*Remove pedras e planta roseiras. Recomeça”*

(Cora Coralina)

**CRIOPRESERVAÇÃO DE TECIDO SOMÁTICO E OBTENÇÃO DE LINHAGENS FIBROBLÁSTICAS DERIVADAS DO PAVILHÃO AURICULAR DE ONÇA-PARDA, *Puma concolor* (LINNAEUS, 1771)**

LIRA, Gabriela Pereira de Oliveira. CRIOPRESERVAÇÃO DE TECIDO SOMÁTICO E OBTENÇÃO DE LINHAGENS FIBROBLÁSTICAS DERIVADAS DO PAVILHÃO AURICULAR DE ONÇA-PARDA, *Puma concolor* (LINNAEUS, 1771). 2021. Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2021.

**RESUMO:** Os bancos de recursos somáticos desempenham um papel crucial na conservação da diversidade genética, permitindo a conservação de amostras biológicas de diferentes populações. As células somáticas de onça-parda podem ser recuperadas desses bancos e usadas em técnicas assistidas para promover sua multiplicação e conservação. Em resposta à redução da população desta espécie de importância ecológica, o presente trabalho teve como objetivos avaliar os danos causados pela criopreservação na formação de bancos de tecidos somáticos (**Etapa 1**), bem como caracterizar linhagens celulares após cultivo prolongado e criopreservação (**Etapa 2**). Para tanto, fragmentos do pavilhão auricular derivados de quatro onças-pardas mantidas em zoológicos da cidade de Fortaleza, Ceará, foram distribuídos em duas etapas. Na primeira etapa, fragmentos criopreservados e não criopreservados foram avaliados quanto à espessura da pele, cartilagem, número de células, número de halos perinucleares, 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, atividade metabólica, estresse oxidativo e níveis de apoptose. Na segunda etapa, células caracterizadas como fibroblastos por imunocitoquímica, foram avaliadas quanto ao período de cultivo (primeira, terceira e décima passagem) e efeitos da criopreservação sobre morfologia, ultraestrutura, viabilidade, atividade proliferativa, metabolismo, níveis de espécies reativas de oxigênio (EROs), potencial de membrana mitocondrial ($\Delta\Psi m$) e níveis de apoptose. Na primeira etapa, a criopreservação aumentou a espessura da camada córnea nos tecidos, o número de halos perinucleares e lacunas vazias. Apesar disso, a criopreservação foi capaz de manter os padrões normais de fibroblastos, mesmo apresentando aumento no percentual de fibras colágenas. Além disso, a criopreservação manteve o potencial proliferativo dos tecidos e dos parâmetros avaliados durante o cultivo *in vitro*, principalmente quanto à viabilidade, atividade proliferativa e níveis de apoptose. Contudo, células de tecidos criopreservados apresentaram diminuição do metabolismo e do $\Delta\Psi m$. Na segunda etapa, as células mostraram uma morfologia fusiforme típica com núcleos ovais localizados centralmente. As células foram identificadas comofibroblastos por marcação com vimentina. O cultivo *in vitro* após a primeira, terceira e décimapassagem não alterou a maioria dos parâmetros avaliados. As células na terceira e décima passagem mostraram uma redução nos níveis de EROs ($P < 0,05$). A ultraestrutura revelou dano morfológico nos prolongamentos e núcleos de células derivadas da terceira e décima passagem. Além disso, a criopreservação resultou em uma redução no $\Delta\Psi m$ em comparação com as células não criopreservadas. Em conclusão, tecidos somáticos de onça-parda submetidos à criopreservação são viáveis e mantêm a integridade do tecido, apresentando alterações mínimas após o aquecimento. Adicionalmente, fibroblastos viáveis podem serobtidos de tecidos somáticos do pavilhão auricular de onça-parda, com pequenas alterações após a décima passagem de cultivo *in vitro* e criopreservação.

**Palavras-chave:** Biodiversidade, criobancos, felídeos silvestres, fibroblastos.

**CRYOPRESERVATION OF SOMATIC TISSUE AND OBTAINING FIBROBLASTIC LINES DERIVED FROM THE PAVILION AURICULAR PAVILION, *Puma concolor* (LINNAEUS, 1771)**

LIRA, Gabriela Pereira de Oliveira. CRYOPRESERVATION OF SOMATIC TISSUE AND OBTAINING FIBROBLASTIC LINES DERIVED FROM THE PAVILION AURICULAR PAVILION, *Puma concolor* (LINNAEUS, 1771). 2021.Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2021.

**ABSTRACT:** Somatic resource banks play a crucial role in the conservation of genetic diversity, allowing the conservation of biological samples from different populations. Puma somatic cells can be recovered from these banks and used in assisted techniques to promote their multiplication and conservation. In response to the reduction in the population of this species of ecological importance, the present study aimed to evaluate the damages caused by cryopreservation in the formation of somatic tissue banks (**Step 1**), as well as to characterize cell lines after prolonged culture and cryopreservation (**Step 2**). For this purpose, fragments of the ear derived from four pumas kept in zoos in the city of Fortaleza, Ceará, were distributed in two stages. In the first stage, cryopreserved and non-cryopreserved fragments were evaluated for skin and cartilage thickness, number of cells, number of perinuclear halos, percentage of collagen matrix, and tissue proliferative activity. Moreover, cells resulting from the cultured fragments were evaluated for morphology, adherence, confluence, viability, proliferative activity, metabolic activity, oxidative stress, and levels of apoptosis. In thesecond stage, cells characterized as fibroblasts by immunocytochemistry were evaluated for the culture period (first, third and tenth passage) and effects of cryopreservation on morphology, ultrastructure, viability, proliferative activity, metabolism, levels of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi m$) and levels of apoptosis. In the first stage, cryopreservation increased the thickness of the corneum layer in the tissues andthe number of perinuclear halos and empty gaps. Despite this, cryopreservation was able to maintain normal fibroblast patterns, even with an increase in the percentage of collagen fibers. Additionally, cryopreservation maintained the proliferative potential of the tissues and parameters evaluated during *in vitro* culture, mainly regarding viability, proliferative activity, and levels of apoptosis. Nevertheless, cells of cryopreserved tissues showed decreased metabolism and $\Delta\Psi m$. In the second stage, the cells showed a typical spindle-shaped morphology with centrally located oval nuclei. The cells were identified as fibroblasts by staining with vimentin. *In vitro* culture after the first, third and tenth pass did not change most of the evaluated parameters. The cells in the third and tenth pass showed a reduction in ROS levels ($P < 0.05$). The ultrastructure revealed morphological damage in the extensions and nuclei of cells derived from the third and tenth passages. Also, cryopreservation resulted in a reduction in $\Delta\Psi m$ compared to non-cryopreserved cells. In conclusion, puma somatic tissues submitted to cryopreservation are viable and maintain the integrity of the tissue, with minimal changes after warming. Additionally, viable fibroblasts can be obtained from somatic tissues of the puma ear, with minor changes after the tenth passage of *in vitro* culture and cryopreservation.

**Keywords:** Biodiversity, cryobanks, wild felids, fibroblasts

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## LISTA DE SÍMBOLOS E SIGLAS

| | |
|---|---|
| ± | Mais ou menos |
| < | Menor |
| > | Menor |
| °C | Graus celsius |
| % | Percentual |
| AgNOR | Região organizadora nucleolar marcada com sais de prata |
| BK | Basal layer |
| $cm^2$ | Centímetro quadrado |
| CL | Congelação lenta |
| CL | Corneum Layer |
| CENAP | Centro Nacional de Pesquisa e Conservação de Mamíferos Carnívoros |
| CEUA | Comitê de Ética no Uso de Animais |
| DNA | cido desoxirribonucleico |
| CS | Cryopreservation solution |
| $CO_2$ | Dióxido de carbono |
| DE | Dermis |
| DMSO | Dimetilsulfóxido |
| DMEM | Dulbecco Modification of Minimum Essential Medium |
| EP | Epidermis |
| FBS | Fetal Bovine Serum |
| GT | Gomory Trichrome |
| h | Hora |
| HE | Hematoxilina-eosina |
| ICMBio | Instituto Chico Mendes de Biodiversidade |
| iPS | Induced Pluripotent Stem Cells |
| IUCN | International Union for Conservation of Nature |
| M | Molar |
| µm | Micrômetro |

| | |
|---|---|
| $\mu m^2$ | Micrômetro quadrado |
| mL | Mililitro |
| $mm^3$ | 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 |
| NORs | Regiões Organizadoras Nucleolares |
| PBS | Solução tampão fosfato |
| SL | Spinosum Layer |
| SUC | Sucrose |
| TNCS | Transferência Nuclear de Célula Somática |
| U/mL | Unidade por mililitro |
| Vs. | Versus |
| VSS | Vitrificação em superfície sólida (SSV: solid-surface vitrification) |
| EROs | Espécies reativas de oxigênio |
| ROS | Oxigen-reactive species |

**SUMÁRIO**

# CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

## 1. INTRODUÇÃO

Uma alta biodiversidade indica no ecossistema a presença de grandes predadores, os quais desempenham importante papel no ecossistema global (MARCHINI et al., 2011). Dentre esses grandes predadores, a onça-parda está inserida como sendo um dos carnívoros de maior importância para os ecossistemas em que habitam. Embora sua população seja considerada mundialmente como menos preocupante (CASO et al., 2015; NIELSEN et al., 2015), uma redução crescente do seu quantitativo populacional tem sido observada, sendo verificada exemplares de onça-parda escassos em determinadas regiões e extintas em outras (AZEVEDO et al., 2013). Entre as razões que justificam essa vulnerabilidade populacional tem-se a redução na disponibilidade de hábitats em virtude do crescimento urbano desordenado (GUSTAFSON et al., 2019). Além disso, o aumento das atividades antrópicas e a diminuição de suas presas, associado as suas populações naturalmente pouco numerosas e de baixa reprodução, proporcionam o seu declínio quantitativo (GUERISOLI et al., 2019).

Diante desse cenário, estratégias de conservação têm sido propostas por meio de ações englobadas no Plano de Ação Nacional para a Conservação da onça-parda (PAN Onça-Parda; Portaria no. 316/2009) elaborado pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). Em 2018, esse plano foi inserido em uma ação maior denominada Plano de Ação para a Conservação dos Grandes Felinos (PAN Grandes Felinos: Portaria no. 612/2018). Contudo, ainda são reduzidos o número de estudos quanto à conservação de onça-parda. Assim, entre as recomendações desse plano de ação, tem-se o desenvolvimento de biotecnologias assistidas, como a formação de bancos de recursos biológicos e biotécnicas reprodutivas, como a inseminação artificial e a produção de embriões.

Recentemente, os bancos de recursos somáticos vêm ganhando cada vez mais importância e têm sido implementados para vários grupos de espécies, visando resguardar genótipos raros e permitindo a exploração do potencial de amostras biológicas (GOLACHOWSKI et al., 2018). Embora bancos de recursos somáticos tenham sido observados em alguns felídeos silvestres em vulnerabilidade, tais como o lince-Ibérico (*Lynx pardinus*, LEÓN-QUINTO et al., 2009) e a onça-pintada (*Panthera onca*, PRAXEDES et al., 2019), não há relatos sobre a formação desses bancos em onça-parda.

Em geral, a formação de bancos de recursos somáticos envolve tanto a conservação de tecidos somáticos (PRAXEDES et al., 2019), quanto de células somáticas (OLIVEIRA et al., 2021) e vem sendo amplamente difundidos em parcerias com zoológicos. Em ambas as situações (conservação do tecido e da célula), as condições essenciais que determinam o sucesso desses bancos consistem na origem das amostras, conhecimento da morfologia da região a ser coletada, os métodos de criopreservação e a caracterização das células em cultivo e após a criopreservação (PEREIRA et al., 2018).

Inicialmente, como fonte de tecidos somáticos, a região do pavilhão auricular tem sido a região de escolha, uma vez que permite uma recuperação menos invasiva no indivíduo e possibilita a colheita de tecidos com abundância de fibroblastos (PRAXEDES et al., 2018). Para tecidos, a vitrificação tem sido empregada como uma técnica promissora na conservação de amostras somáticas, sendo a vitrificação em superfície sólida um método rápido e com bons resultados para a manutenção das características histológicas e celulares (PRAXEDES et al., 2019). Já para a criopreservação de células somáticas, a congelação lenta tem sido a técnica empregada, como já observado em onça-pintada (*P. onca*, OLIVEIRA et al., 2021). Após a conservação dos tecidos somáticos, análises relacionadas à manutenção das características teciduais, bem como a recuperação de células viáveis e seu desempenho após período de cultivo prolongado e criopreservação são passos essenciais na adequação dos bancos de recursos biológicos (PEREIRA et al., 2018).

Portanto, conhecer os efeitos da criopreservação sob as amostras (tecidos e células), bem como identificar as características biológicas e garantir a obtenção de linhagens celulares que garantam o posterior uso dessas células e dos bancos de amostras somáticas (YELISETTI et al., 2017), representa uma etapa a mais no desenvolvimento de estratégias de conservação a partir do conhecimento da biologia felina e melhoria da eficiência de biotecnologias que visam manter e reforçar populações viáveis de felídeos silvestres.

## 2. FUNDAMENTAÇÃO TEÓRICA

### 2.1. IMPORTÂNCIA DA ONÇA-PARDA PARA O ECOSSISTEMA GLOBAL

A onça-parda (**Figura 1**), assim como outros felídeos silvestres, é considerada uma "espécie-bandeira" ou espécie "guarda-chuva" por estar no topo da pirâmide alimentar (DOBROVOLSKI et al., 2013).


**Figura 1.** Exemplares de onça-parda utilizados no presente estudo. **a)** Onça-parda de vida livre. **b)** Onça-parda mantida em zoológico.

Popularmente conhecida como puma, suçuarana, onça-vermelha, onça-vermelha-do-lombo-preto, leão baio, onça-parda ou leão-da-montanha, a *Puma concolor* é a segunda maior espécie de felídeo no Brasil e espécie de mamífero silvestre mais amplamente distribuída do hemisfério ocidental (**Figura 2**). Baseado em diferenças moleculares, são aceitas seis subespécies em toda a área de distribuição (CULVER et al., 2000), em contraste com as 32 já propostas (YOUNG; GOLDMAN, 1946, JACKSON, 1955; CABRERA, 1963).

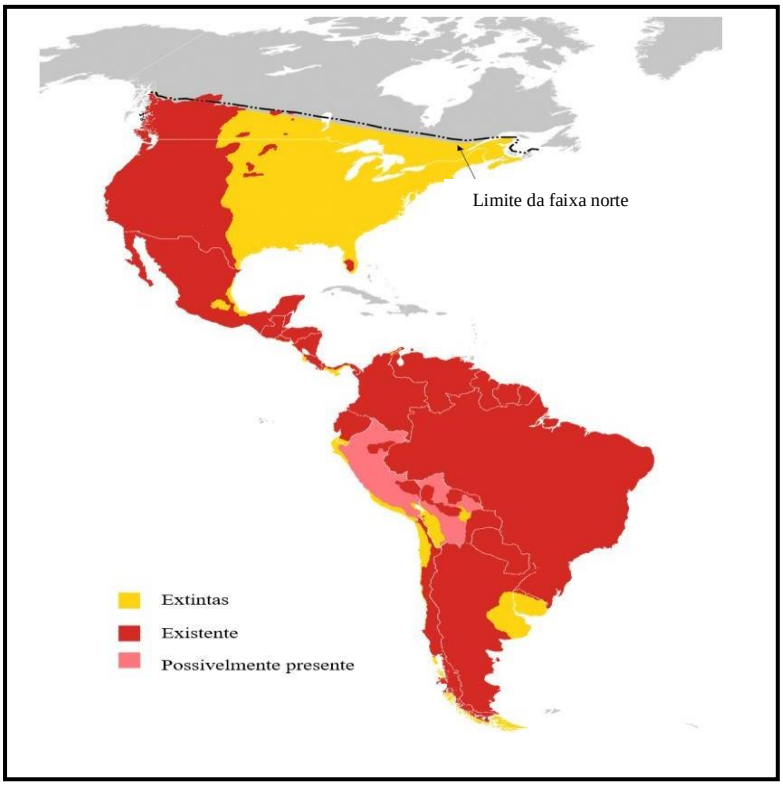


**Figura 2.** Distribuição geográfica de onça-parda. Fonte: IUCN – Lista Vermelha de Espécies Ameaçadas (2021) com modificações.

Em geral, a onça-parda possui hábitos solitários e terrestres, apresentando predomínio de suas atividades no período noturno (AZEVEDO et al., 2020). Seu habitat é variado, incluindo florestas tropicais e subtropicais. A onça-parda é considerada um predador generalista, uma vez que consome uma grande variedade de presas conforme a disponibilidade das mesmas no ambiente (LOGAN; SWEANOR, 2001). Devido à ocorrência de presas de grande porte, na América do Norte, onças-pardas são capazes de matar presas com peso entre 70 e 125 kg, como alces, veados-de-cauda-branca e cabras montanhesas (LOGAN; SWEANOR, 2001, MURPHY; RUTH, 2010). Já as subpopulações que habitam as regiões tropicais ingerem presas de 15 kg em média (POLISAR et al., 2003), como pacas, tatus, quatis, aves e répteis (POLISAR et al., 2003, MARTINS et al., 2008), podendo também ingerir vertebrados de maior porte, como veados, porcos-do-mato, capivaras e jacarés (POLISAR et al., 2003; SIMÁ et al., 2020).

Além disso, as onças-pardas necessitam de grandes áreas, geralmente maiores do que 100 km² e, quando em forrageamento, podem caminhar, em média, 9 km por noite

(PENTEADO et al., 2012). Ainda, são animais que se dispersam por longas distâncias, até mesmo na presença de fragmentação de seu habitat (PENTEADO et al., 2012).

Sua grande importância ecológica está relacionada ao controle que exercem na abundância, distribuição e diversidade das populações e de suas presas, influenciando a dinâmica do ecossistema em que vivem, tanto por predação quanto por mudança no comportamento dessas presas que procuram diferentes habitats, fontes de alimentação ou mudam seus horários de atividade (DEL RIO et al., 2001). Portanto, na ausência desses predadores, todo ecossistema, como os mamíferos, herbívoros, roedores, aves, répteis e insetos, tende a se desequilibrar sendo que populações de algumas espécies podem crescer exponencialmente.

Adicionalmente, a capacidade de dispersão e rápida expansão de distribuição das onças-pardas facilita o fluxo gênico e reduz a subestruturação genética, sendo que sua presença pode ser considerada um indicador da integridade e do potencial de recuperação de um ambiente (GUERISOLI et al., 2019). A capacidade de dispersão e distanciamento do território de nascimento até o local onde o indivíduo estabelece seu território de adulto e se reproduz, é responsável por uma parte do recrutamento de uma população, permitindo que as onças-pardas expandam sua distribuição e recolonizem áreas onde a população foi extinta (LARUE; NIELSEN, 2008). Dessa forma, a presença dessa espécie nos ambientes em que habitam é de central interesse da biologia de conservação e a falta dessa espécie nos ambientes em que habitam podem acarretar grandes prejuízos para o ecossistema global.

## 2.2. AVALIAÇÃO DO RISCO DE EXTINÇÃO DA ONÇA-PARDA

A onça-parda, apesar de ser uma das espécies de mamíferos terrestres mais bem distribuída (GELIN et al., 2017), é tida como uma espécie pouco comum ou rara em diversas regiões (LAUNDRÉ; HERNÁNDEZ, 2007). Elbroch et al. (2018) revelaram populações com sinais de recuperação, principalmente nos habitats onde a caça de onças-pardas é proibida por lei e intensos programas de manejo da espécie têm sido desenvolvidos.

Nas avaliações globais publicadas na Lista Vermelha de Espécies Ameaçadas, elaborada pela União Internacional para a Conservação da Natureza (IUCN), a onça-parda foi considerada Menos Preocupante (LC) na avaliação realizada em 1996, Quase Ameaçada (NT) em 2002, e consta agora na categoria Menos Preocupante (LC, CASO et al., 2008). Contudo, sua população está em declínio (NIELSEN, 2018). Além disso, a subespécie *Puma concolor*

*couguar*, a qual já foi endêmica nos Estados Unidos, em 2011, foi oficialmente declarada como extinta.

