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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL
DOUTORADO EM CIÊNCIA ANIMAL

DENILSA PIRES FERNANDES

**ESTRATÉGIAS PARA CONSERVAÇÃO DE CÉLULAS E TECIDOS SOMÁTICOS
DE TATU-PEBA (*Euphractus sexcinctus*, LINNAEUS, 1758) PARA O
ESTABELECIMENTO DE BIOBANCOS**

MOSSORÓ

2024

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ESTABELECIMENTO DE BIOBANCOS**

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

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal.

Orientadora: Profa. Dra. Alessandra Fernandes Pereira

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*Dedico essa tese aos meus pais, Humberto
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“É justo que muito custe o que muito vale”

Santa Tereza D’Ávila

RESUMO

Os bancos de recursos somáticos são ferramentas efetivas para a conservação *ex situ in vitro* de espécies silvestres, tais como o *Euphractus sexcintus*, um importante mamífero da América do Sul com interesse ecológico, econômico e científico. Para que esses bancos sejam eficientemente desenvolvidos é necessário seu estabelecimento adequado. Nesse contexto, o objetivo foi investigar as condições envolvidas na formação de biobancos de células e tecidos somáticos de *E. sexcintus*, avaliando as condições de obtenção, cultivo *in vitro*, criopreservação e sincronização do ciclo. Para tanto, a tese foi dividida em três etapas. Na primeira etapa, visando o estabelecimento de bancos de tecidos somáticos, fragmentos de pele e cartilagem foram criopreservados comparando dois métodos (congelação lenta [CL] e vitrificação em superfície sólida [VSS]). Além disso, na VSS, duas combinações de crioprotetores (1,5M-EG [etilenoglicol] com 1,5M-DMSO [dimetilsulfóxido] vs. 3M-EG com 3M-DMSO) foram comparadas. A segunda etapa foi dividida em dois experimentos em que, visando o estabelecimento de bancos de células somáticas, as células foram comparadas quanto à duração do cultivo (4ª, 7ª, e 10ª passagem) e criotolerância (experimento 1), e a soluções de criopreservação contendo 10% de DMSO e diferentes concentrações de soro fetal bovino (SFB, 10% vs. 20% vs. 40%), na ausência e presença de 0,2 M de sacarose (experimento 2). Na terceira etapa, visando avaliar a funcionalidade celular, células foram submetidas à métodos de sincronização do ciclo em G₀/G₁ (privação de SFB vs. inibição por contato vs. 15 e 30 µM de roscovitina) por diferentes tempos. Na primeira etapa, CL e VSS (1,5M-EG + 1,5M-DMSO) não alteraram a estrutura dos tecidos quanto à espessura da derme e pele total; porém, somente os grupos VSS garantiram a preservação da espessura da cartilagem. Além disso, todos os tecidos criopreservados apresentaram padrões normais quanto à quantificação das células epidérmicas, melanócitos e fibroblastos, e foram capazes de recuperar células em cultivo, mantendo os parâmetros de qualidade semelhantes aos tecidos não criopreservados. Na segunda etapa, foram identificadas cinco linhagens fibroblásticas por morfologia, cariotipagem e imunofluorescência. O cultivo *in vitro* após todas as passagens, bem como a criopreservação não afetaram a maioria dos parâmetros avaliados, como a viabilidade, atividade proliferativa, atividade metabólica e os níveis apoptóticos. Contudo, células criopreservadas apresentaram um aumento na permeabilidade da membrana mitocondrial e tempo de duplicação celular quando comparado com células não criopreservadas. Quanto à avaliação da criopreservação em diferentes soluções, nenhuma diferença foi observada para a morfologia e viabilidade (> 90%) das células antes e após a criopreservação. Adicionalmente, a adição de sacarose foi positiva na manutenção da atividade metabólica e nos níveis apoptóticos em todas as concentrações de SFB. Contudo, concentrações 10% e 40% de SFB com sacarose apresentaram uma redução no potencial de membrana mitocondrial, enquanto os grupos de 20% de SFB na ausência ou presença de sacarose mantiveram esse parâmetro similar às células não submetidas à criopreservação. Portanto, o grupo 20% de SFB na presença de sacarose promoveu maior conservação das células após a criopreservação. Já na terceira etapa, nenhuma diferença foi observada para a morfologia e viabilidade (~72–97%) entre os métodos de sincronização em G₀/G₁. Os resultados mostraram que a privação de SFB aumentou a porcentagem de células na fase G₀/G₁ de maneira dependente do tempo, onde o grupo 72 h (90,5 ± 0,8%) e 120 h (90,1 ± 0,9%) apresentaram maiores taxas em comparação ao grupo 24 h (86,1 ± 0,9%) e controle (69,7 ± 0,8%). Contudo, a inibição por contato por 24 h, 72 h e 120 h e a roscovitina por 12–24 h não foram hábeis em promover a sincronização em G₀/G₁. Em conclusão, nossos resultados indicam as condições adequadas de obtenção, cultivo *in vitro*, criopreservação e sincronização em G₀/G₁ de amostras somáticas de *E. sexcintus*, visando o estabelecimento de biobancos para a espécie.

Palavras-chave: Mamífero silvestre; recurso somático; cultivo *in vitro*; criopreservação; sincronização do ciclo celular.

ABSTRACT

Somatic resource banks are effective tools for the *ex-situ in vitro* conservation of wild species, such as *Euphractus sexcintus*, an important South American mammal with ecological, economic, and scientific interest. For these banks to be efficiently developed, their proper establishment is necessary. In this context, the aim was to investigate the conditions involved in the formation of biobanks of somatic tissues and cells of *E. sexcintus*, evaluating the conditions for obtaining, *in vitro* culture, cryopreservation, and cycle synchronization. To this end, the thesis was divided into three steps. In the first step, aiming to establish somatic tissue banks, cartilage and skin fragments were cryopreserved comparing two methods (slow freezing [SF] and solid-surface vitrification [SSV]). Furthermore, in SSV, two combinations of cryoprotectants (1.5M-EG [ethylene glycol] with 1.5M-DMSO [dimethyl sulfoxide] vs. 3M-EG with 3M-DMSO) were compared. The second step was divided into two experiments in which, aiming at the establishment of somatic cell banks, cells were compared regarding the duration of culture (4th, 7th, and 10th passage) and cryotolerance (experiment 1), and cryopreservation solutions containing 10% DMSO and different concentrations of fetal bovine serum (FBS, 10% vs. 20% vs. 40%), in the absence and presence of 0.2 M sucrose (experiment 2). In the third step, aiming to evaluate cellular functionality, cells were subjected to cycle synchronization methods in G₀/G₁ (FBS starvation vs. contact inhibition vs. 15 and 30 μ M roscovitine) for different times. In the first stage, SF and SSV (1.5M-EG+1.5M-DMSO) did not change the tissue structure in terms of the thickness of the dermis and total skin; however, only the VSS groups guaranteed the preservation of cartilage thickness. Furthermore, all cryopreserved tissues presented normal standards regarding the quantification of epidermal cells, melanocytes, and fibroblasts, and were able to recover cells in culture, maintaining quality parameters similar to non-cryopreserved tissues. In the second step, five fibroblast lines were identified by morphology, karyotyping and immunofluorescence. *In vitro* culture after all passages, as well as cryopreservation did not affect most of the parameters evaluated, such as viability, proliferative activity, metabolic activity, and apoptotic levels. However, cryopreserved cells showed an increase in mitochondrial membrane permeability and cell doubling time when compared to non-cryopreserved cells. Regarding the evaluation of cryopreservation in different solutions, no difference was observed for the morphology and viability (> 90%) of the cells before and after cryopreservation. Furthermore, addition of sucrose was positive in maintaining metabolic activity and apoptotic levels at all concentrations of FBS. However, concentrations of 10% and 40% of FBS with sucrose showed a reduction in mitochondrial membrane potential, while the groups of 20% of FBS in the absence or presence of sucrose maintained this parameter similar to cells not subjected to cryopreservation. Therefore, the 20% FBS group in the presence of sucrose promoted greater conservation of cells after cryopreservation. In the third step, no difference was observed for morphology and viability (~72–97%) between the synchronization methods in G₀/G₁. The results showed that FBS starvation increased the percentage of cells in the G₀/G₁ phase in a time-dependent manner, where the 72 h (90.5 $\pm$ 0.8%) and 120 h (90.1 $\pm$ 0.9%) groups (%) showed higher rates compared to the 24 h group (86.1 $\pm$ 0.9%) and control (69.7 $\pm$ 0.8%). However, contact inhibition for 24 h, 72 h and 120 h and roscovitine for 12–24 h were not able to promote synchronization in G₀/G₁. In conclusion, our results indicate the appropriate conditions for obtaining, *in vitro* culture, cryopreservation, and synchronization in G₀/G₁ of somatic samples of *E. sexcintus*, aiming to establish biobanks for the species.

Keywords: Wild mammal; somatic resource; *in vitro* culture; cryopreservation; cell cycle synchronization.

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LISTA DE ABREVIATURAS E SIGLAS

°C	Grau Celsius
µg	Micrograma
µL	Microlitro
µM	Micromolar
4th	Fourth
7th	Seventh
10th	Tenth
AgNOR	Argyrophilic nucleolar organizer regions
ART	Artificial Reproductive Technologies
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Cells/mL	Células por mililitro
CEUA	Comite de Ética para uso de animais
CI	Contact inhibition
CL	Congelamento lento
cm	Centímetro
cm ²	Centímetro quadrado
CMXRos	MitoTracker Red [®]
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO ₂	Dióxido de carbono
DMEM	Meio Essencial Mínimo Modificado por Dulbecco (Dulbecco's Modified Eagle Medium)
DMSO	Dimetilsufóxido (<i>dimethyl sulfoxide</i>)
Dr	Doutor
Dra	Doutora
DNA	Deoxyribonucleic acid
EG	Etilenoglicol (<i>ethylene glycol</i>)
g/mol	Grama por mol
FBS	Fetal bovine serum
Fig.	Figure
g	Grama
GRB	Genome resource bank
h	Hora

ICMbio	Chico Mendes Institute for Biodiversity Conservation
IUCN	International Union for Conservation of Nature
iPSCs	Induced pluripotent stem cells
LBA	Laboratório de Biotecnologia Animal (<i>Laboratory of Animal Biotechnology</i>)
LEIAS	Laboratório de Estudos em Imunologia e Animais Silvestres
LN	Liquid nitrogen
M	Molar
min	Minuto
mL	Mililitro
mM	Milimolar
mm	Milímetro cúbico
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
n°	Número
P	Probabilidade de significância
PBS	Phosphate buffered saline
PDT	Population double time
PPGCA	Programa de Pós-Graduação em Ciência Animal
Prof	Professor
Profa	Professora
ROS	Reactive oxygen species
RN	Rio Grande do Norte
SCNT	Somatic cell nuclear transfer
SFB	Soro fetal bovino
SF	Slow freezing
SISBIO	Sistema de Autorização e Informação em Biodiversidade
SS	Serum starvation
SSV	Solid-surface vitrification
VDC	Vitrificação direta em criotubos
VSS	Vitrificação em superfície sólida
Vs.	Versus
TNCS	Transferência nuclear de célula somática
TRA	Tecnologia de Reprodução Assistida
UI	Unidade de Internacional
UECE	Universidade Estadual do Ceará

UFC	Universidade Federal do Ceará
UFERSA	Universidade Federal Rural do Semi-Árido

LISTA DE SÍMBOLOS

@	Arroba
©	Copyright
®	Marca registrada
%	Porcentagem
+	Soma
=	Igual
±	Mais ou menos
<	Menor que
>	Maior que
×	Multiplicação
$\Delta\Psi_m$	Potencial de membrana mitocondrial

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CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

O *Euphractus sexcinctus* é uma das espécies de mamíferos silvestres que ocorrem nos biomas brasileiros (ABBA et al., 2014). Popularmente, a espécie é conhecida por tatu-peba e integra a superordem Xenarthra e à ordem Cingulata (GARDNER, 2005), a qual é particular dos tatus. Esses animais têm origem exclusiva no continente americano e distribuição majoritária na América do Sul (SANTOS et al., 2019).

A espécie é classificada como pouco preocupante quanto à ameaça de extinção (ABBA et al., 2014), e esse status é muitas vezes atrelado à espécie se encontrar amplamente distribuída, por ter uma população estável e ser bastante resistente a mudanças ambientais (ABBA et al., 2014). Apesar disso, a espécie é alvo de atropelamentos, perda de habitat e caça, principalmente em áreas rurais do Brasil (ABBA et al., 2014; TEIXEIRA et al., 2020; BARBOZA et al., 2011), o que tem contribuído para a redução da sua população (SILVA et al., 2015; RIBEIRO et al., 2017; ASCENÇÃO et al., 2022). Em contrapartida, o tatu-peba desperta o interesse em virtude de seu papel ecológico (BONATO et al., 2008; RODRIGUES et al., 2019), sua importância econômica (SANTOS et al., 2018) e biomédica (FROTA et al., 2012; OLIVEIRA et al., 2019), e, somado a sua fácil adaptação, constitui um interessante modelo experimental (SILVA et al., 2015).

Nesse contexto, o tatu-peba é sugerido para o desenvolvimento de técnicas de reprodução assistida (TRAs; AMORIM et al., 2011; SILVA et al., 2015; SOUSA et al., 2016), visando a conservação da própria espécie, como também de espécies do mesmo grupo que se encontram vulneráveis, como o tatu-bola (*Tolypeutes tricinctus*; MIRANDA et al., 2014) e o tatu-canastra (*Priodontes maximus*; ANACLETO et al., 2014). Contudo, no que se refere a estratégias de conservação, os estudos com o *E. sexcinctus* são escassos e, em comparação a outros mamíferos silvestres, pouco se conhece em relação a sua biologia reprodutiva e o estabelecimento de bancos de recursos genéticos (SILVA et al., 2015; SOUSA et al., 2023). Consequentemente, o desenvolvimento de tecnologias de reprodução assistida (TRAs) para a espécie ainda é um desafio. Tal realidade ressalta a necessidade de estudos com foco nessas lacunas e com o objetivo de implementar estratégias de conservação eficientes para a manutenção dos membros existentes desse grupo. Neste cenário, a formação de bancos de recursos genéticos consiste em uma importante estratégia de conservação *ex situ in vitro* que pode ser explorada para a espécie (SOUSA et al., 2023).

Portanto, com esta tese objetivou-se desenvolver protocolos de obtenção, cultivo *in vitro* e criopreservação de tecidos somáticos e de células somáticas da região auricular de *E. sexcintus*, bem como os métodos empregados na sincronização do ciclo celular em G_0/G_1 , visando a formação de biobancos para a espécie e para estudos de reprogramação nuclear, contribuindo assim para programas de conservação dos xenartros.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Características gerais e distribuição geográfica do *Euphractus sexcinctus*

O *Euphractus sexcinctus* (Linnaeus, 1758) é um mamífero silvestre típico da fauna sul-americana, pertencente à superordem Xenarthra (Xenon = estranho + Arthros = articulação), um dos grupos mais antigos de mamíferos placentários, representado pelos tatus, tamanduás e preguiças (LIU et al., 2011; GIBB et al., 2016). A espécie é nativa e restrita ao continente americano, popularmente conhecida por tatu-peba e é o único membro existente de seu gênero, *Euphractus* Wagler, 1830.

O tatu-peba é um animal solitário, tem hábitos diurnos, mas ocasionalmente podem estar ativos à noite (ABBA et al., 2014), e se alimenta principalmente de vegetais, insetos, pequenos vertebrados (REDFORD; WETZEL, 1985) e carcaças (ALVES et al., 2012). Quanto às suas características físicas, esses animais apresentam uma carapaça dura, flexível e de coloração amarelo-pálido, marrom ou marrom-avermelhado, com alguns pelos esbranquiçados e longos (**Figura 1**). Em geral, eles possuem 6 a 8 cintas de placas móveis na região mediana, a cabeça é cônica e achatada e têm uma cauda curta e cilíndrica (REDFORD; WETZEL, 1985). Na idade adulta podem atingir um comprimento corpóreo maior que 40 cm, com uma cauda de 20 a 25 cm, orelhas proporcionalmente longas de 3,5 a 4,1 cm e podem pesar entre 3 e 7 kg (REDFORD; WETZEL, 1985). Eles têm 5 dedos e suas garras são bem desenvolvidas para cavar e construir tocas (REDFORD; WETZEL, 1985).



Figura 1. Exemplar da espécie *E. sexcinctus*. Fonte: IUCN (2024).

O tatu-peba está presente em uma ampla área da América do Sul, ocorrendo em países como Argentina, Bolívia, Paraguai, Suriname, Uruguai e Brasil (ABBA et al., 2014; **Figura 2**). No Brasil, a espécie pode ser encontrada em todos os biomas nacionais – Amazônia, Mata Atlântica, Cerrado, Caatinga, Pantanal e Pampas (WETZEL et al., 2008; PAGLIA et al., 2012). Eles apresentam a maior distribuição geográfica entre os tatus (SANTOS et al., 2019). Em relação ao habitat, os tatus-peba são encontrados em áreas abertas, savanas, matagais e florestas secas e semidecíduas, com o foco no Cerrado e nas áreas alteradas (ANACLETO, 2007). Apresentando essa flexibilidade no uso de habitat, a espécie tem uma tendência populacional estável (ABBA et al., 2014) e, dependendo do bioma, a densidade populacional estimada pode variar (SCHALLER, 1983; DESBIEZ et al., 2010). Por exemplo, apesar da informação ser limitada, foram estimados 1,3 indivíduos por km² na Bolívia e no Brasil foram estimados 14 indivíduos/km² (IUCN SSC ASASG).

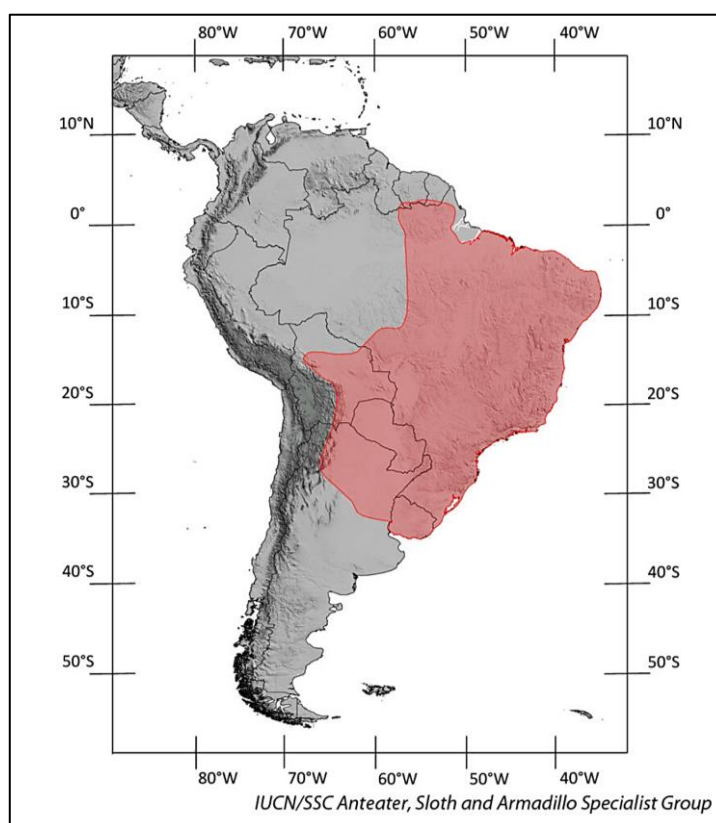


Figura 2. Distribuição geográfica (em vermelho) do tatu-peba (*E. sexcinctus*) na América do Sul. Fonte: IUCN SSC ASASG (2024).

O *E. sexcinctus* é classificado como Menos Preocupante (LC) pela lista vermelha da União Internacional para a Conservação da Natureza (IUCN; ABBA et al., 2014), e esse status

é muitas vezes atrelado à espécie se encontrar amplamente distribuída, por ter uma população estável e ser bastante resistente a mudanças ambientais (ABBA et al., 2014). Mesmo não sendo uma espécie em risco de extinção, o *E. sexcinctus* desperta o interesse em virtude de seu papel ecológico (BONATO et al., 2008; RODRIGUES et al., 2019), sua importância econômica (SANTOS et al., 2018) e biomédica (FROTA et al., 2012; OLIVEIRA et al., 2019), e, somado a sua fácil adaptação, constitui um interessante modelo experimental (SILVA et al., 2015; SOUSA et al., 2023).

2.2 Importância ecológica, econômica e científica do *Euphractus sexcinctus*

O *E. sexcinctus*, além de ser um exemplar único do seu gênero, desempenha um importante papel ecológico na região Neotropical onde a espécie é endêmica. A sua dieta variada e diferente dos outros tatus confere aos tatus-peba um importante elo intermediário nas redes alimentares. Acredita-se que os tatus sejam responsáveis pelo equilíbrio das colônias de cupins e formigas (REDFORD; WETZEL, 1985) e pela ciclagem de nutrientes do solo, ao cavar a matéria orgânica enquanto preparam suas tocas. Ainda, a espécie se mostrou predadora de vertebrados tão grandes quanto uma galinha (FOSTER et al., 2017). Contudo, os tatus-peba ainda dependem muito da matéria vegetal, potencialmente fazendo deles um dos principais agentes de dispersão de sementes entre os tatus (RODRIGUES et al., 2019).

Apesar da espécie não sofrer grandes ameaças, o tatu-peba é caçado extensivamente, principalmente para uso local, para fins comerciais/alimentares e recreacionais (caça esportiva; ALVES, 2012; ABBA; SUPERINA, 2010). No Brasil, a sua caça é ilegal; contudo, a sua carne é apreciada pelos nativos, principalmente nas regiões rurais do Nordeste brasileiro (BARBOZA et al., 2011; SANTOS et al., 2018) sendo popularmente explorado. Além da prática comercial, os tatus-pebas são utilizados para fins medicinais e mágico-religioso (BARBOZA et al., 2011; SOUTO et al., 2018). Os relatos apontam o uso da gordura do animal para o tratamento de várias enfermidades, como picadas de insetos, reumatismo e asma, e da sua cauda para proteção de “mau olhado” (BARBOZA, 2009). A nível laboratorial, foi demonstrada uma atividade anti-inflamatória tópica utilizando o óleo da gordura obtida da espécie (FERREIRA et al., 2014).

Contudo, a utilização insustentável de espécies silvestres para o consumo humano é apontada como uma das principais causas do declínio populacional das espécies caçadas (CAJAIBA et al., 2015). O tatu-peba é uma das principais espécies caçadas no semiárido brasileiro (MENDONÇA et al., 2015; ALVES et al., 2016; CHAVES et al., 2020; BORGES et al., 2021a), e é um dos que apresentam os maiores incidentes de atropelamentos (CÁCERES,

2011; CARVALHO-ROEL et al., 2019). A espécie também se torna alvo em virtude de sua reputação como praga agrícola, uma vez que invadem plantações e prejudicam a dinâmica dos solos por seu hábito de cavar (FALLABRINO; CASTIÑERA, 2006).

Além disso, os tatus se destacam como espécies modelo em pesquisas científicas devido as suas características únicas como boa adaptabilidade a cativeiros, alta resistência a hipóxia e baixa temperatura corporal e taxa metabólica (CÓDON et al., 2001; SILVA et al., 2015). Para fins de pesquisas biomédicas, a espécie desempenha um relevante papel na saúde, como reservatório natural para doenças, como a hanseníase (FROTA et al., 2012), sendo uma das principais espécies alvo dessa pesquisa no Brasil (OLIVEIRA et al., 2019; FERREIRA et al., 2020). A espécie atua como um modelo experimental para a multiplicação do patógeno (*Mycobacterium leprae*) com a finalidade de obter-se material biológico para diagnóstico e pesquisas de vacinas, uma vez que naturalmente podem hospedar e transmitir a doença causada pela bactéria, a hanseníase (FERREIRA et al., 2020). Mesmo nessa área, é destacada a dificuldade da reprodução da espécie em cativeiro como a limitação do seu uso em experimentos (OLIVEIRA et al., 2019). Adicionalmente, há registros da espécie como modelo em estudos de outras enfermidades que acarretam os humanos, como o carcinoma (ALVES et al., 2018). Além das patologias, outras áreas da saúde como a parasitologia, hematologia e imunologia tem a espécie como modelo de estudo (LOUGHRY et al., 2015).

Apesar da sua importância, ainda é observada uma escassez de conhecimento científico em relação a espécie, o que dificulta que ações de conservação sejam bem estabelecidas. Ao mesmo tempo, ações antrópicas sobre suas regiões de ocorrência vêm causando um declínio populacional das espécies (BARBOSA et al., 2022), enfatizando a importância do desenvolvimento de mais pesquisas. Adicionalmente, é importante considerar espécies de tatus que não estejam ainda em situação de risco de extinção como os tatus-pebas para serem usados como modelos experimentais para desenvolver pesquisas para espécies ameaçadas (BRASIL et al., 2020; SOUSA et al., 2023). Desta forma, sugere-se o desenvolvimento de estratégias conservacionistas para a espécie, sendo estas *in situ* ou *ex situ*, visando a manutenção da sua biodiversidade e beneficiando outras espécies da superordem Xenarthra.

2.3 Estratégias de conservação *in situ* e *ex situ* para o *Euphractus sexcinctus*

As estratégias para a conservação da espécie podem ser agrupadas em técnicas de conservação *in situ* e *ex situ* (ANDRABI; MAXWELL, 2007). As estratégias de conservação *in situ* permitem que populações viáveis da espécie sejam mantidas no seu próprio habitat

natural ou em um ambiente próximo ao seu habitat natural (ANDRABI; MAXWELL, 2007). Quanto a este tipo de abordagem, na espécie *E. sexcinctus*, a maioria dos estudos estão relacionados com áreas de conhecimento básico da espécie a fim de conhecer e compreender o comportamento desses animais na natureza, como a sua etnoecologia (MEDRI et al., 2009), dieta (DALPONTE; TAVARES-FILHO, 2004), padrão de atividade (OLIVEIRA et al., 2023), ocorrência e distribuição (BONATO et al., 2008; AYLA et al., 2021; BARBOSA, 2022), comportamento reprodutivo (COSTA et al., 2014; ROMERO et al., 2019) e morfometria (MEDRI, 2009). Além disso, estudos avaliando os efeitos da caça (BARBOZA et al., 2011; MESQUITA et al., 2018; LIMA et al., 2019) e atropelamentos rodoviários (ASCENÇÃO et al., 2022), duas das principais ameaças do *E. sexcinctus*, vem sendo realizados visando implementar medidas mitigatórias à conservação da espécie. Nesse sentido, grupos conservacionistas desenvolvem projetos na América do Sul visando coletar informações para compreender e proteger os xenartros (IUCN SSC ASASG, 2024), incluindo o *E. sexcinctus*.

Embora a conservação *in situ* seja uma estratégia valiosa, estes esforços são por vezes insuficientes para a manutenção da diversidade genética adequada, principalmente considerando a constante destruição de habitats e o ritmo alarmante de extinção de espécies. Assim, sugere-se que a mesma deve ser complementada por estratégias *ex situ* e por uma abordagem integrada para proteger a biodiversidade e os ecossistemas (PRITCHARD et al., 2012). Aqui, destaque-se as estratégias *ex situ*, que permitem a manutenção dos animais fora do seu ambiente (*ex situ in vivo*; PAIVA et al., 2016), sejam em cativeiros ou zoológicos, ou estabelecer uma população pela criopreservação de recursos genéticos, como gametas, embriões e outras amostras de células/tecidos ou DNA, para o uso futuro (*ex situ in vitro*; ANDRABI; MAXWELL, 2007; SOUSA et al., 2016). Os zoológicos e outros cativeiros da Europa, América do Norte, Central e Sul desempenham um papel importante na conservação dos tatus (SUPERINA, 2003). Contudo, ainda existem sérios problemas no cuidado e manutenção dos tatus nessas instalações, o que afeta diretamente na sua reprodução e saúde (SUPERINA, 2003). Nesse contexto, os relatos com os *E. sexcinctus* são escassos, porém, recentemente, visando promover o bem-estar de tatus-pebas mantidos em zoológicos, técnicas de enriquecimento ambiental foram propostas com sucesso (KELLY; ROSE, 2023).