No Brasil, a onça-parda possui distribuição ampla, ocorrendo em todos os biomas; contudo, segundo Azevedo et al. (2013) estima-se que em três gerações, ou 21 anos, poderá ocorrer um declínio de mais de 10% da subpopulação nacional. Em alguns estados brasileiros, a onça-parda consta nas listas de espécies ameaçadas, sendo classificada como vulnerável (VU, BIODIVERSITAS, 2012) e como Em Perigo (EN, FANFA et al., 2011) em outros estados (**Figura 3**).

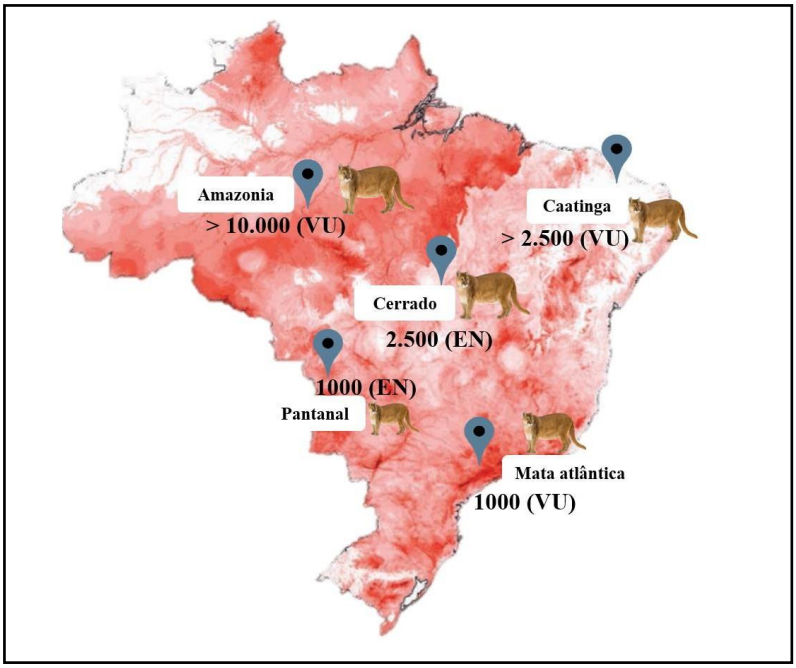


**Figura 3.** Cenário da conservação de onça-parda no Brasil segundo o ICMBio,evidenciando a distribuição remanescentes nos biomas brasileiros e enfatizandoem vermelho as áreas mais favoráveis a ocorrência de onça-parda. Fonte: Azevedo et al. (2013); ICMBio (2020) com modificações.

O histórico de conservação de onças-pardas deve-se, principalmente, a uma das maiores ameaças à sobrevivência dos felídeos silvestres que é a perda de habitats em virtude da expansão urbana, da matriz agropecuária e do conflito com humanos (GUERISOLE et al., 2019). No Brasil, a supressão, fragmentação de habitats, a retaliação por predação de animais domésticos (tanto o abate "preventivo" de onças-pardas quanto o abate após o evento de predação), e os atropelamentos parecem ser as principais causas de perda de indivíduos de onça-parda (AZEVEDO et al., 2013; MONTER et al., 2021). Essas ameaças são recorrentes

em todos os biomas brasileiros em que a onça-parda habita (AZEVEDO et al., 2013). Além disso, há um agravamento em regiões onde a caça esportiva é permitida e ocorre queimadas com frequência, como nos biomas Pantanal. Ainda, as queimadas em fazendas produtoras de cana-de-açúcar na Mata Atlântica e Cerrado e a expansão da matriz energética eólica na Caatinga são causas dessa redução populacional de onça-parda (MAZZOLLI et al., 2000; AZEVEDO et al., 2013).

O avanço agropecuário, principalmente de monoculturas, vem representando uma ameaça às populações de onças-pardas, uma vez que tais empreendimentos, em sua grande maioria, são fonte de retirada maciça e fragmentação de habitats e promove mudanças permanentes na composição dos habitats das regiões, contribuindo para um processo de mudança com inúmeros impactos (AZEVEDO et al., 2013; VALDIVIEZO et a., 2020). Tais fatores expõem espécies, como a onça-parda, a um alto risco, e como consequência disso, tem sido crescente a invasão de espécies de felídeos em zonas urbanas (VALENTI et al., 2019; FERNÁNDEZ et al., 2020).

Finalmente, a caça retaliatória acaba sendo uma das maiores ameaças às populações de onças-pardas em toda sua área de distribuição, devido ao prejuízo financeiro gerado. No Brasil, inúmeros registros de ataques às criações domésticas são reportados (MAZZOLLI et al., 2002; PAVIOLO et al., 2009; PEREIRA et al., 2020; GUERISOLI et al., 2021). Onças-pardas são especialmente vulneráveis porque naturalmente voltam nas carcaças de suas presas abatidas, e estas podem estar envenenadas ou servirem como iscas para caçadores que matam este predador na espera ou usando cachorros especializados em acuá-las em árvores, onde se tornam alvos fáceis (MURPHY; MACDONALD, 2010).

A conservação da onça-parda por ser um predador de grande porte, é considerada um desafio frente ao fato de já ter sido eliminada de grandes áreas de sua distribuição original (CASO et al., 2008). Muitas informações ainda precisam ser levantadas para dar suporte ao manejo desta espécie que, dentre os carnívoros silvestres brasileiros, é um dos que mais se envolve em conflitos diretos com seres humanos (GUERISOLI et al., 2020).

## 2.3. ESTRATÉGIAS *IN SITU* E *EX SITU* DE CONSERVAÇÃO APLICADAS EM ONÇA-PARDA

Diferentes estratégias têm sido adotadas visando à manutenção da fauna existente (ANDRABI; MAXWELL, 2007). Estas podem ser realizadas de maneira *in situ*,

quando são desenvolvidas ferramentas que auxiliam na sobrevivência dos animais na natureza (SANDERSON et al., 2002), bem como de maneira *ex situ*, a qual é subdividida em *in vivo* ou *in vitro*. Assim, enquanto o *in vivo* consiste no transporte de animais para reservas ecológicas ou zoológicos (ARAUJO et al., 2019; GOROSABEL et al., 2020; PEREIRA et al., 2020), o *in vitro* se caracteriza pelo transporte e armazenamento de amostras biológicas na forma de criobancos ou para pesquisas em laboratório (MESTRE-CITRINOVITZ et al., 2016).

Quando pensamos em estratégias de conservação *in situ*, o monitoramento de populações é uma das estratégias iniciais para se entender as correlações adaptativas do genótipo com o ambiente (BABB, 2020; PEREIRA et al., 2020). No decorrer dos últimos anos, em onças-pardas, as estratégias de conservação que visam a manutenção de populações no ecossistema de modo *in situ* vem sendo desenvolvida por meio da proteção de habitats e do manejo das populações na natureza, visando à garantia mínima de variabilidade genética, demográfica e ecológica (PEREIRA et al., 2020).

Assim, com esse intuito, estudos têm buscado esclarecer a densidade populacional e distribuição geográfica de onças-pardas nos diferentes biomas (ASTETE et a., 2017), bem como conhecer os padrões de comportamento e alimentação da espécie em seu habitat (BISCHOFF-MATTSON, 2019). Dessa forma, para realizações de tais avaliações ferramentas não invasivas são empregadas, utilizando armadilhas fotográficas, as quais permite que indivíduos que possuem marcas naturais, parâmetros como dimorfismo sexual, intensidade de coloração, cicatrizes e outros aspectos possam ser identificados (KAUTZ et al., 2008; KELLY et al., 2008).

Além disso, analisar a variabilidade genética faz-se importante para predição dos níveis de familiaridade entre as populações remanescentes (SRBEK-ARAUJO et al., 2018). Para tanto, existem diversos tipos de marcadores moleculares que são empregados na genética da conservação, como aqueles relacionados a íntros do cromossomo Y (CULVER et al., 2000), análise de paisagem, a qual permite inferir o grau em que a paisagem pode afetar o movimento dos organismos (LA RUE; NIELSEN, 2008) e ainda por meio de análises genéticas não invasivas de amostras fecais (PALOMARES et al., 2017).

No Brasil, em cada bioma que essa espécie habita e em decorrência da sua vulnerabilidade, existem alguns projetos e planos que visam a conservação da espécie de forma *in vivo* como o Projeto Onça-Parda do Triângulo Mineiro, realizado pelo Programa de Conservação Mamíferos do Cerrado, que vem levantando informações sobre a ecologia espacial e saúde de onças-pardas em ambientes antropizados desde 2009. Além da pesquisa, o

projeto desenvolve atividades voltadas para a minimização dos conflitos entre os produtores rurais e carnívoros.

O Projeto Puma desenvolve trabalhos com a espécie no bioma Mata Atlântica sobre dieta, área de vida e comportamento. O Projeto Leão Baio, que desenvolve projetos de pesquisa e conservação de carnívoros silvestres nos Aparados da Serra Geral, vem quantificando conflitos entre predadores e seres humanos. O Projeto Onças-pardas, iniciado em 2012, tem como objetivos obter registros da espécie, estudar a movimentação de onças-pardas entre fragmentos de Mata Atlântica e Cerrado, além do uso de habitats, área de vida e ecologia trófica. No bioma da Caatinga, o Projeto Programa Amigos da Onça desenvolvido em 2018, tem como objetivo promover a conservação da onça-pintada e onça-parda, com base nos conhecimentos da ecologia e biologia destas espécies e, ainda, promover a redução dos conflitos entre homens e onças.

Mais recentemente, as organizações governamentais inseriram as ações englobadas no Plano de Ação Nacional para a Conservação da onça-parda (PAN Onça-Parda; Portaria no. 316/2009) elaborado pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) em uma ação maior denominada Plano de Ação para a Conservação dos Grandes Felinos (PAN Grandes Felinos: Portaria no. 612/2018). Entre as recomendações desse plano de ação, tem-se o desenvolvimento técnicas de conservação tanto *in situ* quanto *ex situ in vivo* ou *in vitro* de formas individualizadas ou em conjunto, por meio de biotecnologias assistidas, como a formação de bancos de recursos biológicos e biotécnicas reprodutivas, como a inseminação artificial e a produção de embriões utilizando animais mantidos em zoólogicos.

As estratégias realizadas de maneira *ex situ in vivo* ocorrem por meio do transporte desses animais para reservas, as quais, por serem pequenas áreas protegidas por lei, desempenham um papel fundamental na conservação de grandes espécies como as onças-pardas (BABB, 2020). Outra forma de conservação *ex situ in vivo* são os zoológicos (ARAUJO et al., 2019), que tem objetivo de conservar a espécie com métodos de reprodução, de formar um banco genético visando pesquisas futuras, e de manter a diversidade genética (ARAUJO et al., 2019). Nesses ambientes, os animais são importantes, pois possibilitam a realização de estudos para compreensão de mecanismos fisiológicos e reprodutivos (MIJAHUANCA et al., 2017). Além disso, auxiliam na manutenção de espécies ameaçadas de extinção, com objetivos de reprodução, aumentando as populações e possibilita que aumente o conhecimento acumulado sobre essas espécies. Isso é básico para possibilitar que

se criem programas de reintrodução de animais na natureza, nascidos em cativeiro (HERRING et al., 2020).

Contudo, tanto as estratégias *in situ*, quanto as estratégias *ex situ in vivo* possuem algumas barreiras no seu desenvolvimento. Para conservação *in situ* são requeridos extensos territórios e monitoramento destes, bem como conscientização e contribuição da população (CARVALHO, 2011). Além disso, em decorrência da captura de onças-pardas de vida livre ser de difícil realização, resultados mais eficientes poderiam ser obtidos, conhecendo melhor a biologia dos animais e aspectos reprodutivos das onças-pardas em cativeiro, favorecendo o estabelecimento de métodos *ex situ in vitro*, como os criobancos, que podem ser empregados em conjunto com as estratégias *in situ*e *ex situ in vivo* citadas anteriormente (PEREIRA et al., 2020).

Os criobancos, por meio da criopreservação de amostras biológicas, como gametas, embriões, células e tecidos somáticos, podem garantir a conservação do material genético de espécies (PRAXEDES et al., 2018). Esses bancos podem favorecer a aplicação de outras biotecnologias, como a inseminação artificial, fecundação *in vitro*, produção *in vivo* de embriões; a clonagem por transferência nuclear de célula somática (TNCS) e a produção de células pluripotentes (iPS).

Mundialmente, em onça-parda, algumas técnicas de reprodução assistida (TRAs) já foram empregadas. Inicialmente, em 1990, Miller et al. recuperaram 106 oócitos imaturos de sete fêmeas, maturaram esses oócitos *in vitro* (43,8%) e após a co-incubação com espermatozoides de onça-parda e gato doméstico obtiveram 40% e 26,5% de oócitos fecundados, respectivamente. Posteriormente, em 1994, uma cria saudável foi produzida por inseminação artificial por laparoscopia (BARONE et al., 1994). A partir de 2013, estudos relacionados à criopreservação de sêmen (DECO-SOUZA et al., 2013), incluindo às características morfométricas (CUCHO et al., 2016) e a capacidade fertilizante dos espermatozoides (DUQUE et al., 2017) foram realizados.

Em 2020, Pereira et al. descreveram anatomicamente as glândulas salivares de onça-parda, após morte por atropelamento, visando gerar informações que podem ser utilizadas como ferramenta de estratégias de conservação, tratamentos clínicos e conservação dessa espécie. Diante desses aspectos, é possível perceber que poucos estudos foram desenvolvidos quanto à conservação de onça-parda. Nesse contexto, uma ferramenta ainda a ser desenvolvida nesta espécie consiste no estabelecimento bancos de recursos somáticos.

## 2.4. FORMAÇÃO DE BANCOS DE RECURSOS SOMÁTICOS EM FELÍDEOS SILVESTRES

Os bancos de recursos biológicos, ou biobancos ou criobancos, são definidos como repositórios de material biológico coletado, processado e armazenado em condições adequadas (LEÓN-QUINTO et al., 2009). Seu uso tem sido constantemente proposto uma vez que permite a conservação de material de qualquer espécie, agindo na conservação da diversidade genética atual das populações (COMIZZOLI et al., 2000). Inicialmente, a conservação da biodiversidade era mantida por biobancos de gametas e embriões (ANDRABI; MAWXELL, 2007). Contudo, com a clonagem por TNCS (GOMEZ et al., 2003), e as limitações relacionadas às dificuldades de colheita e processamento dos gametas e embriões, a formação de bancos de tecidos e células somáticas tornou-se uma escolha interessante para os programas de conservação.

Assim, bancos de recursos somáticos têm sido considerados vantajosos, especialmente quando derivados da pele (PRAXEDES et al., 2018), pois possuem como vantagens a possibilidade de recuperação de amostras em ambos os gêneros (THONGPHAKDEE et al., 2010; WITTAYARAT et al., 2013), em fetos ou animais adultos (KITIYANANT et al., 2003; HASHEM et al., 2007) vivos ou após a morte (VERMA et al., 2012; MORO et al., 2015) e a partir de diferentes regiões e tecidos, possibilitando a obtenção de uma ampla amostragem biológica (MESTRE-CITRINOVITZ et al., 2016). Além disso, a pele é um órgão rico em diferentes tipos celulares de diferentes tecidos, podendo 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 TNCS, produção de iPS e obtenção de gametas (PRAXEDES et al., 2018).

Nesse cenário, uma série de felídeos silvestres teve seu material biológico armazenado na forma de bancos de recursos somáticos para atender a todas essas finalidades (**Quadro 1**). No Brasil, há um número bem escasso de bancos de amostras somáticas de felídeos silvestres, conforme monitoramento apresentado pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) – Recursos Genéticos e Biotecnologia. Segundo a EMBRAPA, há mais bancos de recursos somáticos de espécies domésticas ligadas principalmente a produção pecuária. Desde 2010, a Fundação Jardim Zoológico de Brasília (FJZB) juntamente com a EMBRAPA – Cerrados, mantém um Banco de Germoplasma, em um programa de reprodução para a conservação, onde estão armazenadas células somáticas de onça-pintada, jaguarundi

(*Herpailurus yagouaroundi*), gato do mato (*Leopardus geoffroyi*), leão (*Panthera leo*), jaguatirica (*Leopardus pardalis*) e tigre (*Panthera tigris*). Adicionalmente, o número de universidades públicas que mantém material biológico de mamíferos silvestres, especialmente felídeos vem crescendo no país (SILVA et al., 2012; PRAXEDES et al., 2019).

Contudo, apesar de crescente a formação de bancos de amostras somáticas de felídeos silvestres, sabe-se que estudos mais aprofundados envolvendo o desenvolvimento de protocolos de criopreservação dos materiais biológicos, visando à conservação das espécies, são necessários, já que atualmente se conhece pouco a respeito da utilização de biotécnicas em felídeos silvestres, em especial da onça-parda.

**Quadro 1.** Bancos de amostras somáticas aplicados na conservação de felídeos silvestres.

| **Espécie** | **Localização mundial** | **Nível de ameaça** | **Amostra criopreservada** | **Condições do cultivo *in vitro*** | **Condições de criopreservação** | **Autores** |
|---|---|---|---|---|---|---|
| Gato selvagem africano (***Felis silvestris libica***) | África, Ásia, Europa | Ameaçado de extinção | Célula | DMEM suplementado com 10% de SFB e antibióticos a 38 °C, 5% de $CO_2$ por um período de 7-10 dias de cultivo primário. | 10% de DMSO e 10% SFB | Gómez et al. (2003, 2004) |
| Tigre siberiano (***Panthera tigres***) | Ásia | Ameaçado de extinção | Célula | DMEM suplementado com 10% de SFB a 37 °C, 5% de $CO_2$ por um período de 6-8 dias de cultivo primário. | 10% de DMSO | Song et al. (2007) |
| Lince-ibérico (***Lynx pardinus***) | Europa | Ameaçado de extinção | Tecidos e células | DMEM suplementado com 15% de SFB, 1.000U/ml de fator inibidor de leucemia (LIF) e antibióticos a 37 °C, 5% de $CO_2$. | NI | León-Quinto et al. (2009) |
| Lince-ibérico (***Lynx pardinus***) | Europa | Ameaçado de extinção | Tecidos e células | DMEM suplementado com 10%, 15% ou 20% de SFB, 5-10 ng/ml de fator de crescimento epidermal (EGF), 5-10 ng/ml de fator de crescimento fibroblástico (FGF) e antibióticos a 37 °C, 5% de $CO_2$. | 5-10% de DMSO e 0,1-0,2 M de sacarose | León-Quinto et al. (2011) |
| Leopardo (***Panthera uncia***) | Ásia central | Ameaçado de extinção | Células | DMEM suplementado com 10% de SFB e antibióticos a 38,5 °C, 6% de $CO_2$ por um período de 7 dias de cultivo primário. | 10% DMSO e 90% de SFB | Verma et al. (2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Gato marmoreado (***Pardofelis marmorata***) | Sudeste asiático | Quase ameaçado | Células | DMEM suplementado com 20% de SFB e antibióticos a 37 °C, 5% de $CO_2$. | NI | Wittayarat et al. (2013) |
| **Guepardo (*Ancinonyx jabutus*)** | África e sudoeste asiático | Vulnerável | Células | DMEM suplementado com 10% de SFB e antibióticos a 39 °C, 5% de $CO_2$. | 10% DMSO e 10% de SFB | Moro et al. (2015) |
| Onça-pintada (***Panthera onca***) | América | Quase ameaçado | Tecidos e células | DMEM suplementado com 10% de SFB e antibióticos a 37 °C, 5% de $CO_2$ por um período de 10-14 dias de cultivo primário. | 10% de DMSO | Mestre-Citrinovitz et al. (2016) |
| Guepardo asiático (***Ancinonyx jabutus vanticus***) | África e sudoeste asiático | Vulnerável | Células | DMEM suplementado com 10% de SFB e antibióticos a 38 °C, 5% de $CO_2$. | 10% de DMSO e 50% de SFB | Moulavi et al. (2017) |
| Onça-pintada (***Panthera onca***) | América | Quase ameaçado | Tecidos | DMEM suplementado com 10% de SFB e antibióticos a 38,5 °C, 5% de $CO_2$. | 1,5 M de DMSO, 0,25 M de sacarose e 10% de SFB | Praxedes et al. (2019) |

## 2.5. ESTABELECIMENTO DE BANCOS DE TECIDOS SOMÁTICOS EM FELÍDEOS SILVESTRES

A obtenção de um banco de tecidos somáticos depende inicialmente do conhecimento prévio dos componentes e estruturas histológicas da região que estar sendo coletada, além do estabelecimento das condições adequadas de criopreservação tecidual (PEREIRA et al., 2018). Em geral, a pele, órgão mais empregado para a formação desses bancos, possui uma composição e estrutura que variam entre espécies e entre regiões de colheita (HOSSAIN et al., 2016). Na maioria dos estudos em felídeos silvestres, a colheita da pele tem sido em indivíduos vivos anestesiados, e a região tem sido o pavilhão auricular apical (MESTRE-CITRINOVITZ et al., 2016, PRAXEDES et al., 2019). Além disso, o conhecimento da região da pele a ser recuperada torna-se essencial para a definição dos processamentos para a execução da criopreservação (BORGES et al., 2018).

Em felídeos silvestres, Praxedes et al. (2019) avaliaram a estrutura, composição e capacidade de cultivo de pele do pavilhão auricular apical de onça-pintada de pelagem amarela e preta, utilizando métodos qualitativos e quantitativos, com enfoque na espessura da pele, quantificação e distribuição celular, densidade do colágeno, atividade proliferativa e viabilidade. Santos et al. (2021), visando estabelecer as regiões da pele mais adequadas para a conservação do tecido e aumentar a eficiência dos criobancos e o armazenamento de amostras biológicas, avaliaram os efeitos da criopreservação dos tecidos cutâneos das regiões do pavilhão auricular, caudal e femoral de uma onça-pintada *post-mortem* pertencente a um zoológico no Brasil, observando que as regiões do pavilhão auricular e caudal foram as mais adequadas para a conservação dos tecidos somáticos derivados da onça-pintada. Além disso, um maior número de fibroblastos foi encontrado na pele do pavilhão auricular em comparação com outras regiões da pele (Santos et al., 2021).

Após a colheita, amostras de pele são higienizadas e devem ser transportadas ao local do manuseio do material biológico em um menor intervalo de tempo possível (PEREIRA et al., 2018). Posteriormente, as amostras são lavadas em meio de cultivo suplementado com antibióticos, tampões e fontes de proteína, e fragmentados em tamanhos de 1,0 – 9,0 $mm^3$ (WITTAYARAT et al., 2013; PRAXEDES et al., 2019). Após essas etapas, tecidos são criopreservados, usando as técnicas de congelação lenta ou vitrificação, as quais variam na quantidade de crioprotetores e taxas de resfriamentos empregados (GURRUCHAGA et al., 2019).

Dois exemplos de estabelecimento de bancos de tecidos somáticos em felídeos podem aqui ser destacados, os quais León-Quinto et al. (2009) armazenaram tecidos somáticos de diferentes regiões do corpo (músculo, mucosa oral, medula óssea, medula espinal, intestinos) de 69 linces-ibérico (*L. pardinus*), e Mestre-Citrinovitz et al. (2016) que armazenaram tecidos somáticos da pele de onças-pintadas, descrevendo os aspectos envolvidos no estabelecimento de bancos somáticos para conservação da espécie. Assim, os autores descreveram a obtenção de amostras de pele, cartilagem e músculo, abordando condições de transporte, processamento e criopreservação. Assim, a partir do protocolo descrito foi possível armazenar em um biobanco do Zoológico de Buenos Aires amostras de 45 diferentes espécies ameaçadas.

Adicionalmente, podemos citar outros bancos de tecidos somáticas em espécies de felídeos silvestres desenvolvidos para a conservação do gato marmorado (*Pardofelis marmorata*) e do gato-de-cabeça-chata (*Prionailurus planiceps*) na Tailândia (THONGPHAKDEE et al., 2010).

### *2.5.1 Técnicas de criopreservação e eficiência final*

Para o estabelecimento adequado dos bancos de tecidos somáticos a escolha da técnica de criopreservação que promova menores danos ao tecido devem ser estabelecida para a espécie em questão. A congelação lenta ocorre pela redução lenta da temperatura, diminuindo o estresse térmico da solução no estágio de solidificação, e reduzindo a formação de cristais de gelo. Nessa técnica, usam-se concentrações de crioprotetores intracelulares, como o dimetilsulfóxido (DMSO) e o etilenoglicol (EG), variando entre 1,0 a 1,5 M e taxas de resfriamento de -1 °C/min (MAGALHÃES et al., 2017). Já a vitrificação, promove a mudança brusca de temperatura, formando um estado vítreo e minimizando a formação de cristais de gelo. Contudo, necessita de uma quantidade de crioprotetor intracelular mais elevada que o processo anterior para a obtenção de alta viscosidade. Nessa técnica, também utiliza crioprotetores intracelulares, como DMSO e EG, com concentração variando entre 3,0 e 6,0 M (BORGES et al., 2017). Adicionalmente, a vitrificação resulta em uma queda de temperatura >10.000ºC/min (CARVALHO et al., 2012).

Em ambas as técnicas, têm sido observadas a importância do uso de crioprotetores intracelulares, em combinação com crioprotetores extracelulares, e estes podem ser divididos em dois grupos: dissacarídeos, como a sacarose, e proteínas, como o soro fetal bovino (SFB,

LIRA et al., 2020). Esses crioprotetores extracelulares promovem a desidratação celular porque não penetram nas membranas e colaboram para aumentar a osmolaridade, minimizando as possíveis injúrias decorrente da formação de cristais de gelo intra e extracelular (LEÓN-QUINTO et al., 2014). Por tanto, o equilíbrio entre o tipo de crioprotetor escolhido, em combinação ou sozinho, intracelular adicionado de extracelular, e a combinação ideal das concentrações para que não ofereçam danos as amostras biológicas são fatores determinantes para o sucesso da criopreservação e, consequentemente, da formação de bancos de amostras somáticas (LEÓN-QUINTO et al., 2014).

Nesse sentido, León-Quinto et al. (2011) testaram diferentes concentrações de crioprotetores (5%, 7,5%, 10%, 12,5% e 15% de DMSO) em combinação com duas concentrações de sacarose (0,1 M e 0,2 M) e verificaram que a melhor combinação para a criopreservação de tecido somático do lince-ibérico, seria o DMSO a 10% com 0,2 M de sacarose. Mestre-Citrinovitz et al. (2016) relataram o uso da congelação lenta na criopreservação da pele de onças-pintadas, no entanto, atualmente tem se observado para tecidos somáticos de pele o uso da vitrificação na criopreservação como sendo mais eficiente do que a congelação lenta (LEÓN-QUINTO et al., 2009), resultando no emprego da vitrificação para a conservação de tecidos somáticos em diferentes espécies (BORGES et al., 2018; PRAXEDES et al., 2018).

Em 2019, Praxedes et al. demonstraram um efeito positivo da vitrificação em superfície sólida (VSS) na manutenção das características teciduais e celulares da pele, quando comparada a vitrificação direta em criotubos (VDC) e congelação lenta. Os autores observaram que entre os efeitos positivos da VSS estavam a praticicidade, o baixo custo para execução, menor formação de cristais de gelo e a menor exposição do material biológico a solução de criopreservação, evitando toxicidade de crioprotetores intracelulares e seus metabólitos. Assim, os crioobancos pode ser realizada para criopreservação de amostras de animais que estão em zoológicos (PRAXEDES et al., 2019), desde que sejam adequadamente estabelecidos quanto à identificação dos danos gerados após a criopreservação.

## 2.6. ESTABELECIMENTO DE BANCOS DE CÉLULAS SOMÁTICAS EM FELÍDEOS SILVESTRES

Independente da formação de bancos de tecidos somáticos, os bancos de células são essenciais, pois funcionam como matéria-prima de uso imediato nas biotecnologias aplicadas

(PEREIRA et al., 2018). Para tanto, células são inicialmente caracterizadas quanto aos seus requerimentos nutritivos e demais condições ambientais de cultivo (SANTOS et al., 2015).

Além disso, a criopreservação de células somáticas e a qualidade dessas células após a descongelação consiste numa etapa importante para a otimização da conservação da espécie (LEÓN-QUINTO et al., 2011). Em geral, na criopreservação de células derivadas da pele de felídeos selvagens, a congelação lenta é a metodologia mais empregada (GÓMEZ et al., 2008; MESTRE-CITRINOVITZ et al., 2016), sendo 10% de DMSO, 0,2 M de sacarose e 10–35% de SFB, a combinação de crioprotetores mais utilizada (LEON-QUINTO et al., 2011).

Oliveira et al. (2021) avaliaram os efeitos de diferentes crioprotetores intracelulares na ausência ou presença da sacarose como solução de criopreservação de células somáticas de onças-pintadas, sendo esses efeitos avaliados por meio da morfologia, confluência, viabilidade e metabolismo. Neste estudo, as células criopreservadas com DMSO em associação com a sacarose ou na ausência de sacarose mantiveram sua atividade metabólica após a descongelação.

Arantes et al. (2021), visando estabelecer protocolos de criopreservação que sejam adequados para cada tipo de célula, avaliaram a criopreservação e caracterizou fibroblastos de onça-pintada, gato-do-mato e gato-dos-pampas (*Leopardus colocolo*), avaliando diferentes soluções crioprotetoras (2,5%, 10% DMSO ou CryoSOfree). Os autores observaram que os protocolos usando CryoSOfree resultaram em uma diminuição da viabilidade de fibroblastos de onça-pintada, e as células de *L. colocolo* e *P. onca* apresentaram características fusiformes e *L. tigrinus* esféricas e todas as células apresentaram projeções citoplasmáticas. As diferenças encontradas na eficiência dos protocolos de criopreservação de acordo com o tipo de crioprotetor indicaram que as espécies reagiram de forma diferente à criopreservação.

Após a criopreservação, à obtenção das células, ocorre a partir de subcultivos, os quais permitem que as células sejam caracterizadas quanto aos seus aspectos morfológicos, condições de crescimento, viabilidade, atividade funcional e metabólica, além da homogeneidade da população celular, estresse oxidativo, níveis de apoptose bem como a confirmação do tipo celular utilizado (SILVA et al., 2021).

### *2.6.1. Estabelecimento de linhagens celulares e eficiência final*

Além de protocolos de criopreservação adequado, outro passo importante para a aplicação dos criobancos, é ter linhagens celulares definidas que garantam que essas células

possam ser armazenadas para uso futuro (LEÓN-QUINTO et al., 2011). Nesse sentido, o estabelecimento de uma linhagem celular, garante o conhecimento seguro dos parâmetros que conferem qualidade à célula armazenada. Tais parâmetros consistem na manutenção de um cultivo homogêneo, sem contaminações e dentro de um número de passagens estabelecido que permitam a estabilidade genética da amostragem (SHARMA et al., 2018). Além disso, para realizar biotecnologias como a clonagem por TNCS, bem como para produzir iPS, é necessário estabelecer linhagens celulares devidamente caracterizadas.

Atualmente, as linhagens de células fibroblásticas derivadas da pele têm sido amplamente utilizadas (JYOTSANA et al., 2016; SIENGDEE et al., 2018; BORGES et al., 2020). Assim, inicialmente, para o estabelecimento de uma linhagem, as células são avaliadas de acordo com diferentes tempos de cultivo, denominados de passagens e geralmente, as avaliações para o estabelecimento da linhagem celular ocorrem na terceira e décima passagem, pois já foram observados que tais passagens são momentos interessantes para a caracterização celular, uma vez que são as passagens mais frequentemente empregadas na reprogramação nuclear (KUBOTA et al., 2000).

Sharma et al. (2018) ao estabelecer uma linhagem de células fibroblásticas de *Camelus bactrianus*, visando armazenar as células desses animais, observaram com o cultivo *in vitro*, que nas primeiras passagens havia a ocorrência de células epiteliais e de fibroblastos. Contudo, os fibroblastos superaram as células epiteliais nas passagens subsequentes e na terceira passagem ocorreu a predominância dos fibroblastos. Além disso, esses autores observaram que com o aumento do número de passagens, o cultivo fica envelhecido e a atividade e a taxa de proliferação de células diminuem, sendo aconselhado o seu uso até a décima passagem.

No estabelecimento de uma linhagem de células, caracterizar a morfologia das células é uma das mais importantes abordagens qualitativas, em que se associa com outras avaliações, como da integridade da membrana, perfil proliferativo, ultraestrutura da célula, ausência de contaminação, atividade funcional, metabólica por meio da análise do potencial de membrana, e criotolerância a partir da criopreservação (BORGES et al., 2020). Adicionalmente, outras análises podem ser realizadas por meio de teste que confirmam o tipo celular, usando-se da imunocitoquímica e imunofluorescência (AMOLI et al., 2017), em que células são incubadas, com anticorpos primários que se ligam às moléculas desejadas, ou, com anticorpos secundários conjugados ou não com fluoróforos, sendo possível avaliar a viabilidade celular, pela presença de moléculas relacionadas a funções fisiológicas (CETINKAYA et al., 2014).

Silva et al. (2021) avaliaram os efeitos do cultivo *in vitro* e da criopreservação no estabelecimento de fibroblastos derivados de onças-pintadas. Nesse estudo, eles mostraram que fibroblastos viáveis podem ser estabelecidos a partir da pele do pavilhão auricular e que, embora essas células não apresentem viabilidade e atividade proliferativa alteradas, sofrem danos durante o cultivo estendido e criopreservação, sugerindo restringir o cultivo de fibroblastos a menos de dez passagens.

Finalmente, linhagens de células somáticas bem caracterizadas pode possibilitar uma melhor aplicação dos bancos de células somáticas em biotecnologias como já observado em estudos realizados por Thongphakdee et al. (2010), que usaram células somáticas de tecidos epiteliais e musculares de gatos marmorizados (*Pardofelis marmorata*) e gatos de cabeça chata (*Prionailurus planiceps*) em TNCS. Os autores relataram que os genomas de ambas as espécies estavam preservados desde 2003 no Genome Resource Bank, que foi desenvolvido em conjunto pela Organização do Parque Zoológico.

Além disso, como um uso potencial de células somáticas mantidas nesses criobancos, a clonagem por TNCS interespecífica (TNCSi) pode ser usada na conservação de felídeos (LOI et a. 2011). Gómez et al. (2004) relataram o primeiro nascimento de um gato selvagem por TNCSi usando embriões clonados utilizando o núcleo de fibroblasto de gato selvagem africano com citoplastos de gato doméstico. Posteriormente, o mesmo grupo (Gómez et al., 2008) relataram o nascimento de outra prole, neste caso entre diferentes espécies, utilizando o gato da areia como doador de núcleo e o gato doméstico como doador de citoplasma. Além disso, uma transferência intergenérica do núcleo foi realizada usando os fibroblastos de gatos de cabeça chata e os citoplastos de gatos domésticos, resultando em blastocistos. Em todos as biotecnologias desenvolvidas pelo grupo de Gómez, os fibroblastos utilizados estavam armazenados em um criobanco.

Adicionalmente, os avanços na área de pluripotência induzida também foram alcançados para felídeos silvestres. As iPS podem fornecer uma fonte de células pluripotentes para uso na conservação da vida selvagem por criopreservação de recursos genéticos, transferência nuclear usando células de doadores reprogramadas e diferenciação dirigida de gametas (Verma et al., 2012). Assim, Verma et al. (2012) caracterizaram células iPS de leopardo da neve (*Panthera uncia*), a partir de fibroblastos da pele auricular de animais de zoológico, em que o conhecimento da linhagem celular foi fundamental para a eficiência final da técnica. Em onça-parda, consta apenas um estudo de caracterização de linhagens de células, realizado por Echeverry et al. (2020) que caracterizou uma população celular isolada

de tecido adiposo abdominal de uma onça-parda, a fim de determinar seu potencial para terapia celular nesta espécie.

## 3. JUSTIFICATIVA

A redução populacional de onças-pardas a níveis nacional e internacional tem resultado no desenvolvimento de estratégias voltadas para a conservação desta espécie, a qual atua como importante papel ecológico e ainda há poucos estudos que auxiliem na sua conservação. Nesse sentido, inúmeras ferramentas podem ser empregadas, como a formação de bancos de recursos somáticos. Esses bancos são importantes em virtude da possibilidade dessas células serem empregadas na clonagem por transferência nuclear de célula somática, produção de células pluripotentes e obtenção de gametas.

Em se tratando de colheita de amostras somáticas, a pele e tecidos adjacentes especialmente do pavilhão auricular consiste num órgão adequado para a recuperação de células somáticas, uma vez que permite uma colheita menos invasiva e obtenção de amostras eficientes. Para garantir uma formação eficiente de bancos de recursos somáticos, alguns passos tornam-se essenciais, tais como conhecer a estrutura a qual deseja armazenar, avaliar o efeito da criopreservação sobre as amostras, estabelecer e caracterizar uma linhagem que possa ser utilizada em biotecnologias futuras.

Por tanto, inicialmente, as amostras somáticas devem ser caracterizadas quanto ao efeito da criopreservação tecidual, visando à obtenção de um protocolo que garanta a manutenção de uma maior viabilidade dos tecidos após o aquecimento. Até a presente data, nenhum estudo foi realizado quanto à criopreservação de tecidos somáticos derivados da pele desses animais, bem como não há informações acerca das características morfológicas dos tecidos desses animais.

Além disso é essencial o isolamento, caracterização e criopreservação de fibroblastos para o uso posterior dessas células. Assim, o referido estudo estabeleceu de forma descritiva passos importantes e necessários para o estabelecimento de um banco de recursos somáticos de onça-parda. Portanto, esta proposta pretendeu contribuir de forma significativa nas pesquisas relacionadas à conservação das onças-pardas.

## 4. HIPÓTESES CIENTÍFICAS

I – A vitrificação em superfície sólida e a congelação lenta promovem uma manutenção adequada das características histológicas e celulares, respectivamente, de acordo com os parâmetros de viabilidade, atividade metabólica e funcional;

II – Linhagens fibroblásticas podem ser obtidas adequadamente a partir da pele do pavilhão auricular apical de onças-pardas;

III – O número elevado de passagens realizadas durante o cultivo *in vitro* de fibroblastos de recuperados do tecido da região auricular apical de onças-pardas pode influenciar negativamente na viabilidade, metabolismo e funcionalidade celular;

IV – A criopreservação celular pode afetar negativamente o metabolismo e a funcionalidade de linhagens fibroblásticas de onças-pardas.

## 5. OBJETIVOS

### 5.1. OBJETIVO GERAL

Caracterizar células somáticas isoladas a partir do pavilhão auricular de onças-pardas e avaliar os danos causados pela criopreservação em amostras somáticas.

### 5.2. OBJETIVOS ESPECÍFICOS

- Caracterizar os efeitos da criopreservação tecidual sobre a viabilidade dos tecidos somáticos de onça-parda e qualidade das células recuperadas após cultivo *in vitro*;

- Avaliar os danos gerados pelo tempo de cultivo, da criopreservação sobre o estabelecimento de linhagens de células somáticas de onça-parda.

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# CAPÍTULO 2 – EFFECTS OF SOMATIC TISSUE CRYOPRESERVATION ON PUMA (*Puma concolor* LINNAEUS, 1771) TISSUE INTEGRITY AND CELL PRESERVATION AFTER *IN VITRO* CULTURE

**Artigo experimental:** Effects of somatic tissue cryopreservation on puma (*Puma concolor* Linnaeus, 1771) tissue integrity and cell preservation after *in vitro* culture

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# Effects of somatic tissue cryopreservation on puma (*Puma concolor* Linnaeus, 1771) tissue integrity and cell preservation after in vitro culture

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**ABSTRACT**

Somatic resource banks play a crucial role in the conservation of genetic diversity, allowing for the preservation of biological samples from different populations. Puma somatic cells can be recovered from these banks and used in assisted techniques toward enhancing their multiplication and conservation. In response to the population reduction of this ecologically importance species, we aimed to evaluate the capacity of cryopreservation of somatic tissues on the maintenance of the integrity and quality of the cells recovered after culture, with the aim of establishing a somatic tissue bank that will allow for the safeguarding of a wide genetic sampling of pumas. Cryopreservation increased the thickness of the corneum layer in the tissues, and the number of perinuclear halos and empty gaps. Nevertheless, cryopreservation was able to maintain normal fibroblast patterns, even showing an increase in the percentage of collagen fibers. Cryopreservation maintained the proliferative potential of the tissues and the parameters evaluated during *in vitro* culture, mainly regarding the viability, proliferative activity, and apoptosis levels. Nevertheless, cells from cryopreserved tissues showed decreased metabolism and mitochondrial membrane potential. In summary, we demonstrated for the first time that puma somatic tissues subjected to cryopreservation are viable and maintain tissue integrity, featuring minimal changes after warming. Although

viable somatic cells are obtained from these tissues, they undergo alterations in their metabolism and oxidative stress. Improvements in the conservation conditions of somatic samples are needed to increase the quality of somatic tissue banks in this species.