Além disso, para o desenvolvimento de estratégias de conservação *ex situ*, incluindo principalmente a manutenção em cativeiro de espécies silvestres, torna-se necessário conhecer a fisiologia reprodutiva da espécie. Contudo, o que se refere as estratégias de conservação *ex situ* relacionados a biologia reprodutiva dos *E. sexcinctus*, também pouco se tem relatado comparado a outros mamíferos silvestres (SILVA et al., 2015). Adicionalmente, é crucial

considerar que aprimorar o conhecimento básico de uma espécie a nível biológico é um pré-requisito para o desenvolvimento de técnicas de reprodução assistida (TRAs), que são o foco dos programas de conservação.

Em machos, foram desenvolvidos estudos sobre comportamentos reprodutivos (FERNANDES et al., 2014), métodos de coleta de sêmen, avaliação das características espermáticas (SERAFIM et al., 2010), refrigeração (AMORIM et al., 2011) e criopreservação espermática (SOUSA et al., 2016). Em fêmeas, estudos relacionados à anatomia reprodutiva (REZENDE et al., 2013), comportamentos reprodutivos (COSTA et al., 2014), monitoramento do ciclo estral e mensuração hormonal (CAMPOS et al., 2016), caracterização da população folicular ovariana (BRASIL, 2020) foram desenvolvidos.

Além disso, o emprego de amostras genéticas, além dos gametas, como alternativa para a manutenção da diversidade genética destes animais representa uma abordagem interessante para o conhecimento e conservação da espécie. Assim, estudos usando amostras somáticas representam uma abordagem importante na formação de bancos de recursos genéticos.

2.4 Formação de bancos de recursos somáticos para a conservação da fauna silvestre

A criopreservação de células e tecidos somáticos constitui uma importante estratégia de conservação da biodiversidade no sentido de conservar o material genético a longo prazo, garantindo o seu uso não apenas em TRAs, mas também para estudos toxicológicos, genéticos e epidemiológicos (COMIZZOLI, 2017). O interesse nas amostras somáticas é atrelado às vantagens que essas amostras apresentam em comparação a conservação dos gametas, uma vez que amostras somáticas podem ser recuperadas independente do sexo da espécie (WITTAYARAT et al., 2013), idade, estando os animais vivos ou após a sua morte (SIENGDEE et al., 2018), como também a partir de diferentes regiões e tecidos (MESTRE-CITRINOVITZ et al., 2016). Além disso, a coleta de amostras somáticas é geralmente realizada a partir de regiões pouco invasivas, como é o caso do pavilhão auricular, fornecendo células em boa quantidade e qualidade (MESTRE-CITRINOVITZ et al., 2016; PRAXEDES et al., 2019, 2021).

Nesse contexto, diferentes protocolos e ajustes metodológicos para formação de bancos de células e tecidos somáticos de mamíferos silvestres vem sendo propostos, principalmente em relação as fontes de obtenção das amostras, aos tipos de técnicas de criopreservação que podem ser empregadas, aos crioprotetores e as concentrações dos mesmos, além das diferentes técnicas de análise da qualidade após o seu processamento (PEREIRA et al., 2018).

2.4.1 Estabelecimento e formação de bancos de tecidos somáticos

Os bancos de tecidos somáticos são coleções organizadas que conferem a conservação e manutenção de amostras, geralmente em condições criogênicas, por um tempo indeterminado. Ainda que seja objetivo final o isolamento de células somáticas para a sua posterior aplicação, o estabelecimento e formação de bancos de tecidos é uma alternativa necessária em casos de ausência de um protocolo para as células somáticas, ou até mesmo quando não seja possível o processamento imediato, permitindo assim que os fragmentos teciduais possam ser conservados e mantidas viáveis até o uso. Nesse sentido, é importante o desenvolvimento de um protocolo de conservação tecidual para tatu-peba.

Diversos órgãos podem constituir fontes viáveis de amostras somáticas, apresentando vários tipos celulares que são utilizados na conservação. Entre estes, a pele de diferentes regiões do animal é o órgão que tem sido bastante empregado nesse intuito, mediante as suas vantagens supracitadas, e ainda por ser uma fonte abundante de fibroblastos, que são células de interesse em reprogramação nuclear (VERMA et al., 2012; PEREIRA et al., 2018).

Nos mamíferos silvestres, três técnicas de conservação de tecidos somáticos foram desenvolvidas, a criopreservação por congelamento lento (PRAXEDES et al., 2019), por vitrificação (BORGES et al., 2017; COSTA et al., 2020; VIANA et al., 2023) e a refrigeração a 4–6°C (QUEIROZ NETA et al., 2018). A refrigeração confere apenas uma conservação a curto prazo do tecido e é utilizada em caso de coleta do material em regiões de difícil acesso e com limitação de materiais e condições laboratoriais adequadas (PEREIRA et al., 2018). Assim, para a conservação a longo prazo, as técnicas de criopreservação são as alternativas de maior interesse para a formação dos biobancos (**Quadro 1**).

A criopreservação consiste no conjunto de etapas que compreende desde a exposição das amostras aos crioprotetores, redução da temperatura, armazenamento, descongelamento e remoção dos crioprotetores das mesmas (JANG et al., 2017). Após a obtenção do tecido, estes podem ser submetidos a dois métodos de criopreservação: a congelamento lento (MAGALHÃES et al., 2017; BORGES et al., 2020) e a vitrificação (BORGES et al., 2017; COSTA et al., 2020). Esses se distinguem principalmente de acordo com o tempo de exposição a solução crioprotetora e a forma como a temperatura é reduzida com relação ao tempo (BORGES; PEREIRA, 2019).

A congelamento lento se baseia na redução da temperatura, havendo uma desidratação gradual dos tecidos e células. Por essa razão, necessita de baixas concentrações de

crioprotetores para reduzir a formação de cristais de gelo, que podem danificar e causar perda de viabilidade nas amostras biológicas (JANG et al., 2017). A congelação lenta consiste na elaboração de uma curva de resfriamento, onde a solução de criopreservação e a amostra biológica passam por fases de exposição e estabilização a temperaturas decrescentes para somente então serem expostas ao nitrogênio líquido (SILVA et al., 2018).

Já a vitrificação tem como princípio uma redução instantânea da temperatura tanto durante o congelamento onde os tecidos alcançam um estado vítreo, como durante o aquecimento, para que reduza a formação de cristais de gelo com as mudanças de temperatura (JANG et al., 2017). Em contraste à congelação, são utilizadas altas concentrações de crioprotetores com o intuito de aumentar a viscosidade do meio e reduzir os possíveis danos gerados pela queda abrupta da temperatura (JANG et al., 2017).

Assim como a congelação, a vitrificação possui algumas variações metodológicas que se distinguem pelas etapas do processo (BORGES; PEREIRA, 2019). Dentre estes tipos podem ser citados a vitrificação em superfície sólida (VSS), na qual o fragmento é exposto aos crioprotetores e dispostas sobre uma superfície de metal em contato com nitrogênio líquido para, posteriormente, ser transferido para criotubos e armazenados em nitrogênio líquido (BORGES et al., 2018a). Ambos os procedimentos, congelação lenta e vitrificação, já foram empregados na conservação da pele em diferentes mamíferos silvestres (**Quadro 1**).

Em relação a eficiência das técnicas de criopreservação aplicadas em tecidos somáticos de mamíferos silvestres, foi observada que a técnica por VSS resultou em melhores resultados na manutenção das características originais dos fragmentos originais nas espécies de cateto (BORGES et al., 2017), cutia (COSTA et al., 2020) e onça-pintada (PRAXEDES et al., 2019), constituindo uma metodologia promissora para outras espécies. Adicionalmente, a vitrificação tem por vantagem ser uma ferramenta de baixo custo, ser prático e gerar menos formação de cristais de gelo nas células em comparação com a congelação lenta, e, como vem sendo comprovado, mantém a eficiência das células após a descongelação. Por outro lado, eficientes protocolos de criopreservação de pele foram definidos sob uma taxa de congelamento mais lenta, avaliados em mamíferos silvestres como o urso-pardo (*Ursus arctos*; CAAMAÑO et al., 2008) e o lince-ibérico (*Lynx pardinus*; LÉON-QUINTO et al., 2011).

319 **Quadro 1.** Estabelecimento de bancos de tecidos somáticos em mamíferos silvestres usando técnicas de criopreservação e crioprotetores.

Espécie	Fonte	Técnica de criopreservação	Crioprotetores	Principais conclusões	Autores
Urso-pardo (<i>Ursus arctos</i>)	Pele femoral	CL x VDC	10% DMSO + 10% SFB (CL); 20% EG + 20% DMSO + 20% SFB (VDC).	CL permitiu uma maior e mais rápida proliferação celular comparada a VDC.	Caamaño et al. (2008)
Lince-ibérico (<i>Lynx pardinus</i>)	Pele de diferentes regiões	CL	5%, 7,5%, 10%, 12,5% e 15% DMSO, na ausência e presença de 0,1 M ou 0,2 M sacarose.	DMSO a 10% associado ou não com 0,2 M sacarose foi mais apropriada.	León-Quinto et al. (2011)
Cateto (<i>Pecari tajacu</i>)	Pele auricular	VSS x VDC	20% EG + 20% DMSO + 0,25 M sacarose + 10% SFB.	VSS foi o método mais adequado.	Borges et al. (2017)
Cateto (<i>Pecari tajacu</i>)	Pele auricular	VSS	3,0 M EG ou 3,0 M DMSO ou 1,5 M EG + 1,5 M DMSO (+ 10% SFB, na ausência ou presença de 0,25 M sacarose).	O melhor resultado foi a combinação de 3,0 M EG e 0,25 M sacarose com 10% SFB.	Borges et al. (2018b)
Onça-pintada (<i>Panthera onca</i>)	Pele auricular	CL x VSS x VDC	1,5 M DMSO + 0,25 M sacarose + 10% SFB.	VSS foi considerada o método mais eficiente.	Praxedes et al. (2019)
Cutia (<i>Dasyprocta leporina</i>)	Pele auricular	VSS x VDC	20% EG + 20% DMSO + 0,25 M sacarose + 10% SFB.	VSS foi considerada o método mais eficiente.	Costa et al. (2020)

Peixe-boi-marinho (<i>Trichechus manatus manatus</i>)	Pele abdominal	VSS	3,0 M EG ou 3,0 M DMSO ou 3,0 M EG + 3,0 M DMSO ou 1,5 M EG + 1,5 M DMSO (+ 10% SFB + 0,25 M sacarose).	Nenhum dos fragmentos criopreservados nas diferentes soluções foi capaz de produzir células <i>in vitro</i>	Nascimento et al. (2022)
Jaguatirica (<i>Leopardus pardalis</i>)	Pele auricular	VSS x VDC	3,0 M DMSO + 10% SFB + 0,25 M sacarose	VSS garantiu uma maior qualidade celular após o cultivo <i>in vitro</i> dos tecidos	Viana et al. (2023)

320 CL: Congelação lenta; VDC: vitrificação direta em criotubos; VSS: vitrificação em superfície sólida; EG: Etilenoglicol; DMSO: Dimetilsulfóxido;

321 SFB: Soro Fetal Bovino.

Independente da técnica de criopreservação aplicada, são utilizados dois tipos de crioprotetores: intracelulares ou penetrantes e extracelulares ou não penetrantes. Esses crioprotetores são usados em diferentes concentrações e combinações com o intuito de manter a integridade e viabilidade tecidual após a criopreservação. Tanto a presença como a escolha dos crioprotetores a serem utilizados num protocolo de criopreservação são fatores importantes que ditarão a eficiência da técnica (BORGES et al., 2018b). Devido ao mecanismo de ação distinto dos crioprotetores, a combinação de intracelulares e extracelulares tem como intuito a atuação sinérgica, visando maximizar a proteção da estrutura celular e tecidual (ELLIOT et al., 2017). Em mamíferos silvestres, os crioprotetores intracelulares mais comumente utilizados são o dimetilsulfóxido (DMSO) e o etilenoglicol (EG), que agem se ligando a moléculas de água, reduzindo a formação de cristais de gelo. Os extracelulares mais utilizados são a sacarose e o SFB, que atuam na manutenção do equilíbrio osmótico, reduzindo a formação de cristais de gelo (WENG et al., 2018).

A combinação de crioprotetores já foi empregada com sucesso na criopreservação dos tecidos somáticos em mamíferos silvestres. Na criopreservação por VSS da pele auricular da Jaguatirica (*Leopardus pardalis*), a combinação de 3,0 M de DMSO com 0,25 M de sacarose e 10% de SFB garantiu a conservação do tecido quanto a manutenção do seu potencial de cultivo *in vitro* após a descongelação (VIANA et al., 2023). Em catetos, a associação de 3,0 M de EG com 0,25 M de sacarose e 10% de SFB resultou em uma melhor conservação dos tecidos somáticos durante a VSS (BORGES et al., 2018a; 2018b). Costa et al. (2020) obtiveram bons resultados em relação a qualidade do tecido auricular de cutias após a sua criopreservação por VSS utilizando uma combinação de 20% de DMSO e 20% de EG, 0,25 M de sacarose e 10% de SFB.

Apesar dos ajustes realizados em protocolos de criopreservação tecidual a partir da aplicação da técnica em mamíferos silvestres, em xenartros, a criopreservação de tecidos somáticos ainda não foi relatada.

2.4.2 Estabelecimento e formação de bancos de células somáticas

Os fibroblastos oriundos de tecido auricular são notoriamente as células de eleição para aplicação em TRAs, em virtude da sua obtenção e manutenção em cultivo *in vitro*, além de uma grande quantidade de células a partir de um único explante (SANTOS et al., 2015). Em xenartros, Yang et al. (2006) empregaram uma linhagem de fibroblastos oriundos de uma biópsia de pele de preguiça (*Choloepus didactylus*) e tamanduá (*Tamandua tetradactyla*), além

de um macho pangolin (*Meloidogyne javanica*). Contudo, a abordagem do estudo foi sobre a organização filogenética desses mamíferos silvestres mediante mapeamento do genoma.

A formação dos bancos de células somáticas depende do estabelecimento de linhagens celulares pela sua avaliação de acordo com o tempo de cultivo e condições de criopreservação. Durante o cultivo *in vitro* é importante acompanhar o desenvolvimento das células, as suas características biológicas e ajustar as condições que elas são submetidas. Nesse contexto, o número de passagens pode causar danos nucleares e citoplasmáticas nas células em cultivo (MAGALHÃES et al., 2017). Dessa forma, em protocolos de cultivo se propõe a avaliação da qualidade celular ao longo de diferentes passagens, e assim definir até que passagem as células não sofrem alterações negativas. Em geral, as passagens avaliadas podem variar da terceira a décima passagem (MAGALHÃES et al., 2017; SELOKAR et al., 2018; BORGES et al., 2020).

Magalhães et al. (2017) relataram que fibroblastos criopreservados de veado-catingueiro (*Mazama gouazoubira*) apresentaram boa viabilidade (> 80%) após a quarta e sétima passagem, porém reduzindo a sua qualidade na décima passagem. Siengdee et al. (2018), com fibroblastos oriundos do tecido auricular de elefantes-asiáticos (*Elephas maximus*), obtiveram bons resultados na quarta e na sétima passagem. Adicionalmente, a primeira passagem das células tem sido avaliada; contudo, nessa fase podem ser observadas tanto fibroblastos como células epiteliais, e, por outro lado, na terceira passagem apenas os fibroblastos são visualizados (BORGES et al., 2020). No mesmo estudo (BORGES et al., 2020) não foi verificada diferença nas taxas de viabilidade celular entre a primeira, terceira e décima passagem.

Além disso, uma boa eficiência da criopreservação das células é acessada quando a técnica promove a conservação da sua morfologia, integridade do genoma, viabilidade e funcionalidade. Dessa maneira, alguns fatores como as taxas de decréscimo de temperatura e descongelação, o tipo e a concentração de crioprotetores e o tipo celular influenciam na eficiência da técnica. Em mamíferos silvestres, a técnica de criopreservação por congelamento lento tem sido usualmente empregada (**Quadro 2**).

A congelamento lento é caracterizada pela diminuição gradual da temperatura, utilizando um freezer programável ou recipientes de congelamento (Mr. Frosty), ressuspensão das células às soluções com baixas concentrações de crioprotetores (BORGES et al., 2020). Siengdee et al. (2018) obtiveram 95,5% de células viáveis após descongelamento, usando a congelamento lento.

387 **Quadro 2.** Estabelecimento de bancos de células somáticas derivados da pele auricular em mamíferos silvestres comparando diferentes
 388 crioprotetores.

Espécie	Passagem celular	Crioprotetores	Principais conclusões	Autores
Veado-catingueiro (<i>Mazama gouazoubira</i>)	4 ^a , 7 ^a e 10 ^a	10% DMSO + 10% SFB	Taxas de viabilidade superiores a 80% após a criopreservação.	Magalhães et al. (2017)
Camelo-asiático (<i>Camelus bactrianus</i>)	4 ^a -6 ^a	10% DMSO + 20% SFB	90% de viabilidade e o material genético conservado após a congelação.	Sharma et al. (2018)
Búfalo-asiático (<i>Bubalus bubalis</i>)	5 ^a -10 ^a	10% DMSO + 20% SFB	Criopreservação não afetou a produção <i>in vitro</i> de embriões clonados.	Selokar et al. (2018)
Elefante-asiático (<i>Elephas maximus</i>)	2 ^a -4 ^a	10% DMSO + 40% SFB	Viabilidade foi mantida após a criopreservação.	Siengdee et al. (2018)
Dromedário (<i>Camelus dromedarius</i>)	2 ^a -4 ^a	10% DMSO + 40% SFB	Fibroblastos mantiveram a sua capacidade de proliferação após a criopreservação.	Saadeldin et al. (2019)
Cateto (<i>Pecari tajacu</i>)	3 ^a	10% DMSO + 10% SFB + 0,2 M sacarose	Criopreservação não influenciou a viabilidade, nem a atividade metabólica e PDT. Contudo, alterou os níveis de EROs e $\Delta\Psi_m$.	Borges et al. (2020)
Onça-pintada (<i>Panthera onca</i>), gato- do-mato-pequeno	2 ^a -3 ^a	2,5% DMSO + 10% SFB ou 10% DMSO +	CryoSOfree, 2,5% e 10% DMSO foram eficientes para <i>L. tigrinus</i> e <i>L. colocolo</i> . Para <i>P. onca</i> , apenas as soluções com 2,5%	Arantes et al. (2021)

(<i>Leopardus tigrinus</i>) e colocolo (<i>Leopardus colocolo</i>)		10% SFB ou e 10% de DMSO apresentaram eficiência semelhante.	
Cutia (<i>Dasyprocta leporina</i>)	2 ^a , 5 ^a e 8 ^a	10% DMSO + 10% SFB + 0,2 M sacarose	Criopreservação não influenciou a viabilidade, atividade metabólica, PDT e nem os níveis de EROs, porém teve um efeito negativo sobre o $\Delta\Psi_m$. Praxedes et al. (2021)
Puma (<i>Puma concolor</i>)	1 ^a , 3 ^a e 10 ^a	10% DMSO + 10% SFB + 0,2 M sacarose	Criopreservação não afetou a viabilidade, metabolismo ou atividade proliferativa. Lira et al. (2022)
Puma (<i>Puma concolor</i>)	3 ^a	2,5% DMSO ou 10% DMSO ou 2,5% EG ou 10% EG (+ 10% SFB, na ausência ou presença de 0,25 M sacarose)	Todas as combinações de DMSO ou EG em diferentes concentrações (2,5% e 10%), com ou sem sacarose, foram eficientes. Rodrigues et al. (2022)
Cutia (<i>Dasyprocta leporina</i>)	3 ^a	10% DMSO + (10%, 40%, 90%) SFB, na ausência ou presença de 0,2 M sacarose	A combinação de 10% DMSO, 90% de SFB e 0,2 M de sacarose promoveu maior conservação da qualidade das células após o descongelamento Praxedes et al. (2023)

389 DMSO: Dimetilsulfóxido; SFB: Soro Fetal Bovino; PDT: Tempo de Duplicação da População; ERO: Espécies Reativas de Oxigênio; $\Delta\Psi_m$:
390 potencial da membrana mitocondrial.

Em relação aos crioprotetores, as soluções são compostas por 10% DMSO em combinação com o SFB em diferentes concentrações (**Quadro 2**; MAGALHÃES et al., 2017; SIENGDEE et al., 2018; SAADELDIN et al., 2019; PRAXEDES et al., 2023). Embora as concentrações de 10–20% de SFB são usualmente testadas, no *E. maximus* foi observado que 40% SFB resultou em uma melhor viabilidade das células pós-criopreservação (SIENGDEE et al., 2018). Adicionalmente, a sacarose, que também atua como um crioprotetor extracelular, foi testada na concentração de 0,2 M na congelação lenta de fibroblastos, onde foi observado um aumento nas taxas de viabilidade celular pós-descongelação (LEÓN-QUINTO et al., 2011; BORGES et al., 2020; PRAXEDES et al., 2023). Diante de tais variações, principalmente em relação a concentração de SFB e presença de sacarose, é necessária a criopreservação adequada de fibroblastos de tatu-peba, avaliando a composição da solução de criopreservação.

2.4.2.1 Estudos de reprogramação nuclear de células somáticas

O interesse em células somáticas na formação de biobancos teve o seu início com os avanços significativos de tecnologias, como a clonagem por transferência nuclear de célula somática (TNCS; IQBAL et al., 2021) e geração de células-tronco pluripotentes induzidas (iPSCs; SWEGEN et al., 2023), sobretudo pela sua capacidade de reprogramação nuclear (MASTROMONACO et al., 2014). Apesar da TNCS ter suas limitações e apresentar reduzida eficiência, a partir dessa metodologia foi comprovada que a pluripotência de núcleos de células adultas diferenciadas pode ser “recuperada” (CHEN et al., 2007).

Para a reprogramação nuclear adequada, as células somáticas precisam ser submetidas à sincronização do ciclo em G_0/G_1 de modo a permitir a remoção de alterações epigenéticas reversíveis adquiridas durante a diferenciação celular (MALIN et al., 2022). Alguns tratamentos de sincronização, como a inibição por contato, privação de soro (SFB) e agentes químicos, vêm sendo aplicados em células de mamíferos silvestres (SELOKAR et al., 2012; PEREIRA et al., 2019; BORGES et al., 2021). Em geral, o método varia de espécie para espécie. Em roedores, como os ratos, a inibição por contato não mostrou diferença na taxa de blastocistos gerada por TNCS usando células somáticas sincronizadas em G_0/G_1 , em comparação com a privação de soro (HAYES et al., 2001), método este adotado em búfalo-d'água-selvagem (*Bubalus arnee*; PRIYA et al., 2014), macaco-cinomologo (*Macaca fascicularis*; LIU et al., 2018). Adicionalmente, o método de privação de soro resultou em aumento significativo da porcentagem de células em G_0/G_1 nas primeiras 24 h de tratamento, conforme relatado em espécies silvestres, como o panda gigante (*Ailuropoda melanoleuca*;

87,8%; HAN et al., 2003), bisões (*Bos Gaurus*; 84,1%; MAHESH et al., 2011) e espécies de felinos (74,6–91,3%; WITTAYARAD et al., 2012). Em felídeos silvestres tem sido aplicada a privação de soro a 0,5% por diferentes períodos (24–120 h; VERAGUAS et al., 2017; MOULAVI et al., 2017; MŁODAWSKA et al., 2022).

Além dos métodos supracitados, Wittayarat et al. (2013) avaliaram a roscovitina por 24 h em três concentrações (7,5, 15 e 30 μ M) na sincronização do ciclo celular em quatro espécies de felídeos silvestres, onde concluíram que os fibroblastos dessas espécies respondem diferentemente a cada tratamento, sendo que o tratamento com mais de 7,5 μ M aumentou a proporção de células G₀/G₁ em uma das espécies avaliadas. Em concordância, Selokar et al. (2012) concluíram que o tratamento com 30 μ M de roscovitina resultou numa maior quantidade de células em estágio G₀/G₁ em comparação ao método por privação de soro ou inibição por contato. Até o momento, nenhuma informação foi relatada sobre a sincronização do ciclo em fibroblastos de tatus. Com esta tese, objetivou-se avaliar pela primeira vez os métodos supracitados na sincronização do ciclo em G₀/G₁ em fibroblastos de tatus-pebas, visando a sua obtenção para estudos de reprogramação nuclear.

3 JUSTIFICATIVA

Os tatus representam o grupo mais diverso entre os xenartros, e são considerados importantes para o entendimento da história evolutiva e a relação filogenética da superordem. Mesmo diante da sua importância, o grupo é pouco explorado pela comunidade científica. Essa escassez de informações dificulta a aplicação de estratégias de conservação para algumas espécies que se encontram vulneráveis ou em perigo de extinção.

Dessa maneira, considerando que a formação de bancos de recursos genéticos constitui uma relevante estratégia de conservação *ex situ in vitro* de mamíferos silvestres, torna-se necessária a definição de protocolos eficientes de cultivo e criopreservação de amostras somáticas em tatu-peba, haja vista a escassez de referência da técnica em qualquer xenartro existente. Além disso, a espécie pode ser utilizada como modelo experimental em diversas pesquisas, pois além de apresentar uma população estável na natureza, tem maior disponibilidade de exemplares em cativeiro. Nesse contexto, a formação de bancos de recursos somáticos permite disponibilizar material genético de alto valor e recurso celular para pesquisas científicas básicas e em diversas áreas de estudo, incluindo a filogenética, biologia evolutiva e fisiologia. Essa conservação de células viáveis confere uma fonte renovável de material genético que pode ser cultivado após longos períodos ou até mesmo indefinitivamente.

Finalmente, sabe-se que as células somáticas diferenciadas possuem a capacidade de serem reprogramadas *in vitro* em estágio de indiferenciação, semelhante às células embrionárias. Dessa maneira, os estudos com células somáticas permitem a sua aplicação em outras biotécnicas, como a clonagem por TNCS e geração de iPSCs a partir de células somáticas. Especialmente se tratando de animais silvestres, essas novas abordagens oferecem oportunidades importantes na conservação das espécies, como o *E. sexcinctus*, aumentando a disponibilidade de biotecnologias para a reprodução assistida.

4 HIPÓTESES CIENTÍFICAS

I – A vitrificação em superfície sólida é mais adequada para a criopreservação da pele e cartilagem auricular de tatus-peba, quando comparada à congelação lenta, promovendo uma maior manutenção das características histológicas e da funcionalidade celular após o aquecimento;

II – A redução das concentrações de etilenoglicol (EG) e dimetilsulfóxido (DMSO) em combinação resulta em uma maior proteção tecidual e celular após a vitrificação em superfície sólida da pele e cartilagem auricular de tatus-pebas;

III – Células somáticas de tatus-pebas podem ser cultivadas por mais de quatro passagens, mantendo suas características fenotípicas e genotípicas normais;

IV – Concentrações maiores de 10% de SFB e a adição de sacarose na criopreservação de células somáticas de tatus-pebas promove uma maior manutenção da qualidade celular após a descongelação;

V – A sincronização de células somáticas de tatus-peba usando privação de soro por 24 h garante uma maior quantidade de células em G_0/G_1 , quando comparado ao uso da roscovitina e inibição por contato.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Estabelecer as condições para a formação de banco de células e tecidos somáticos de tatus-pebas, visando contribuir para a conservação da espécie.

5.2. OBJETIVOS ESPECÍFICOS

Comparar técnicas de criopreservação (congelamento lento vs. vitrificação em superfície sólida) e avaliar a combinação de crioprotetores intracelulares (EG + DMSO) em diferentes concentrações (3 M ou 6 M, para vitrificação em superfície sólida) na conservação da pele e cartilagem auricular de tatus-pebas;

Obter, isolar e caracterizar linhagens celulares de tatus-pebas avaliando o efeito do cultivo *in vitro* prolongado até a décima passagem;

Criopreservar células somáticas de tatus-pebas e avaliar o efeito de soluções de criopreservação contendo 10% de DMSO acrescido de diferentes concentrações de SFB (10%, 20% ou 40%) na ausência ou presença de 0,2 M de sacarose;

Avaliar diferentes métodos de sincronização (privação do soro vs. inibição por contato vs. roscovitina) de células somáticas de tatus-pebas no estágio G₀/G₁;

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**CAPÍTULO 2 – *EX SITU* APPROACHES FOR THE CONSERVATION OF GENETIC
RESOURCES IN THE SUPERORDER XENARTHRA (Artigo de revisão)**

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***Ex Situ* Approaches for the Conservation of Genetic Resources in the Superorder Xenarthra**

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Running title: *Ex situ* Conservation of Xenarthrans

Abstract

Xenarthra—a superorder of placental mammals endemic to the Neotropics—is represented by
armadillos, anteaters, and sloths. Considering their long history in the Americas, extant xenarthrans
represent an important group for understanding the impact of past environmental changes on
species diversification and serve key ecological functions as ecosystem engineers. Unfortunately,
most wild xenarthran populations are at risk due primarily to anthropogenic activities, necessitating
urgent conservation efforts. Moreover, the paucity of information on some species has rendered
population estimation and, consequently, conservation management challenging. Additionally,
relatively few groups are researching this superorder, perhaps because fieldwork with armadillos,

anteaters, or sloths and their captive care are challenging tasks. Nevertheless, dedicated research and efforts to ensure the long-term conservation of these animals are deemed essential. In this context, cryobanks are a practical approach for breeding and maintaining genetic diversity in wildlife, and they are important tools for assisting and improving both *ex situ* and *in situ* conservation strategies. Therefore, cryopreservation of biological resources may be a promising strategy for conserving xenarthrans. Specifically, semen cryopreservation, which has already been applied in some species, may be the most effective strategy for this group. The present article provides an overview of *ex situ* conservation of xenarthrans, which will contribute to the development and implementation of additional strategies for protecting these unique mammals.

Keywords: Wildlife conservation, Cingulata, Genome resource banking, Pilosa, Threatened species

Introduction

Storage of genetic material in cryobanks may assist and improve the long-term conservation for several species.¹ Cryobanks, or genome resource banks (GRBs), constitute reserves of gametes, embryos, and gonadal or somatic tissues, representing an important long-term biodiversity conservation strategy through the preservation of genetic material of species for several purposes, such as the development of artificial reproductive technologies (ARTs) and application in basic biological, toxicological, genetic, and epidemiological studies.²

Typically, *ex situ* conservation involves the maintenance of live populations outside of their natural habitat (*ex situ in vivo* conservation) or preservation of genetic material in cryobanks (*ex situ in vitro* conservation). The consensus among conservationists is that habitat preservation is the most

effective measure for preserving biodiversity and saving wild populations. However, these *in situ* approaches are not practical for certain species; thus, *ex situ* actions are worthwhile and, at times, the last resort to establish viable populations and preserve genetic material.² Unfortunately, while cryopreservation is common in *ex situ* programs for some species, it remains a major challenge for xenarthrans.

In xenarthrans—a unique group of placental mammals—certain conservation strategies have been implemented, including political actions, practical interventions, and educational campaigns.^{3,4} Specifically, to establish GRBs for this group, semen cryopreservation may be the most effective approach. However, cryopreservation has been applied only in some xenarthran species. In addition, this group has lost substantial genetic information, as many species are already extinct, and some extant ones are on the brink of extinction.

Xenarthra—a superorder of mammals endemic to the Neotropics—includes both extinct and extant species of armadillos, anteaters, and sloths. These mammals are further divided into two monophyletic orders, namely Cingulata (armadillos) and Pilosa (anteaters and sloths)⁵, together comprising 37 described extant species, all endemic to the Americas.^{6,7} The name Xenarthra is derived from the characteristics of these animals, such as xenarthric joints, fused pelvic bones,⁸ slow metabolic rate, and low and variable body temperature, which differentiate them from other mammals.⁹

Xenarthrans predominantly inhabit South America, while a few species are present in Central and North America. Some species, such as the nine-banded armadillo (*Dasypus novemcinctus*), occur throughout the Americas.⁵ Owing to their evolutionary history in the American continent

(approximately 60–100 Mya),¹⁰ xenarthras constitute a promising model for studies on the impact of past environmental changes on species diversification.^{10,11} Yet, compared with other mammalian species, xenarthrans remain understudied.^{12,13,14}

The majority of the published studies on xenarthrans focused on the fundamental knowledge of their ecology, diet, habitat, distribution, activity pattern, evolution, and genetics,^{4,15} mostly aimed at developing effective conservation strategies. To this end, the present review seeks to provide an overview of *ex situ* conservation of xenarthrans and emphasize the need for further research to establish GRBs, particularly for species threatened with extinction—the reality of some xenarthran species whose populations continue to dwindle.

Why preserve xenarthrans?

Xenarthrans are essential components of their ecosystems. By virtue of their unique dietary habits, they aid insect control and nutrient cycling. In addition, these mammals, particularly armadillos, serve as ecosystem engineers and, thus, as indicators of environmental quality.¹⁶ Furthermore, the fossorial activities of xenarthrans for foraging and constructing burrows benefit other animals, including other xenarthrans, by serving as stable shelters and possible food sources.¹⁷ In addition to being specialized predators, xenarthrans are prey for other species, such as jaguars and wolves.¹⁸

Xenarthrans have attracted attention because of their importance as experimental models in biomedical research. For instance, armadillos are natural reservoirs of *Mycobacterium leprae*, which is the primary causative agent of leprosy. *Mycobacterium leprae* cannot grow in artificial media, and armadillos and primates are the only mammals other than humans known to be natural hosts of this bacterium.¹⁹ At present, *Dasypus novemcinctus* is considered the most suitable model

species for research on leprosy.¹⁹ Additionally, sloths and anteaters serve as pathological models in studies on leishmaniasis.²⁰ Meanwhile, in many regions, such as northeastern Brazil, meat and other products from xenarthrans, particularly armadillos, are consumed, indicating the close relationship between humans and these animals.²¹

In terms of the global conservation status, some xenarthran species is facing some risk of extinction.^{22,23} Currently, over 20% of xenarthran species are threatened by extinction.²⁴ In particular, pygmy three-toed sloth (*Bradypus pygmaeus*) is a critically endangered (CR) species (3%), while giant anteater (*Myrmecophaga tridactyla*), giant armadillo (*Priodontes maximus*), maned three-toed sloth (*Bradypus torquatus*), and Brazilian three-banded armadillo (*Tolypeutes tricinctus*) are vulnerable (VU) species. Maned three-toed sloth and Brazilian three-banded armadillo are endemic to Brazil, while pygmy three-toed sloth is endemic to Panamá. Five species (14%) are classified as data deficient (DD), and their conservation status remains unassessed. Furthermore, nine species (24%) are classified as not evaluated (NE), as these are considered new species. Overall, the current conservation status of nearly 40% of extant xenarthrans remains unknown, and the population trend for most species is either decreasing or unknown (Fig. 1).

Furthermore, the xenarthran species that are considered threatened globally are also endangered locally in countries where they are present.⁴ Even species that are listed globally as least concern (LC) are threatened in at least one country in which they are present. For instance, northern tamandua (*Tamandua mexicana*) is threatened in 42% of the countries in which it is present.⁴ This inconsistency may be reflected in taxonomic issues⁴ and may consequently delay the formulation of conservation strategies. Other factors attributed to the current conservation status of these species include habitat loss,²⁵ illegal trafficking, and roadkills.⁴

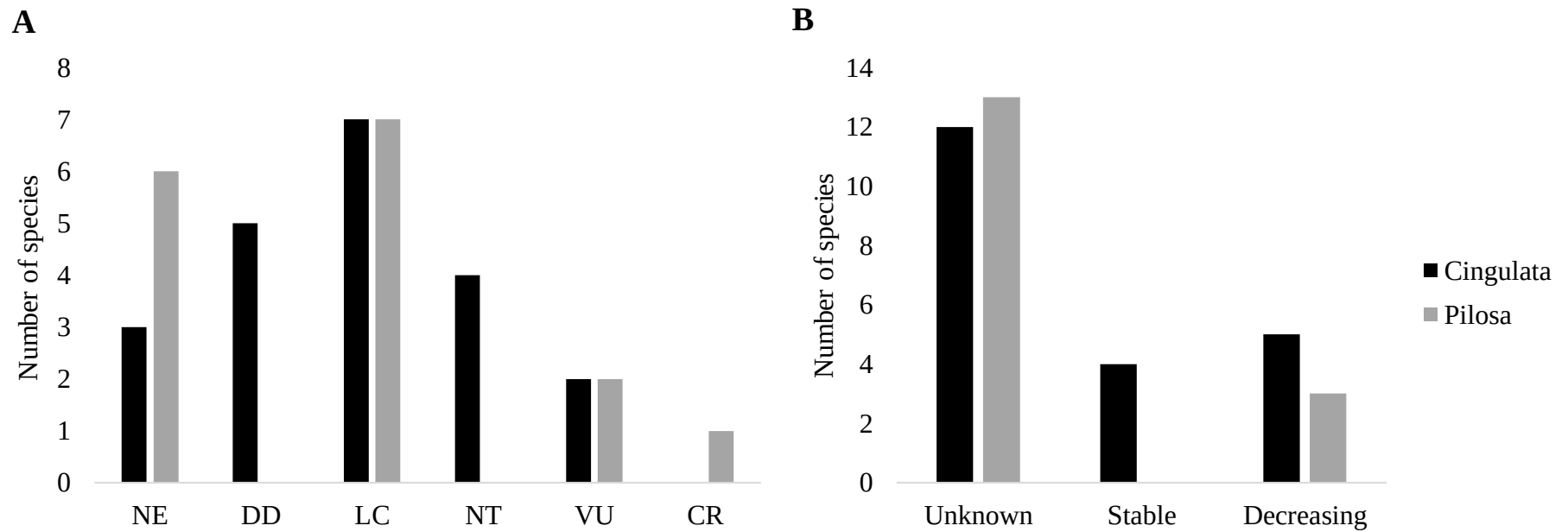


FIG. 1. Conservation status and population trend of xenarthrans. **(A)** Conservation status according to the division of xenarthrans in Cingulata and Pilosa. Conservation categories listed are NE, Not evaluated; DD, Data Deficient; LC, Least Concern; NT, Near Threatened; VU, Vulnerable; CR, Critically Endangered. **(B)** Population trend of Cingulata and Pilosa species.^{4,6,7,24}

1190 ***Ex situ* conservation of xenarthras**

1191 *In situ* conservation aims at maintaining the animals alive in their natural environment or an
 1192 environment close to their natural habitats.²⁶ Therefore, this is undoubtedly the most commonly
 1193 used strategy for conserving xenarthrans. Much information on the various xenarthran species has
 1194 been collected through *in situ* conservation.¹⁵ Table 1 lists several projects and programs developed
 1195 and implemented in American countries with the aim of collecting information for the conservation
 1196 of these mammals.

1197
 1198 Although habitat preservation is, according to conservationists, the most appropriate strategy to
 1199 conserve wildlife diversity, this approach alone does not suffice to prevent species extinction in the
 1200 face of the current global reality and uncertain future.^{1,27} In light of this, *ex situ* conservation
 1201 strategies offer essential knowledge and techniques for immediate intervention, primarily
 1202 considering the instability of species at the risk of extinction. Additionally, zoos and other facilities
 1203 around the Americas support most of the conservation projects for xenarthrans, playing a key role
 1204 in their preservation by maintaining the last individuals of most threatened species and collecting
 1205 genetic samples to establish GRBs.

1206
 1207 The establishment of GRBs benefits wildlife²⁸ and is, therefore, a promising strategy that should
 1208 be pursued in future studies on xenarthrans. Here, we curated studies reporting the establishment
 1209 of cryobanks for the conservation of xenarthrans (Table 2). All studies used male gametes of six-
 1210 banded armadillo (*Euphractus sexcinctus*)^{29,30} or giant anteater.^{31,32}

1211

1212 **TABLE 1.** Examples of organizations and ongoing projects committed to the conservation of xenarthrans.

Institution/Program/Project	Country	Started	General goals
Anteater, Sloth and Armadillo Specialist Group	International alliance	1980	Evaluation of the conservation status of xenarthran species for the IUCN Red List of Threatened Species
Fundación AIUNAU	Colombia	1996	Study and conservation of sloths
Green Heritage Fund Suriname – The Xenarthra Program	Suriname	2005	Shelter and Rehabilitation of Xenarthra; Education and Information; Habitat protection
Tamandua Institute	Brazil	2005	<i>In situ</i> and <i>ex situ</i> conservation of xenarthrans
Projeto Tatu canastra – The Giant Armadillo Project	Brazil	2010	Conservation of <i>Priodontes maximus</i>
Projeto Bandeiras e Rodovias – The Anteaters and Highways Project	Brazil	Not informed	Assesment the impact of roads on <i>Myrmecophaga tridactyla</i> populations in the Brazilian Cerrado
Tamanduaí Conservation Program in Northeast Brazil	Brazil	2011	Identify priority areas for the conservation of the species, restore degraded mangrove areas
Armadillo Conservation Programme	Colombia	2012	Conservation and management of armadillos in the Orinoco Llanos of Colombia
Saving the Pygmy Sloth from Extinction Project	Panama	2012	Improve the understanding of <i>Bradypus pygmaeus</i> population and threats to the species
Programa Tatu-bola – Armadillo program	Brazil	2013	Research and conservation of <i>Tolypeutes tricinctus</i> and protect its habitat.

	Armadillos de Chile – Chile Armadillos	Chile	2016	Promote research and initiate strategies for the three armadillo species (<i>Chaetophractus vellerosus</i> , <i>Zaedys pichiy</i> , <i>Chaetophractus villosus</i>)
	Projeto Preguiças – Sloth Project	Brazil	2019	Conservation of <i>Bradypus variegatus</i> on Bahia coast
1213	International Union for Conservation of Nature and Natural Resources			
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1235 **TABLE 2.** Protocols employed for semen cryopreservation among xenarthrans.

Species	Technical approach	Used solutions	Main results	Authors
<i>Euphractus sexcinctus</i>	Short-term preservation at 5 °C	Tris vs powdered coconut water (ACP®) extender	No immediate effect on sperm motility or viability; nevertheless, sperm motility was reduced after 3 h of storage.	Amorim et al. ²⁹
<i>Euphractus sexcinctus</i>	Fast freezing and storage in N ₂	Tris, 20% of egg yolk and 3% of glycerol	Significant decline in all semen parameters, in which 6.1% motile sperm were found; A variable resistance to cryoinjuries was observed.	Sousa et al. ³⁰
<i>Myrmecophaga tridactyla</i>	Cooling and storage at 5 °C	BotuCrio extender without cryoprotectant	Sperm motility index decreased from 0 to 24 h, and vigor did not change in the first 6 h and decreased after 18 h.	Luba et al. ³¹
<i>Myrmecophaga tridactyla</i>	Fast freezing and storage in N ₂	OptiXcell® medium	There was a decrease in vigor and sperm motility in post-thawing sample (3 and 30%, respectively) compared to fresh semen (4 and 70%, respectively).	Silva et al. ³²

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Over the years, interest in the preservation of somatic samples in GRBs as part of species conservation programs has increased.³³ This can be attributed to the advantages somatic samples offer over gametes. As such, somatic samples can be recovered regardless of sex, age, and seasonality from both live and dead animals as well as from different tissue sources.³⁴ Moreover, somatic samples are typically collected through minimally invasive procedures, such as skin biopsy. A successful long-term *in vitro* culture of skin cells from a six-banded armadillo was documented in a recent study.³⁵ Additionally, the rate of cell recovery and viability over long-term culture were high. This proved an important step toward establishing cryobanks and using these cells in ARTs.³⁵ Unfortunately, cryopreservation of somatic resources from xenarthrans has not been reported yet, highlighting the importance of testing protocols for preserving somatic tissues from these species.

Although all xenarthrans are assigned to a single super group, they differ in terms of morphological and ecological aspects. Examples of morphological and ecological differences among xenarthras include digging as the primary method of acquiring food in anteaters and armadillos,⁵ arboreal or suspensory locomotion in sloths, and the unique protective body armor of armadillos.³⁶ Additionally, these species have received differential scientific attention, with armadillos being the most studied xenarthran species, perhaps due to their biomedical importance.^{12,15} To better guide xenarthran conservation efforts, we have divided the discussion below by their groups.

Conservation perspectives on armadillos

Armadillos represent the most diverse group of xenarthrans. *Dasypus* is the only extant genus characterized by the monozygotic polyembryonic form of reproduction. Unlike other members of the superorder, armadillos possess a hard and flexible carapace composed of hundreds of

osteoderms.³⁶ These are semi-fossorial animals,³⁷ and their diet are one of the most diverse among xenarthrans. Armadillos are essential for the ecosystem due to their roles as predators, ecosystem engineers, seed dispersers, invertebrate vectors, and nutrient reserves.³⁹ Within Xenarthra, armadillos are the most studied species¹⁵, although species-specific studies are scarce.¹²

A review on armadillos by Superina et al. identified a large gap in the conservation of these species.¹² According to the authors, between 1660 and 2011, 3,117 articles on armadillos were published, and the most frequently cited research topic was healthcare, including studies on leprosy, parasitology, and pathology. Of the 21 extant armadillo species, four species, namely *Dasypus novemcinctus*, *Chaetophractus villosus*, *Chaetophractus vellerosus*, and *Euphractus sexcinctus*, account for two-thirds of all publications. Additionally, most studies were conducted in the United States, Brazil, Argentina, and Bolivia and were mostly performed in the laboratory, or field. The authors proposed that future research prioritize studies on the ecology, conservation, population genetics, reproduction, and threats of armadillos, particularly hairy long-nosed armadillo (*Dasypus pilosus*), Brazilian three-banded armadillo, and pink fairy armadillo (*Chlamyphorus truncatus*).

A good example of armadillo conservation efforts is the Armadillo Conservation Program initiated in 2012 in Orinoco Llanos, Colombia³ (Table 1). The program developed multiple strategies to raise awareness regarding five armadillo species, namely southern naked-tailed armadillo (*Cabassous unicinctus*), nine-banded armadillo, greater long-nosed armadillo (*Dasypus kappleri*), northern long-nosed armadillo (*Dasypus sabanicola*), and giant armadillo. The program achieved excellent engagement of local communities and encouraged restaurants to avoid selling wild armadillo meat. Moreover, the program addressed the topic of armadillo

conservation in various educational settings, published pamphlets and books, and established breeding and rehabilitation facilities for maintaining confiscated armadillos.

Regardless of the limited information available, armadillos are used as experimental models for developing ARTs considering their adaptability to captive environments.³⁰ Although the reproductive biology of armadillos has not been completely elucidated, some studies have offered deeper insights. Semen collection using electroejaculation from the Brazilian three-banded armadillo, a species listed as NT, and subsequent ejaculate analysis³⁹ determined that the semen samples contained abundant morphologically normal spermatozoa. However, the sperm concentration and motility were low, and urine contamination was a persistent problem. Additionally, Howell-Stephens et al. assessed the gonadal hormone activity of the Brazilian three-banded armadillo in a zoo, and the results of the analysis may aid with management decisions in such facilities.⁴⁰

The giant armadillo is the largest extant representative of the order Cingulata^{41,42} and is classified as VU.⁴³ A study monitored and collected reproductive behavior data, such as gestation, inter-birth interval, litter size, and parental care, of male and female giant armadillos in Brazilian Pantanal.⁴¹ The data indicated a low population rate, underscoring the high risk of local extinction. Therefore, further studies on this species are imperative.

Moreover, the sperm cells of a giant armadillo collected *post-mortem* from the epididymis were morphologically similar to those of most mammals. In particular, these sperm possessed a large oval head, similar to those in other members of the order Cingulata,⁴⁴ including six-banded armadillo⁴⁵ and large hairy armadillo.⁴⁶ In a recent study by Luba et al.,⁴⁷ the age of sexual maturity of giant armadillo was determined, and the characteristics of the captured sexually

mature males were described, providing information that can assist studies on *in situ* populations.

Semen collection using electroejaculation has been reported in captive six-banded armadillos.⁴⁸ Other parameters, such as sperm morphology, morphometry, ultrastructure,⁴⁵ and viability, in different semen extenders have been evaluated in this species.⁴⁹ Moreover, semen conservation has been studied in some species. The first attempt to preserve the male gametes of armadillos was reported by Amorim et al. in six-banded armadillos²⁹ (Table 2). The authors observed that the sperm were sensitive to refrigeration at 5°C. Subsequently, a sperm cryopreservation protocol using a solution containing 20% egg yolk and 3% glycerol was established.³⁰ However, except for this pioneering study, cryopreservation protocols for the sperm of other armadillo species have not been established to date.

For females, *ex situ in vitro* conservation approaches are even scarcer, and no studies on the cryopreservation of ovarian tissue or oocyte recovery have been conducted in armadillos. However, studies on female armadillo reproduction, including reproductive anatomy, have been conducted.⁵⁰ The estrous cycle of six-banded armadillos has been monitored based on vaginal cytology, ultrasound examination, and hormonal measurements.⁵¹ In general, the reproduction of armadillos is seasonal, with most species producing a single litter annually. Except for pichi (*Zaedyus pichiy*), which is known to hibernate during winters, other armadillo species remain active throughout the year.¹³

Additionally, blood and somatic tissue samples have been collected from armadillos for taxonomic⁷ and molecular phylogenetic analyses¹³ as well as biomedical studies⁵²; however,

such samples have not been used for *ex situ* species conservation through cryopreservation as yet.

Conservation perspectives on anteaters

Anteaters belong to the suborder Vermilingua of the order Pilosa, and although they are not the most abundant or diverse xenarthrans, these mammals exhibit a characteristic set of morphological adaptations.⁵³ Anteaters are the only toothless xenarthrans, despite the nomenclature “Edentata” (meaning toothless) formerly used to refer to xenarthrans. Anteaters possess a long tongue that they use to capture ants, termites, and other insects.⁴ The number of known anteater species has increased following the description of six newly recognized species of the genus *Cyclopes* in the family Cyclopedidae,⁶ which previously had only one representative—silky anteater (*Cyclopes didactylus*). These recent discoveries indicate that anteater populations warrant further research, particularly for establishing conservation and management practices, as these mammals occur in highly fragmented habitats, such as the Atlantic Forest.^{6,53} The other anteater family Myrmecophagidae, represented by three species, namely *Myrmecophaga tridactyla*, *Tamandua mexicana*, and *Tamandua tetradactyla*, is a group requiring cryobank establishment since the giant anteater is categorized as VU.⁵⁴

Regarding male reproductive traits, anteaters (similar to all xenarthrans) possess internal testes and disproportionate sperm heads.⁵⁵ Rossi et al.⁵⁵ described the histology of the male reproductive tract and the process of spermatogenesis in lesser anteaters. Following complete spermatogenesis, two individuals were azoospermic and showed considerable differences in the size of their genital tract organs, although some other characteristics were similar to those in other xenarthrans. The authors recommended further studies with more individuals from their natural environment to establish whether lesser anteaters exhibit reproductive seasonality.

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1365 Investigation of female reproductive traits, such as the estrus cycle, can allow for the
1366 implementation of ARTs in captive animals. Related research in southern tamandua revealed
1367 bloody vaginal secretion approximately 3 weeks before estrus, which may be a useful signal to
1368 monitor the ovarian cycle of the species.⁵⁶ Major advances in the non-invasive tracking of
1369 progesterone and estrogen metabolites in fecal samples have allowed for monitoring the estrous
1370 cycle in this species.⁵⁷

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1372 There are no records of an ovarian cycle in northern tamandua. Meanwhile, the ovarian cycle
1373 (51.4 days) of the giant anteater has been monitored through the analysis of hormones in the
1374 feces of captive females.⁵⁸ Nonetheless, knowledge of the ovarian cycle and semen and sperm
1375 of anteaters remains limited.

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1377 Regarding semen conservation in giant anteaters, Mendonça et al.⁵⁹ described the physical,
1378 chemical, morphological, and functional characteristics of semen collected using
1379 electroejaculation from captive animals. Semen was successfully collected from 62.2% of the
1380 analyzed males, and although the results varied among individuals, these differences were
1381 attributed to age, captive stress, and nutritional and health management. In another study, Luba
1382 et al.³¹ reported a rapid decline in the sperm quality of giant anteaters following dilution with a
1383 commercial semen extender (Botucio[®], Botupharma Ltd, Botucatu, Brazil) and storage at 5°C
1384 for 18 h. However, low resistance to cold temperatures may be characteristic of xenarthrans.³⁰
1385 Similar results were observed by Silva et al.,³² who reported a reduction in sperm vigor and
1386 motility following semen cryopreservation in giant anteaters (Table 2). Nonetheless, semen
1387 collection with medetomidine performed in that study was efficient in terms of sperm
1388 concentration, motility, and vigor.

Ultimately, efforts for the conservation of anteaters, focusing on their reproduction and germplasm preservation, are imperative to ensure their multiplication both in captivity and the wild. Such interventions may decelerate the loss of valuable genes in these species. Threatened and endangered species, such as the giant panda (*Ailuropoda melanoleuca*), have directly benefited from biobanking programs, highlighting the importance of systematic biobanking in species conservation.⁶⁰

Conservation perspectives on sloths

Sloths, similar to anteaters, belong to the order Pilosa and are divided into two families, namely Bradypodidae (genus *Bradypus*), represented by three-toed sloths,⁶¹ and Megalonychidae (genus *Choloepus*), represented by two-toed sloths.⁶² Sloths are arboreal and adapted for climbing.⁶³ All sloth species are herbivorous. *Bradypus* species are obligate folivores, while *Choloepus* species consume a more diverse diet, including fruits, flower buds, young stems, and some animal materials.⁴ Their global range is restricted to forested areas ranging from Honduras to Central Bolivia and Brazil.⁴ Sloths are known for their slow movements due to their restrictive diet.⁶⁴ While three-toed sloths are exclusively folivorous, two-toed sloths feed on leaves and fruits and, occasionally, animal matter. They are characterized as being solitary with parsimonious movements.⁶⁵ Additionally, sloths exhibit a very charismatic behavior, garnering much interest internationally.⁶⁴

In terms of conservation, sloths are the xenarthrans with the most critical conservation status. Of the six extant species, two are categorized as threatened.^{66,67} Nevertheless, all sloth species are directly affected by the destruction and fragmentation of forests, particularly since their population growth rate is low⁶³ and they are strictly arboreal species depending on trees

throughout their life. Research on sloths is scarce, even among xenarthrans. For instance, the vertebral processes common to all members of the group, which allow these animals to stand upright on the tripod formed by their hind limbs and tail, were only recently described in sloths (*Bradypus variegatus*),⁶⁸ as opposed to their relatively early descriptions in armadillos (*Dasypus novemcinctus*)⁶⁹ and anteaters (*Myrmecophaga tridactyla*).⁷⁰ Moreover, it is difficult to locate sloths in the forest, and even more so for untrained or inexperienced people,⁶³ rendering studies on these mammals in their natural habitats challenging. Another challenge is the low adaptability of *Bradypus* species to captive environments,⁶³ as they cannot survive on substitute diets that seem to lack certain trace elements, are very susceptible to cold, and are not immune to even common diseases.⁷¹

In this context, improving knowledge of sloth species through fundamental research on their reproductive parameters is an important step toward improving and understanding the reproductive functioning of this group to develop ARTs.^{63,65} In an attempt to gather deeper basic information on sloths, semen of free-living *Bradypus tridactylus* was successfully collected using electroejaculation, and the seminal characteristics of this species were described.⁶³ However, the low volume of the ejaculate limited the possibility of seminal analysis. The samples showed high viscosity and low motility and vigor. Satisfactory results were obtained by monitoring the ovarian cycle of female *Bradypus variegatus* through the analysis of hormones present in fecal samples.⁷² Nevertheless, further studies are warranted to characterize the hormonal cycle, spermatogenesis, estrus cycle, and gestational period of sloth species.