**KEYWORDS**: Wildlife; somatic resource banks; vitrification; somatic cells.

## 1. INTRODUCTION

The puma is an important predator of species such as collared peccary (*Pecari tajacu*), deer (*Mazzama ssp.*), paca (*Cunuculus paca*), coati (*Nasua nasua*), and capybara (*Hydrochoerus hydrochaeris*), and they are essential in controlling the density of these species that carry out the ecological processes of consumption and dispersion of seeds [1]. Although the puma is the most widespread felid in the American continent, its population has become extinct in part of the United States and in some areas of South America [22]. In Brazil, one study indicated that the species is in a vulnerable stage with the possibility of a population reduction of 10% in the next 21 years, accentuated in some biomes such as the Caatinga, where a population of only 2500 individuals is identified [1].

According to Borling [6], the main reason for this population reduction is human occupation and anthropic actions such as hunting and burning, particularly the latter, which currently is responsible for the loss of biodiversity of felids, including the puma in Pantanal, Brazil. Therefore, conservation tools that guarantee the maximum preservation of genetic and biological diversity are required for pumas. In this scenario, somatic resource banks represent a valuable strategy for the conservation of the current genetic diversity of populations [23,26], providing conservation opportunities by using these samples in assisted biotechnologies, such as somatic cell nuclear transfer and pluripotency-induced cell generation [21].

Somatic resource banks are sources of somatic tissues and cells after collection, processing, and cryopreservation for long periods [12]. These banks have the advantage of being easily obtained and not restricted to the gender and age of the animal, allowing for a wide sampling [25]. A sequence of wild felids has had their biological material stored in somatic tissue banks for different purposes. Three examples can be highlighted: León-Quinto [12] stored somatic samples from different regions of the body from 69 Iberian lynx (*Lynx pardinus*), while Mestre-Citrinovitz [18] and Praxedes [28] stored tissues of the skin of the jaguar (*Panthera onca*).

Establishing a well-planned somatic sample bank requires the identification of adequate conditions for the cryopreservation of somatic tissues and the study of cryoinjury [24]. Interestingly, vitrification using the cryoprotectants dimethyl sulfoxide, sucrose, and fetal

bovine serum has promoted adequate conservation of somatic tissues for the Iberian lynx [12], jaguar [28], and domestic cat [17]. Nevertheless, the evaluation of cryopreservation conditions consists of assessing the tissue of interest [4]. Currently, there is no information on the efficiency of vitrification in the conservation of puma somatic tissues.

Thus, the establishment of tissue cryobanks has been suggested as a practical approach to the conservation of species and, when performed in conjunction with assisted techniques, can provide a means for the reproduction of endangered species [24]. Therefore, the aim of this study was to evaluate the capacity of cryopreservation of somatic tissues to maintain the integrity and quality of the cells recovered after culture, with the aim of establishing a somatic tissue bank for pumas.

## 2. Materials and methods

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

### *2.1. Bioethics and animals*

All experiments were carried out with the ethical approval of the Committees of the Rural Federal University of Semi-Arid (no. 23091.010755/2019-32) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71804-1). A total of four pumas were used, one female and three males of 3.7 ± 1.5 years of age. All animals belonged to zoos in northeastern Brazil (Sargento Prata Municipal Zoo and Ecologic Park Ecopoint).

### *2.2. Somatic tissue collection and experimental design*

Peripheral ear samples (1–2 $cm^2$) were collected after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, SP, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, São Paulo, SP, Brazil) and mechanical containment [28]. The somatic tissues were transported to the laboratory in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution at 4 °C for 4 h.

In the laboratory, fragments were trichotomized, cut into fragments (9.0 $mm^3$) using a sterile surgical blade, and washed twice with DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution. Eight fragments per animal were randomly distributed into non-cryopreserved and cryopreserved groups. Thus, non-cryopreserved and cryopreserved/warmed fragments were evaluated for morphological analysis with emphasis on epidermal, dermal, and perichondrium thickness, quantification of normal or degenerate chondrocytes, empty or filled gaps, cell and perinuclear halo, collagen matrix, and tissue proliferative activity. In addition, tissues were evaluated ultrastructurally. Other samples were submitted to primary tissue culture and subcultures for up to one passage. The cells were analyzed for morphology, adhesion, subconfluence, viability by trypan blue, metabolic activity by 3-(4.5-dimethylthiazole-2yl)-2.5-diphenyl tetrazoline bromide (MTT),proliferative activity through cell growth curve and determination of population doubling time (PDT), analysis of oxidative stress by quantification of the levels of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi m$), and evaluation of levels of apoptosis,as described below.

*2.3. Cryopreservation for somatic tissue conservation*

The cryopreservation procedure used was solid-surface vitrification (SSV), according to a study developed with jaguar [25]. A cryopreservation solution (CS) comprising DMEM with 3.0 M dimethyl sulfoxide (DMSO), 0.25 M sucrose (SUC), and 10% FBS was used. Briefly, fragments were exposed to 1.8 mL of CS for 5 min, and the excess solution was removed with absorbent paper. Subsequently, the fragments were placed individually on a metal surface partially immersed in liquid nitrogen (-196 °C), transferred to cryovials, and stored in liquid nitrogen.

After two weeks, all cryovials were maintained for 1 min at 25 °C and immersed in a water bath at 37 °C. For removal of CS, fragments were washed three times for 5 min in DMEM plus 10% FBS and SUC at decreasing concentrations (0.50 M, 0.25 M, and without SUC).

*2.4. Evaluation of the somatic tissues by histological analysis*

Fragments derived from non-cryopreserved and cryopreserved tissues were fixed in 4% paraformaldehyde for 7 days, processed in paraffin, and sectioned at 5.0 µm. Fragments of each treatment were stained with (i) hematoxylin–eosin (HE), (ii) Gomori trichrome, and (iii)

silver nitrate. HE was used to quantify the thickness of the epidermis, dermis, and perichondrium (µm), and the number of fibroblasts, keratinocytes, melanocytes, epidermal cells, chondrocytes, gaps, and perinuclear halos [26]. For this analysis, 12 images per animal were evaluated using the ImageJ software (US National Institutes of Health, Bethesda, MA, USA).

Gomori trichrome was used to analyze the collagen matrix and quantify the collagen fibers in the dermis. The percentage of collagen fibers was calculated as the total area of collagen divided by the total area of the analyzed image [26]. For this analysis, 10 images per animal were acquired for each treatment, using the threshold color plugin of the ImageJ software (US National Institutes of Health, Bethesda, MA, USA), converted to the RGB 32-bit format.

The AgNOR were stained with silver nitrate for the analysis of tissue proliferative potential with the AgNOR assay, and dark spots marked by silver nitrate bound to nuclear proteins were counted according to the cell location [26]. In each image, 100 randomly selected stained fibroblast nuclei were counted, and the AgNOR number/cell and AgNOR area/cell were quantified using Image Pro Plus software. Twelve images/animal were used for this analysis, totaling 48 images per group.

*2.5. Evaluation of the somatic tissues by ultrastructural analysis*

Fragments were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for five days. After this period, tissues were post-fixed in 1% osmium tetroxide diluted in distilled water and dehydrated with increasing concentrations of ethanol [7]. The dehydrated sample was taken to the carbon dioxide critical point (K850 Critical Point Dryer, Quorum Technologies, United Kingdom), mounted on the scanning electron microscopy (SEM) sample holder ("stub"), and metallized with a thin layer in gold. Finally, the tissue ultrastructure was visualized using a SEM (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

*2.6. In vitro culture and evaluation of somatic cells derived from tissues*

The fragments were cultured in a medium of DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution, distributed in four in a polystyrene plate, at 38.5 °C and 5% $CO_2$. Cultures were monitored every 24 h with the total replacement of the culture medium.

Once cell growth started around the fragments, the medium was changed every 48 h [28]. When the explants were surrounded by a considerable layer of cells, they were removed, leaving only the cells.

The culture was analyzed from before reaching confluence to the 70%–80% confluence level. Using an inverted microscope (Nikon TS100, Tokyo, Japan), cells were evaluated for the following parameters: morphology, number of attached explants, number of explants with subconfluence, days of attached explants, number of explants that grew to subconfluence, days of subconfluence of explants, and total time for subconfluence [28].

The subconfluent cells were washed in PBS and trypsinized with a trypsin/EDTA solution (0.25%/0.2%) for 7 min and centrifuged at 600×g for 10 min. The supernatant was removed, the cell pellet was resuspended in culture medium, and the cells were evaluated for morphology, viability, proliferation, metabolism, oxidative stress, and apoptotic activity.

*2.6.1. Analysis of morphology, and viability*

The morphological characteristics of the cells were assessed in terms of cell adherence and confluency using an inverted microscope. Morphological characteristics were observed throughout the *in vitro* culture of the cell and nuclear forms and cytoplasmic extensions.

Cell viability was analyzed using trypan blue assay. Briefly, an aliquot of suspended cells was stained with 0.4% trypan blue (in PBS) in a 1:1 ratio and counted using a Neubauer chamber. Unstained cells were considered viable, whereas cells stained with trypan blue were considered non-viable. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted [28].

*2.6.2. Analysis of proliferative activity and metabolism*

The proliferative activity of cells was quantified according to the growth curve and determination of PDT. Cells ($1 \times 10^4$ cell/mL) were plated in 24-well dishes, trypsinized, counted, and recorded at 24–168 h intervals. The average of the counts at intervals of 24 h was used for elaboration of the growth curve, and PDT was estimated [29], according to the following equation:

PDT = T ln2/ln (Xe/Xb)

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

For the evaluation of metabolism, cells were subjected to the MTT assay. Briefly, cells ($5 \times 10^4$ cells/mL) were cultured for 5 days in 5% $CO_2$ at 38.5 °C. After this period, cells were incubated with 5 mg/mL MTT for 3 h at 38.5 °C and 5% $CO_2$. Absorbance was measured at 595 nm.

*2.6.3. Quantification of intracellular ROS levels and ΔΨm*

For the evaluation of oxidative stress, cells were marked with the 2′,7′chlorohydrofluorescein diacetate ($H_2$DCFDA) probe diluted in DMSO for 30 min at 38.5 °C and 5% $CO_2$, protected from light, and visualized with fluorescence microscopy (excitation: 495 nm and emission: 520 nm), to quantify ROS levels. To evaluate ΔΨm, cells were stained with 500 nM MitoTracker Red® (CMXRos) for 30 min and then visualized under a fluorescence microscope (excitation: 579 nm and emission: 599 nm [14,30].

In both tests, images were obtained using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The background signal strength was subtracted from the values obtained for the treated samples. The measured mean value of the micrograph was obtained from the cells of the *in vitro* culture of non-cryopreserved fragments and used as a calibrator. The relative expression levels (arbitrary fluorescence units) were generated by dividing the measured value of each micrograph for the cells derived from cryopreserved tissues by the mean of the calibrator.

*2.6.4. Apoptosis assay*

An aliquot of 50 µL of cells was resuspended in 2 µg/mL acridine orange and 10 µg/mL ethidium bromide. Subsequently, cells were evaluated under fluorescence microscopy at 480 nm, and 300 cells were counted at 200× magnification. For the classification, 1200 (300 cells/animal) cells were analyzed for each experimental treatment. Cells were classified as viable cells, with a uniform light green nucleus; cells in initial apoptosis, with non-uniform

green nucleus; cells in late apoptosis, with non-uniform bright orange nuclei; and necrotic cells, with uniform orange nuclei. A fluorescence microscope was used to observe apoptotic changes in the stained cells, which were quantified using the ImageJ software.

*2.7. Statistical analysis*

Data from the four pumas were expressed as mean ± standard error (one animal/repetition) and analyzed using GraphPad software (GraphPad Software Inc., La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity using Levene's test. The data from the trypan blue test on metabolic activity and apoptosis did not show a normal distribution, but were arcsine-transformed. Data from morphometric analysis were analyzed by ANOVA followed by Tukey's test. Results of AgNOR analysis, and cell and perinuclear halo numbers were analyzed by Kruskal-Wallis and Dunn tests. All other *in vitro* culture data were analyzed using ANOVA followed by an unpaired t-test. Significance was set at $P < 0.05$.

## 3. Results

*3.1. Evaluation of the somatic tissues by histological analysis*

We evaluated the somatic tissues by staining with hematoxylin-eosin for visualization of morphological characteristics in non-cryopreserved or cryopreserved samples (Fig. 1a-c). Regarding the effects of cryopreservation on tissue morphometry, cryopreserved tissues showed a greater thickness (4.9 ± 1.8 µm) of the corneum layer when compared to non-cryopreserved tissues (3.5 ± 0.7 µm, Fig. 1d).

No difference was observed in the number of fibroblasts, keratinocytes, chondrocytes, or melanocytes between cryopreserved and non-cryopreserved tissues (Table 1). Nevertheless, a greater number of perinuclear halos were observed in cryopreserved tissues than in non-cryopreserved tissues ($P < 0.05$, Table 1). Moreover, in cryopreserved tissues, there was an increase in the number of empty gaps ($P < 0.05$, Table 1). Additionally, cryopreserved tissues presented a higher percentage of collagen fibers compared to non-cryopreserved tissues (Fig. 2a-a'-b).

Finally, no difference was observed in the proliferative potential between non-cryopreserved and cryopreserved tissues (Fig. 3a-a'-b-c). The AgNOR assay demonstrated that cryopreservation maintained the proliferative activity of the tissues after warming.

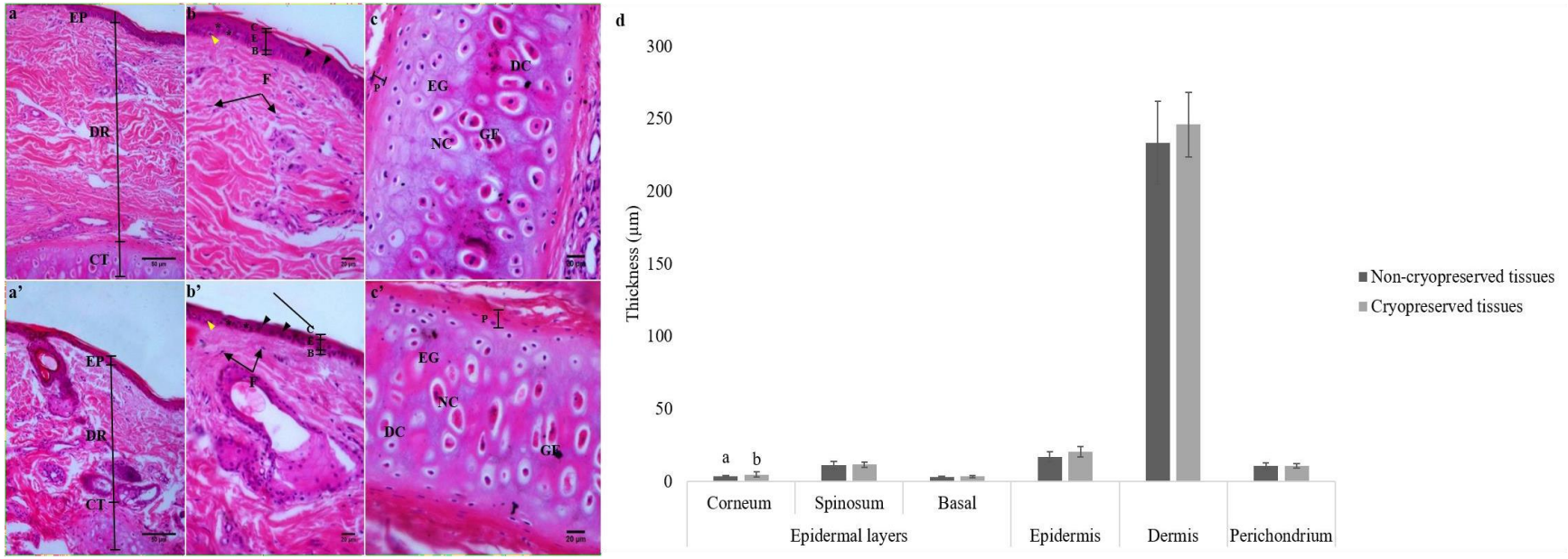


**Fig. 1.** Evaluation with hematoxylin-eosin of non-cryopreserved somatic tissues (**a, b, c**) and cryopreserved somatic tissues (**a', b', c'**) derived from pumas. **a, a')** Overview of the tissues, identifying epidermis (EP), dermis (DR) and cartilaginous tissue (CT). **b, b')** Epidermal and dermal layers and cells, identifying corneum (C), spinous (E), basal (B), melanocytes (*), keratinocytes (black triangle), perinuclear halos (yellow triangle), fibroblasts (F). **c, c')** Perichondrium (P), normal chondrocyte (NC), degenerate chondrocyte (DC), empty gap (EG), filled gap (GF). **d)** Morphometric analysis with the bars indicating the standard error. $^{a,b}$ Values with different differ ($P < 0.05$).

1566 **Table 1.** Evaluation of the somatic tissues derived from pumas for quantification of cell, chondrocytes, gaps, and perinuclear halos

1567

| Tissues | Melanocytes | Keratinocytes | Epidermal cells | Fibroblasts | Perinuclear halos | Normal chondrocytes | Degenerated chondrocytes | Gaps filled | Empty gaps |
|---|---|---|---|---|---|---|---|---|---|
| **Non-Cryopreserved** | 29 ± 3.8[a] | 25 ± 2.9[a] | 17 ± 2.7[a] | 52 ± 5.1[a] | 2 ± 1.1[a] | 39 ± 5.8[a] | 15 ± 2.9[a] | 31 ± 3.7[a] | 8 ± 2.0[a] |
| **Cryopreserved** | 30 ± 3.9[a] | 24 ± 2.9[a] | 19 ± 2.7[a] | 53 ± 3.4[a] | 3 ± 1.2[b] | 30 ± 6.7[a] | 15 ± 3.8[a] | 30 ± 4.9[a] | 10 ± 2.6[b] |

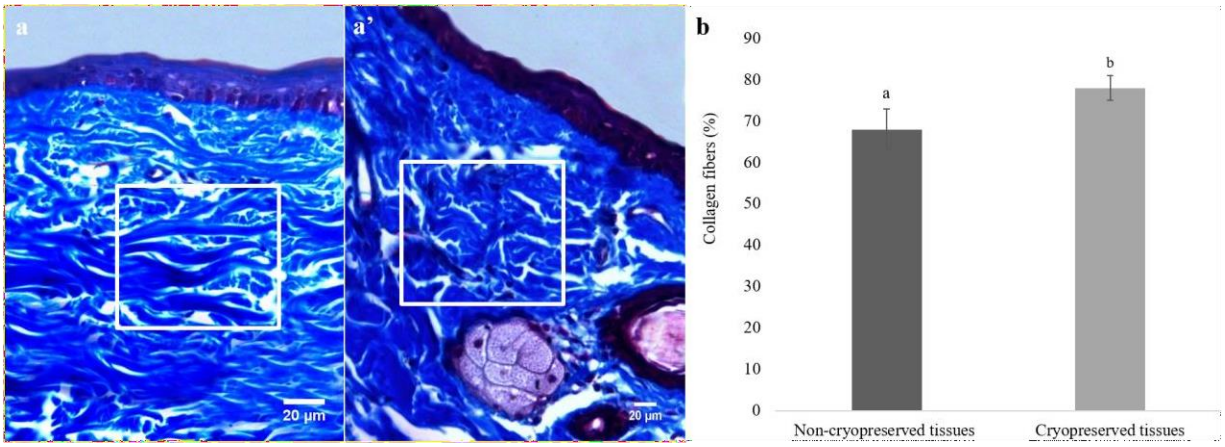


**Fig. 2.** Histological sections of non-cryopreserved and cryopreserved tissues derived from puma using Gomori trichrome. **a**) Non-cryopreserved and **a'**) Cryopreserved samples, **b**) Quantification of the collagen fiber matrix. The white square indicates the space where the blue collagen fibers are shown. 40×, Scale Bar = 20 µm.