Bradypus pygmaeus, recognized as a distinct species in 2001,⁶¹ is the smallest sloth species; unfortunately, it is the world's most endangered xenarthran and, hence, a global conservation

priority. Further research on this species is urgently required to establish conservation measures for the following reasons: (1) *Bradypus pygmaeus* is classified as CR; (2) its range is extremely limited, as it is endemic to a single island, Escudo de Veraguas, in Panamá; (3) its population trend is declining; (4) it is directly affected by habitat loss; (5) it exhibits lower genetic diversity than its putative population; and (6) there is growing interest in its collection for captive rearing.⁶⁴ In a similar situation is *Bradypus torquatus*,⁶⁶ a species also at high risk of extinction for almost the same reasons cited for *Bradypus pygmaeus*. Silva et al.⁷³ have highlighted the need for protecting *Bradypus* populations, which inhabit areas with high fragmentation and exhibit low genetic diversity.

Future perspectives and conclusions

Challenges arising from the limited knowledge of xenarthrans and the lack of attention in terms of research have hindered the conservation of these unique mammals, particularly with regard to the development of *ex situ* strategies. Semen cryopreservation in armadillos and anteaters may represent the first step toward conserving biological resources from xenarthrans. Nevertheless, these protocols are only initial attempts, and further optimization is warranted, particularly considering the low cryotolerance reported for xenarthran sperm.

Further, to overcome the limitations encountered when working with sperm and oocytes, the use of somatic cells may be an optimal alternative. In this regard, somatic tissues and cell banks must be explored as potential resources for the conservation of xenarthrans. These genetic resources are important for the development and application of advanced technologies, such as somatic cell nuclear transfer and induced pluripotent stem cell culture, as well as for the generation of information for fundamental and applied research on understudied species. To the best of our knowledge, comprehensive somatic cell banks for xenarthrans are lacking, although

fibroblast lines have been established from skin biopsies of anteaters (*Tamandua tetradactyla*) and sloths (*Cyclopes didactylus*).⁷⁴ Nevertheless, previous studies focused on the phylogeny of these mammals through genome mapping rather than their *ex situ in vitro* conservation.

Much remains unknown regarding the reproductive physiology of xenarthrans. Long-term storage of somatic cells as a potential conservation strategy can aid conservation by contributing to fundamental scientific research and ART development. Therefore, cryopreservation of genetic resources from xenarthrans holds immense potential in *ex situ* conservation programs and can serve as an invaluable tool in conservation, assisted reproduction, evolutionary biology, and wildlife medicine. To preserve the remaining genetic diversity of Xenarthra, threatened species, including pygmy sloth, maned sloth, giant armadillo, Brazilian three-banded armadillo, and giant anteater, must be prioritized in conservation studies, although this important strategy must cover all xenarthran species.

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**CAPÍTULO 3 – INFLUENCE OF CRYOPRESERVATION TECHNIQUES AND LOW
CONCENTRATIONS OF PERMEATING CRYOPROTECTANTS ON THE
CONSERVATION OF EAR CARTILAGE AND SKIN DERIVED FROM SIX-BANDED
ARMADILLOS (*Euphractus sexcinctus* LINNAEUS, 1758) (Artigo experimental)**

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Influence of cryopreservation techniques and low concentrations of permeating cryoprotectants on the conservation of ear cartilage and skin derived from six-banded armadillos (*Euphractus sexcinctus* Linnaeus, 1758)

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Abstract

Considering the importance of efficiently obtaining somatic resource banks as an *ex-situ* conservation strategy for wild mammals, we evaluated two techniques (slow freezing - SF and solid-surface vitrification - SSV) for the cryopreservation of ear cartilage and skin from six-banded armadillos. Additionally, we analyzed the effects of two combinations of intracellular cryoprotectants (3.0 M or 6.0 M ethylene glycol - EG and dimethyl sulfoxide - Me₂SO) on SSV. Tissues not subjected to cryopreservation were used as controls. All samples were evaluated for morphological analysis and cell ability during culture. The thickness of the basal layer was similar to the control only for tissues derived from SF, while SSV ensured the preservation of the cartilage thickness. Moreover, fragments derived from SF and SSV, especially in the 3.0 M EG-Me₂SO group, resulted in dermis thickness and total skin similar to the control. All cryopreserved techniques maintained normal patterns of the fibroblasts, epidermal cells, and melanocytes. While only SF-derived fragments maintained the number of degenerated chondrocytes similar to the control, no difference was observed between groups for normal chondrocytes and lacunae. Moreover, SSV maintained the collagen fibers percentage of the tissues even after warming. After culture, SF and SSV techniques were efficient for the recovery of the somatic cells in all parameters evaluated, except the day of subconfluence, which was greater for the SSV group with 6.0 M EG-Me₂SO. In summary, SSV, especially with 3 M EG-Me₂SO, was as efficient as SF in preserving ear skin and cartilage derived from six-banded armadillos.

Keywords: Wildlife; Xenarthrans; Cryobank; Vitrification; Somatic tissue; *In vitro* culture

1. Introduction

The six-banded armadillo, *Euphractus sexcinctus* (Linnaeus, 1758), is one of the wild mammals representing the superorder Xenarthra with significant socio-economic and biomedical interest [1,12]. In 2014, the species was globally listed as Least Concern (LC) by the International Union for Conservation of Nature (IUCN) [1]. Nevertheless, particularly in Brazil, the species faces several threats driven by the high hunting pressure [1,3,42], habitat destruction and roadkills [28,36], which has contributed to the reduction of the species in its habitat. In this scenario, species conservation is essential for maintaining its biodiversity, especially considering the lack of knowledge concerning armadillos' species which represents a challenge to successfully implement conservation strategies [12].

One of the conservation strategies that can be employed involves using biological sample banks through cryopreservation [12], including cartilage and skin cryobanks. During cryopreservation, cryoinjuries can occur but are also reduced by proper choice of cryopreservation technique (e.g., slow freezing - SF and solid-surface vitrification - SSV), use of cryoprotectants, and temperature control equipment (for SF) [39]. The SF is a well-documented technique used for the cryopreservation of mammalian tissues [37,22] and one benefit of it is the need of lower concentrations of cryoprotectants to minimize ice crystal formation comparing to SSV [2]. However, vitrification techniques are preferable to SF [22,24] for not requiring equipment or being more time-consuming; it is easier to perform in field research and reduces intracellular ice formation by using higher concentrations of cryoprotectants followed by a short exposure [15].

In general, cryopreservation techniques and the various cryoprotectants, their concentration, and the combination added can have different outcomes depending on the types of biological

samples and species studied [22,34,35,37]. Combining these cryoprotective additives is advantageous since the toxic properties of individual cryoprotectants can be nullified, and at the same time, they might act synergistically in the system [32]. The use of ethylene glycol (EG) and dimethyl sulfoxide (Me₂SO) as permeating cryoprotectants, and sucrose and fetal bovine serum (FBS) as non-permeating cryoprotectants [47] is prevailing for many cryopreservation protocols of wild species tissues [7,5,11]. The combination of 3 M EG, 3 M Me₂SO, 0.25 M sucrose, and 10% FBS resulted in good outcomes on red-rumped agouti (*Dasyprocta leporina*) ear skin cryopreservation by SSV technique compared to other vitrification techniques [11]. Later, we evaluated the SSV technique following exposure to different combinations of intracellular cryoprotectants (3.0 M EG, 3.0 M Me₂SO or 1.5 M EG + 1.5 M Me₂SO) and 0.25 M sucrose and found that the 3.0 M EG-Me₂SO plus sucrose in combination is better than Me₂SO or EG alone in preserving the somatic tissue characteristics of this species [29]. Moreover, the combination of 6.0 M EG-Me₂SO was beneficial in terms of maintaining the tissue ultrastructure characteristic, dermis thickness, and proliferative activity in Antillean manatees (*Trichechus manatus manatus*) species compared to other vitrification solutions after SSV. However, none of the cryopreserved tissues could outgrowth cells *in vitro* [22].

The variable responsiveness to the cryopreservation technique and cryoprotectant concentration is species and tissue-specific and must be considered in order to develop a successful cryopreservation protocol [2]. Therefore, we aimed to evaluate the cryopreservation of ear cartilage and skin from six-banded armadillos comparing (1) conventional slow freezing versus solid-surface vitrification and (2) two concentrations of intracellular cryoprotectants (3.0 M or 6.0 M EG-Me₂SO) during solid-surface vitrification.

2. Material and methods

2.1. Chemicals and media

Sigma provided all the chemicals, except when indicated otherwise (Sigma Chemical Co., St. Louis, USA).

2.2. Bioethics and animals

The experiment was conducted with the ethical approval of the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 36/2020) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Five six-banded armadillos were used, consisting of two males and three females, and each individual was considered a replicate. These animals belonged to the Laboratory of Studies in Immunology and Wild Animals (LEIAS/UFERSA). They were kept in individual enclosures with daily feeding of pet food (Pedigree[®], MARS, Campinas, Brazil), vegetables, and water *ad libitum*.

2.3. Skin and cartilage collection and experimental design

For skin and cartilage biopsies, the animals were previously anesthetized with xylazine (1 mg/kg; Sedomin[®], König S.A., Buenos Aires, Argentina), ketamine (7 mg/kg; Injectable Ketamine, Vetnil[®], São Paulo, Brazil) intramuscularly, and propofol (5 mg/kg; Propovan[®], Cristalia, Fortaleza, Brazil) intravenously [38]. Subsequently, samples (1–1.5 cm²) from the ear region (one of each ear) were collected with surgical scissors, washed rapidly with 70% alcohol, and deposited in tubes containing Dulbecco's Modified Minimal Essential Medium (DMEM; Gibco-BRL, Carlsbad, USA) plus 10% FBS (Gibco-BRL, Carlsbad, USA) and 2% antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B; Gibco-BRL, Carlsbad, USA) and transported within 1 h to the laboratory.

In the laboratory, the skin tissues were sectioned into 6.0 mm³ using a scalpel blade and tweezers, and 32 fragments per animal were randomly assigned among the non-cryopreserved and cryopreserved groups (8 fragments to each group per animal; Fig. 1). The fragments were cryopreserved by slow freezing (SF group) and solid-surface vitrification (SSV) testing two cryoprotectant solutions; 1.5 M EG + 1.5 M Me₂SO (SSV-3M group) and 3.0 M EG + 3.0 M Me₂SO (SSV-6M group), with a base solution composed of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, and 0.25 M sucrose. After thawing, the tissues were evaluated by histological analysis and their capacity for *in vitro* culture.

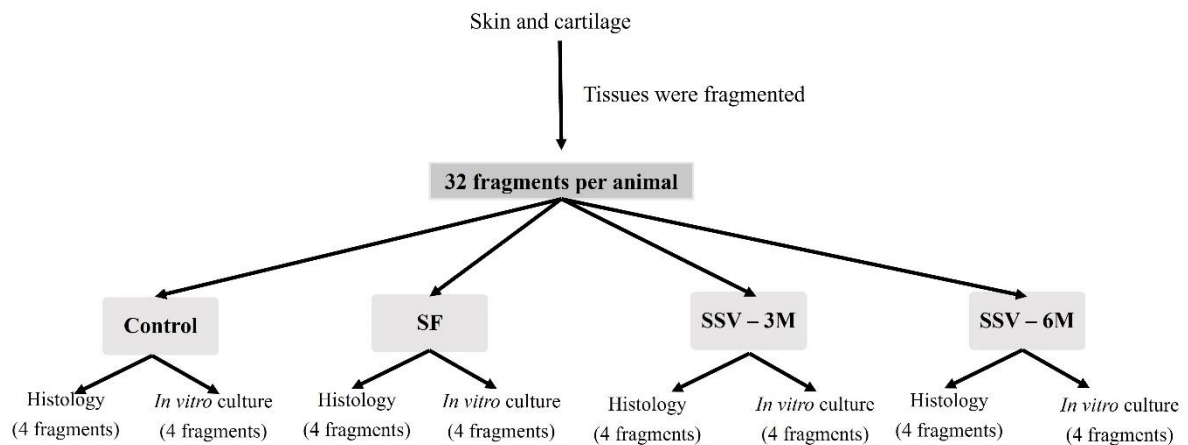


Fig. 1. Distribution of tissue fragments to assess the cryopreservation of ear cartilage and skin from six-banded armadillos comparing conventional slow freezing versus solid-surface vitrification and two concentrations of intracellular cryoprotectants (3.0 M or 6.0 M EG-Me₂SO) during solid-surface vitrification.

2.4. Cryopreservation by slow freezing

For SF, fragments were stored in cryovials containing 2.0 mL of the freezing solution composed of DMEM supplemented with 10% FBS, 0.75 M EG, 0.75 M Me₂SO, and 0.25 M sucrose [24], and transferred within 30 min to a freezer at -80 °C in Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, USA) for 12 h, using a cooling rate of 1°C/min. Subsequently, all cryovials were kept in liquid nitrogen (-196 °C).

2.5. Cryopreservation by solid-surface vitrification

For SSV, a vitrification solution consisting of DMEM supplemented with 10% FBS and 0.25 M sucrose was used, containing different concentrations of intracellular cryoprotectants: 1.5 M EG + 1.5 M Me₂SO (SSV-3M group) and 3.0 M EG + 3.0 M Me₂SO (SSV-6M group) [5]. Two fragments at a time were exposed to 1.8 mL solution for 5 min and dried on absorbent paper. Subsequently, they were placed individually on a thin rectangular aluminum recipient (9 × 12 × 3 cm) kept directly over the liquid nitrogen cooled to around -150 °C to -180 °C, transferred to cryovials, and stored in liquid nitrogen (-196°C).

2.6. Thawing of cryopreserved tissues

The cryopreserved fragments were thawed after two weeks. Initially, they were exposed for 1 min at room temperature and then immersed in a water bath at 37 °C for 30 s. To remove cryoprotectants, fragments were washed three times for 5 min in DMEM supplemented with 10% FBS and sucrose in decreasing concentrations (0.50 M, 0.25 M, and 0.0 M). Finally, the fragments were destined for the evaluations described below.

2.7. Evaluation of the skin and cartilage by histological analysis

The fragments were conditioned in a 4% paraformaldehyde solution for up to 2 weeks, followed by histological processing [25], sectioned at 5.0 µm using a microtome (Leica RM2125 RT, Leica Microsystems, Wetzlar, Germany), and stained with hematoxylin-eosin, Gomori's trichrome, or the silver-stained nucleolar organizer region (AgNOR) for analysis of morphometric aspects, collagen matrix, and tissue proliferative activity, respectively. To obtain the images, all samples were visualized using microscopy (Leica DM500, Leica Microsystems, Wetzlar, Germany) with an attached camera (Leica ICC50 HD, Leica Microsystems, Wetzlar, Germany).

For morphometric analysis and cell quantification and distribution, the following parameters were evaluated: the thickness of the epidermis, dermis, cartilage, total skin, and epidermal layers (in μm), number of epidermal cells, fibroblasts, keratinocytes, melanocytes, perinuclear halos, normal and degenerate chondrocytes, and number of filled and empty lacunae [29]. Additionally, fibers in the dermis were quantified and presented in percentage to analyze the collagen matrix, dividing the area of collagen deposits by the total area of the analyzed image [29].

To analyze tissue proliferative activity, dark spots marked by silver nitrate bound to nuclear proteins were counted according to cell location [25]. The AgNORs (discrete black dots) were measured in 100 nuclei of stained and randomly selected fibroblast cells in the dermis using the ImageJ software, and the AgNOR number/cell and AgNOR area/cell were quantified by the Image Pro Plus software [24].

2.8. Evaluation of the skin and cartilage by in vitro culture

Cryopreserved and non-cryopreserved tissues were placed in 60×15 mm culture dishes and cultured in DMEM containing 10% FBS and 2% antibiotic-antimycotic solution at 38.5°C in a humidified atmosphere of 5% CO_2 [33]. The dishes were monitored daily under an inverted microscope (Nikon TS100, Tokyo, Japan), and the culture medium was replaced every 1–2 days to verify cell outgrowth and any contamination. The tissues were evaluated for their morphology, number of attached explants, number of subconfluent explants, the day on which all explants were attached, the day at which explants reached subconfluence, the total time to reach subconfluence, and total culture duration [24].

After the outgrowth cells reached 70–80% confluence, after approximately 30 days, they were detached with 0.25% Trypsin-EDTA solution and evaluated for their morphology, viability, proliferative activity, metabolism, mitochondrial membrane potential, and apoptosis levels.

2.8.1. Assessment of cell viability

Live cells were estimated using 0.04% trypan blue dye, applying the traditional cell counting method [6]. For each animal per group, two replicates were performed, where dead cells were identified (stained in blue) by membrane rupture that allowed the permeabilization of the dye (non-viable cells) and those alive as colorless, without membrane rupture (viable cells).

2.8.2. Assessment of proliferative and metabolic activity

To determine the proliferative capability, the cells were cultured at a concentration of 1.0×10^4 cells/mL into 24-well culture plates, and the count was taken every 24 h for seven days. The population doubling times (PDT) were estimated according to the following formula [21]:

$$\text{PDT} = T \ln 2 / \ln (X_f / X_i),$$

where T is the incubation time (in hours), X_i is the initial cell count, X_f is the final cell count, and ln is the Naperian logarithm. The growth curve for seven days was plotted using cell numbers only.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoline bromide (MTT) assay was performed to evaluate the metabolic functionality. For this, 5.0×10^4 cells/mL was incubated at 38.5 °C and 5% CO₂. After five days, the MTT solution (5 mg/mL in DMEM) was added, and the plates were incubated for 3 h at 38.5 °C. Subsequently, Me₂SO was added as an MTT solubilization solution, and the reading was performed at an absorbance of 595 nm [5].

2.8.3. Assessment of mitochondrial membrane potential and apoptosis level

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined using the MitoTracker Red probe (CMXRos) diluted in Me₂SO for 30 min at 38.5 °C, 5% CO₂ [6]. Cells were washed with PBS and incubated with 500 nM CMXRos for 30 min at 38.5 °C, 5% CO₂, visualized under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and the intensity of the fluorescence signal (pixels) was quantified. Images were evaluated using the ImageJ software. The apoptotic cell death was analyzed according to Lira et al. [18], where cells were stained with acridine orange (AO, 1.0 µg/mL) and ethidium bromide (EB, 1.0 µg/mL) and evaluated under a fluorescence microscope. Images obtained were analyzed using the Image J software (National Institutes of Health, NIH, USA) to quantify the fluorescence signal intensity (pixels). The viable cell had a uniform light green nucleus, an early apoptosis cell had a non-uniform green nucleus, a late apoptotic cell had bright orange areas, and a necrotic cell had a uniform orange nucleus.

2.9. Statistical analyses

Data were expressed as mean $\pm$ standard error and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at $p < 0.05$.

3. Results

3.1. Effects of skin and cartilage cryopreservation on histomorphological analysis

The morphological characteristics of non-cryopreserved (control) and cryopreserved (SF, SSV-3M, SSV-6M) skin explants obtained by hematoxylin-eosin stain are shown in Fig. 2.

Considering the epidermis layers, all cryopreservation techniques, SF ($96.5 \pm 16.8 \mu\text{m}$), SSV-3M ($98.1 \pm 23.6 \mu\text{m}$), SSV-6M ($93.4 \pm 18.3 \mu\text{m}$), reduced the thickness of the spinosum compared to the control group ($127.3 \pm 26.2 \mu\text{m}$), while only the thickness of the basal layer for tissues derived from SF ($50.0 \pm 16.9 \mu\text{m}$) remained similar to the control ($54.8 \pm 11.4 \mu\text{m}$; Fig. 3a). No difference was observed among cryopreserved (SF: $316.5 \pm 45.4 \mu\text{m}$, SSV-3M: $352.8 \pm 74.0 \mu\text{m}$, and SSV-6M: $334.8 \pm 58.8 \mu\text{m}$) and non-cryopreserved tissues ($363.8 \pm 56.0 \mu\text{m}$) for epidermis thickness. The dermis and total skin thickness were similar to the control ($1339.2 \pm 114.1 \mu\text{m}$ and $1702.9 \pm 125.8 \mu\text{m}$) for the SF ($1339.3 \pm 125.3 \mu\text{m}$ and $1655.8 \pm 136.3 \mu\text{m}$) and SSV-3M groups ($1408.8 \pm 185.3 \mu\text{m}$, and $1761.6 \pm 218.4 \mu\text{m}$), while the SSV-6M group ($1588.9 \pm 109.7 \mu\text{m}$, and $1923.6 \pm 109.6 \mu\text{m}$) presented an increase in both parameters ($P < 0.05$). Moreover, the cartilage thickness was preserved only after the evaluated vitrification techniques (SSV-3M: $273.5 \pm 28.6 \mu\text{m}$ and SSV-6M: $276.5 \pm 31.5 \mu\text{m}$), compared to the control ($286.4 \pm 26.3 \mu\text{m}$).

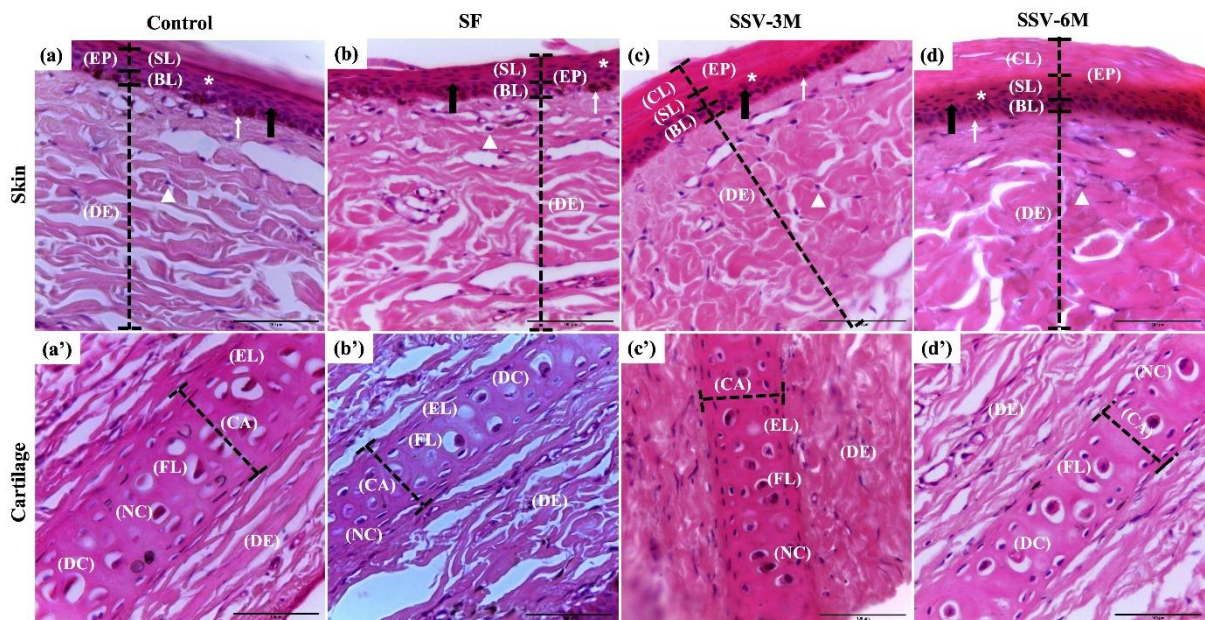


Fig. 2. Histological sections of ear skin layers (a-d) and cartilage (a'-d') of non-cryopreserved and cryopreserved tissues derived from six-banded armadillos using hematoxylin-eosin. (a, a') non-cryopreserved (control); and cryopreserved tissues by (b, b') slow freezing, (c, c') solid-

surface vitrification at 3.0 M concentration of intracellular cryoprotectants, and **(d, d')** solid-surface vitrification at 6.0 M concentration of intracellular cryoprotectants. BL, basal layer; CA, cartilage; CL, corneum layer; DC, degenerated chondrocytes; DE, dermis; EL, empty lacunae; EP, epidermis; FL, filled lacunae; NC, normal chondrocytes; SL, spinosum layer. Epidermal cells (*), melanocyte (white arrow), keratinocyte (black arrow), and fibroblast (triangle). Scale bar: 100 μ m. 40 $\times$ magnification.

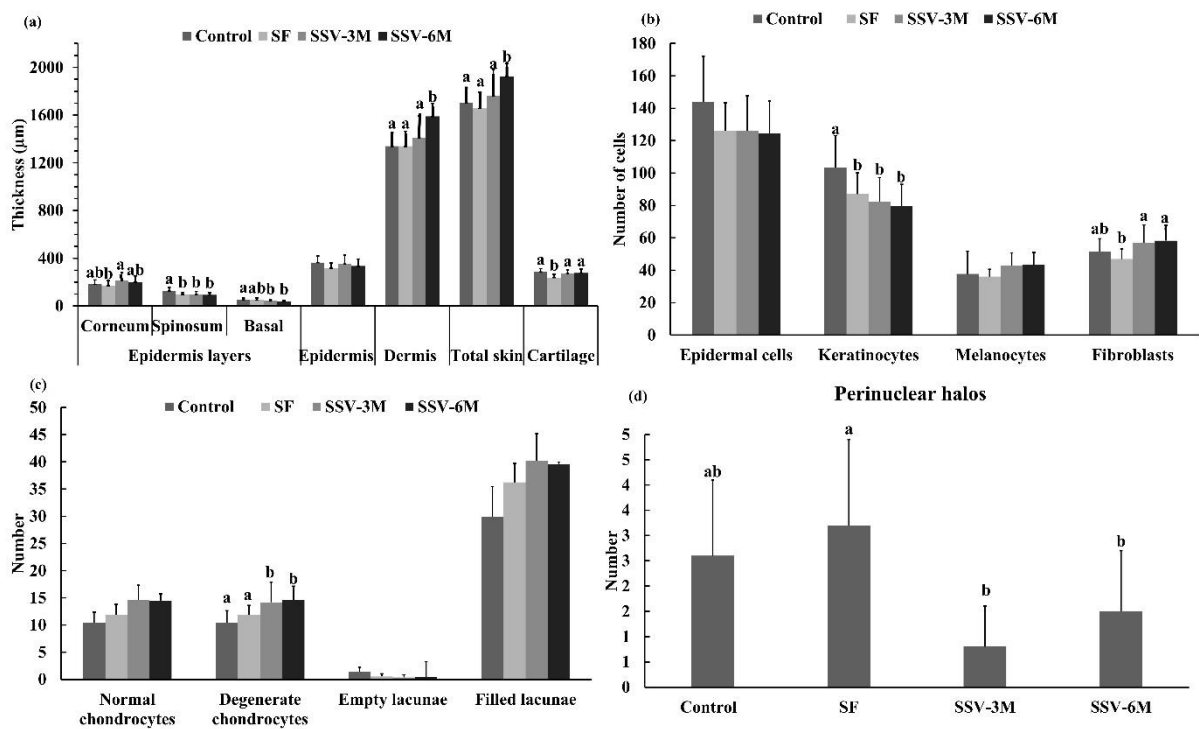


Fig. 3. Thickness of skin layers and cartilage and main cell quantification of non-cryopreserved and cryopreserved tissues derived from six-banded armadillos. **(a)** Thickness of the epidermis layers, epidermis, dermis, total skin, and cartilage; **(b)** Mean number of epidermal cells, keratinocytes, melanocytes, and fibroblasts; **(c)** Mean number of normal chondrocytes, degenerate chondrocytes, filled lacunae and empty lacunae in cartilage; **(d)** Mean number of perinuclear halos in the epidermis.

1986 As a result of main skin cells quantification, the epidermal cells and melanocytes number alone
 1987 were similar among evaluated groups, but the number of keratinocytes was lower in
 1988 cryopreserved groups (SF: 87.0 ± 13.1 ; SSV-3M: 82.4 ± 14.8 ; SSV-6M: 79.5 ± 13.5) when
 1989 compared to the control (103.5 ± 19.7 ; Fig. 3b). Moreover, comparing the result with the control
 1990 group (51.6 ± 7.9), the fibroblast number remained through cryopreservation (SF: 46.9 ± 6.3 ;
 1991 SSV-3M: 57.0 ± 11.0 , SSV-6M: 58.3 ± 9.5). Although only the SF group showed a similar
 1992 value of degenerated chondrocytes when compared to the control, we found no difference in
 1993 normal chondrocytes and filled, and empty lacunae count between groups (Fig. 3c).
 1994 Nevertheless, the tissues from both SSV groups (SSV-3M: 0.8 ± 0.8 , SSV-6M: 1.5 ± 1.2)
 1995 presented fewer perinuclear halos when compared to the SF group (3.2 ± 1.7), but these results
 1996 did not differ from the non-cryopreserved tissues (2.6 ± 1.5 ; Fig. 3d).
 1997
 1998 Furthermore, we evaluated the collagen matrix from non-cryopreserved (control) or
 1999 cryopreserved tissues by Gomori trichrome stain (Fig. 4a-d). The vitrified groups (SSV-3M: 72
 2000 $\pm 0.03\%$; SSV-6M: $73 \pm 0.03\%$) showed collagen matrix percentage similar to the control group
 2001 ($74 \pm 0.03\%$); however, the percentage from the SF group ($78 \pm 0.04\%$) was significantly higher
 2002 (Fig. 4e). Although the AgNOR number per fibroblast cell did not alter after cryopreservation
 2003 and thawing, the cryopreserved groups showed an increase in AgNOR area per cell (Fig. 5).

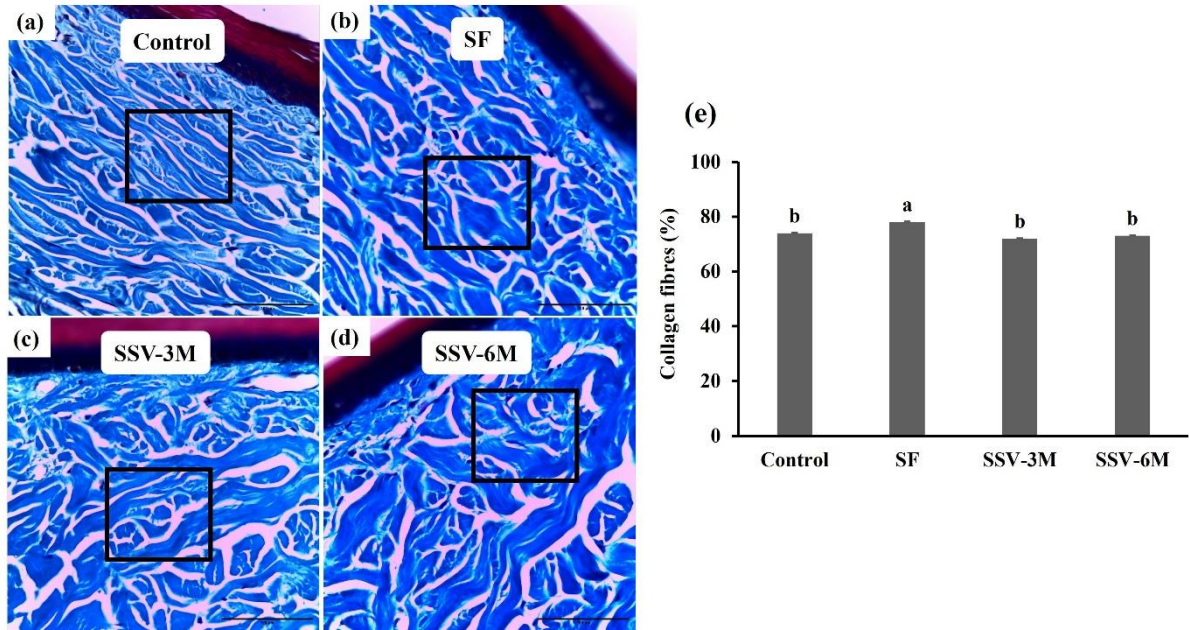


Fig. 4. Evaluation of collagen density from ear skin of six-banded armadillos using Gomori trichrome **(a)** Staining of collagen fibers in the dermis from non-cryopreserved tissues; and cryopreserved tissues by **(b)** slow freezing (SF), **(c)** solid-surface vitrification at 3 M concentration of intracellular cryoprotectants (SSV 3M), **(d)** solid-surface vitrification at double concentration of intracellular cryoprotectants (SSV 6M). **(e)** Percentage of collagen fibers in the dermis from non-cryopreserved and cryopreserved tissues. Square exemplifies the dermal area of evaluation of collagen matrix. Bars represent standard error. Scale bar: 100 μm 40× magnification.

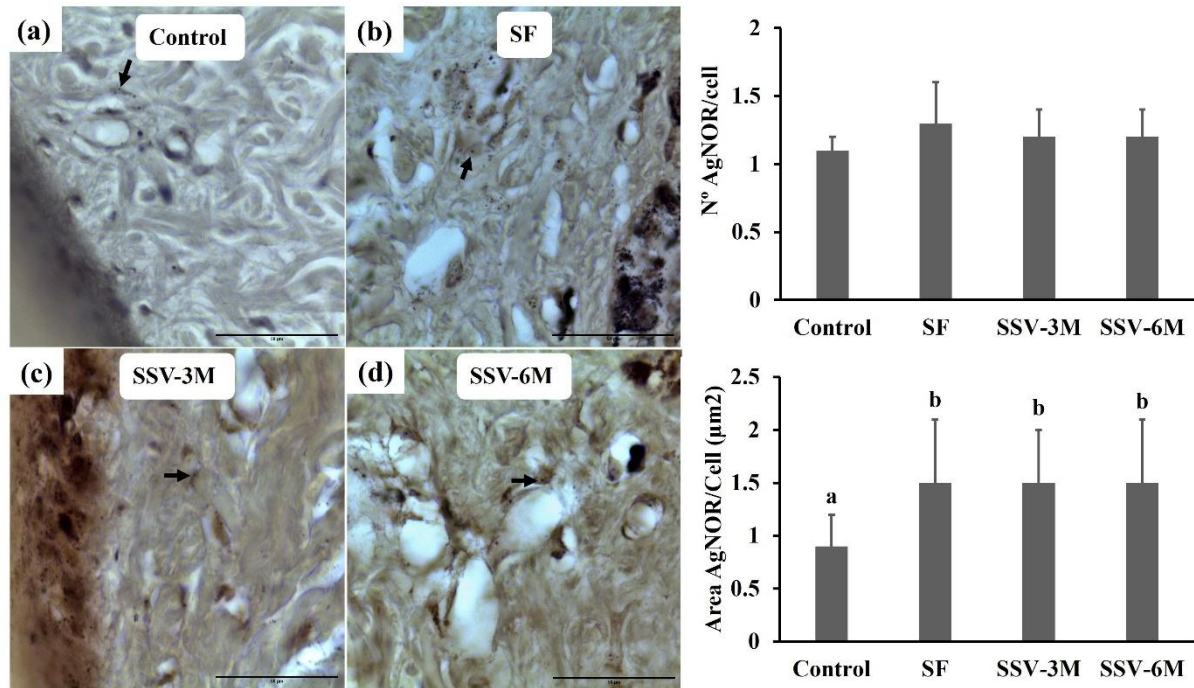


Fig. 5. Proliferative activity of non-cryopreserved and cryopreserved skin derived from six-banded armadillos submitted to different techniques. **(a)** Staining of AgNOR in the fibroblasts from fresh skin (control); **(b)** slow freezing, **(c)** solid-surface vitrification at 3.0 M concentration of intracellular cryoprotectants, and **(d)** solid-surface vitrification at 6.0 M concentration of intracellular cryoprotectants. **(e)** Quantification of AgNOR number/cell. **(f)** Quantification of AgNOR area/cell. SF: slow freezing. SSV: solid-surface vitrification. Bars represent standard error. Arrow indicates fibroblast nuclei stained with AgNOR. Scale bar: 50 μm 100× magnification.

3.2. Effect of skin and cartilage cryopreservation on primary culture and subculture

According to the *in vitro* culture evaluation, both cryopreserved and non-cryopreserved tissues were able to adhere and detach somatic cells until they reached subconfluence (70–80% of confluence; Fig. 6). As shown in Fig. 6, the cells presented a star or spindle-shape with prolonged cytoplasmic projections like fibroblasts. All explants were attached within 1–7 day

of culture, and the migration of primary cells was seen in the first days. Still, the monolayer of epithelial-like and fibroblast-like cells around all explants was observed at 12.6, 9.8, 15.0, and 14.5 days in the non-cryopreserved, SF, SSV-3M, SSV-6M groups, respectively (Table 1). Additionally, all explants showed subconfluence, but the day they reached subconfluence was longer in SSV-6M compared to the control and SF group, yet not differing from the same vitrification technique with a higher concentration of intracellular cryoprotectants (SSV-3M). The duration of culture was similar between groups (Table 1).

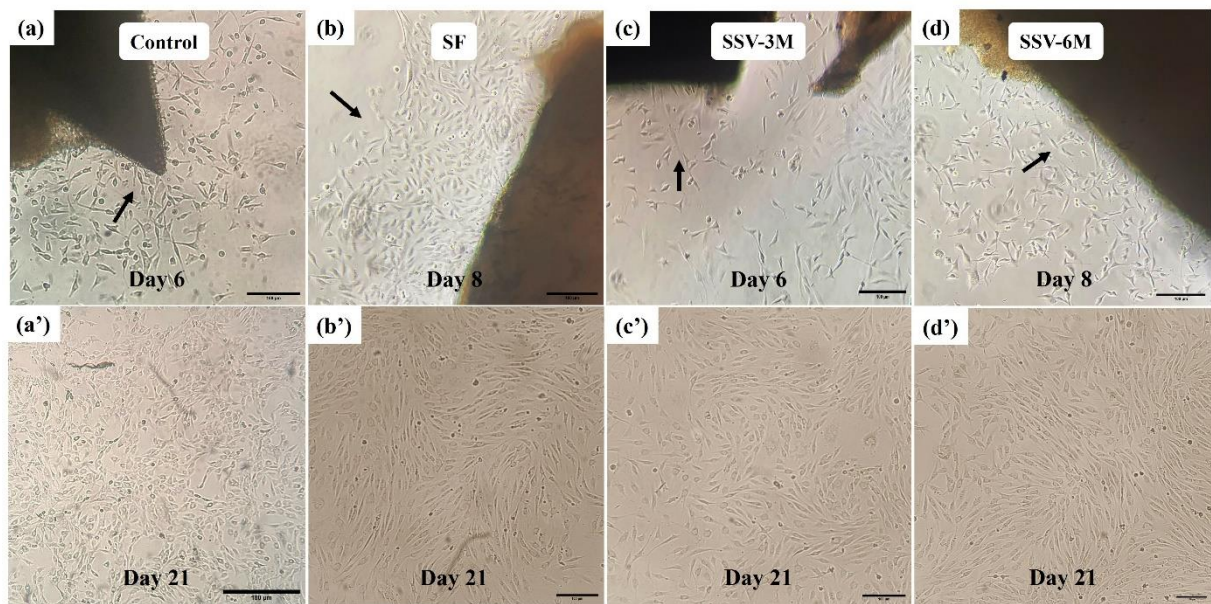


Fig. 6. Examples of cell outgrowth from six-banded armadillos skin tissue explants. **(a)** and **(a')** cells from non-cryopreserved tissues; and cryopreserved tissues by **(b)** and **(b')** slow freezing (SF), **(c)** and **(c')** solid-surface vitrification at 3.0 M concentration of intracellular cryoprotectants (SSV-3M), **(d)** and **(d')** solid-surface vitrification at higher concentration of intracellular cryoprotectants (SSV-6M). Arrow indicates the beginning of cell detachment in primary cultures. Scale bar: 100 μ m. Magnification 10 $\times$.

2042 **Table 1.** Effect of cryopreservation techniques and solutions on primary cultures of skin tissues derived from six-banded armadillos.

Groups	Attached explants (%)	Day of all attached explants $\pm$ S.E.	Day of all explants with cell growth $\pm$ S.E.	Grow to subconfluence (%)	Subconfluence day $\pm$ S.E.	Duration of culture (days) $\pm$ S.E.
Control	20/20 (100) ^a	1.4 $\pm$ 0.4 ^a	12.6 $\pm$ 1.0 ^a	23/24 (96) ^a	15.6 $\pm$ 1.2 ^a	36.8 $\pm$ 1.2 ^a
SF	20/20 (100) ^a	6.0 $\pm$ 1.8 ^a	9.8 $\pm$ 1.2 ^a	24/24 (100) ^a	14.8 $\pm$ 1.2 ^a	28.0 $\pm$ 2.1 ^a
SSV-3M	19/20 (95) ^a	6.8 $\pm$ 2.6 ^a	15.0 $\pm$ 1.5 ^a	13/16 (81) ^a	20.0 $\pm$ 1.0 ^{ab}	31.8 $\pm$ 1.7 ^a
SSV-6M	20/20 (100) ^a	3.5 $\pm$ 2.5 ^a	14.5 $\pm$ 2.5 ^a	7/8 (88) ^a	24.0 $\pm$ 5.0 ^b	34.0 $\pm$ 6.0 ^a

2043 SF: slow freezing. SSV: solid-surface vitrification. S.E.: standard error. ^{a,b}: P < 0.05.

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After the primary culture reached subconfluence, the cells were detached and submitted to quality analysis. The cell viability, metabolic and proliferation activity, and growth curve results are shown in Fig. 7. The viability and metabolic activity were high (over 85% and 70%, respectively) in all groups and remained similar after cryopreservation, regardless of the technique or solution (Fig. 7a-b). The population doubling time did not change between cryopreserved and control groups (Fig. 7c). Additionally, the graph to observe the cell growth pattern was plotted (Fig. 7d). The non-cryopreserved group showed an increase in the number of cells, increasing exponentially after one day of culture, and decreasing on the seventh day. In general, the cryopreserved groups showed lower cell concentration than the control group but similar growth patterns between them, except in the SSV-6M group at Day 7, where cell growth continued and did not decrease.

Our results showed that the intercellular $\Delta\Psi_m$ level remained similar in cryopreserved and non-cryopreserved groups (Fig. 7e). The signs of apoptosis were evaluated using AO/EB dual staining. The results revealed a high incidence of viable cells and a low rate of cells in the apoptotic stage. The necrotic cells were rarely observed in all groups, and all types of cells, such as viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells, showed similar percentages among the groups (Fig. 7f).

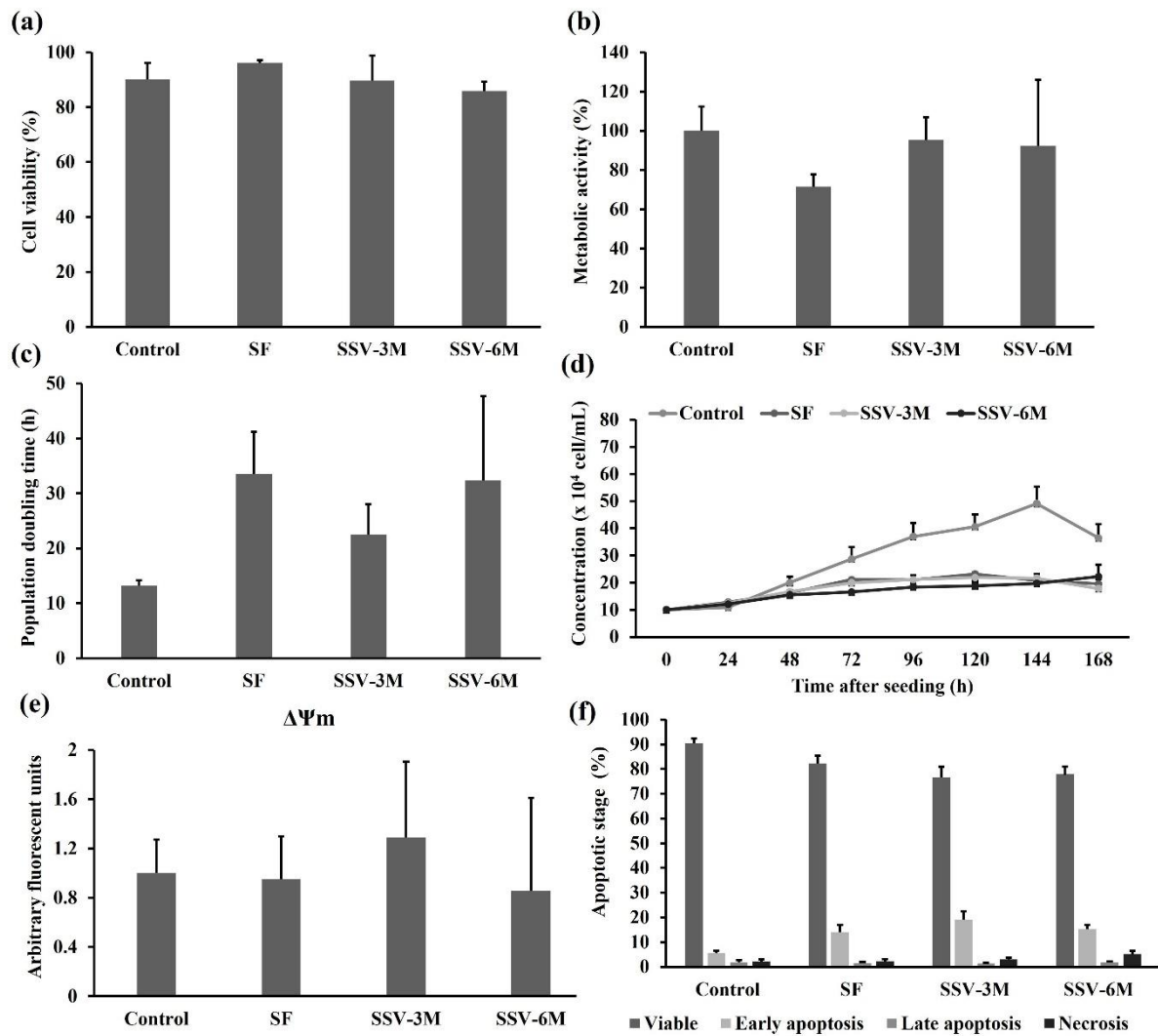


Fig. 7. Survival rate, functionality, grow dynamics, oxidative stress and apoptosis levels of six-banded armadillos fibroblast-like cells from non-cryopreserved and cryopreserved tissues. **(a)** viability, **(b)** metabolic activity, **(c)** population double time and **(d)** growth curve, **(e)** measurement of intracellular mitochondrial membrane potential ($\Delta\Psi_m$) and **(f)** apoptosis levels. Bars represent standard error. $P > 0.05$.

4. Discussion

In this study, we report the first attempt to preserve ear skin and cartilage of six-banded armadillos by assessing three cryopreservation protocols and evaluating the post-thawed tissue histological aspects and *in vitro* culture for cell recovery. The results demonstrated that the SSV

cryopreservation technique, especially with 3 M EG-Me₂SO, was as effective as SF in conserving the tissue (skin and cartilage) in terms of histological characteristics, highlighting both groups as an ideal cryopreservation method and solution in maintaining tissue and cell functionality unchanged after culture. Nevertheless, the SSV is a fast, cheap, and practical cryopreservation technique [9], outstanding as a more suitable alternative to be performed in field work compared to SF, and therefore recommended for routine use in assisted reproduction for wildlife species conservation.

The armadillo skin is thick [16]. In this study, the average thickness of the total skin in the non-cryopreserved group was 1702.9 µm, and the cryopreservation process did not shrink the total skin. Moreover, the epidermis layers (corneum, spinosum, and basal layer), epidermis, dermis, and cartilage were identified and measured separately in skin samples from six-banded armadillos. The thickness of the basal and spinosum layers decreased only after vitrification. This change can be explained by the thermal stress effect due to nonuniform changes in tissue temperature that might occur during cryopreservation since it has been reported that the tissue layers tend to shrink, in this case, more in the inside layers than the outside [26]. The same alteration was seen in ocelot (*Leopardus pardalis*) skin basal and spinosum layers after SSV [44]. Further, no retraction was observed in the epidermis, dermis, and total skin after cryopreservation, possibly because of the presence and concentration of intra- and extracellular cryoprotectants that reduce cryoinjuries and prevents tissue alterations. However, even briefly, 6 M of permeating cryoprotectants in the SSV-6M group were not adequate for preserving the tissues since the dermis and total skin thickness were greater than in the non-cryopreserved fragments. This result is explained by using a higher concentration of cryoprotectants, triggering a greater risk of cryoprotectant toxicity during the addition or removal of cryoprotectants and causing a change in the tissue due to excessive osmotic forces [17,31].

On the other hand, only the SSV groups did not differ from the control group in cartilage thickness. The shorter cartilage thickness in the SF group can be attributed to the cooling rate used since the intracellular water is effluxed from the cell in the slow cooling process, reducing the intracellular water amount, which does prevent interior ice formation and further damage. Still, there is a high risk of extracellular ice formation in SF [15,32], which could alter the tissue histomorphology compared to vitrification and non-cryopreserved tissues. In SSV, due to the rapid cooling, the intracellular water has less time to move to extracellular space and gets supercooled, which may cause intracellular ice formation instead [32]. We used higher concentrations of cryoprotectants to prevent this injury. Accordingly, cryoprotective additives and the cooling rate adjustment are critical for successful vitrification [32]. A similar response was observed in the jaguar, in which the ear skin tissue was better conserved in the SSV group after histological evaluation compared to SF [24]. Our data demonstrate that the SSV-3M group ensured the conservation of tissue integrity.

Additionally, the cryopreservation by SSV positively affected the dermis and the cartilage with greater fibroblast when evaluating the histological quantification of cells. In the dermis, the fibroblasts are the primary cell type. They are desired for genetic material conservation and research studies, being the most frequently used somatic cells in cloning by nuclear transfer [46] and favorable for wild species [11]. The vitrification groups (SSV-3M and SSV-6M) showed lesser perinuclear halos than SF but no difference between cryopreserved and non-cryopreserved tissues. The perinuclear halos were measured as a number of apoptotic cells and are visualized in the epidermal as retractions of the nucleus from the cytoplasm [4]. Our result showed that the cryopreservation techniques preserved the tissues from this damage.

Nevertheless, while the SSV caused an increase in degenerated chondrocytes, the SF maintained a similar number compared to the control, indicating low cell degeneration. The slow cooling rate may explain this result, along with using a lower concentration of permeating cryoprotectants since the intracellular ice formation is reduced during slow freezing, preventing cell degeneration [45]. Alternately, the percentage of collagen fibers was only unchanged in the SSV groups, according to the knowledge that vitrification promotes better maintenance of structural and extracellular matrix of tissues due to the rapid vitreous state, causing less risk of freeze injury [15,44].

Further, evidencing the efficiency of the techniques and the combination of cryoprotectants, the tissue proliferative activity was maintained through cryopreservation, proved by the measure of AgNORs. AgNOR has been used as histochemical pointers of cell multiplication, a good marker of proliferative action, and is moderately inexpensive and direct in understanding [43]. Moreover, during the *in vitro* culture, we observed that the primary cell morphology was both epithelial-like and fibroblast-like cell types at the first migration. The fibroblasts present a different behavior in *in vitro* environment; they detach faster after trypsin digestion and can attach more easily and readily than epithelial cells [13,46]. Thus, after a few days of culture, both cryopreserved and non-cryopreserved groups showed a predominant cell type, fibroblast-like cells, growing as monolayers. In general, these cells showed rapid proliferation and good *in vitro* cell performance, with no difference observed among the control, SF, or SSV groups. The combination of both intra and extracellular cryoprotectants may be the cause of that since the use of Me₂SO and EG as intracellular cryoprotectants and sucrose and FBS as extracellular cryoprotectants had proved to be effective in reducing the possible damages in the cryopreservation process and maintained the tissues and cells viable [7,24]. Me₂SO is the most used cryoprotectant; it penetrates the cells and prevents ice crystal formation and membrane

rupture [45]. EG is considerably less toxic, but both cryoprotectants have been proven reliable for vitrification with tolerable toxicity at high concentrations. Moreover, vitrification immediately in liquid nitrogen can reduce the cytotoxic effect.

Accordingly, the cell quality was preserved by comparing the standard slow freezing technique and vitrification (by SSV) and the vitrification solutions set to 3.0 M or 6.0 M of intracellular cryoprotectants. Cell viability was high in control tissues (90.2%) and maintained in non-cryopreserved tissues, corroborating other studies [18,24]. The PDT value did not alter, with a maximum of 34 h in the SF group. These cells' PDT rates in armadillos deferred from other wild mammals after cryopreservation, considering the maximum value, 66 h in collared peccaries [7], 26 h in jaguar [24], and 46 h in puma (*Puma concolor*) [18]. The growth curve from the control group showed high exponential growth reaching a maximum cell concentration of 49×10^4 cells/mL at 144 h of culture. The cryopreserved groups presented a very similar profile, showing lower concentrations of cells during culture, but still with normal proliferation capacity regardless of the cryopreservation process.

The cells also showed no changes in metabolic activity through the MTT assay, and the $\Delta\Psi_m$ were not affected by the cryopreservation. The $\Delta\Psi_m$ value was evaluated as a hallmark of mitochondrial dysfunction compared with the control group. The apoptosis rate was low in all groups with no statistical difference, confirmed by the AO/EB dual staining and considering early apoptosis, late apoptosis, and necrosis stage. The EB stain can only damage cells and gives orange-red fluorescence when bound to the DNA; different color tones indicate apoptotic stages. The green fluorescent resulting from the AO stain indicates viable cells. The control group had 90% of viable cells after culture, and this parameter was also unaffected by the cryopreservation protocols.

5. Conclusions

Although both cryopreservation techniques were able to conserve the somatic tissue with minor changes compared to the control group, we highlight the SSV technique, especially using permeating cryoprotectants in combination at lower concentrations (3 M). This vitrification process is more practical in the field, less time-consuming, requires minimal equipment, and can preserve cells closer to their natural state. Regarding the vitrification solutions, the SSV-3M is preferable for less concentration of EG-Me₂SO, reducing the chances of cell toxicity. Our finding supports that the efficiency of the technique depends on the use of lower concentrations of permeating cryoprotectants at 3 M. We highlight the importance of an adequate cryopreservation protocol for the six-banded armadillo genetic resource bank that can be used in assisted reproduction techniques for the species conservation and others phylogenetically closely related species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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**CAPÍTULO 4 – LONG-TERM PRESERVATION OF ESTABLISHED FIBROBLAST
LINES FROM SIX-BANDED ARMADILLOS (*Euphractus sexcintus*, LINNAEUS, 1758)
BY EXTENDED PASSAGE AND CRYOPRESERVATION (Artigo experimental)**

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2409 **Long-term preservation of established fibroblast lines from six-banded armadillos**
 2410 **(*Euphractus sexcinctus*, Linnaeus, 1758) by extended passage and cryopreservation**

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2432 **Running head:** Establishment of fibroblast cell lines.