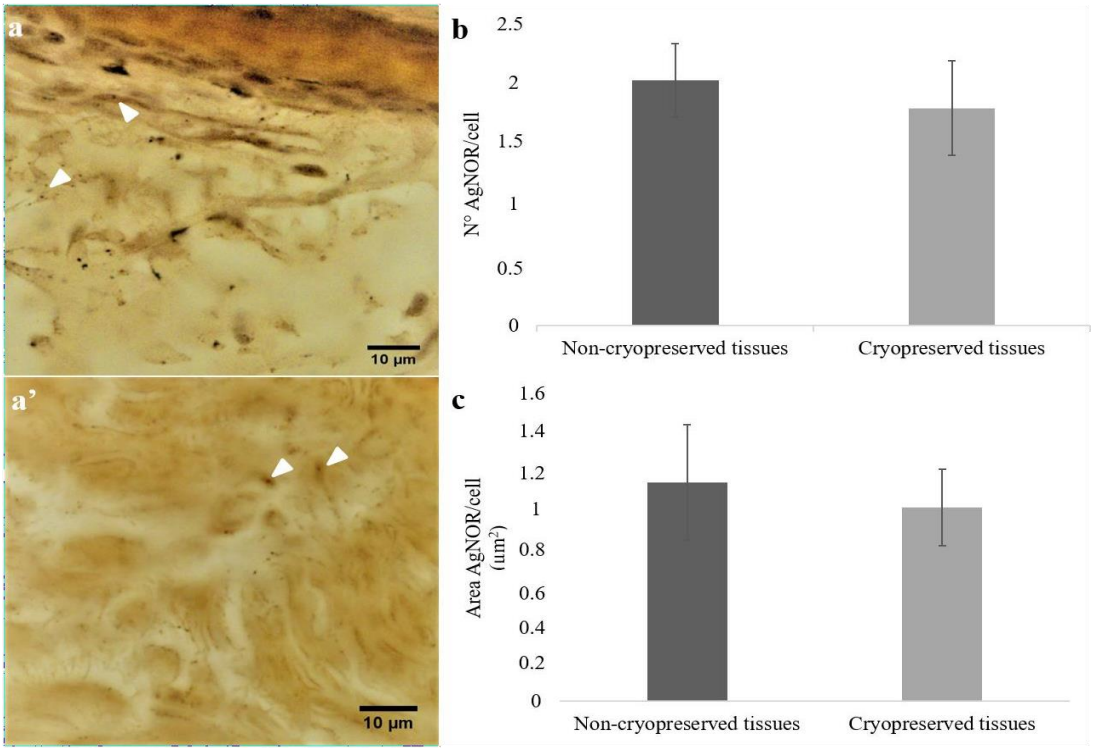


**Fig. 3.** Proliferative potential of non-cryopreserved and cryopreserved somatic tissues derived from puma. **a**) Staining of AgNOR in the fibroblast of non-cryopreserved and **a'**) cryopreserved tissues. Nucleus organizing regions (NORs, white triangle). AgNOR present in the fibroblast nucleus (arrow). **b)** Quantification of the AgNOR number/cell. **c)** Quantification of the AgNOR area/cell. 100×, Scale Bar = 10 µm.

*3.2. Evaluation of the somatic tissues by ultrastructural analysis*

Using scanning electron microscopy, ultrastructural analysis of non-cryopreserved (Fig. 4a-b-c) and cryopreserved tissues revealed similar structural patterns (Fig. 4a'-b'-c'). In the epidermis of cryopreserved tissues, greater cellular detachment from the corneum layer was observed (Fig. 4b').

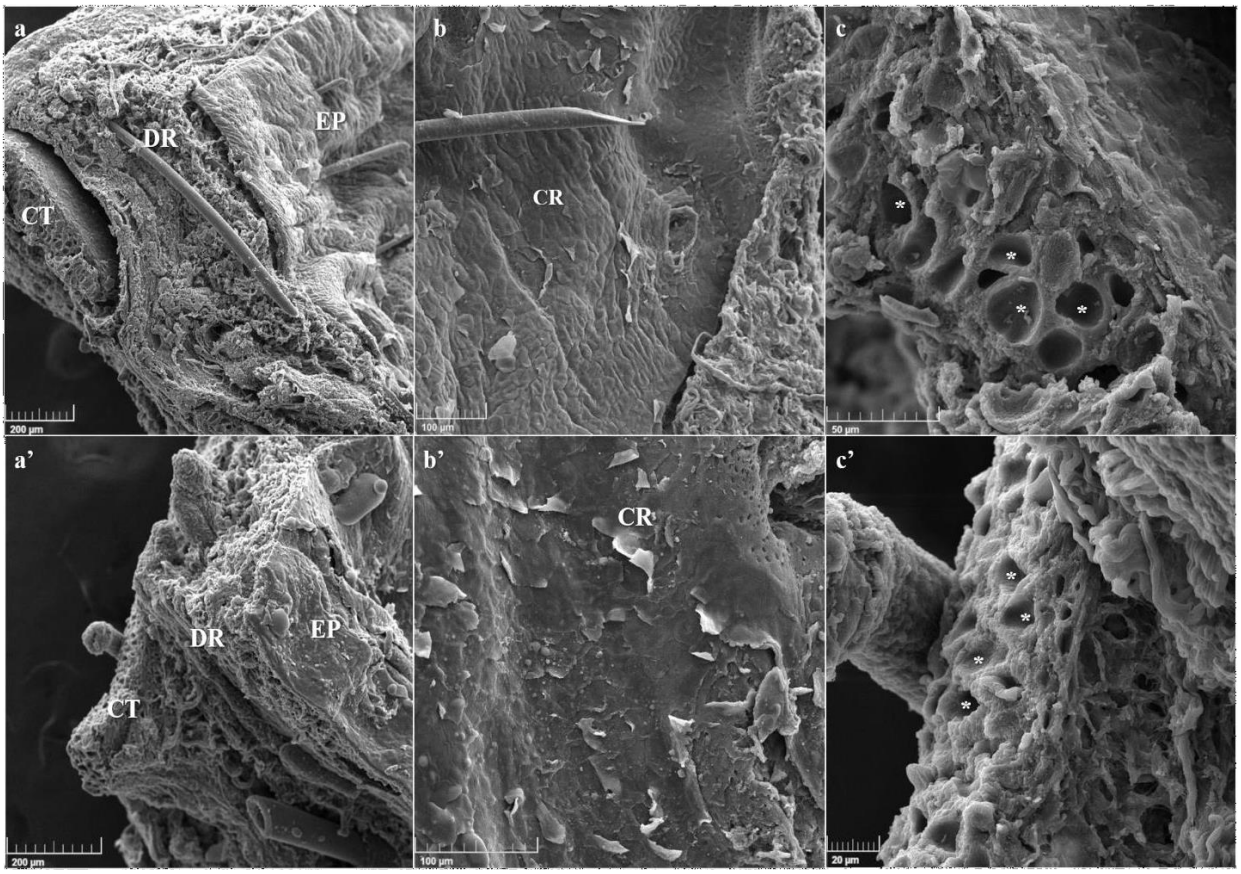


**Fig. 4.** Ultrastructure of non-cryopreserved (**a-b-c**) and cryopreserved **(a'-b'-c')** tissues derived from pumas. Epidermis (EP), dermis (DR) and cartilaginous tissue (CT), corneum layer of the epidermis (CR), gaps (*).

*3.3. Evaluation of somatic cells derived from non-cryopreserved and cryopreserved tissues*

After collection (Fig. 5a), processing (Fig. 5b-c), cryopreservation, and warming, tissues were cultured *in vitro*. All fragments showed adherence between the first and second days of culture (Fig. 5d-d'). There was no difference in tissue adherence and cell confluence between the non-cryopreserved and cryopreserved samples (Table 2).

1592 **Table 2.** Establishment of the primary culture of the puma ear fragments cultured for 35 days

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| Tissues | Attached Samples (%) | Day all attached explants ± S.E. | Day all cell grow explants ± S.E. | Subconfluence samples (%) | Subconfluence total time (days) ± S.E. |
|---|---|---|---|---|---|
| **Non-cryopreserved** | 16/16 (100) | 1.0 ± 0.0 | 8.0 ± 1.6 | 16/16 (100) | 14.0 ± 0.6 |
| **Cryopreserved** | 16/16 (100) | 1.25 ± 0.3 | 8.3 ± 1.4 | 16/16 (100) | 15.0 ± 0.0 |

Cell growth around explants occurred within 4 days, and cell subconfluence was observed after 16 days. Fibroblast-like cells migrated from non-cryopreserved and cryopreserved tissue fragments after 8 days. The morphological characteristics of cells presented a fusiform shape, central oval nucleus, and similarity in fibroblasts (Fig. 5e-e').

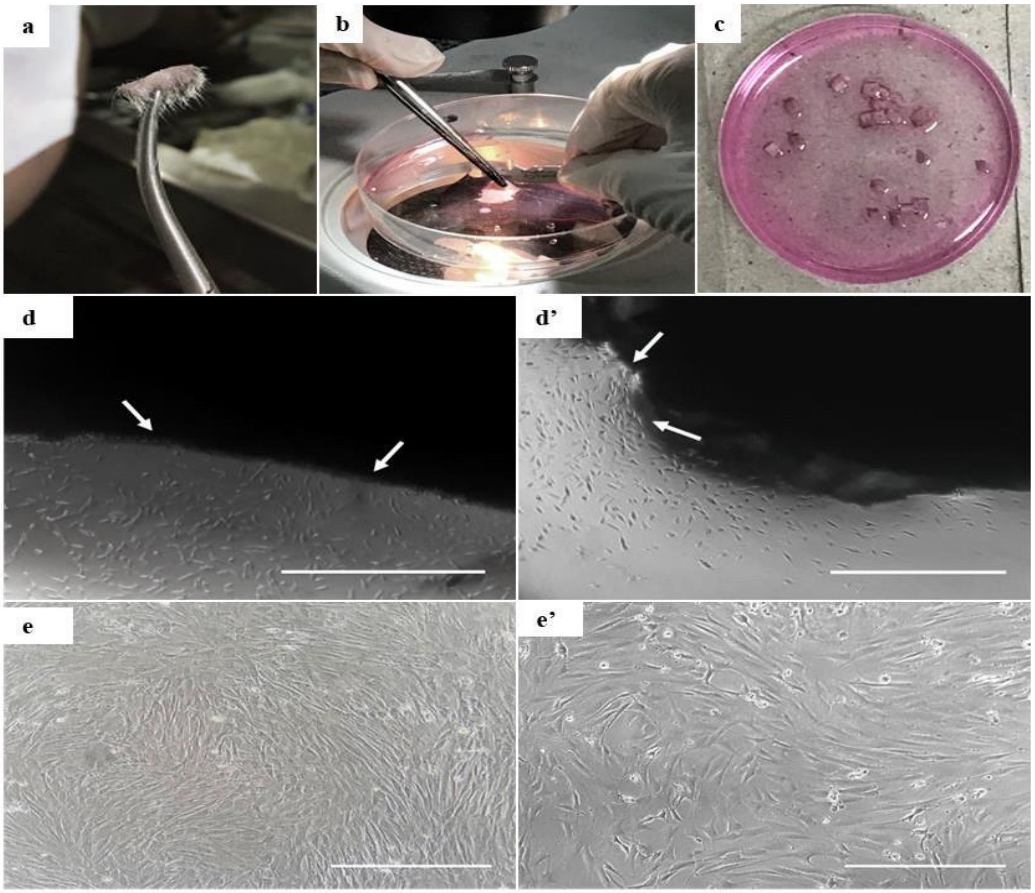


**Fig. 5.** *In vitro* processing and culture of non-cryopreserved and cryopreserved somatic tissues derived from pumas. **a**) Fragments collected from the ear skin. **b**) Sterilization and trichotomy of the tissues. **c**) Tissue fragments (9.0 $mm^3$). **d**) Non-cryopreserved fragments cultured with somatic cell detachment after 4 days. **d'**) Cryopreserved fragments cultured withsomatic cell early detachment. **e**) Secondary culture and cell morphology of non- cryopreserved tissues. **e'**) Secondary culture and cell morphology of cryopreserved tissues. The arrow indicates the start of cell separation in primary cultures (10×. Scale Bar = 50 µm).



Viability did not differ (Fig. 6a) for cells obtained from non-cryopreserved (79.2% ± 5.2%) and cryopreserved tissues (65.1% ± 4.9%), nor did the cell proliferative activity, with a maximum time of 46 h for cryopreserved cells (Fig. 6b-c). The cells derived from the

cryopreserved tissues showed changes in the cell growth curve between 96 and 120 h. The non-cryopreserved and cryopreserved treatments differed ($P < 0.05$), showing a negative effect of cryopreservation on the metabolic activity of cryopreserved tissue cells (Fig. 6d).

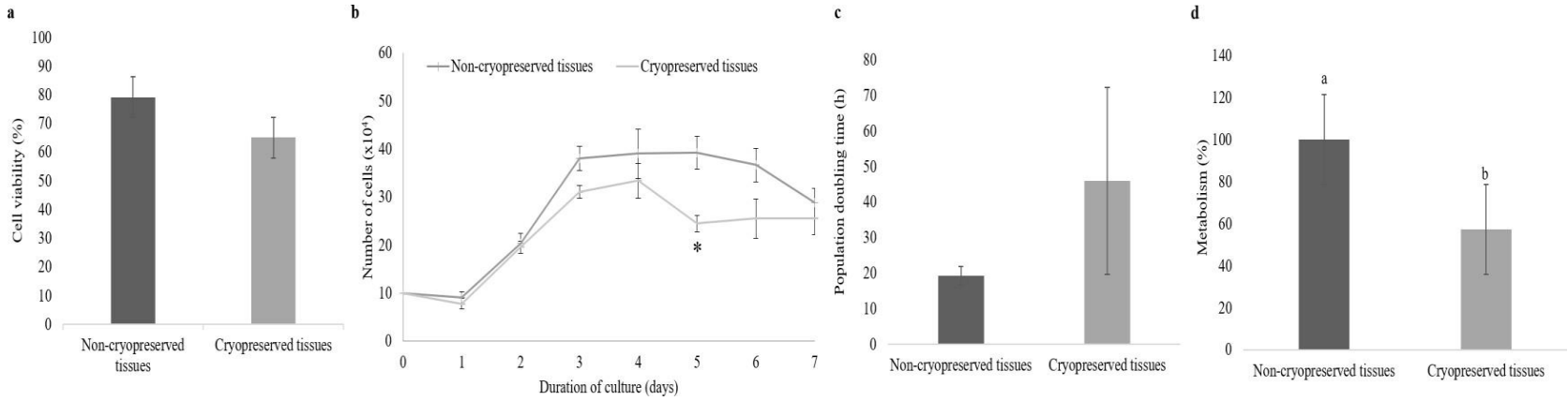


**Fig. 6.** *In vitro* culture assessment of non-cryopreserved and cryopreserved somatic tissues derived from pumas. **a)** Viability. **b)** Curve of cells derived from of the non-cryopreserved and cryopreserved somatic tissues. **c)** Proliferative, and **d)** Metabolism. The bars indicate the standard error. *Values differ in same time ($P < 0.05$). [a,b]: $P < 0.05$.

Finally, cells derived from the non-cryopreserved group showed similar levels of ROS (Fig. 7a, a', d) when compared to the cryopreserved tissues. Nevertheless, a change in ΔΨm was observed for cells derived from cryopreserved tissues (0.6 ± 0.0) when compared to cells derived from non-cryopreserved tissues (1.0 ± 0.0, Fig. 7b, b', e). Regarding the quantification of the levels of apoptosis (Fig. 7c, c', f), there was a difference only in cells in initial apoptosis, in which the cryopreserved tissues (1.25% ± 0.2) showed an increase in apoptosis compared to cells in the non-cryopreserved tissues (0.17% ± 0.1%).

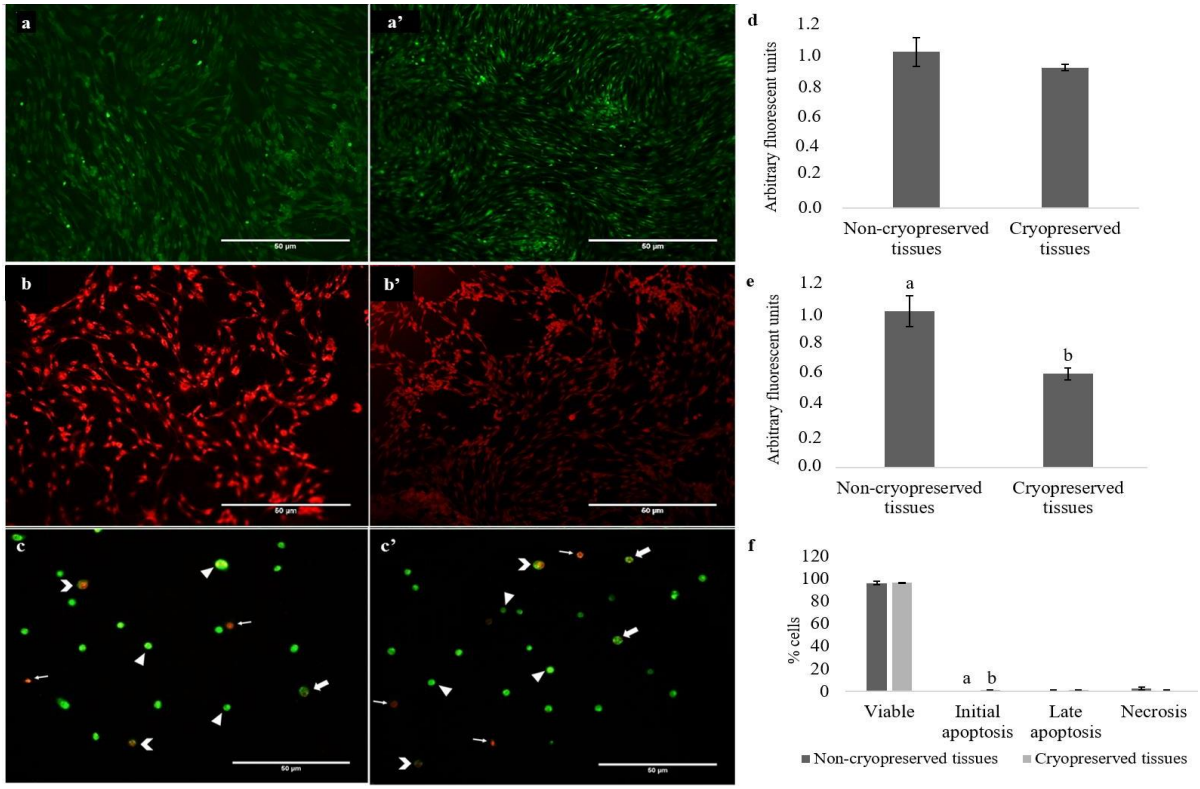


**Fig. 7.** Evaluation of reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm) and apoptosis rates. Fluorescent $H_2$DCFDA in cells derived from **a**) Non-cryopreserved tissues and **a'**) Cryopreserved. Fluorescent MitoTrackerRed® in cells derived from **b**) Non-cryopreserved tissues and **b'**) Cryopreserved. Evaluation of apoptosis levels in cells derived from **c**) Non-cryopreserved tissues and **c'**) Cryopreserved. Viable cells: uniform green nucleus (triangle); early apoptotic cells: non-uniform green nucleus (thick arrow); late apoptotic: nucleus with orange areas (arrowhead); necrotic cells: orange nucleus (thin arrow). Quantification of **d**) ROS, **e**) ΔΨm levels. **f)** Quantification of cell levels of apoptosis. $^{a,b}$: $P < 0.05$. 40×, Scale Bar = 10 and 50 µm.

## 4. Discussion

This study is the first to evaluate the cryopreservation capacity of puma somatic tissues. Based on the information obtained in this study, it was possible to establish a biobank to enhance conservation strategies for puma and other wild felids. Some assisted reproduction techniques have been employed for puma conservation (*in vitro* fertilization [19], artificial insemination [2], and semen cryopreservation [8,9]). Nevertheless, to date, no study has demonstrated cryopreservation and establishment of a somatic resource cryobank for this species. Therefore, the development of a bank of somatic tissues in this species offers an efficient and alternative tool for the conservation of genetic material and its application [28].