2433

Abstract

Establishing new somatic cell cultures has raised significant attention as an effective and convenient way to preserve genetic samples for different applications. Although many lines have been established in model animals, none derived from six-banded armadillo species is currently available. We report the successful isolation and characterization of fibroblasts from six-banded armadillos, evaluating the cell quality after extended culture and cryopreservation. Initially, we collected ear skin from five captive adult individuals and identified fibroblast lines by morphology, karyotyping, and immunophenotyping assays. The isolated fibroblasts were evaluated after several passages (fourth, seventh, and tenth passages) and cryopreservation by slow freezing. Cell morphology, viability, metabolism, proliferative activity, mitochondrial membrane potential, and apoptosis levels were analyzed. The skin explants had great adhesion, and cell outgrowth could be seen after 3–6 days. The cells were verified as fibroblasts at the fourth passage by vimentin expression and normal karyotype ($2n=58$). The viability remained high ($>87\%$) and constant from the fourth to the tenth passage ($p > 0.05$). The passages did not change the cell morphology and metabolic and growth rates. Moreover, cryopreservation did not affect most evaluated parameters; post-thawed cells maintained their viability, growth, metabolism, and apoptosis levels. Nevertheless, cryopreservation increased mitochondrial membrane permeability and cell population doubling time compared to non-cryopreserved cells ($p < 0.05$). In summary, viable fibroblasts can be obtained from six-banded armadillo skin while conserving their quality as the number of passages increases and featuring few changes after cryopreservation.

Keywords: Xenarthra; genetic resource banks; *in vitro* culture; somatic cells.

2459 **Introduction**

2460 The six-banded armadillo (*Euphractus sexcintus* Linnaeus, 1758) is a wild mammal native to
2461 the Americas and found in all biomes in Brazil (Paglia et al. 2012; Superina and Abba 2018).
2462 The species is an important ecosystem engineer, mainly for seed dispersal, soil aeration, and
2463 pest control services (Abba et al. 2014; Rodrigues et al. 2019). Moreover, the six-banded
2464 armadillo arouses interest in studies due to their scientific classification as a natural reservoir
2465 of zoonoses and economical due to carcass, meat, and medicine value (Abba et al. 2014;
2466 Teixeira et al. 2020).

2467

2468 It is the armadillo species with the largest geographic distribution (Santos et al. 2019), being
2469 very diverse and not in danger of extinction like other armadillo species (Abba et al. 2014).
2470 Despite their resistance to environmental alterations, the six-banded armadillo population
2471 suffers from anthropological pressures, such as habitat degradation, hunting, and roadkill
2472 incidents (Barbosa and Oliveira 2022). Furthermore, the species is easily adapted to captivity;
2473 they are often globally transported to various zoos as ornamental animals (Guo et al. 2022) and
2474 represent an ideal experimental model for reproductive biotechnology for the species and other
2475 threatened members of the Xenarthra superorder (Sousa et al. 2016), which comprehends more
2476 than 20% of xenarthrans (Fernandes et al. 2023a). Such information gives strong arguments
2477 about the need to develop research on conservation strategies and expand knowledge about this
2478 species.

2479

2480 Cell line storage is considered an effective and promising tool for biodiversity conservation
2481 (Borges et al. 2020; Wang et al. 2020). The long-term conservation of genetic materials and
2482 their original characteristics and functions offers enormous possibilities. In this case, using
2483 fibroblast skin cells is straightforward due to their inexpensive recovery, isolation, and

maintenance in culture (Praxedes et al. 2018; Dua et al. 2021). Moreover, fibroblasts are suitable as donor cells for advanced reproductive technologies, like somatic cell nuclear transfer (SCNT) and induced pluripotent stem cell (iPSC) techniques (Praxedes et al. 2018; Sharma et al. 2018). In general, three essential steps must be performed for the successful establishment of somatic cell lines: (1) identify and establish the cell line, (2) characterize the cell during extended culture, and (3) evaluate the effects of cryopreservation (Praxedes et al. 2021).

Extended culture and cryopreservation can stress the cells or even cause their senescence (Magalhães et al. 2017; Praxedes et al. 2021). Skin fibroblast cells derived from different species have been cultured in up to ten passages, maintaining their functionality (Borges et al. 2020; Wang et al. 2020). Nevertheless, earlier passages (fourth and seventh passages) demonstrated the better quality of fibroblasts from brown brocket deer (*Mazama gouazoubira*) compared to the tenth passage. This was also evidenced in cryopreserved cells within the same passage (Magalhães et al. 2017). In subsequent studies, skin fibroblast viability and metabolic and proliferative activities were not affected by cryopreservation in red-rumped agouti (*Dasyprocta leporina*; Praxedes et al. 2021) and puma (*Puma concolor*; Rodrigues et al. 2023). A similar result was obtained in skin fibroblasts taken post-mortem from the ear of an Asian elephant (*Elephas maximus*), where the cells had high viability and post-freezing recovery rates (Siengdee et al. 2018). Cryopreservation induced changes on mitochondrial membrane potential ($\Delta\Psi_m$) in fibroblasts derived from collared peccaries (*Pecari tajacu*; Borges et al. 2020) and puma (*P. concolor*; Lira et al. 2022).

We initiated a protocol for skin fibroblast cryobanks of six-banded armadillos, considering the need for developing somatic cell resource banks for this species. Therefore, we aimed to (1) isolate, identify, and establish cell lines, evaluating the effects of (2) extended culture time

(fourth, seventh, and tenth passages) and (3) cryopreservation on the quality parameters of somatic cells derived from ear skin of six-banded armadillos kept in captivity.

Materials and methods

Chemicals and media

The Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin solutions were obtained from Gibco-BRL (Carlsbad, USA). The fluorescent probes were purchased from Invitrogen (Carlsbad, USA), and the mouse anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor 488, Warrington, USA) were purchased from Abcam (Cambridge, USA). The other chemicals were obtained from Sigma-Aldrich (St. Louis, USA). Media were filtered using a 0.22- μ m system (Corning, New York, USA).

Bioethics and animals

All experiments were approved by the Animal Ethics Committee of the Rural Federal University of Semi-Arid (CEUA/UFERSA, no. 36/2020) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Five adults, 3 females and 2 males, six-banded armadillos were selected for the tissue biopsies (Fig. 1a). They were kept in individual enclosures at the Laboratory of Studies in Immunology and Wild Animals (LEIAS/UFERSA) with daily feeding of pet food (Pedigree[®], MARS, Campinas, Brazil) plus vegetables and water *ad libitum*.

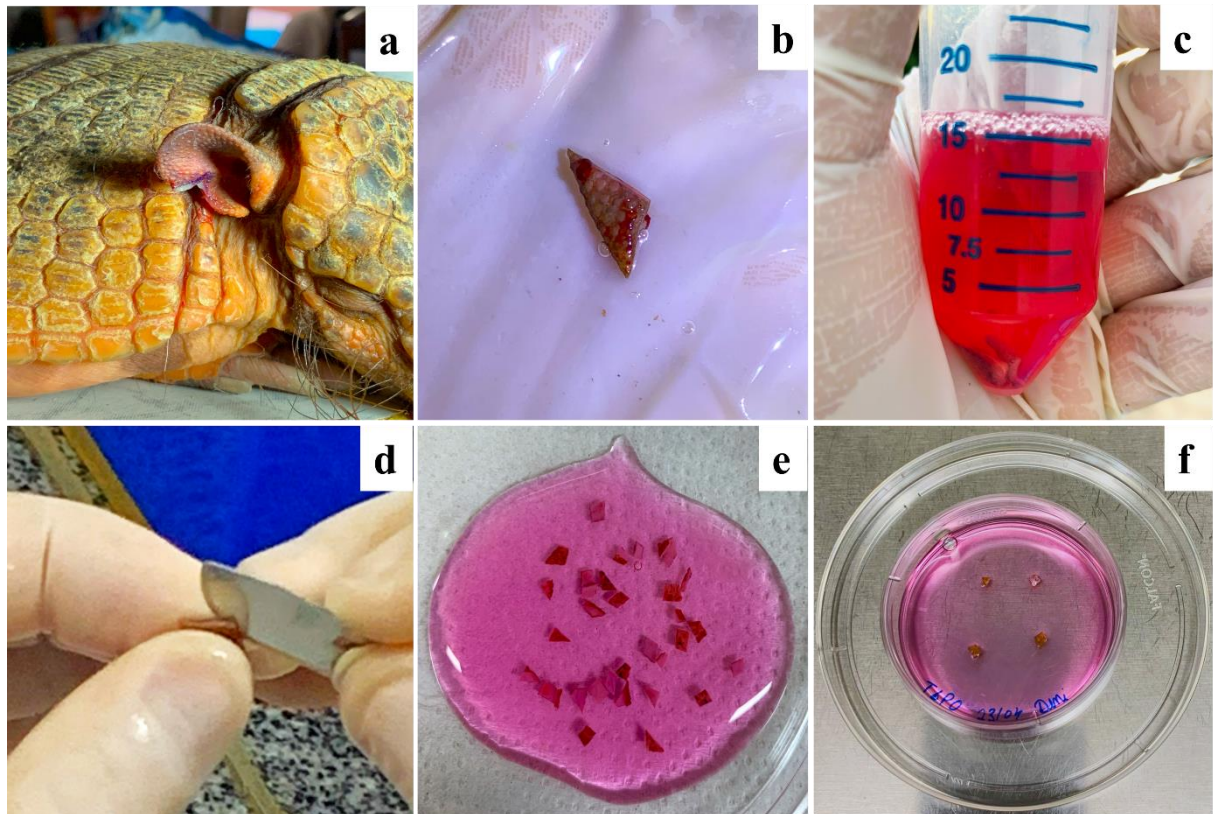


Fig. 1 Ear explant collection and preparation. (a-b) Ear sample cut from a six-banded armadillo, and (c) transferred in a sterile 50 mL tube containing DMEM, 10% FBS and 2% antibiotic-antimycotic. (d) The front and back of the excised skin sample were separated, (e) cut into pieces approximately 6.0 mm^3 in size, washed, and (f) four pieces were placed in a 60-mm-diameter culture dish with DMEM, 10% FBS and 2% antibiotic-antimycotic for culture.

Tissue collection and explant preparation

Skin tissue biopsies ($1\text{--}1.5 \text{ cm}^2$) from the basal region of the ear were collected using surgical scissors (Fig. 1b). After the collection, the tissues were washed rapidly with 70% alcohol, deposited in tubes containing DMEM plus 10% FBS and 2% antibiotic-antimycotic solution, and transported immediately to the laboratory (Fig. 1c).

In the laboratory, the biopsies were chopped in small pieces (Fig. 1d), approximately 6.0 mm^3 in size per piece (explants) and washed quickly in sequential baths of (1) DMEM supplemented

with 10% FBS and 10% antibiotic–antimycotic solution, (2) alcohol, and (3) DMEM plus 10% FBS and 2% antibiotic–antimycotic solution (Fig. 1e). Four explants per animal (Fig. 1f) were placed in polystyrene culture dishes with a culture medium consisting of DMEM plus 10% FBS and 2% antibiotic–antimycotic solution and cultured at 38.5 °C under a controlled environment with 5% CO₂ and 95% air (Borges et al. 2020).

Experimental design

We divided the experiment into three steps to establish and generate cell lines derived from the tissues. At first, the cells were grown under primary culture conditions and analyzed regarding the *in vitro* characteristics of explant culture and cell morphology. Moreover, upon the fourth passage, the cells were subjected to an immunofluorescence assay to confirm the cell type identity and karyotype analysis to assess chromosomal stability. In the second step, the cells were cultured and evaluated on the fourth (P4), seventh (P7), and tenth (P10) passages for *in vitro* culture effects on morphology, viability, proliferative activity, and metabolic activity. Finally, the cells (passage 4) were submitted to cryopreservation, evaluating the process's effect by analyzing viability, metabolism, proliferative activity, mitochondrial membrane potential ($\Delta\Psi_m$), and apoptosis levels. The cryopreserved cells' performance post-thawing was compared to those of non-cryopreserved cells.

Primary culture evaluation and subcultures

The medium was changed every 24 h for the primary culture. The cultures were observed daily, distinguishing the following parameters: number of samples, number of adhered samples, day of sample adherence, day of subconfluent growth of the samples, and total time to reach 70–80% confluence (Fernandes et al. 2023b). When most of the explant area were surrounded by outgrowing cells at 70%–90% confluence, they were removed, leaving only the cells; once the

cells reached 70%–80% confluence over the whole dish, they were trypsinized and subcultured. Cell morphology and growth were also noted. For trypsinization, the cells were previously washed with phosphate-buffered saline (PBS), incubated with trypsin/EDTA solution (0.25%/0.2%) for 7 min, and centrifuged at 600×g for 10 min. The resultant supernatant was discarded, the cell pellet was resuspended in a culture medium, and the cell suspension was transferred to another dish for subculturing. They were monitored daily, and the medium was replaced with fresh medium every other day.

Moreover, the cells were cultured without using antibiotics and antimycotics to ensure the absence of contamination. Subsequently, after characterization, the influence of the number of passages (fourth, seventh, and tenth passage) and the effects of slow freezing cryopreservation were analyzed.

Characterization of cells by morphological characteristics

Morphological characteristics were observed under a light microscope throughout the *in vitro* culture period to trace cellular and nuclear shapes and cytoplasmic extensions.

Characterization of cells by karyotype analysis

For the karyotype evaluation, the cells were subjected to a protocol adapted from the methodology described by Mehrabani et al. (2014) and Praxedes et al. (2021). Initially, the cells at the fourth passage were exposed to colchicine solution at a final concentration of 0.1%, followed by incubation at 38.5 °C for 2 h. The medium was removed, the cells were trypsinized, resuspended in DMEM, and transferred to a tube. After centrifugation (600×g for 10 min), 5 mL of hypotonic solution (0.075 M KCl) was added to the pellet, mixed gently, and incubated for 10 min at 37 °C. After this period, the tube was centrifuged (600×g for 10 min), and the

supernatant was discarded. Then, 5 mL of cold fixative solution (3:1 solution of methanol: acetic acid) was added, mixed gently, and centrifuged at 600×g for 10 min. The cell pellet was suspended by flicking the bottom of the tube. The fixation step was repeated three times. After the final fixation step, 1.0 mL of fixative solution was added to the suspended pellet. Two to three drops of the cytogenetic suspension were placed on a slide. Once the slides had dried, they were stained for 5 min using Giemsa, washed in distilled water, and air dried for subsequent analysis. At least ten metaphases were analyzed (Olympus BX51TF, Tokyo, Japan) at 100× magnification.

Characterization of cells by anti-vimentin immunofluorescence staining

The cell line's identity was confirmed by immunofluorescence staining according to the protocol described by Selokar et al. (2018), using antibodies against vimentin (a marker of fibroblasts). After the fourth passage, the cells were cultured until 70–80% confluence, fixed in 4% paraformaldehyde for 10 min at 25 °C, and washed three times with chilled PBS. Subsequently, they were incubated with antigen retrieval buffer (100 mM Tris, 5% urea, pH 9.5) for 24 h, washed three times with PBS, and permeabilized for 1 h in 0.4% Triton X-100. Afterward, the cells were incubated in a solution composed of 5% bovine serum albumin (BSA) in PBS and 0.1% Tween 20 for 1 h to block the non-specific binding of antibodies. Finally, the cells were immuno-stained with mouse anti-vimentin antibody (ab8979, 1:200) for 24 h at 4 °C, followed by incubation with secondary antibody (Alexa Fluor® 488-coupled goat anti-mouse IgG, ab150113, 1:400) for 1 h at 25 °C in the dark. Nuclei were counter-stained with 1.0 µg/mL Hoechst 33342 for 1 min and observed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 20× magnification.

Confirmation of the absence of bacterial and fungal contamination

To confirm the absence of possible contamination in the cell line, cells at fourth passage were also cultured for 28 days in DMEM containing 10% FBS without an antibiotic–antimycotic solution at 38.5 °C, 5% CO₂, and 95% air. The daily evaluation was performed under light microscopy to identify bacterial and fungal contamination. After this, the cells were discarded.

Passage influence on the quality of fibroblast lines

Subculturing of the cells was performed up to the tenth passage (from P0 to P10). We started the evaluation on the fourth passage precisely because only fibroblasts were visualized, and immunofluorescence confirmed the cell identity. Thus, when the cells reached 70–80% of confluence, they were resuspended after trypsinization during the fourth, seventh, and tenth passages and then subjected to further analyses performed immediately after cell resuspension. These are described below.

Cryopreservation influence on cell quality

After characterization, cells (at P4) were cryopreserved using a slow freezing method as previously described (Selokar et al. 2018; Praxedes et al. 2021). Briefly, 100% confluent cells were trypsinized, resuspended in DMEM to obtain a final concentration of 1.0×10^5 cells/mL, and then submitted to cryopreservation solution containing DMEM, 10% FBS, 10% dimethyl sulfoxide (Me₂SO), and 0.2 M sucrose. The cells were maintained in contact with the cryopreservation solution for 15 min at 4 °C. Later, they were stored in cryotubes and transferred to the freezing container Mr. Frosty system[®] (Thermo Scientific Nalgene, Rochester, USA) for 12 h, and subsequently, to -80 °C freezer while maintaining a cooling rate of 1 °C/min. After reaching the desired temperature, the samples were stored in liquid nitrogen (-196 °C).

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

Cell viability and metabolic activity

Cell viability was evaluated using the trypan blue assay, according to the methodology described by Praxedes et al. (2021). The cells were stained with 0.4% trypan blue in PBS (1:1 ratio) and counted on a hemocytometer. Unstained cells were considered viable; non-viable cells became blue due to the cell's ruptured membrane and consequent dye penetration. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

Subsequently, the cells were analyzed for metabolic activity using the 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay, according to the method described by Borges et al. (2018). A 5.0×10^4 cells/mL concentration was grown in 12-well polystyrene plates treated for cell adhesion. After five days, 1.5 mL of the MTT solution (5 mg/mL in DMEM) was added and incubated for 3 h. The MTT solution was then removed, and 1.0 mL of Me₂SO was added for 5 min under slow stirring to solubilize the MTT. After the total dissolution of formazan crystals, samples were analyzed in a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) at an absorbance wavelength of 595 nm.

Proliferative activity

The proliferative activity of the cells was described by growth curve modeling and by determining the population doubling time (PDT) over 168 h. Cells at 1.0×10^4 cells/mL were plated in 24-well dishes, trypsinized, counted, and recorded. Average counts at regular intervals of 24 h were used for determining the growth curve, and PDT was estimated (Mehrabani et al. 2016) according to the following equation:

$$PDT = T \ln 2 / \ln (X_e / X_b)$$

where PDT is the doubling time of the culture (in h), T is the incubation time, X_b is the number of cells at the beginning of the incubation period, and X_e is the number of cells at the end of the incubation period.

Measurements of $\Delta\Psi_m$

After having reached 70–80% confluence, the cells were exposed to 500 nM of MitoTracker Red (CMXRos) for 30 min at 5% CO₂ and 38.5 °C and then analyzed under a fluorescence microscope (excitation: 579 nm and emission: 599 nm) to determine the $\Delta\Psi_m$, obtaining ten images for each type of probe at 40× magnification. Images were evaluated using Image J software (National Institute of Health, Bethesda, USA) for quantifying the fluorescence intensities of the pixels, according to Lira et al. (2022).

Apoptosis levels

An aliquot of cells was resuspended in 2 µg/mL acridine orange and 10 µg/mL ethidium bromide. Subsequently, the cells were evaluated at 480 nm using a fluorescence microscope, where 300 cells were counted at 40× magnification. The cells were classified into (i) viable cells, with a uniform light green nucleus; (ii) cells in initial apoptosis, with a non-uniform green nucleus; (iii) cells in late apoptosis, with a non-uniform bright orange nucleus; and (iv) necrotic cells, with a uniform orange nucleus (Praxedes et al. 2021). A fluorescence microscope was

used to observe apoptotic changes in the stained cells, which were quantified using ImageJ software (National Institutes of Health, Bethesda, USA).

Statistical analyses

Data were expressed as mean $\pm$ standard error (one animal/ repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro–Wilk test and homoscedasticity by Levene’s test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at $p < 0.05$.

Results

Primary culture quality and cell morphology

The total culture time reached 81 days up to the tenth passage, except for one animal that lasted 61 days of culture. The 20 skin tissue fragments (four fragments per animal) had fast adhesion in dishes, and the first cells appearance was seen on day 3. We observed that all fragments attached to the dishes detached cells and showed great proliferative capacity during *in vitro* culture. All the samples adhered in a couple of days (1.4 ± 0.4 days). The cells around adhered fragments reached confluence within 15.6 ± 1.2 days (Table 1), and the total primary *in vitro* culture time was 36.8 days. The cells had fibroblast-like morphology, a fusiform shape, and oval nuclei and could quickly replace the epithelial cells that also appeared in the first days of culture (Fig. 2a).

2715 **Table 1** Establishment of primary culture of somatic cells derived from six-banded armadillos ear skin explants.

Animals	No. samples		No. attached samples				Total primary culture time (days)
	Initial	Attached (%)	Day all attached explants	Day cell outgrow	Confluent* (%)	Days to reach confluence*	
A1	4	4/4 (100)	3	3	4/4 (100)	17	34
A2	4	4/4 (100)	1	6	4/4 (100)	17	39
A3	4	4/4 (100)	1	6	4/4 (100)	15	39
A4	4	4/4 (100)	1	3	4/4 (100)	11	38
A5	4	4/4 (100)	1	3	4/4 (100)	18	34
Mean ± S.E.	4	4/4 (100)	1.4 ± 0.4	4.2 ± 0.7	4/4 (100)	15.6 ± 1.2	36.8 ± 1.2

2716 *Confluence 70–80%. S.E.: standard error.

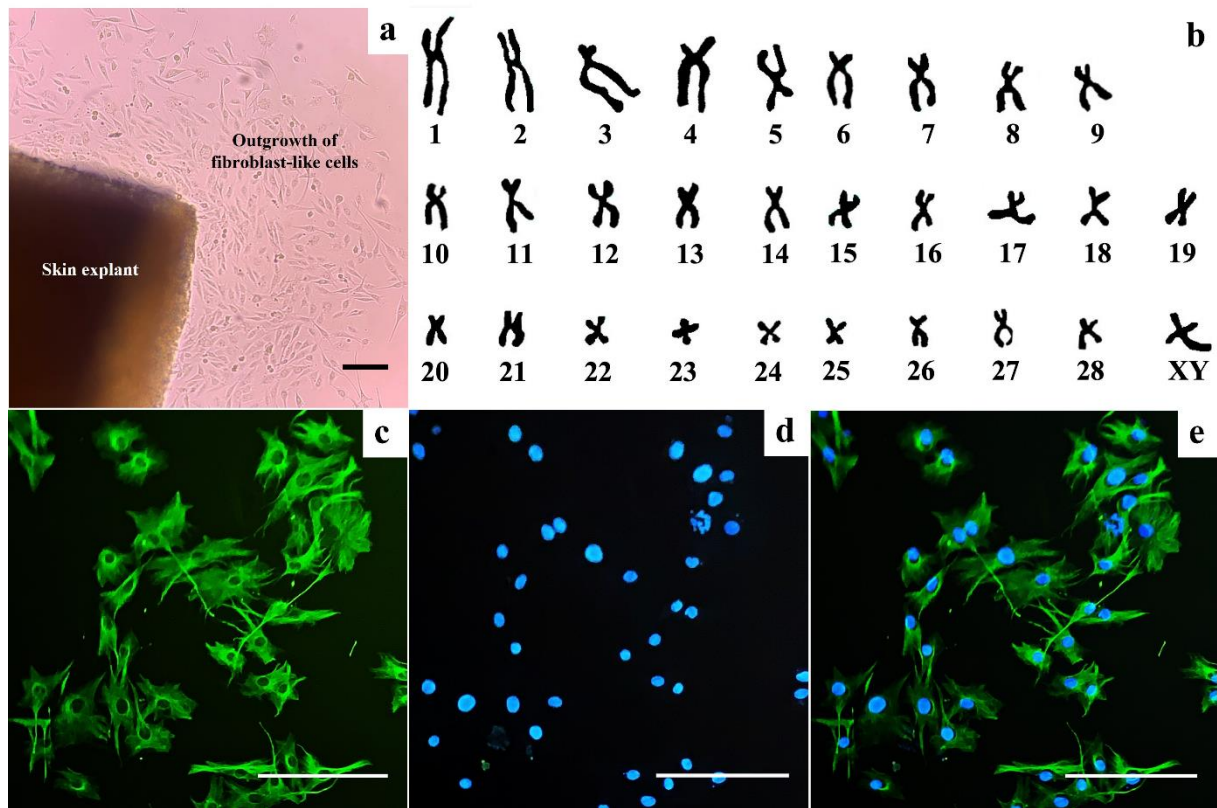


Fig. 2 Primary culture of ear skin tissues of six-banded armadillos. Line identification and cell type confirmation using a primary antibody (anti-vimentin). (a) Cell detachment and morphological aspects of fibroblasts on Day 7. Scale bar 50 μm , 10 $\times$ magnification. (b) Karyotype representation of six-banded armadillos ($2n = 58$). (c) Cells stained with vimentin antibody (green). (d) Cells labeled with Hoechst 33342 (blue), and (e) Image overlapping. Scale bar 50 μm , 20 $\times$ magnification.

Karyotyping and cell type confirmation

Our analysis of the six-banded armadillo karyotype was consistent with previous reports on this species; $2n = 58$ chromosomes were observed, of which 28 pair were autosomal, plus XY sex chromosomes. Moreover, the analyzed cells were of male origin, and the morphological characterization showed acrocentric, metacentric, and submetacentric stages of chromosomes (Fig. 2b). No chromosomal aberration was observed.

2732 Additionally, we confirmed the cells as fibroblasts by vimentin immunofluorescence (Fig. 2c–
2733 e). The identification was evident since the cells exhibited a high expression of vimentin that
2734 marked the cytoplasm completely (Fig. 2c), indicating the presence of intermediate filaments.
2735 The Hoechst labeling highlighted the spindle-like shape and ovoid nucleus (Fig. 2d).

2737 *Confirmation of the absence of bacterial and fungal contamination*

2738 No sign of contamination (turbidity, colony, or hyphal growth) was observed for 28 days of
2739 culture in media without antibiotics and antifungals. Visually, the culture medium did not show
2740 any change in appearance, and the cells proliferated to confluence and still maintained a steady
2741 flow in a confluent condition, indicating an absence of contamination.

2743 *Influence of the passage number on the quality of fibroblast lines*

2744 The prolonged *in vitro* culture time did not alter the fibroblast shape, with no changes observed
2745 after the tenth passage (Fig. 3a–c). The cells reached confluence in every passage; for the
2746 primary culture, they took approximately 37 days and only four days in the subsequent
2747 subcultures. Moreover, the cell viability in P4 was $90.6 \pm 3.3\%$, in P7 $95.1 \pm 2.3\%$, and in P10
2748 $87.1 \pm 3.6\%$ (Fig. 4a), remaining high throughout passages and showing no significant
2749 difference ($p > 0.05$). Additionally, the metabolic activity was maintained in all evaluated
2750 passages, with high values of 100%, 99.2%, and 100% for the fourth, seventh, and tenth
2751 passages, respectively ($p > 0.05$, Fig. 4b).