The epidermis comprises an initial skin barrier, specifically the corneum layer, which is one of the most important barriers for the penetration of cryoprotective agents, the entry and exit of water during vitrification and warming, and the formation of ice crystals, since the lipid matrix provides resistance to friction and impermeability to water [11,20]. In both the ultrastructural and morphometric analyses, an increase in the thickness and detachment of cells was evident in the corneum layer of cryopreserved tissues when compared to non-cryopreserved tissues. This is because the corneum layer is the most superficial and therefore the most exposed, suggesting that cryopreservation increased the thickness of this layer. Cryopreservation can lead to an alteration and cellular deformation due to the efflux of water from inside the cells to the extracellular medium [14]. Thus, this efflux of water could explain the increase in the thickness of the corneum layer, and consequently, an increase in the perinuclear halos of the puma skin. The number of perinuclear halos showed a subtle increase in cryopreserved tissues. These halos are structures that signal the beginning of apoptosis and are formed by the separation of the nucleus from the cytoplasm [3]. Nevertheless, as the cells of interest for subsequent studies are fibroblasts in the dermis [22], altering the epidermis and increasing halos would not be a limiting factor.

In the cells of the cryopreserved cartilaginous tissue, there was a subtle increase in the number of empty gaps, signifying an increase in cell death. This can be the result of cryopreservation by SSV, in which high cryoprotective concentrations are used, resulting in an increase in the toxicity of cryoprotective solutions, which can reach different degrees in the extracellular matrix, particularly in chondrocytes.

For the collagen matrix, there was an increase in the percentage of collagen fibers in the cryopreserved tissues, indicating a greater stimulation of the fibers, since after warming, cells synthesize more fibers to repair possible tissue damage resulting from the cryopreservation [26]. This may explain why there was no difference in the number of fibroblast cells in the puma skin between non-cryopreserved and cryopreserved tissues. An increase in the number of collagen fibers in cryopreserved tissues favored the production of fibroblasts, which remained similar to the non-cryopreserved tissues. Additionally, the tissue proliferative activity assessed by AgNOR was not affected by cryopreservation. Thus, it can be assumed that all cryopreservation procedures for the establishment of the bank may allow maintenance of the proliferative activity of the tissues.

These variations in the outermost layers of the tissue show the importance of the extracellular and intracellular cryoprotective agents, which are used in cryopreservation to reduce the formation of ice crystals, and the possible damage to the tissue resulting from cryoinjuries [28]. The protocol used in this study was able to maintain the thickness of the skin in the intermediate and basal layers, epidermis, dermis, and perichondrium and promote adequate maintenance of the number of melanocytes, keratinocytes, and fibroblasts. In the present study, DMSO was used as an intracellular cryoprotectant with low toxicity and high membrane permeability because of its low molecular weight and high hydrophilicity [12]. Moreover, its combination the extracellular cryoprotectants FBS and SUC aided in maintaining tissue morphology after warming [27]. Additionally, the efficient use of this combination has been observed in other wild cats [13,27].

During *in vitro* culture, it was observed that the tissue adhesion capacity, growth, culture time, cell isolation, and viability were not affected by cryopreservation. It is likely that the composition of the culture medium used was an auxiliary factor in maintaining these parameters through the supply of substances that favor tissue adhesion and cell growth, such as FBS, which proves growth factors, proteins, vitamins, antioxidant properties, trace elements, and hormones, which are essential for cell growth and maintenance [14].

Cell viability after the first passage was similar between cryopreserved and non-cryopreserved tissues, corroborating the values of León-Quinto [13] after cryopreservation of Iberian lynx skin, and Praxedes [28], after skin vitrification derived from the jaguar. The PDT values did

not change, with a maximum of 46 h, doubling those found for other wild cats, such as Bengal tiger (*Panthera tigris tigris*) at 28 h [10] and Siberian tiger (*Panthera tigris altaica*) at 24 h [15], indicating the need for specific protocols for each species, considering that tissue and cellular requirements may differ among species. The cell growth curve demonstrated the stages of cell growth, latency, and exponential and stable phases [10,15]. In contrast, cells showed a decrease in cell concentration at 96 and 120 h, altering the curve, but this reduction was mitigated after the cells stabilized, demonstrating that *in vitro* culture showed efficiency in maintaining cell quality parameters during the culture, and even with the reduction of cells in that period, the culture managed to restore the proliferative activity of these cells.

Cells obtained from cryopreserved tissues showed a reduction in metabolism; thus, it can be inferred that metabolic activity was affected by cryopreservation. Low metabolic activity may be associated with the ΔΨm of the cells, which was reduced in cryopreserved tissues. This can be explained by the increased permeability of the cell membrane, which results in the release of apoptotic factors and, consequently, a decrease in the membrane potential [25]. Additionally, storage at cryogenic temperatures can cause a reduction in protein synthesis, as well as intracellular molecular transport [28].

With low evidence of mitochondrial activity, cellular respiration decreases, increasing the production of ROS necessary for the performance of important cellular functions, since ROS production is related to mitochondrial respiration [31]. Therefore, the increase in oxidative stress may be mainly due to a lower ΔΨm in the cells of cryopreserved tissues, suggesting that the optimization of cryopreservation methods is related to the minimization of an altered ΔΨm.

Cryopreserved tissues showed high viability when evaluating the levels of apoptosis; nevertheless, in the non-cryopreserved tissues, there was an increase in cells in the initial apoptosis. This increase can be attributed to the formation of ice crystals inside the cell, the inability of cells to recover from the damage induced by cryopreservation, and the increase in apoptotic factors released because of reduced metabolic activity [16]. This result does not directly influence the efficiency of cells that recovered from cryopreserved tissues, since, in our findings we demonstrated an excellent index of cell viability, confirming the efficiency of the cryopreservation and *in vitro* culture protocol used. Additionally, such results may be

related to the fact that the evaluations were carried out on the first passage, since the cells need a longer culture time to resume their normal biological functions [5].

## 5. Conclusions

This study was the first to describe the cryopreservation of puma somatic tissues, demonstrating that samples submitted to cryopreservation were viable and maintained the integrity of the tissue, showing minimal changes after warming. Moreover, it was possible to isolate somatic cells such as viable fibroblasts from cryopreserved tissues, and although these cells undergo changes in their metabolism and oxidative stress, they demonstrated goodperformance in *in vitro* culture.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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# CAPÍTULO 3 – ESTABELECIMENTO E CARACTERIZAÇÃO DE UMA LINHAGEM DE CÉLULAS FIBROBLÁSTICAS DERIVADAS DA PELE DE *Puma concolor* (LINNAEUS,1758) ATÉ A 10ª PASSAGEM – UMA ABORDAGEM PARA O ESTABELECIMENTO DE UM BANCO DE CÉLULASSOMÁTICAS

**Artigo Experimental:** Morphological, ultrastructural, and immunocytochemical characterization and assessment of puma (*Puma concolor* Linnaeus, 1771) cell lines after extended culture and cryopreservation

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# Morphological, ultrastructural, and immunocytochemical characterization and assessment of puma (*Puma concolor* Linnaeus, 1771) cell lines after extended culture and cryopreservation

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**Running title:** Characterization of puma fibroblasts

**ABSTRACT**

Cell lines are valuable tools to safeguard genetic material from species threatened with extinction mainly due to human action. In this scenario, the puma constitutes a species whose population is being rapidly reduced in the ecosystems it inhabits. For the first time, we established and characterized puma-skin-derived cell lines, and assessed these cells after extended culture and cryopreservation. Initially, we identified and characterized four dermal fibroblastic lines using morphology, ultrastructure, and immunophenotyping assays. These lines were subjected to two other experiments. We evaluated the effects of culture time (first, third, and tenth passages) and cryopreservation on their morphology, ultrastructure, viability, metabolism, proliferative activity, reactive oxygen species (ROS) levels, mitochondrial membrane potential ($\Delta\Psi m$), and apoptosis. The cells showed a typical spindle-shaped morphology with centrally located oval nuclei. The cells were identified as fibroblasts by staining for vimentin. In vitro culture after the first, third, and tenth passages did not alter most of the evaluated parameters. Cells in the third and tenth passages showed a reduction in ROS levels ($P < 0.05$). The ultrastructure revealed morphological damage in the prolongments, and nuclei of cells derived from the third and tenth passages. Moreover, cryopreservation resulted in a reduction in $\Delta\Psi m$ compared with that of non- cryopreserved cells. In conclusion, we found that viable fibroblasts could be obtained from puma skin, with slight changes after the tenth passage in in vitro culture and cryopreservation. This is the first report on the development of cell lines derived from pumas.

**KEYWORDS:** Animal conservation, biological characterization, cryobanking, wild felids.

## 1. Introduction

The puma (*Puma concolor* Linnaeus, 1771), the second largest felid in the Americas, belongs to the family Felidae and genus *Puma*. These animals are the most widely distributed carnivores in the western hemisphere; they offer important ecological benefits, as their predation can influence the vital rates of terrestrial ungulates (Caruso et al., 2020). Puma populations have reduced in size mainly due to hunting in retaliation for the predation of domestic animals, burning, and being run over (Balbuena-Serrano et al., 2020). Although some populations of pumas have expanded their reach and present new management challenges, other populations are small, isolated, or in danger, and may need conservational intervention (Azevedo et al., 2013).

Given this scenario, conservation strategies, including assisted technologies such as *in vitro* fertilization (Miller et al., 1990), artificial insemination (Barone et al., 1994), semen cryopreservation (Deco-Souza et al., 2013), and pharmacological collection (Araujo et al., 2020), have been proposed for pumas. A tool that is yet to be developed for this species is the establishment of somatic resource banks, such as cryobanks of somatic cells. These banks are important sources of cells for use in the conservation and multiplication of wild species by means of somatic cell nuclear transfer, and the production of cells that can be induced to pluripotency (Pereira et al., 2018). Therefore, it is necessary to preserve somatic cells to meet future needs.

To obtain a bank of somatic cells, the isolation, characterization, and cryopreservation of these cells are essential for adequate application (Borges et al., 2020). During these steps, all the procedures must be performed with the necessary efficiency to guarantee cell quality (Praxedes et al., 2018). In general, cells are obtained from primary and subcultures, which allow cells to be characterized in terms of their morphological aspects, growth conditions, viability, functionality, and metabolic activity, in addition to the homogeneity of the cell population (Santos et al., 2015). To this end, the establishment of cell lines ensures complete knowledge of the parameters that impart quality to the cell. These parameters consist of maintaining a homogeneous culture, without contamination, and within an established number of passages that allow genetic stability (Sharma et al., 2018).

In general, the number of passages in an *in vitro* study can modify the cellular epigenetic state (Sharma et al., 2018). Assessments for the establishment of a cell line are conducted in the third and tenth passages, as it has been observed that such passages are interesting moments for cell characterization, since they are the passages most frequently employed in nuclear reprogramming (Kubota et al., 2000). Moreover, cryopreservation and maintenance of the quality of cells after thawing is an indispensable step in the establishment of cell banks, since the identification of the damage that occurs during cryopreservation can affect cellular parameters, including survival, functionality, and the cytoskeleton (León-Quinto et al., 2011).

Therefore, to present a greater genetic representation of population biodiversity and facilitate conservation studies, it is important to acquire and store the biological resources of the puma. Hence, for the first time, we established and characterized four cell lines derived from pumas and evaluated these cells after extended culture and cryopreservation.

## 2. Materials and methods

### *2.1. Chemicals and media*

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, USA). Fluorescent probes were purchased from Invitrogen (Carlsbad, CA, USA). Anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, USA). Osmium tetroxide, glutaraldehyde, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### *2.2. Ethics statement and animals*

This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.010755/2019-32) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71804-1). Three males and one female puma of 3.7 ± 1.5 years of age were used. These animals belonged to

Ecologic Park Ecopoint (Fortaleza, CE, Brazil) and Sargento Prata Municipal Zoo (Fortaleza, CE, Brazil).

*2.3. Skin somatic cell collection and experimental design*

Peripheral ear samples (1–2 $cm^2$) were collected after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, SP, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, São Paulo, SP, Brazil), and mechanical containment (Silva et al., 2020). After the collection, trichotomy of the tissues was performed, followed by sterilization in 70% alcohol. Then, the samples were transported to the laboratory in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 4°C within 4 h, according to the protocol of Praxedes et al. (2019).

In the laboratory, sample fragments (9.0 $mm^3$) were washed sequentially under laminar flow conditions using the following media and solutions: (i) DMEM with 10% FBS and 10% antibiotic-antimycotic solution; (ii) alcohol; and (iii) DMEM plus 10% FBS and 2% antibiotic-antimycotic solution. The samples were then fragmented and placed in dishes (4 fragments/dish), with the latter medium being used for culturing the tissues. Skin tissues were cultured at 38.5°C under 5% $CO_2$ and 95% air (Praxedes et al., 2019).

The study was divided into three experiments. The first experiment aimed to establish cell lines; cells were grown under primary culture conditions, and analyzed with regard to primary culture quality, cell morphology, and ultrastructure. Moreover, after the third passage, the cells were subjected to an immunofluorescence assay to confirm the cell type. In the second experiment, cells were cultured and evaluated after the first, third, and tenth passages with respect to the effects of the culture on morphology, ultrastructure, viability, metabolism, proliferative activity, reactive oxygen species (ROS) levels, mitochondrial membrane potential ($\Delta\Psi m$), and apoptosis. Finally, in the third experiment, cryopreserved cells were evaluated for damage occurring in the processes assessed using the aforementioned parameters. The characteristics of the cryopreserved cells were compared with those of non-cryopreserved cells.

*2.4. Primary culture, cell isolation, and subcultures*

Primary and subcultures were maintained in DMEM with 10% FBS and 2% antibiotic-antimycotic solution at 38.5°C under 5% $CO_2$. The culture medium was changed every 24 h. The cells were harvested when they attained 70% confluence and were subcultured in new dishes. Subconfluent cells were evaluated using an inverted microscope (Nikon TS100, Tokyo, Japan), trypsinized, and passaged. Primary cultures and visual appearance of cell monolayers were evaluated using the following parameters: number of attached explants, number of subconfluent explants, day on which all explants were attached, day on which the explants attained subconfluence, and total time required to attain subconfluence.

*2.5. Morphological, ultrastructural, and immunocytochemical characterization of cells*

Morphological characteristics were observed under a bright-field microscope throughout the *in vitro* culture to trace cellular and nuclear shapes, and cytoplasmic extensions. The cells were evaluated based on their size, aspect, shape, and adherence patterns. The obtained data were recorded and assessed based on the morphological patterns.

To determine their ultrastructure, cells were fixed in 4% glutaraldehyde in phosphate buffered saline (PBS) for 12 h, washed thrice with PBS, and post-fixed in 1% osmium tetroxide for 1 h. The cells were then washed and dehydrated in increasing concentrations of ethyl alcohol (15–100%), as described by Ribeiro et al. (2012).

For cell type confirmation, the cells were subjected to the immunocytochemistry protocol of Borges et al. (2020). Cells were fixed using 4% paraformaldehyde for 10 min at 25°C, and then washed with ice-cold PBS. Subsequently, the cells were incubated with an antigen recovery buffer (100 mM Tris, 5% urea, pH 9.5), permeabilized for 1 h in 0.4% Triton X-100, and then incubated in 0.1% Tween-20 for 1 h to block non-specific binding of antibodies. Finally, the cells were immunoassayed with anti-mouse vimentin antibody (ab8979, 1:200) for 24 h at 4°C, and incubated with the secondary antibody (goat anti-mouse IgG, Alexa Fluor®488, ab150113, 1:400) for 1

h at 25°C in the dark. The nuclei were counterstained with Hoechst for 1 min and observed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan).

Additionally, the cells were cultured for 15 days in DMEM containing 10% FBS in the absence of an antibiotic-antimycotic solution at 38.5°C under 5% $CO_2$ and 95% air. Daily evaluation was performed using light microscopy to identify bacterial and fungal contamination.

*2.6. Influence of* passage *number on cell quality*

Cells were subcultured until the tenth passage. When the cells attained 70% confluence, they were resuspended after trypsinization during the first, third, and tenth passages, and subjected to analyses, which were performed immediately after cell resuspension, as described below.

*2.7. Cell cryopreservation*

Cells were cryopreserved by slow freezing in 10% dimethyl sulfoxide (DMSO), 10% FBS, and 0.2 M sucrose to a concentration of $1.0 \times 10^5$ cells/mL (Borges et al., 2020). Briefly, cells were in contact with a cryopreservation solution lacking sucrose for 15 min at 4°C, and then with one containing sucrose for another 15 min at 4°C. The cells were stored in cryotubes, transferred to a Mr. Frosty system® freezing container (Thermo Scientific Nalgene, Rochester, NY, USA), incubated for 12 h, and then stored in a −80°C freezer that maintained a cooling rate of 1°C/min. After this period, the samples were stored in liquid nitrogen (−196°C).

After 2 weeks, the cells were thawed by incubation for 1 min at 25°C and 4 min at 37°C, added to DMEM plus 0.2 M sucrose, and kept at 4°C for 15 min. The cell suspension was centrifuged for 10 min at 600 × *g*, diluted in DMEM, and maintained at 25°C for up to 15 min, after which it was centrifuged again. At the end of thawing, the cells were subjected to the analyses described below.

*2.8. Cell assessment*

*2.8.1. Membrane integrity, proliferative activity, and metabolic activity*

Cell viability was evaluated by measuring membrane integrity using the trypan blue assay. Briefly, cells were stained with 0.4% trypan blue in PBS, and dead cells were identified (blue-colored) by rupture of the membrane, which allowed the permeabilization of the dye (non-viable cells); live cells were colorless, without rupture of the membrane (viable cells). The viability rate was calculated according to the formula: (number of living cells/total number of cells) × 100.

To analyze proliferative activity, cells seeded in 24-well plates at a density of $1.0 \times 10^5$ cells/mL were trypsinized and counted at intervals for 8 days. The average cell counts recorded every 24 h were used to construct the cell growth curve. PDT was estimated (Roth, 2006) using the formula, PDT = T ln2/ln (Xe/Xb), where T is the incubation time, Xb is the number of cells at the beginning of the incubation time, Xe is the number of cells at the end of the incubation time, and ln is the Naperian logarithm.

Subsequently, the cells were analyzed for metabolic activity using the MTT assay (Praxedes et al., 2019). Cells ($5.0 \times 10^5$ cells/mL) were cultured in 12-well plates. After 5 days, 1.5 mL of MTT solution (5 mg/mL in DMEM) was added and the plates were incubated for 3 h. The MTT solution was removed and 1.0 mL DMSO was added over 5 min with slow stirring to solubilize MTT. After the total dissolution of the formazan crystals, the samples were analyzed using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) at an absorbance wavelength of 595 nm.

*2.8.2. Oxidative stress and apoptosis levels*

To assess oxidative stress by quantifying intracellular ROS levels, cells that attained 70% confluence were stained with the 2′,7′-dichlorohydrofluorescein diacetate ($H_2$DCFDA) probe diluted in DMSO, according to the method described by Lira et al. (2020). Cells were washed with PBS, placed in plates containing 5 μM $H_2$DCFDA diluted in DMSO, and incubated at 38.5°C under 5% $CO_2$ for 30 min. The stained cells were washed with PBS, placed on glass slides, and photographed under a fluorescence microscope (Olympus BX51TF), and the fluorescence signal intensity (pixels) was quantified. The images obtained were evaluated using ImageJ software (version 1.49,

Java 1.8.0_201, Wayen Rasband, US National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html).

To assess ΔΨm, cells were stained with 500 nM fluorescent probe MitoTracker Red® (CMXRos), according to the method described by Lira et al. (2020). The staining procedure, incubation, and image evaluation were performed as described for the quantification of ROS.

Apoptosis levels were assessed by binding with the following fluorescent markers: acridine orange (2.0 µg/mL) and ethidium bromide (10 µg/mL); acridine orange was absorbed by viable and non-viable cells, which then emitted green fluorescence, whereas ethidium bromide was absorbed only by non-viable cells, which then emitted red fluorescence. After labeling, the cells were evaluated under a fluorescence microscope and the images were analyzed using ImageJ software (National Institutes of Health, NIH, USA) to quantify the fluorescence signal intensity. Cells were classified as viable when they had a light green nucleus and uniform characteristics, as early or early apoptosis when the nucleus was green and with non-uniform aspects, as late apoptosis when they presented bright orange areas, and finally, as necrotic when the core was uniform orange.

*2.9. Statistical analysis*

All data were expressed as means ± standard errors (1 animal/repetition) and analyzed using StatView 5.0 software (GraphPad Software Incorporation, La Jolla, CA, USA). The normality of all the results was verified using the Shapiro–Wilk test, and the homoscedasticity using the Levene test. The levels of ROS, ΔΨm, viability, and metabolism were altered with arcsine and analyzed using ANOVA followed by Tukey's test. PDT was compared using ANOVA followed by the unpaired t-test. Statistical significance was set at $P < 0.05$.

## 3. Results

*3.1. Morphological, ultrastructural, and immunocytochemical characterization of cells*

The total culture time was 58 days, with the cells being evaluated until the tenth passage. The adhesion of the fragments, detachment of cells, and proliferative capacity were observed in all the explants until they attained confluence (and later, subconfluence) around the adhered fragments (Fig. 1a, Table 1). All the explants exhibited adhesion ability and attained subconfluence. The time taken for each explant to achieve 100% tissue adherence, grow around the explants, and attain subconfluence was 1, 16, and 14 days, respectively.

**Table 1.** Ability of ear skin tissues derived from puma after *in vitro* culture

| Characterization of *in vitro* cultured tissues | Values |
|---|---|
| Attached samples (%) | 16/16 (100) |
| Day all attached explants ± S.E. | 1.0 ± 0.0 |
| Day all cell grow explants ± S.E. | 8.0 ± 1.7 |
| Subconfluence samples (%) | 16/16 (100) |
| Subconfluence total time (days) ± S.E. | 14.0 ± 0.6 |

S.E.: standard error

Monolayers of cells with fibroblast-like morphology and normal growth were observed in the cultures. In general, the cells had oval nuclei and spindle-shaped extensions and showed rapid growth (Fig. 1b). The morphology of the fibroblast-like cells in the initial culture was confirmed to identify the cell type of fibroblasts labeled with anti-vimentin, using fluorescence microscopy (Fig. 1d–f). The cells exhibited high expression of vimentin that marked the cytoplasm, and a fusiform shape, and nuclei were evidenced by Hoechst staining (Fig. 1e). Therefore, efficient isolation and identification of fibroblasts were performed.

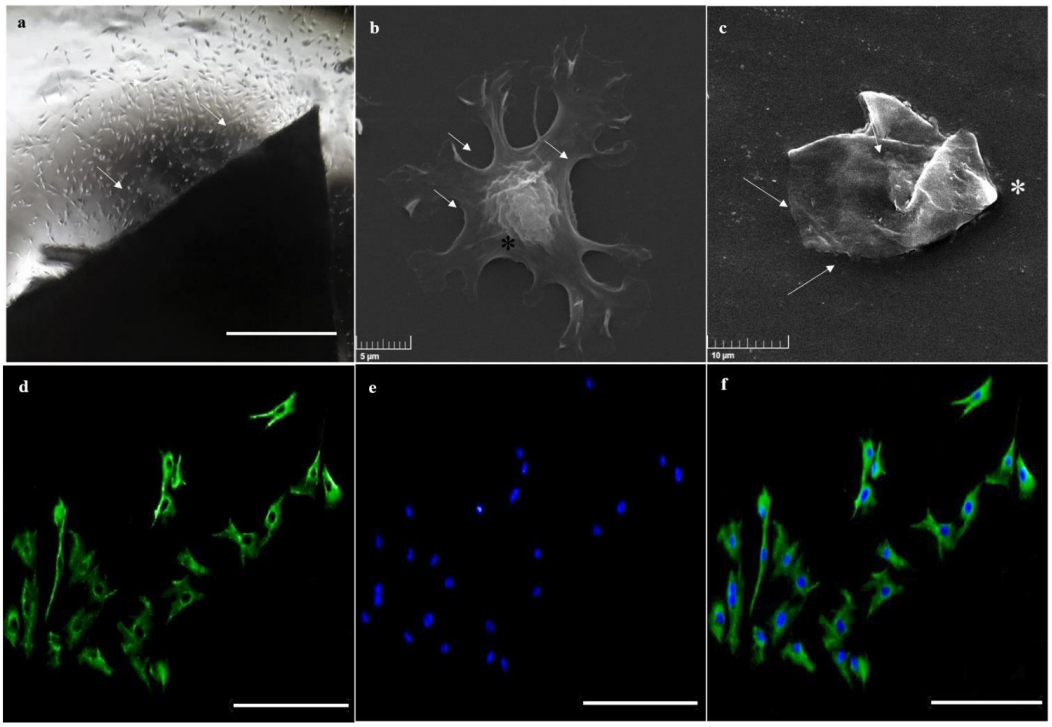



**Figure 1.** Morphological, ultrastructural, and immunocytochemical characterization in puma cells cultured *in vitro*. (a) Primary culture showing detachment of cells (arrow) derived from explants after 5 days of culture, 10× magnification, scale bar = 50 µm. (b) Ultrastructure evaluation showing surface characteristics of cells in third passage. (c) Ultrastructure evaluation showing surface characteristics of cells in tenth passage. In both images, cytoplasmic extensions (arrow), and nucleus (*). (d) Immunofluorescence of vimentin protein (green) for confirmation of fibroblasts. (e) Cell nucleus stained with Hoechst 33342 (blue). (f) Merged images, 20× magnification, scale bar = 50 µm.

No indications of contamination were observed for 15 days in the culture without antibiotics and antifungals. The culture medium showed no change in appearance when observed under an optical microscope. We did not observe turbidity or any specific odors. Additionally, there were no changes in the biological characteristics of growth and proliferation.

*3.2. Influence of passage number on cell quality*

The fibroblasts of the first passage (Fig. 2a) were smaller, with the cytoplasmic extensions being very evident and their locations being more widely spaced. In the third passage (Fig. 2b), the cells showed a more accelerated growth and an increase in their size, becoming more ovoid. In the tenth passage (Fig. 2c), the cells showed a reduction

in their growth, an increase in cell detachment, which may have been indicative of cell death, and a reduction in their cytoplasmic prolongations; these characteristics were different from those of the cells obtained in the first and third passages.

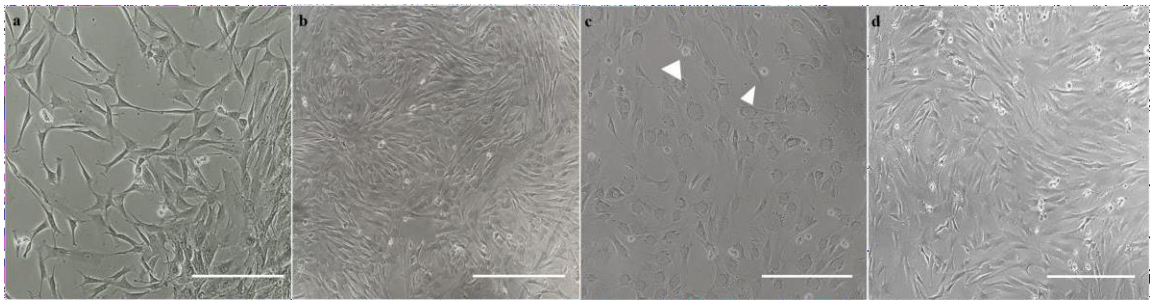



**Figure 2.** Subcultures of cells derived from puma skin. (a) Cells in the first passage. (b) Cells in the third passage. (c) Cells in the tenth passage. Detached and dead cells (triangle). (d) Cells after freezing/thawing and 7 days of *in vitro* culture. 10× magnification, scale bar = 50 µm.

The ultrastructure revealed an evident difference between the fibroblasts of the third and tenth passages (Fig. 1b and c). In the fibroblasts of the third passage (Fig. 1b), the nucleus was large and spindle-shaped (ovoid), presenting one or more evident nucleoli, and extensions were normal and quite extensive. In contrast, fibroblasts from the tenth passage (Fig. 1c) demonstrated a complete retraction of their extensions, which overlapped the nucleus, completely altering the morphology of the cells, and making thenucleus less evident.

There was no difference in cell viability after different passages (79.2% ± 5.2% *vs*. 73.8% ± 7.6% *vs*. 72.0% ± 6.8%, Fig. 3a). The metabolism (Fig. 3b) of the fibroblasts remained close to that expected until the tenth passage, varying from 100% ± 13.7% to 76.2% ± 14.6%, and showing no difference for the various passages.

We generated a growth curve (Fig. 3c and d) to analyze the proliferative activity, and regardless of the number of passages, cells showed a typical "S" pattern of the 8-day cell culture. The latency time was 2 days, followed by an exponential phase until the fourth day, except for cells in the tenth passage, which showed exponential growth until the fifth day. In contrast, cells of the tenth passage did not enter the stationary phase, similar to the cells of the first passage, which remained in the stationary phase until the

seventh day. From the fifth day, cells in the tenth passage entered the declining phase, indicating a reduction in cell growth (Fig. 3d). There were no differences in the PDT (31.4 h ± 8.5 h *vs.* 38.8 h ± 9.2 h *vs.* 64.3 h ± 37.8 h).

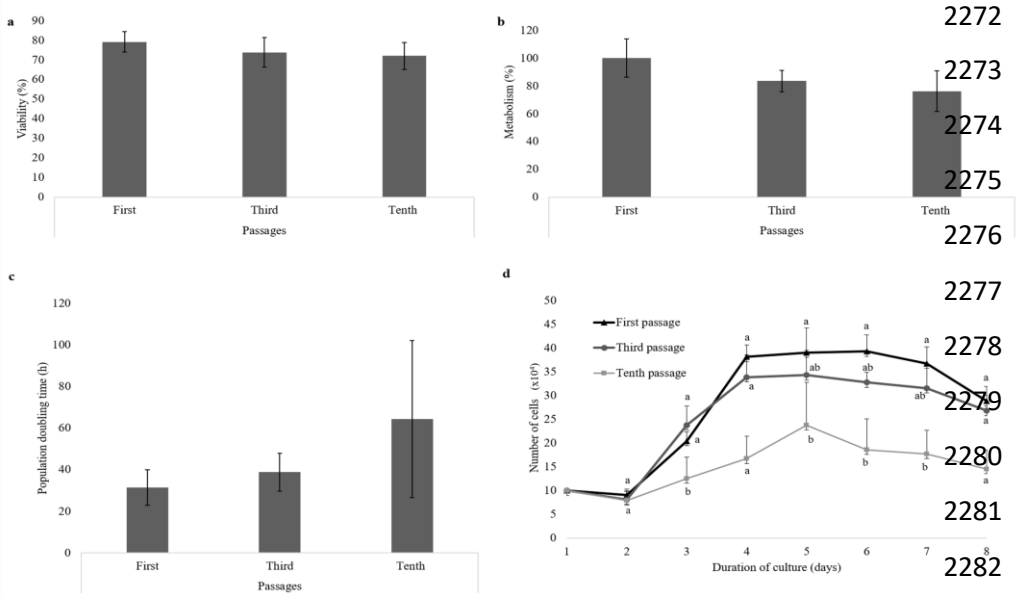



**Figure 3.** Influence of the passage number on viability, metabolic activity, and proliferative activity of puma cells. (a) Viability. (b) Metabolism. (c) Proliferativeactivity evaluated by population double time. (d) Growth curve. Bars indicate standard error. [a,b]: Differ within each time ($P < 0.05$).

The levels of intracellular ROS decreased with an increase in the number of passages; cells of the first passage (1.00 ± 0.09) showed higher levels of ROS compared with those of the third and tenth passages (0.7 ± 0.07 to 0.3 ± 0.01, Fig. 4a–d). Moreover, thecells showed no difference in ΔΨm until the tenth passage (Fig. 4e–h). There were no differences in the levels of apoptosis (96.33% ± 1.5% *vs.* 96.50% ± 0.3% *vs.* 96.91% ± 1.9%), and proportions of early apoptotic cells (0.17% ± 0.00% *vs.* 0.50% ± 0.00% *vs.* 2.42% ± 0.01%), late apoptotic cells (0.83% ± 0.00% *vs.* 1.83% ± 0.01% *vs.* 0.50% ± 0.00%), and necrotic cells (2.67% ± 0.01% *vs.* 1.17% ± 0.00% *vs.* 0.17% ± 0.00%), during the aging of the *in vitro* culture (Fig. 4 i–l).

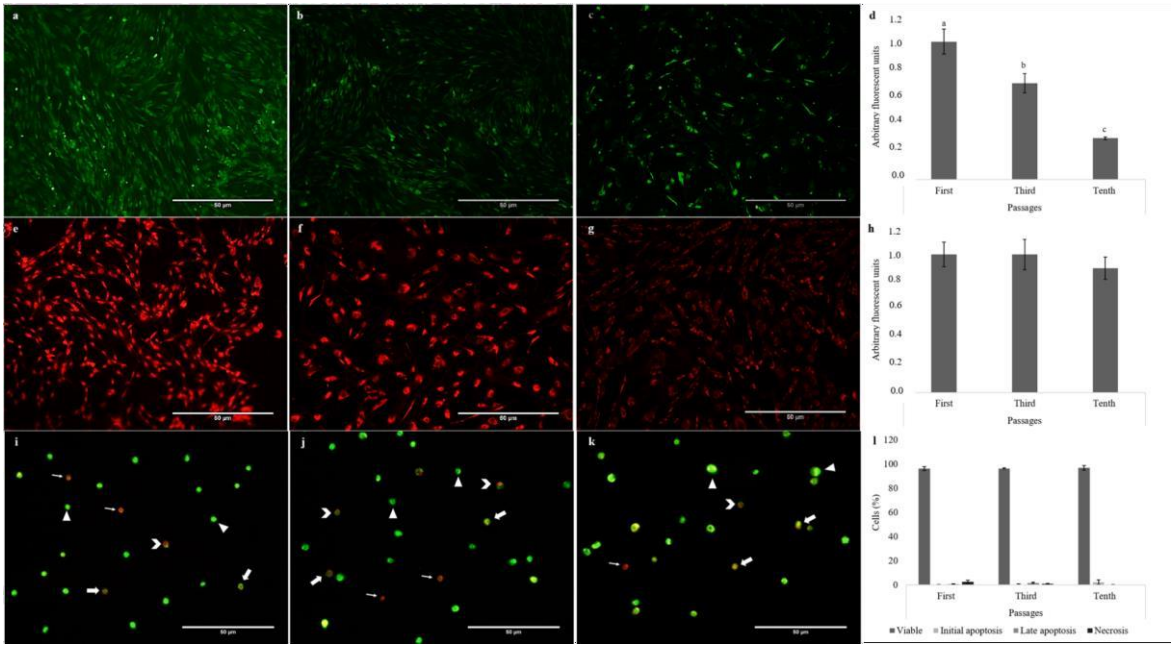
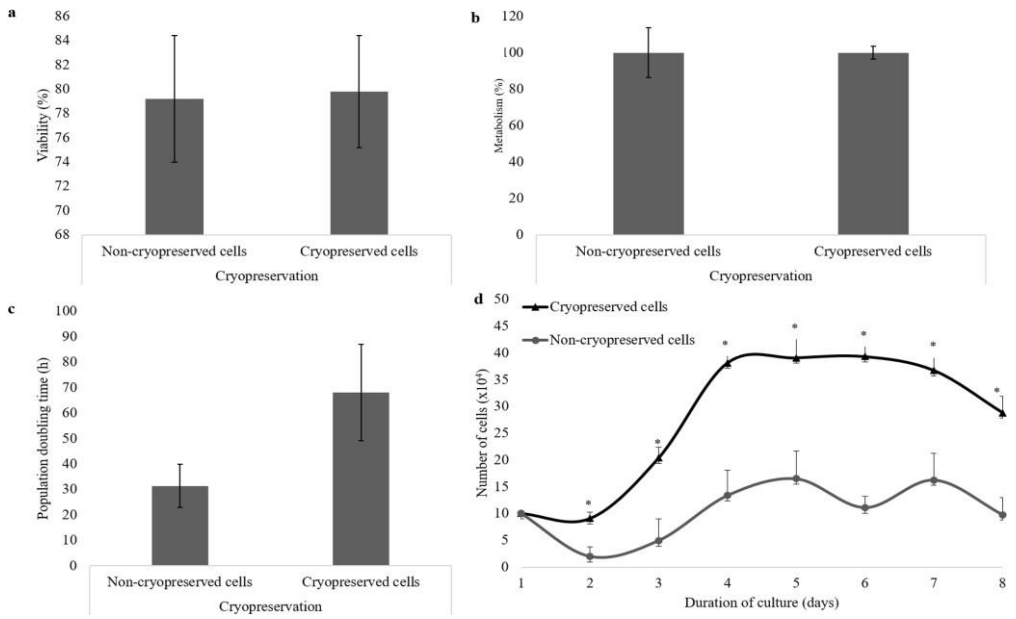



**Figure 4.** Influence of the passage number on oxidative stress and apoptosis levels of puma cells. (a) Cells in the first passage for evaluation of reactive oxygen species (ROS) levels. (b) Cells in the third passage for evaluation of ROS levels. (c) Cells in the tenth passage for evaluation of ROS levels. (d) Quantification of ROS levels. (e) Cells in the first passage for evaluation of mitochondrial membrane potential (ΔΨm). (f) Cells in the third passage for evaluation of ΔΨm. (g) Cells in the tenth passage for evaluation of ΔΨm. (h) Quantification of ΔΨm. (i) Cells in the first passage for evaluation of apoptosis levels. (j) Cells in the third passage for evaluation of apoptosis levels. (k) Cells in the tenth passage for evaluation of apoptosis levels. (l) Quantification of apoptosis levels. Viable cell (triangle). Cell undergoing initial apoptosis (fat arrow). Cell in late apoptosis (arrowhead). Necrosis cell (thin arrow). Bars indicate standard error. $^{a,b}$ $P < 0.05$. 10× magnification

*3.3. Influence of cryopreservation on cell quality*

Cryopreservation (Fig. 2d) did not affect the viability (79.2% ± 5.2% vs. 79.8% ± 4.6%) and metabolism (100% ± 13.7%) of cells (Fig. 5a and b). However, all the growth phases were different in the non-cryopreserved and cryopreserved cells (Fig. 5d). The latency time of cryopreserved and non-cryopreserved cells was 2 days, followed by an exponential phase until the fourth day, stationary phase until the seventh day, and plateau phase from the eighth day (Fig. 5d). There were no differences in the PDT of cryopreserved and non-cryopreserved cells (31.4 h ± 8.5 h vs. 68.1 h ± 18.9 h, Fig. 5c).




2319 **Figure 5**. Influence of the cryopreservation on viability, metabolic activity, and
2320 proliferative activity of puma cells. (a) Viability. (b) Metabolism. (c) Proliferativeactivity
2321 evaluated by population double time. (d) Growth curve. Bars indicate standard error.
2322 *They differ between cryopreserved and cryopreserved cells at the same time ($P < 0.05$).
2323
2324 The ROS levels in cryopreserved cells (1.0 ± 0.1) did not differ (Fig. 6a–c) from those
2325 in non-cryopreserved cells (1.0 ± 0.04). When comparing non-cryopreserved cells (1.0
2326 ± 0.09) with cryopreserved cells (0.7 ± 0.04), a reduction in ΔΨm was observed (Fig. 6f).
2327 Finally, there was no difference between non-cryopreserved and cryopreserved cellswith
2328 respect to the levels of apoptosis (Fig. 6g–i).
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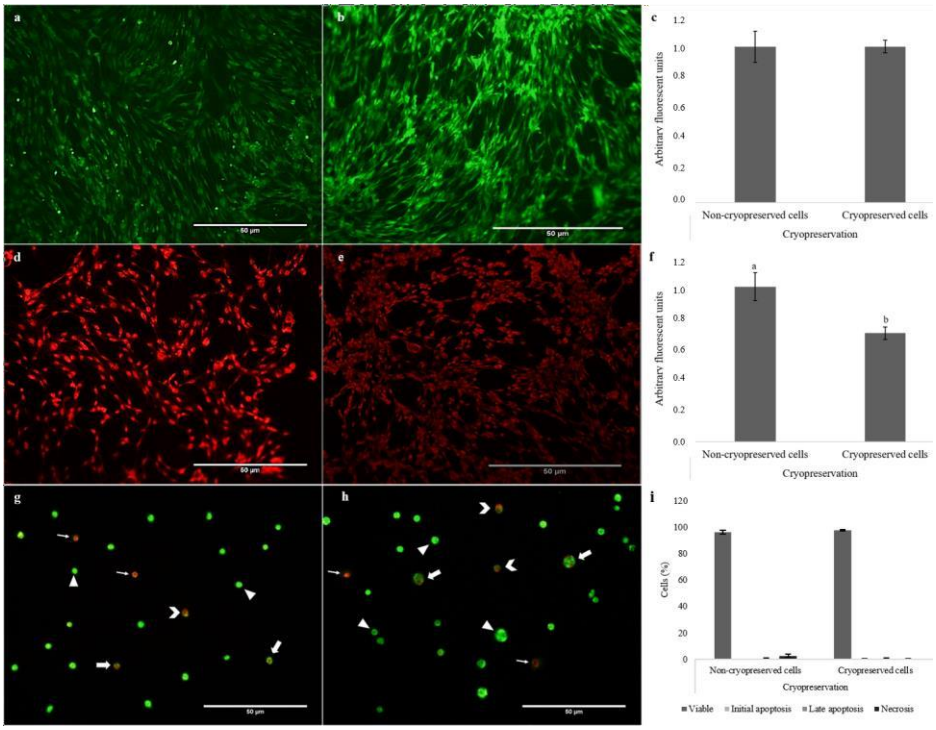


**Figure 6.** Influence of the cryopreservation on oxidative stress and apoptosis levels of puma cells. (a) Non-cryopreserved cells for evaluation of reactive oxygen species (ROS) levels. (b) Cryopreserved cells for evaluation of reactive oxygen species (ROS) levels. (c) Quantification of ROS levels. (d) Non-cryopreserved cells for evaluation of mitochondrial membrane potential (ΔΨm). (e) Cryopreserved cells for evaluation of ΔΨm. (f) Quantification of ΔΨm. (g) Non-cryopreserved cells for evaluation of apoptosis levels. (h) Cryopreserved cells for evaluation of apoptosis levels. (i) Quantification of apoptosis levels. Viable cell (triangle). Cell undergoing initial apoptosis (fat arrow). Cell in late apoptosis (arrowhead). Necrosis cell (thin arrow). Bars indicate standard error. $^{a,b}$ $P < 0.05$. 10× magnification.