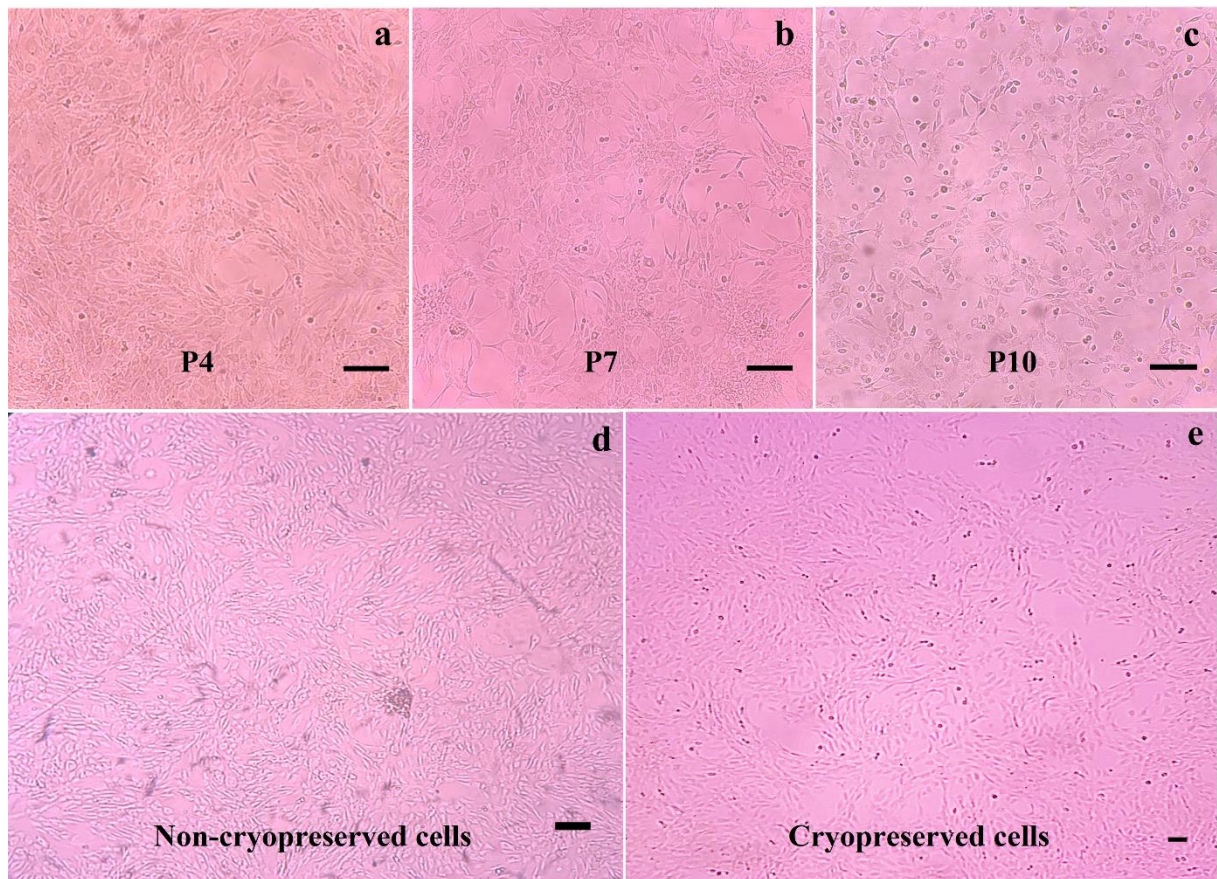


Fig. 3 Morphological characteristic of six-banded armadillos fibroblast cells. Cells were evaluated during the (a) Fourth passage, (b) Seventh passage and (c) Tenth passage, (d) Before, and (e) After freezing, and nine days of *in vitro* culture. Scale bar 50 μm, 10× magnification (a–c) and 4× magnification (d–e).

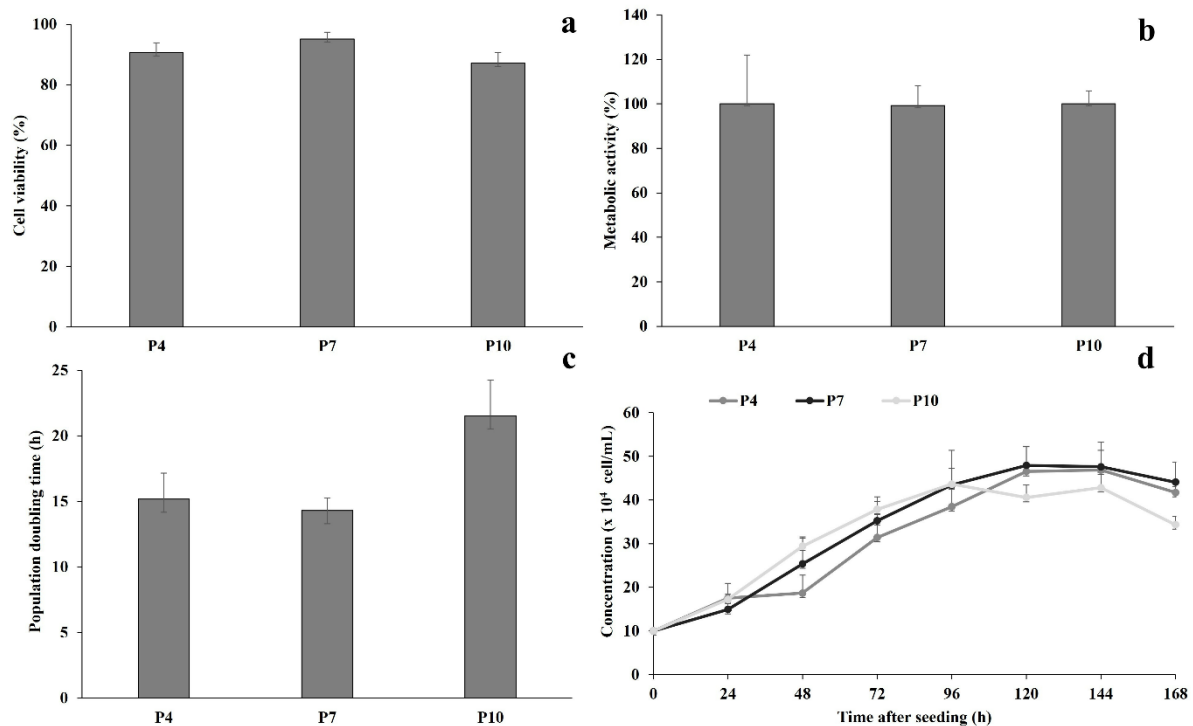


Fig. 4 Survival rate, functionality, and growth dynamics of skin fibroblast cells of six-banded armadillo after prolonged *in vitro* culture. (a) Viability, (b) Metabolic activity, (c) Population doubling time, and (d) Growth curve. Bars represent standard error. $p > 0.05$.

Overall, the cells took a short time to double their population. No differences were detected between the P4 (15.2 ± 2.0 h), P7 (14.3 ± 0.9 h), and P10 (21.5 ± 2.7 h) regarding their PDT value ($p > 0.05$, Fig. 4c). The cell growth curves of the groups were plotted by counting the total number of cells every 24 h for seven days. Accordingly, we observed the latency, exponential, and stationary phases (Fig. 4d). Cells in P7 and P10 showed exponential growth in the first 24 h, which lasted until 96 h, later the cells in the fourth passage showed exponential growth after 48 h of culture up to 120 h. The stationary phase and eventual population decrease started on the sixth day of culture in all groups. Moreover, we noticed in P4 and P7 that the cells had maximum cellular growth, $47 \pm 6.4 \times 10^4$ cells/well and $48 \pm 4.3 \times 10^4$ cells/well, respectively.

Effect of cryopreservation on cell quality

The cryopreservation did not change the cell morphology (Fig 3d–e). Moreover, no difference was observed in the cell viability ($98.7 \pm 0.3\%$) when compared to the non-cryopreserved group ($90.2 \pm 5.9\%$; $p > 0.05$, Fig 5a).

Also,

no alteration of cryopreserved cells' metabolic activity was observed ($89.3 \pm 4.4\%$ vs. $100.0 \pm 12.3\%$; $p > 0.05$, Fig 5b). Nonetheless, they took more time to double the cellular population since the PDT values were higher (16.2 ± 1.2 h) when compared to the non-cryopreserved cells (13.1 ± 1.0 h; $p < 0.05$, Fig. 5c). Moreover, we compared the growth dynamic between cryopreserved and the non-cryopreserved cells, the latter showed latency, and exponential and plateau phases. Cryopreserved cells exhibited exponential growth on the first day until the fifth day, when the population started to decrease; on the last day, both groups diminished (plateau phase; Fig. 5d). It was also observed that maximum cellular growth was noticed in the group of cryopreserved cells ($67 \pm 5.1 \times 10^4$ cells/well vs. $49 \pm 6.2 \times 10^4$ cells/well).

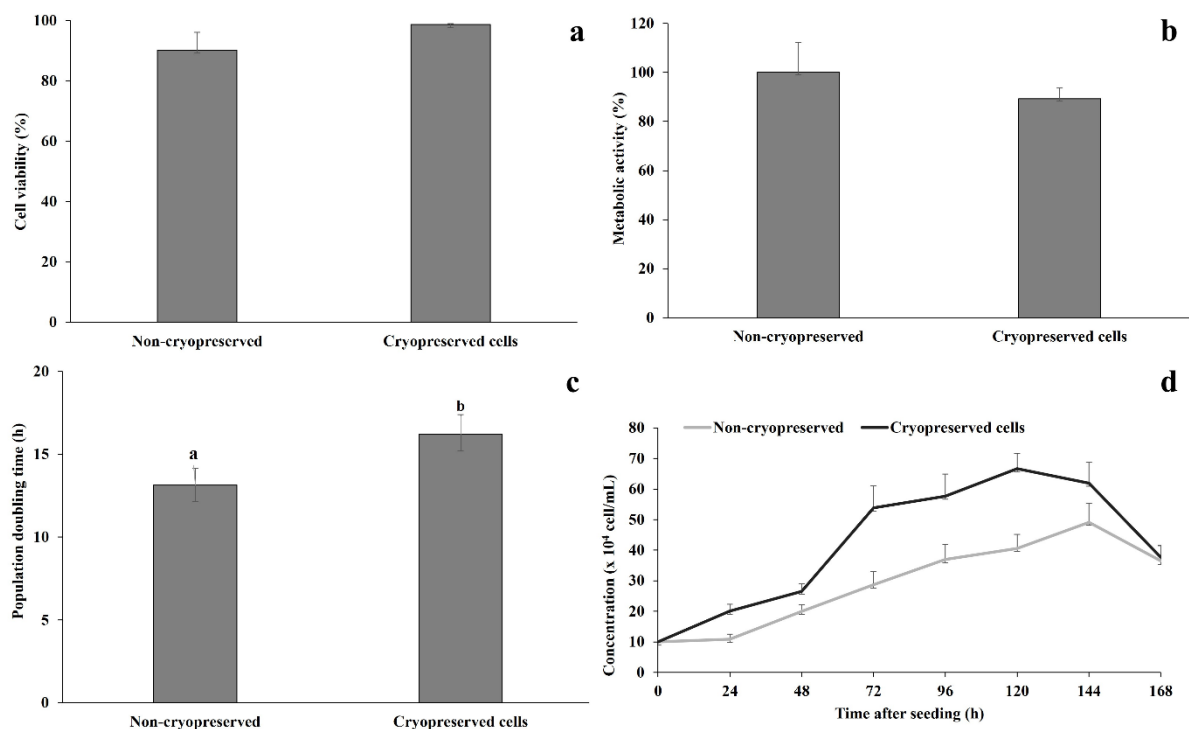


Fig. 5 Survival rate, functionality, and growth dynamics of skin fibroblast cells of six-banded armadillo before and after cryopreservation and seven days of *in vitro* culture. (a) Viability, (b) Metabolic activity, (c) Population doubling time, and (d) Growth curve. Bars represent standard error. ^{a,b}; $p < 0.05$.

Furthermore, the cryopreservation changed the $\Delta\Psi_m$ in arbitrary fluorescence units when compared to that of the non-cryopreserved cells; the value was significantly lower (0.6 ± 0.1 vs 1.0 ± 0.3 , $p < 0.05$, Fig. 6a–c). Nevertheless, the number of viable and non-viable cells in different apoptotic stages (early apoptosis, late apoptosis, and necrosis) did not differ statistically between non- and cryopreserved cells ($p > 0.05$), and both groups presented great number of viable cells with values above 80% (Fig. 6d–f).

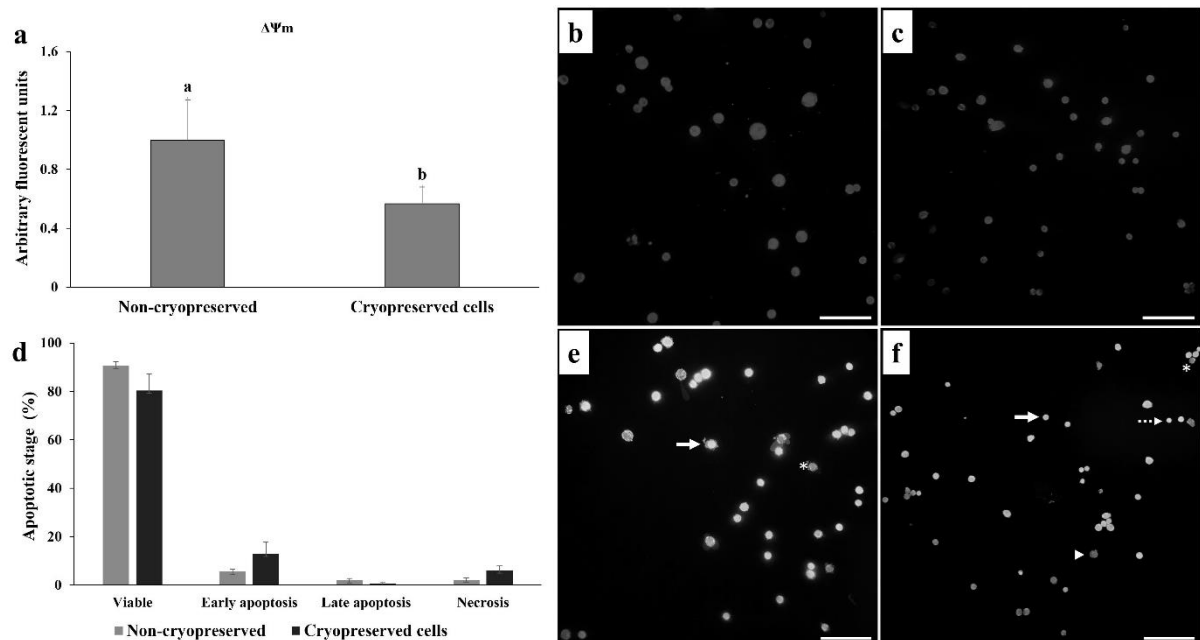


Fig. 6 Influence of cryopreservation on oxidative stress and apoptosis levels in fibroblast cells of six-banded armadillos. (a) Measurement of intracellular mitochondrial membrane potential ($\Delta\Psi_m$) with MitoTracker and d) Apoptosis levels. (b), (e) Before, and (c), (f) After cryopreservation. Bars represent standard error. Viable cells (arrow); initial apoptotic cells

(dotted arrow); late apoptotic cells (asterisk); necrotic cells (triangles). Scale bar 50 μm , 20 $\times$ magnification. ^{a,b}: $p < 0.05$.

Discussion

In this study, we successfully isolated, characterized, and evaluated the effects of serial passages and cryopreservation processes on the quality preservation of fibroblast cells derived from the skin tissues of six-banded armadillos. The fibroblast cells maintained their viability from the fourth to tenth passages and their metabolism and growth patterns. They were also efficiently cryopreserved, showing few changes in their biological characteristics *in vitro*. Establishing the fibroblast cell line in this species is essential for future use in genetic studies, cryobanking, cloning, or another reproductive research. These results have been achieved in many wild mammals aiming to preserve materials from valuable species, including species that occur in the worldwide biomes, such as collared peccaries (*P. tajacu*, Borges et al. 2020), red-rumped agouti (*D. leporina*, Praxedes et al. 2021), jaguar (*Panthera onca*, Oliveira et al. 2021), and puma (*P. concolor*, Rodrigues et al. 2023). Studies in armadillo fibroblast culture are very scarce, and there are no reports detailing establishing this cell line from six-banded armadillo species.

The six-banded armadillo ear fragments were thick, and the tissue was separated in two (front and back) to isolate fibroblasts for culture. The adherent method for primary cell culture was preferable in this study because it is simpler and avoids tissue damage compared to other methods, such as enzyme digestion (Sharma et al. 2018). In the first's days of primary culture, the medium was carefully replaced to prevent the detachment of the explants. However, some fragments still detached after manipulation, which delayed their firm attachment. Therefore, some samples took more days to liberate cells than others. Overall, the skin explants had early

and great adherence (1.4 ± 0.4 days), and the cells of the samples quickly grew (4.2 ± 0.7 days). Similar results were observed in other wild animals, such as in collared peccary ear skin culture, where tissues adhered in 1.3 ± 0.5 days and cell growth was seen at day 6.6 ± 1.6 (Borges et al. 2017). Siengdee et al. (2018) also reported the early outgrowth around explants in skin fragments from the Asian elephant (*Elephas maximus*; 4–12 days). The Asian elephant was proved to have a closer phylogenetic relationship with the nine-banded armadillo (*Dasypus novemcinctus*; Sreekumar et al. 2007). Therefore, the protocol and results reported by Siengdee et al. (2018) were a meaningful basis for comparison.

Moreover, in our study, the cells around explants took two weeks to reach confluence, but it is worth mentioning that some fragments reached confluence faster than others in the same dish. This observation indicates that the cells had a fast proliferation rate in the primary culture. In accordance, the primary fibroblast cells from the killer whale (*Orcinus orca*) skin tissue culture appeared in 4–7 days after biopsy, and cell confluence (90–95%) around explants was observed after 13–14 days (Bjørneset et al. 2023).

Overall, the total primary culture time was shorter than in other studies with similar conditions (Borges et al. 2020; Praxedes et al. 2021), indicating that the cells were grown in good culture conditions. Initially, epithelial and fibroblast cells grew together during explant culture. However, over time, fibroblast cells overgrew the epithelial cells since fibroblasts grow faster, have greater adherence, require less nutritional support in *in vitro* environment, and respond fast to trypsinization (Selokar et al. 2018; Siengdee et al. 2018; Wang et al. 2020; Praxedes et al. 2021). After the first passage, the fibroblast cells grew rapidly; within four days, they reached 80% confluence, and subsequent passages occurred.

Morphologically, it was easy to identify the fibroblast cells, especially with low confluence. At the P4, when the cells were characterized, fibroblast monolayer was predominant in culture dishes, and the purity of these cells was then confirmed by vimentin staining. The anti-vimentin antibody binds to intermediate filaments, a typical structure among fibroblasts, proving that the cells have a mesenchymal origin.

When establishing cell lines, it is essential to confirm the normal genetic makeup of the animal by karyotyping, especially while conserving animal germplasm (Sharma et al. 2018). The cells under *in vitro* conditions should maintain the same diploid character as those *in vivo*. Our results supported that the chromosome karyotyping showed normal diploid ($2n = 58$) numbers, validating the stability of these cells (Liu et al. 2010). Furthermore, it is worth mentioning that we could not find many cells containing separate chromosomes, which would allow better visualization of individual chromosomes under a light microscope. This result was also observed in Chinese muntjac (*Muntiacus reevesi*) fibroblasts karyotype analysis (Wang et al. 2020) when they collected the fibroblast cells after 80 h culture; however, the cell culture collected after 68 h of seeding, when they were highly proliferative, presented separated chromosomes for the analysis. The authors pointed that is important to evaluate when to collect the cells for the karyotyping to find more cells containing separated chromosomes at metaphase of mitosis. Nevertheless, even so that the chromosomes were paired in our result, they were well condensed, visible and in correct number, allowing us to conclude that the genetic properties of six-banded armadillo were maintained.

Bacterial and fungal contaminations are detected easily with the naked eye (Sharma et al. 2018). Our study confirmed the absence of bacterial or fungal contamination by examining the cell line in culture medium without antibiotics and antifungals under daily observation with light

microscope. Fortunately, the culture medium remained clear during the 28-day assay, and no other alterations (like moving granules or fungal structures) were noted, suggesting that the cells were free from microbial contaminations. However, despite best efforts, microbial contaminations are quite frequent during mammalian cell culture (Malik et al. 2023). Therefore, antibiotics and antimycotics are still recommended, and combined with good laboratory working practices, will ensure the absence of contamination (Borges et al. 2020).

Considering the prolonged culture time evaluation, adequate results were obtained, and the cells showed no changes in their morphology, viability, metabolism, and proliferative activity parameters. These findings are consistent with other studies that evaluated longer culture time up to the fourth passage for wild species, supporting the idea that the fibroblast cell line was stable. We imaged P4, P7, and P10 fibroblasts to monitor possible morphology changes due to serial passaging, but no significant difference was observed. Liu et al. (2022) reported that giant panda (*Ailuropoda melanoleuca*) fibroblasts changed their morphology (began to enlarge) and had their proliferation rate decrease dramatically from P9. Magalhães et al. (2017) also evaluated the quality of brown brocket deer (*Mazama gouazoubira*) fibroblasts after P4, P7, and P10; however, they noticed a significant decrease in cell survival rate at P10, compared with earlier passages. Here, the cell viability remained high (> 87%) in earlier and later passages. The percentage of viable cells at P10 was higher when compared with fibroblasts cultured from collared peccary (84%; Borges et al. 2020) and Chinese muntjac (85%; Wang et al. 2020), considering the same passage.

The extended culture also did not affect the cell's metabolism or proliferative activity. To evaluate the PDT, we manually counted the cell suspension for seven days daily and plotted a cell growth curve with a Napierian logarithm. The growth was similar between the serial

passages, and the growth curve was noted as parallel. As shown in Wang et al. (2020) report, the cells proliferated rapidly after a short latency phase and entered the exponential growth phase. In this study, the cells at P4 had an initial lag phase of 48 h for adaptation, followed by an exponential phase between 48–120 h. As expected, cell proliferation started to slow and eventually decreased their population at six days because of contact inhibition; the same was observed in P7 and P10 groups and camel's fibroblasts at P5 (Sharma et al. 2018). The established cell line revealed PDT of 15.2 h in P4, 14.3 h in P7, and 21.5 h in P10. Similar estimates of PDT have been obtained from several studies (21.3 h at P3: Wang et al. 2020; 26.1 h at P5: Sharma et al. 2018; 25.0 h at P6: Siengdee et al. 2018; 22.4 h at P10: Jenuit et al. 2021).

On the other hand, it has been documented that wild species present a higher PDT value than domestic animals, as observed in giant pandas (33.8 h: Yu et al. 2015) and brown brocket deer (90.0 h: Magalhães et al. 2017). The latter showed that the fibroblast cells had different metabolism and proliferative activity between passages, the PDT of cells from P10 was higher, and the cell proliferation rate was lower than that from P4 and P7. In addition, the cells at P10 exhibited lower metabolic activity compared with P4 cells (Magalhães et al. 2017). Jenuit et al. (2021) also evaluated primary fibroblast cells from Sumatran rhinoceros (*Dicerorhinus sumatrensis*) throughout P5, P10, and P15, reporting that cell senescence level increased significantly as the passage number increased. They also observed significant changes in morphology, growth, and viability with an increasing number of passages. No significant changes in our cell line throughout passages suggests that our experimental protocol was highly efficacious, providing six-banded armadillo fibroblast cells with excellent number and quality.

The cryopreservation of fibroblast cells is usually performed in early passages. This is recommended to obtain cells with conserved genetic characteristics (Borges et al. 2020). In this

study, we chose the fourth passage, in which the cells were characterized, and the purity of the fibroblast cell line was confirmed. Regarding survival rate, the cryopreserved cells showed excellent viability (98.7%) after being thawed and cultured, not differing from the non-cryopreserved cells, as observed in Asian elephant (Siengdee et al. 2018) and Sumatran rhinoceros (Jenuit et al. 2021).

Meanwhile, the growth of non- and cryopreserved cells was different; according to the growth curve, the comparison showed a higher proliferation rate in cryopreserved cells, but the PDT value was higher compared to non-cryopreserved cells (16.2 h vs. 13.1 h). This delay might have been due to the freezing process, but it did not induce cell growth reduction. Furthermore, the after-effects of cryopreservation might cause mitochondrial structural abnormalities (Borges et al. 2020). Therefore, $\Delta\Psi_m$ is a global indicator of mitochondrial function. Here, we observed an effective decrease in $\Delta\Psi_m$ after cryopreservation, a sign of cellular stress that eventually leads to apoptosis.

In contrast, apoptosis levels and their metabolic rate remained constant after cryopreservation. Overall, our fibroblast cell line showed high tolerance to cryopreservation since most of the quality parameters were unchanged. This implied that the slow freezing technique and a cryopreservation medium composed of 80% DMEM, 10% FBS, 10% Me₂SO, and 0.2 M sucrose were suitable for conserving fibroblast cells of six-banded armadillo. The addition of both intra- (Me₂SO) and extracellular cryoprotectants (FBS and sucrose) protected the cells from significant damage associated with the freezing process. This result agrees with other studies (Praxedes et al. 2021; Lira et al. 2022).

Conclusions

In conclusion, we demonstrated that the isolation, characterization and cryopreservation of stable fibroblast cell lines derived from six-banded armadillos is possible. Moreover, the cell viability was maintained until the tenth passage and after cryopreservation. The cell lines were of high quality and confirmed to have no contamination, and the protocol provided useful information to extend their storage efficiently. Additionally, establishing the fibroblast cell lines from this species offers manifold possibilities, preserving the genetic diversity of six-banded armadillo and providing excellent biological resources for future research studies, such as cloning and other somatic cell reprogramming technologies.

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Conflict of interests

The authors declare no conflict of interests.

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3102 **CAPÍTULO 5 – NON-PERMEABLE CRYOPROTECTANTS’ INFLUENCE ON**
3103 **FIBROBLAST SLOW FREEZING IN SIX-BANDED ARMADILLOS (*Euphractus***
3104 ***sexcinctus* LINNAEUS, 1758) (Artigo experimental)**

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3108 **SUBMETIDO PARA A CRYOLETTERS**

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3112 **Qualis 2017–2020: B1**

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**NON-PERMEABLE CRYOPROTECTANTS' INFLUENCE ON FIBROBLAST SLOW
FREEZING IN SIX-BANDED ARMADILLOS (*Euphractus sexcinctus* LINNAEUS,
1758)**

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Abstract

BACKGROUND: Determining an appropriate cryopreservation solution is crucial for
establishing somatic resource biobanks. **OBJECTIVE:** Here, we proposed a cryopreservation
protocol to optimize the preservation of skin-derived fibroblasts from six-banded armadillos by
comparing different concentrations of fetal bovine serum (FBS) in the absence or presence of
sucrose as non-permeable cryoprotectants. **MATERIALS AND METHODS:** Cells were
cryopreserved by slow freezing with different solutions containing Dulbecco's modified
Eagle's medium (DMEM) with 10% dimethyl sulfoxide (DMSO), varying concentrations of
FBS (10, 20%, and 40%) without or with 0.2 M sucrose, totaling six comparison groups. Cells
not subjected to cryopreservation were used as a control. Cells were evaluated for
morphological characteristics, viability, metabolism, apoptosis levels, proliferative activity, and
mitochondrial membrane potential ($\Delta\Psi_m$). **RESULTS:** Cells maintained similar fusiform
morphology and demonstrated high viability (> 90%) before and after cryopreservation in all

groups. Cryopreserved cells with 10% and 40% of FBS without sucrose showed lower metabolism, but interestingly, when sucrose was added, this parameter was maintained as in the control group. This effect was not observed in the 20% FBS groups in the absence or presence of sucrose, with both similar to the non-cryopreserved group. The addition of sucrose maintained apoptosis levels, while 20% and 40% FBS without sucrose groups showed alteration in viable, early apoptosis, and necrosis stages. Nevertheless, all cryopreserved groups showed lower proliferative activity with a higher population doubling time (16.2–19.9 h) than the non-cryopreserved group (15.2 h). Finally, 20% FBS groups in the absence or presence of sucrose maintained the $\Delta\Psi_m$. **CONCLUSION:** We demonstrated that 20% FBS with sucrose was the most suitable cryopreservation solution for six-banded armadillo skin-derived fibroblast lines, promoting a greater cell survival rate after thawing.