## 4. Discussion

In this study, we took another step in the conservation of the puma; we cultured (*in vitro*), isolated, characterized, and cryopreserved fibroblasts derived from puma skin, and recovered viable cells until the tenth passage and after cryopreservation. Thus, we established lines of fibroblasts derived from these animals and stored them in a somatic cell bank for use in studies that can guarantee genetic diversity. The establishment and use of fibroblast biobanks have been instrumental in the development of basic research

and are indispensable for long-term storage, especially for wild felids (*Panthera tigris altaica* – Hashem et al., 2007; *Pardofelis marmorata* – Thongphakdee et al., 2006; *Panthera uncia* – Verma et al., 2012; *Pardofelis temminckii, Pardofelis marmorata,* and *Panthera pardus* – Wittayarat et al., 2013; *Panthera leo persica*, *Panthera tigris tigris,* and *Panthera pardus fusca* – Yelisetti et al., 2016). For other species, somatic cell banks are becoming quite promising, allowing for the recovery of endangered species (Hildebrandt et al., 2018; Stanton et al., 2019).

In this study, all explants adhered to the plate in 1 day, with cell growth around the explant in 8 days, demonstrating confluence in 5–14 days after the start of the culture and after the first passage. In addition, cells needed 7 days to attain confluence for the next passage. These characteristics of explants during *in vitro* culture were similar to those of explants derived from other felids (*Felis catus* – Kitiyanant et al., 2003; *Panthera onca* – Praxedes et al., 2019; *Leopardus tigrinus* and *Leopardus colocolo* – Arantes et al., 2021). In somatic jaguar skin, muscle, and cartilage tissues, the growth of cells around the tissue took between 10 and 14 days, and after each passage, the cells needed 3–6 days to attain 90% confluence (Mestre-Citrinovitz et al., 2016). In the somatic tissues of jaguar skin, all explants adhered in 2 days, fibroblast-like cells could be observed migrating from the sides of explants within 8–11 days after adhesion, and 12 days were sufficient for cells to attain confluence (Praxedes et al., 2019). The similarity of these data may be related to the culture medium, since in most of these studies, DMEM was used with antibiotics and 10% or 20% FBS at 38.5°C under 5% $CO_2$ (Liu et al., 2010; Praxedes et al., 2019).

We could isolate fibroblast-like cells with normal detachment patterns and predominant homogeneous growth of fibroblasts, without the need for an enzymatic method that is used for some species (Saadeldin et al., 2019). The predominant growth of fibroblasts from the first to the tenth passage was possible; this finding was positive because, for other wild mammals (*Bubalus bubalis* and *Pecari tajacu*), it has been demonstrated that the appearance of other cell types, such as epithelial cells, may occur in the first passage (Jyotsana et al., 2016; Borges et al., 2020).

In this study, cells from the third passage were confirmed as fibroblasts using immunofluorescence analysis; these cells were identified using vimentin, an intermediate filament that indicates the mesenchymal origin of cells (Yajing et al., 2018), confirming that puma ear skin was a safe source of fibroblast cells. Additionally, cell-type confirmation was performed on the third passage, as this is the passage most frequently used in nuclear reprogramming (Kubota et al., 2000).

The number of passages can reduce the rate of metabolic activity and cell proliferation (Li et al., 2009). Studies have shown that after several passages, the genetic characteristics of cells can be modified by the culture conditions; therefore, a minimum number of passages has been recommended to conserve cellular characteristics (Mehrabani et al., 2014). In our study, puma cells proved to be more resistant and tolerant; despite the aging of the culture, the cells remained viable and metabolically active, without differences until the tenth passage.

With regard to the morphological aspects of the cells, it was observed that puma fibroblasts are adherent and elongated cells, with abundant cytoplasm, rich in endoplasmic reticulum and well-developed Golgi apparatus; in addition to innumerable extensions, they have a large and fusiform nucleus (ovoid), with one or more evident nucleoli. Furthermore, in different passages, the patterns that characterize cells as fibroblasts, such as size, appearance, shape, and length (Borges et al., 2020), were similar to those found in jaguar fibroblasts (Oliveira et al., 2021; Praxedes et al., 2019). These aspects were fundamental in observing the change in cell morphology with an increase in the number passages. In the tenth passage, it was possible to observe damage in the morphology of the cells, such as the retraction of the prolongations and a flattening, indicating that with an increase in the number of passages, cells age and there is a change in their morphology (Kubota et al., 2000; Li et al., 2009). These findings were confirmed by analyzing the ultrastructure of the cells in the third and tenth passages.

Another important step in the establishment of a cell line is the confirmation of the absence of biological contamination, since, when working with a cell culture, it is

necessary to pay attention to all stages of preparation of the medium, handling, and external environment so that no external factors contaminate the culture (Guan et al., 2010). Therefore, it is important to maintain adequate sterilization conditions for each stage (Liu et al., 2010). Bacterial and fungal contaminations are easily detected with the naked eye or by means of an optical microscope, as they cause alterations in the medium due to the accumulation of metabolites, changes in the color and smell of the medium, as well as the appearance of colonies in some cases (Sharma et al., 2011). During 15 days of culture of puma fibroblasts, no visible contamination was observed; hence, fibroblasts were grown without any biological contamination, even in the absence of antibiotic/antimycotic solutions in the culture medium.

The growth curves of cells in different passages presented a similar profile, showing normal proliferative capacity. Nevertheless, there was a change in the proliferative profile of the cells of the tenth passage that differentiated it from the profiles of cells of the first and third passages: the absence of the plateau phase and the occurrence of early cell death phase, compared with that in the previous passages.

The mechanism of ATP production, which involves the electron transport chain, was determined to measure oxidative stress. A greater ΔΨm indicates a greater synthesis of mitochondrial ATP. ROS generation depends on ΔΨm. Therefore, a high mitochondrial respiratory chain ΔΨm is a significant producer of ROS. This is due to the leakage of electrons (from the mitochondrial membrane) that can bind to $O_2$, forming ROS (Zorova et al., 2018). Thus, a higher ΔΨm can lead to more ROS production. In this study, ΔΨm remained constant until the tenth passage. Nevertheless, there was less ROS formation; this can be explained by the aging of the culture and fibroblasts, which reduces their proliferation, metabolic activity, and ATP synthesis in mitochondria (Dluska et al., 2019). In addition, the increase in the number of passages did not alter the levels of apoptosis in the cells. Therefore, puma fibroblasts are more resistant than those of other species of wild mammals (Sharma et al., 2018; Borges et al., 2020).

The cells of the third passage were cryopreserved to analyze their cryosensitivity to the cryogenic temperatures and cryoprotectants used. Slow freezing is the most commonly

used method for the cryopreservation of cells derived from the skin of wild cats, e.g., *Felis margarita* (sand cat) (Gómez et al., 2008) and jaguar (Mestre-Citrinovitz et al., 2016). The combination of intracellular and extracellular cryoprotectants is used as it offers greater protection, because it protects the cell membrane by binding to phospholipid groups, reducing osmotic shock, and controlling the entry of water into the cell (León-Quinto et al., 2011). Additionally, it can help reduce the oxidative stress of cells after thawing (Lira et al., 2020). Thus, the cryopreservation protocol used did not alter the cell viability, metabolic activity, or proliferative activity of the cryopreserved cells. The cryopreservation protocol proved to be efficient, and the cells demonstrated high resistance to cryopreservation, since there was no change in the cryopreserved cells in any of the evaluations described above.

Cryopreserved cells presented a different growth curve profile compared with non-cryopreserved cells, indicating lower proliferative capacity after cryopreservation. This factor demonstrates the importance of ideal *in vitro* culture conditions that influence the recovery from cell damage caused by freezing (Guan et al., 2010). The failure in maintaining the ΔΨm may have been due to the oxidative stress generated by cryopreservation (Magalhães et al., 2012). The cryopreserved cells showed a reduction in ΔΨm, which may have occurred because of the cessation of the metabolic activities of the cells; therefore, after cryopreservation, mitochondria needed time to recover and regulate their synthesis of ATP and their ΔΨm.

Finally, parameters such as cryovariables, including cooling and thawing rates, type, and concentration of the cryoprotectant, and cell type and shape, can affect the success of cryopreservation (Chatterjee et al., 2017). This suggests that the optimization of cryopreservation methods for puma fibroblasts is essential. There is a great peculiarity in the establishment of the protocol for each species in question, considering that the needs may be different for the different species of wild felids.

## 5. Conclusions

This is the first report on the isolation, establishment, and cryopreservation of cell lines derived from pumas. We have shown that the adherent culture was efficient for

obtaining fibroblasts. The fibroblastic cells of the pumas were confirmed to have no contamination, guaranteeing the efficiency of the culture conditions and cell line characterization. The cell viability could be maintained until the tenth passage. In addition, cryopreservation did not affect the viability, metabolic activity, or proliferative activity of the fibroblasts after slow freezing. Nevertheless, cryopreservation changed ΔΨm, indicating the need for optimization of the cryopreservation protocol.

## Conflict of interest

None of the authors have conflict of interest.


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## CONCLUSÕES GERAIS E PERSPECTIVAS

O presente trabalho descreveu pela primeira vez os parâmetros histológicos do pavilhão auricular apical de onças-pardas submetidas a criopreservação. Assim, o pavilhão auricular apical de onças-pardas não apresentou variações quando comparada com a mesma região criopreservada em relação a espessura das camadas da pele, densidade de matriz colágena, número de melanócitos e fibroblastos, e análise ultraestrutural. Portanto, o protocolo de criopreservação utilizado demostrou ser eficiente no estabelecimento de bancode tecidos somáticos, de acordo com os parâmetros histológicos e celulares.

Além disso, foi realizada pela primeira vez a caracterização das células recuperadas de tecidos oriundos do pavilhão auricular apical, sendo, portanto, confirmada o tipo celular, fibroblastos. Adicionalmente, os fibroblastos foram analisados até a décima passagem, onde as células em cultivo conseguiram manter uma adequada viabilidade, atividade proliferativa e metabólica mesmo em cultivo prolongado. Contudo, quando avaliamos a morfologia por meio da ultraestrutura foi possível observar alterações nos prolongamentos citoplasmáticos dos fibroblastos nas células na décima passagem.

Finalmente, esse trabalho compreendeu as primeiras etapas, visando o uso dessas amostras para as diferentes finalidades, desde os estudos voltados para a multiplicação de indivíduos por clonagem usando a transferência nuclear de célula somática a produção de células pluripotentes e obtenção de gametas a partir dessas células.

# ANEXOS

ANEXO – A: COMPROVANTE DE SUBMISSÃO DO ARTIGO: EFFECTS OF SOMATIC TISSUE CRYOPRESERVATION ON PUMA (*Puma concolor* LINNAEUS, 1771) TISSUE INTEGRITY AND CELL PRESERVATION AFTER IN VITRO CULTURE À REVISTA CRYOBIOLOGY 28 – MARÇO – 2021

Journal: Cryobiology

Title: Effects of somatic tissue cryopreservation on puma (Puma concolor Linnaeus, 1771) tissue integrity and cell preservation after in vitro culture

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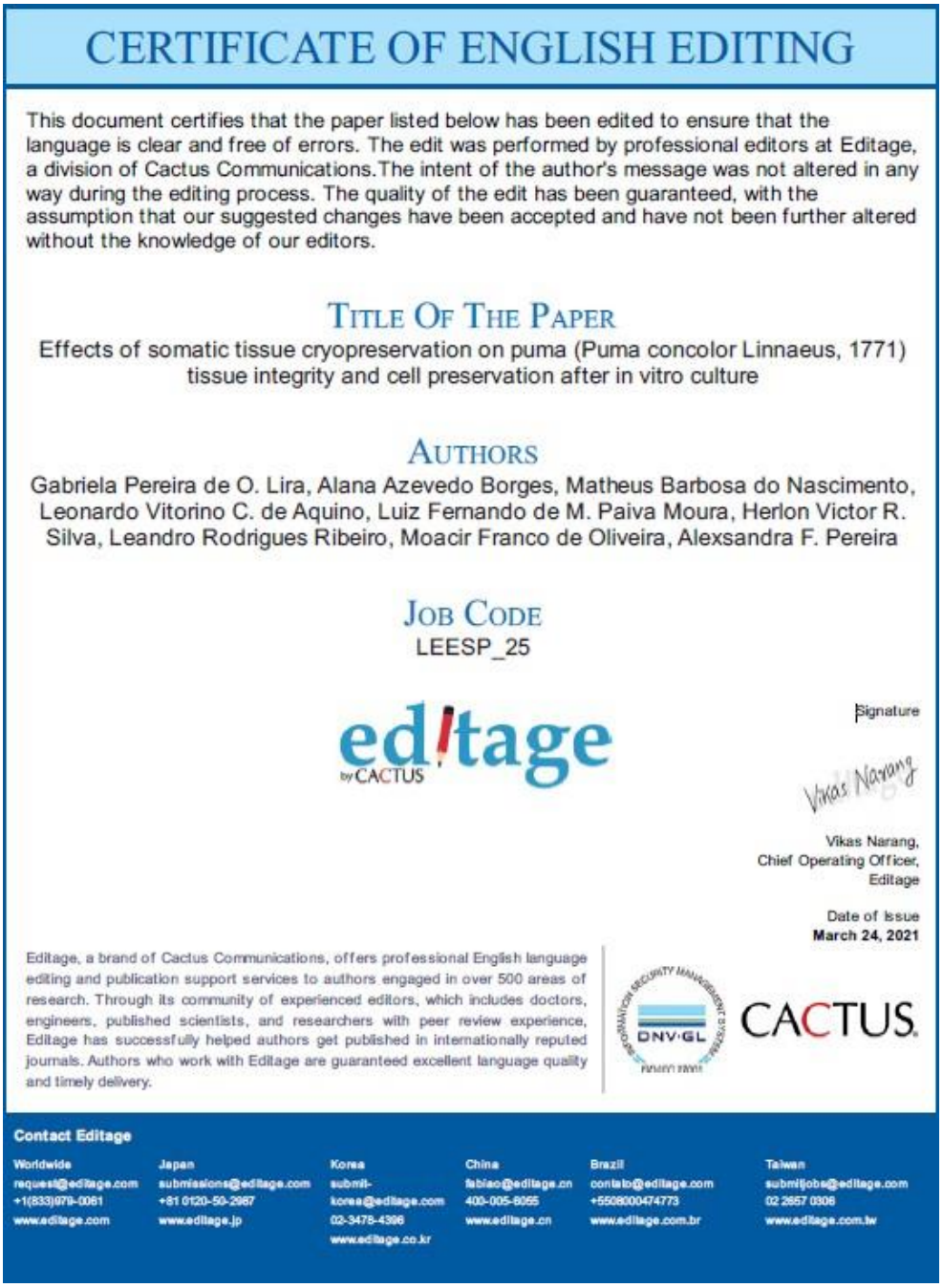
# APÊNDICES

**APÊNDICE A**: CERTIFICADO DE REVISÃO DO INGLÊS: EFFECTS OF SOMATIC TISSUE CRYOPRESERVATION ON PUMA (*Puma concolor* LINNAEUS, 1771) TISSUE INTEGRITY AND CELL PRESERVATION AFTER *IN VITRO* CULTURE



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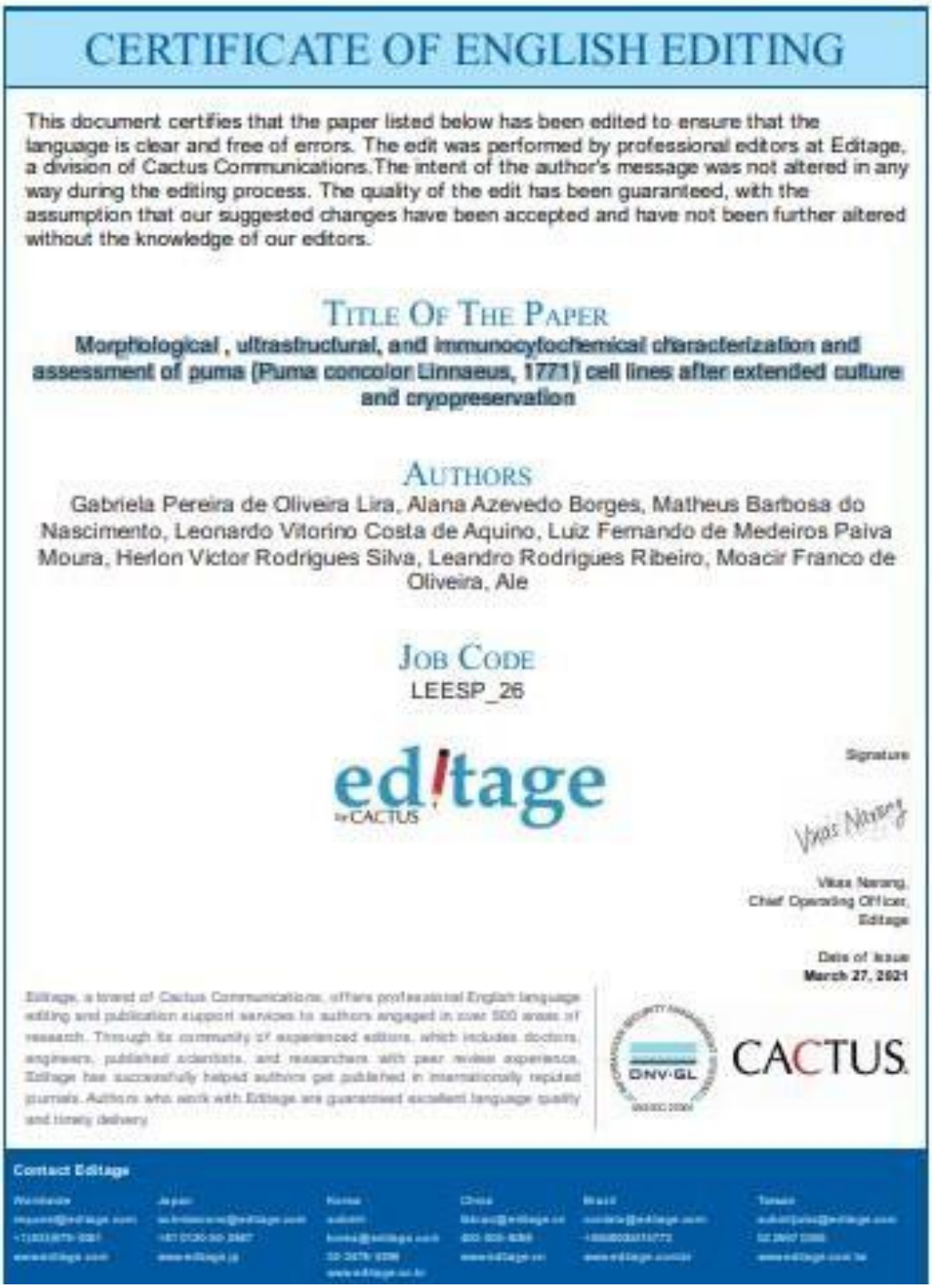

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