Keywords: cryobanking; cell cryopreservation; fetal bovine serum; long-term conservation; somatic cell; sucrose

INTRODUCTION

Cryobanking somatic cells from wild mammals has become a widespread measure to develop *ex situ* biodiversity conservation (1). Skin fibroblasts are the most common somatic cell type deposited in cryobanks because they are technically easy to obtain and maintain in culture (2) and used for cloning and generating induced pluripotent stem cells (1, 3). In this matter, significant progress has been made towards the creation of somatic cryobanks of several wild mammals using skin biopsies, such as collared peccary (*Pecari tajacu*; 4), Sumatran rhinocero (*Dicerorhinus sumatrensis*; 5), and African savanna elephant species (*Loxodonta Africana*; 6). These studies aim to successfully establish and preserve cell lines for further research and reproductive efforts.

Establishing new cryobanks in biodiversity-rich regions is encouraged (7), and for any species, it is essential to store the biological sample efficiently. This includes finding the most suitable cryopreservation technique, cryoprotectant agents, and correct dilutions (8, 9). Recently, we started the construction of a somatic biobank for six-banded armadillo species, establishing the appropriate method and solution for cartilage and skin tissue cryopreservation (10). The six-banded armadillo is a native American wild mammal of the superorder Xenarthra with wide distribution and a presumed large population (11, 12). At the same time, their population has been suffering from severe habitat loss, roadkill, and hunting, especially in Brazilian territory (12, 13, 14). The species play a key role as ecosystem engineers, arousing great economic and biomedical interest (15) and serving as valuable experimental models for endangered Xenarthra species conservation (16, 17). Nevertheless, little information relevant to the conservation of this species is available, and no biobanked cell lines have been reported.

The standard approach for fibroblast cryopreservation includes slow freezing, dimethyl sulfoxide (DMSO) use, and rapid thawing at 37 °C (18). Studies have revealed that combining permeating cryoprotectants, such as DMSO, and non-permeating cryoprotectants, like fetal bovine serum (FBS) and sucrose, offer the highest recovery rates after cryopreservation (4, 9). DMSO at 10% has remained the gold standard cryoprotectant. Still, the composition of the solution can vary with differences in the concentration of FBS (from 10–90%) and the presence or absence of sucrose (6, 19, 20, 21). How the FBS acts is not completely elucidated, but it protects the cells from osmotic injury by stabilizing cell membranes and adjusting the intra- and extracellular osmotic pressure (9). Although concentrations of 10–20% FBS are usually tested, in Asian elephants (*Elephas maximus*) it was observed that 40% FBS resulted in great post-cryopreservation cell viability above 95% (20). In addition to protecting membranes, other studies have shown that the combination of high concentration of FBS in the presence of 0.2 M sucrose improved the post-thaw survival of collared peccary (4) and red-rumped agouti

(*Dasyprocta leporina*; 9) fibroblast after slow freezing. Sucrose also acts as a powerful osmoprotectant; therefore, we can hypothesize that both cryoprotectants work synergistically to increase extracellular cell protection.

In this context, this study sought to determine the most suitable cryopreservation protocol for six-banded armadillo skin-derived fibroblast cell lines by evaluating different concentrations of FBS in the presence or absence of sucrose. We aimed to enhance the efficiency of storing this material in cryobanks and help preserve the biodiversity of the *Xenarthra* species.

MATERIALS AND METHODS

The Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, No. 36/2020) approved the study in compliance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio, No. 76655-1). Dulbecco's modified Eagle medium (DMEM), FBS, and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA), and the fluorescent probes were purchased from Invitrogen (Carlsbad, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Media were filtered using a 0.22- μ m system (Corning, New York, USA).

Sample collection, primary culture, and subculture

Skin biopsies (1.0–1.5 cm²) were collected from five adult six-banded armadillos, three females and two males, kept in individual enclosures at the Laboratory of Studies in Immunology and Wild Animals (LEIAS/UFERSA). The samples were transported to the laboratory in conical tubes containing DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution within 30 min at 37 °C, as described by Fernandes et al. (10).

In the laboratory, the tissues were fragmented (6.0 mm³) and submitted to *in vitro* culture at 38.5 °C and 6.5% CO₂. The primary culture was monitored daily, and the medium was

3226 replaced with fresh medium every other day. When 70–80% of the plate was covered by cells,
3227 they were trypsinized and passaged until the fourth passage (10).

3228 ***Experimental design***

3229 To assess the effects of different concentrations of FBS in the presence or absence of
3230 sucrose, cells at fourth passage were cryopreserved with DMEM containing 10% DMSO and
3231 (i) 10% FBS (10FBS group), (ii) 10% FBS and sucrose (10FBS + sucrose group), (iii) 20%
3232 FBS (20FBS group), (iv) 20% FBS and sucrose (20FBS + sucrose group), (v) 40% FBS (40FBS
3233 group), and (vi) 40% FBS and sucrose (40FBS + sucrose group). Cells not subjected to
3234 cryopreservation were used as a control (non-cryopreserved group). All cells were evaluated
3235 regarding morphological characteristics, viability, metabolism, apoptosis levels, proliferative
3236 activity, and mitochondrial membrane potential ($\Delta\Psi_m$).

3237 ***Cryopreservation and thawing protocols***

3238 Cells were cryopreserved by slow freezing, as described previously (22). After reaching
3239 subconfluence (70–80%) in culture, cells were resuspended by trypsinization, and the pellets
3240 were dissolved in the freezing medium as described in the experimental design and transferred
3241 into 1.5 mL cryogenic vials with 1.0×10^5 cells/mL. For the groups cryopreserved with sucrose
3242 in the medium, cells were first put in contact with the cryopreservation solution (DMEM plus
3243 DMSO and FBS) without sucrose for 15 min at 4 °C, and then for another 15 min at 4 °C in
3244 cryopreservation solution with sucrose. Cells were subjected to slow freezing at a rate of 1.0
3245 °C/min to -80 °C in freezing containers (Mr. Frosty™ freezer, Thermo Scientific Nalgene,
3246 Rochester, NY, USA) overnight. Finally, samples were stored in liquid nitrogen (-196 °C).

3247 After two weeks, cryovials were thawed by holding the vials for 1 min at room temperature
3248 in air (~25 °C), followed by immersion in a water bath at 37 °C for 4 min and transferring the
3249 contents of the tubes into the solution to remove cryoprotectants as per a method described

previously (22). Initially, for the groups with sucrose, the first wash was performed with DMEM and 10% FBS containing 0.2 M sucrose at 4 °C for 15 min and centrifuged for 10 min at 1300×g. Subsequently, the second wash was performed using only DMEM and 10% FBS, maintained at 25 °C for 15 min, and centrifuged again. Two centrifugations in the same condition were performed for the group without sucrose using only DMEM and 10% FBS. Finally, thawed cells were subjected to analysis.

Cell morphology and viability by membrane integrity assay

Cell morphology and confluence were observed daily. Cell viability was performed by analyzing cell membrane integrity using the trypan blue staining method (10), where dead cells were identified (stained in blue) by membrane rupture that allowed the permeabilization of the dye (non-viable cells) and those alive as colorless, without membrane rupture (viable cells). Immediately after cell resuspension, an aliquot (20 µL) of cells was stained with 0.4% trypan blue in phosphate-buffered saline (PBS) at a 1:1 ratio and counted in a Neubauer chamber. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted ×100 (23).

Metabolism and apoptosis levels

Metabolism was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoline bromide (MTT) assay. Briefly, cells were seeded at a 5.0×10^4 cells/mL density in a 12-well plate at 38.5 °C with 6.5% CO₂. After 5 days, MTT solution (5 mg/mL in DMEM) was added, and the dishes were incubated for an additional 3 h. Subsequently, the MTT solution was removed, and DMSO was added as an MTT solubilization solution. The absorbance was then measured at a wavelength of 595 nm (10).

The apoptotic state of cells was examined according to Lira et al. (24), where cells were stained with acridine orange (AO, 2.0 µg/mL) and ethidium bromide (EB, 10.0 µg/mL) and

evaluated under a fluorescence microscope. Images obtained were analyzed using the Image J software to quantify the fluorescence signal intensity (pixels). Thus, 300 cells were counted in each group, where the viable cell had a uniform light green nucleus, an early apoptosis cell had a non-uniform green nucleus, a late apoptotic cell had bright orange areas, and a necrotic cell had a uniform orange nucleus.

Proliferative activity

To determine the proliferative activity, the cells were cultured in 24-well dishes at a concentration of 1.0×10^4 cells/mL, trypsinized, and counted every 24 h for seven days. The average of the counts at regular intervals of 24 h was used for preparing the growth curve, and population doubling time (PDT) was estimated (23) according to the following equation: $PDT = T \ln 2 / \ln (X_e/X_b)$, where PDT is the time of the culture (in hours), T is the incubation time, X_b is the number of cells at the beginning of the time incubation, X_e is the number of cells at the end of the incubation time, and ln is Napierian logarithm.

Mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined using the MitoTracker Red probe (CMXRos; 22). Cells were washed with PBS and incubated with 500 nM CMXRos diluted in DMSO for 30 min at 38.5 °C, 6.5% CO₂, visualized under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) (excitation: 579 nm and emission: 599 nm), and the intensity of the fluorescence signal (pixels) was quantified. The ImageJ software (National Institutes of Health, NIH, USA) obtained and evaluated ten images.

Statistical analysis

All data were expressed as mean $\pm$ standard error and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro–Wilk test and homoscedasticity by Levene’s test. Since data did not

show a normal distribution, they were transformed with arcsine and analyzed using variance analysis (ANOVA) followed by the Tukey or unpaired t-test. Significance was set at $P < 0.05$.

RESULTS

As shown in Fig. 1, the cryopreserved cells had a typical fibroblast-like morphology in all groups, with a fusiform shape, elongated cytoplasmic extensions, and centered oval-shaped nuclei, similar to the non-cryopreserved group. The cryopreservation conditions also did not alter the cells' ability to grow and reach 80% confluence, which was achieved in approximately 5 days of culture after thawing in all groups tested.

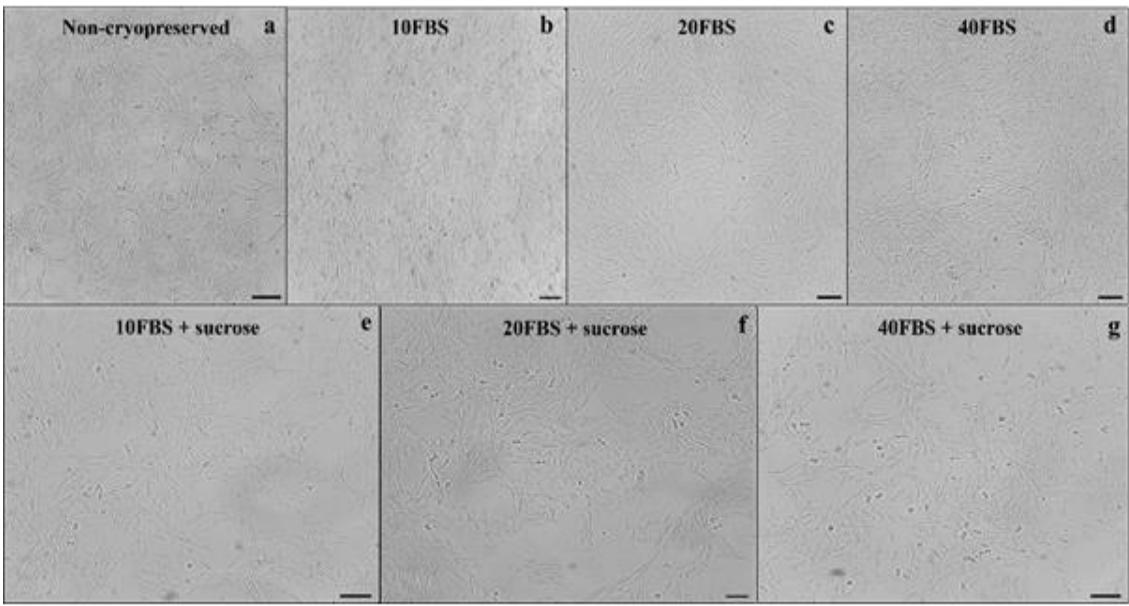


Figure 1. Morphologic characteristics of six-banded armadillos fibroblast cells before and after cryopreservation using different combinations of extracellular cryoprotectant solutions. **(a)** non-cryopreserved group, **(b, c, d)** cryopreserved cells with 10%, 20%, and 40% FBS, and **(e, f, g)** 10%, 20%, and 40% FBS with sucrose, respectively. *Fetal bovine serum (FBS). Scale bar 100 μm .

Furthermore, all groups showed high viability after cryopreservation, with values ranging between 93.1 and 99.1%, and found no difference compared to the non-cryopreserved group

3314 (90.6%; $P > 0.05$; Fig. 2a). Regarding the metabolic activity, only 10FBS and 40FBS groups
3315 presented a shorter value compared to the other cryopreserved groups and the non-
3316 cryopreserved group (Fig. 2b). This effect was not observed in the 20% FBS groups in the
3317 absence or presence of sucrose, both being similar to the non-cryopreserved group. Moreover,
3318 all the cryopreserved groups with sucrose showed similar results regarding the apoptosis levels
3319 compared with the non-cryopreserved group (Fig. 2c). However, the 20FBS without sucrose
3320 presented a lower proportion of viable cells ($60.1 \pm 10.3\%$) and a higher proportion of early
3321 apoptosis cells ($33.5 \pm 9.4\%$), differing from the non-cryopreserved cells ($90.5 \pm 1.9\%$ and 5.5
3322 $\pm 1.0\%$, respectively; $P > 0.05$; Fig. 2c). Also, higher concentration of 40% FBS without
3323 sucrose presented higher percentage of necrotic cells ($7.5 \pm 0.8\%$) compared to non-
3324 cryopreserved cells ($2.1 \pm 0.9\%$; $P > 0.05$; Fig 2c).

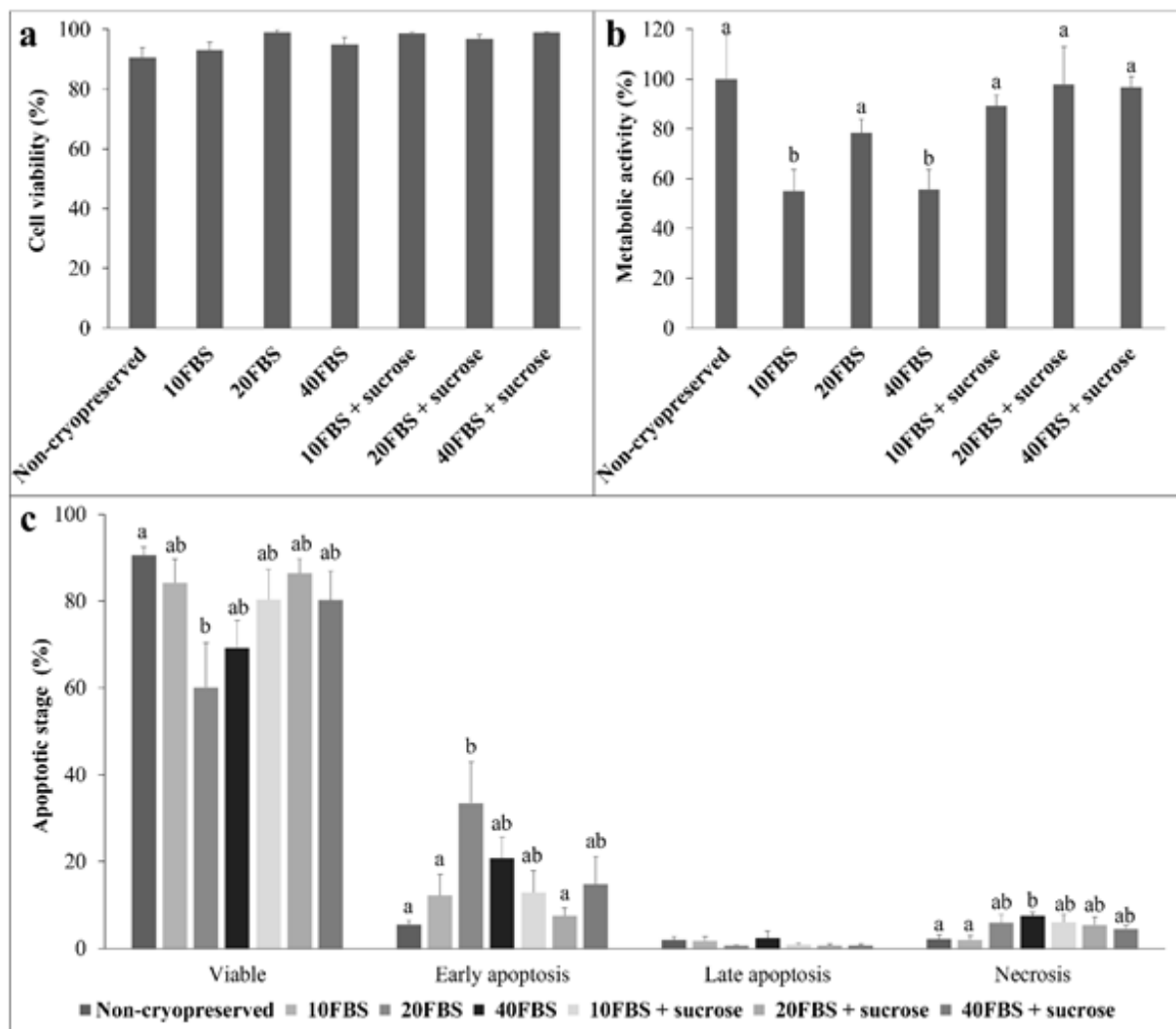
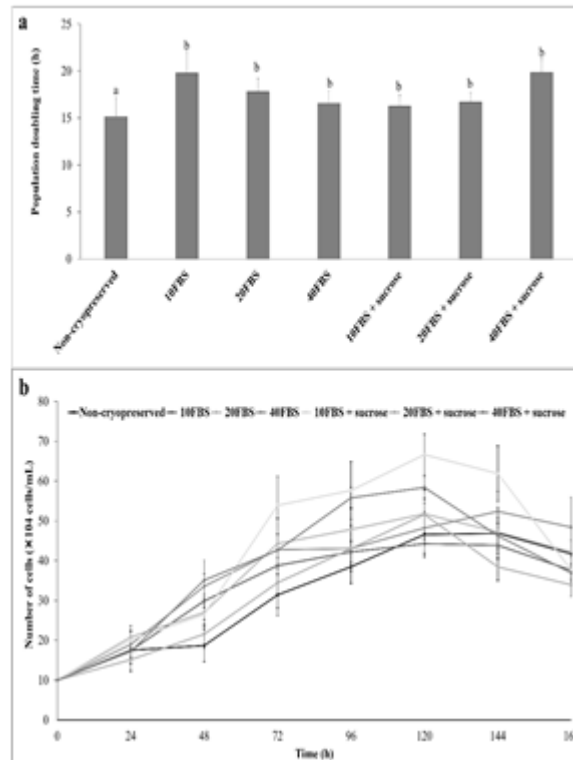


Figure 2. Influence of extracellular cryoprotectants on six-banded armadillos fibroblast cells (a) survival rate, (b) metabolism, and (c) apoptosis levels. *Fetal bovine serum (FBS). Standard error (S.E.). Bars represent standard error. a,b: $P < 0.05$.

Although cryopreservation reduced the proliferative activity showed by a higher PDT value (> 16 h), no differences were found among cryopreservation solutions ($P > 0.05$; Fig. 3a). Evaluating the cells' growth curve, in the first 24 h, the initial adaptation phase was similar in all groups, but, afterward, the groups presented a different proliferation rate, corresponding to the exponential growth phase (Fig. 3b). In between 72 h and up to 144 h, the group 10FBS with sucrose had a more significant number of cells in culture than other groups. Overall, the cells reached stationary growth after 120 h and notably decreased their population, except for

the 40FBS group, which presented a more prolonged proliferation phase and only started to decrease the population rate on the last day of culture (168 h).



3338

Figure 3. Influence of extracellular cryoprotectants on six-banded armadillos fibroblast cells proliferative activity. **(a)** Population doubling time. **(b)** Growth curve. *Fetal bovine serum (FBS). Bars represent standard error. a,b: $P < 0.05$.

When analyzing the $\Delta\Psi_m$, among the cryopreserved groups with sucrose, 20% FBS groups in the absence or presence of sucrose maintained the $\Delta\Psi_m$ (Fig 4). The 10FBS + sucrose and 40FBS + sucrose groups presented a decreased value compared to the non-cryopreserved group ($P > 0.05$; Fig. 4).

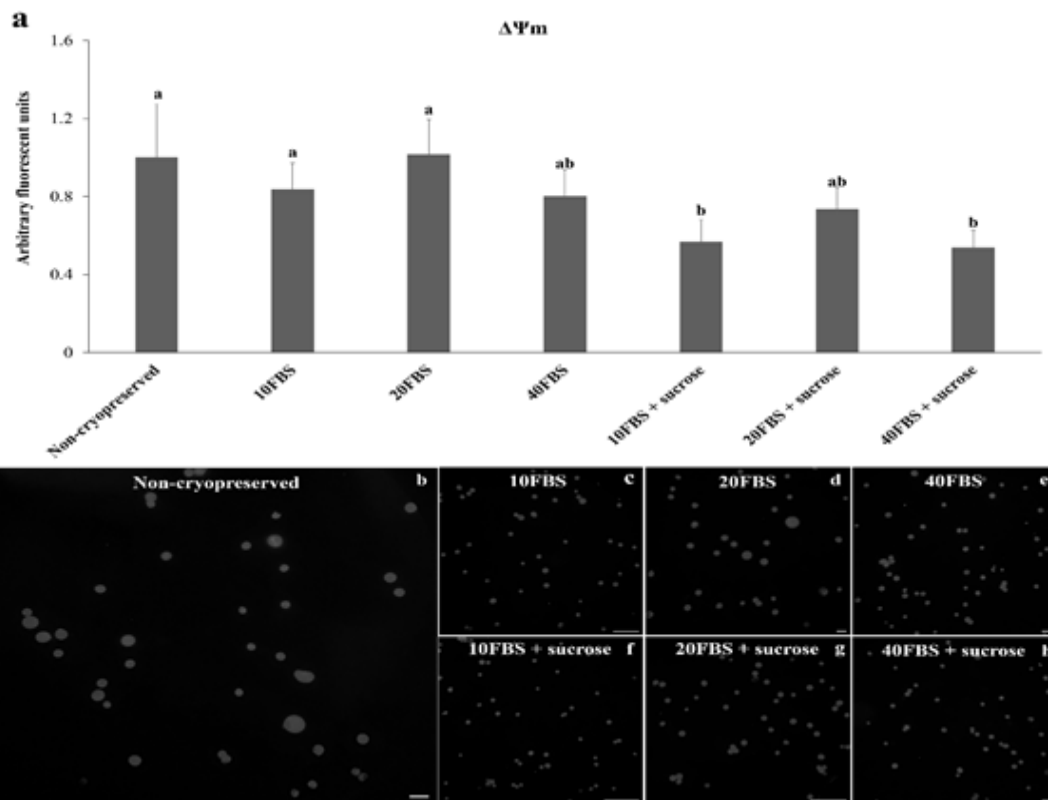


Figure 4. Influence of extracellular cryoprotectants on intracellular mitochondrial membrane potential using MitoTracker Red in six-banded armadillos fibroblast cells. **(a)** Measurement of $\Delta\Psi_m$ levels using different cryopreservation solutions. **(b)** Non-cryopreserved group, **(c, d, e)** cryopreserved cells with SFB 10%, 20%, 40%, and **(f, g, h)** SFB 10%, 20%, 40% with sucrose, respectively. Scale bar 100 μ m. *Fetal bovine serum (FBS). Bars represent standard error. a,b: $P < 0.05$.

DISCUSSION

Cryopreservation has become an essential tool in wild mammal conservation studies for allowing the banking of their valuable biological material for an undetermined period, especially semen, oocytes, embryos, fibroblasts, and tissues (25, 26). Adjusting the use and concentration of cryoprotectants continues to be an area of active research, and success is strictly dependent on the maintenance of viable cells post-thawing. This study shows that the

3359 skin fibroblast of six-banded armadillos was successfully preserved after slow freezing
3360 cryopreservation using 10% DMSO, 20% FBS, and 0.2 M sucrose. This combination resulted
3361 in better cell quality in terms of maintaining the morphology, viability, metabolic activity,
3362 apoptosis levels, and $\Delta\Psi_m$.

3363 The cryopreservation and thawing process did not affect the cells' morphology and
3364 confluence aspect; therefore, all cells presented a long spindle shape like fibroblast cells, similar
3365 to those of six-banded armadillo skin-derived fibroblasts observed in our study from primary
3366 cultures (10). Cells attached rapidly after thawing and were quite fast at reaching subconfluence
3367 (80–90%).

3368 Regardless of the FBS concentration or the presence of sucrose, the cells' viability rates
3369 remain very high with values above 93%, more than cryopreserved somatic cells from other
3370 wild mammals using 10% DMSO plus 90% FBS (84%, 6) or 10% DMSO plus 10% FBS and
3371 0.2 M sucrose (68%, 27). Our findings suggest that the cryopreservation conditions and
3372 solutions presented here were efficient in promoting cell recovery with optimal viability and
3373 corroborate with a previous report, where different concentrations of FBS (10%, 40%, and
3374 90%) with and without sucrose promoted great viability of cryopreserved fibroblast cells from
3375 red-rumped agouti, a wild rodent, to as high as our result and with no difference among groups
3376 (9).

3377 It is important to understand that the assessment of cell viability by trypan blue assay
3378 allowed us to accurately distinguish between active and dead cells only concerning membrane
3379 integrity. Other techniques are additionally recommended to infer cell recovery rates after
3380 thawing (9). Therefore, cells' metabolic activity and apoptosis levels were also assessed as
3381 other viability parameters, indicating the influence of cryoprotectants on the functionality of
3382 the cell and its organelles.

We found that adding sucrose to the cryoprotectant solution positively influenced the cells after metabolic activity analysis, where significantly lower values were observed only in cryopreserved groups without sucrose (10FBS and 40FBS). This parameter was maintained stable when using the same concentration of FBS and adding sucrose. The results from all cryopreserved groups with sucrose also stand out regarding the apoptosis level. The changes were observed only in 20FBS and 40FBS without sucrose groups. Similar alteration was observed in red-rumped agouti species when using 10% and 40% FBS without sucrose for fibroblast cryopreservation, resulting in fewer viable cells. Still, no harm was observed when sucrose was added (9).

In this study, 10% FBS without sucrose did not affect the cell regarding this parameter. Still, higher concentrations of FBS (20% and 40%) were only suitable when sucrose was added, confirming previous findings that adding sucrose at 0.2 M in a medium with DMSO and FBS increased cryopreserved fibroblast cells survival rate in wild species, such as Iberian lynx (*Lynx pardinus*, 28), collared peccary (4), and red-rumped agouti (9). Sucrose is a well-known naturally occurring cryoprotectant, important in maintaining osmotic balance and, therefore, preventing cell rupture. Its use is also beneficial during thawing (29, 27).

Extensive studies have exploited combinations of cryoprotectants to mitigate cryoprotectant toxicity and reduce cryoinjuries, especially mixing both permeating and non-permeating cryoprotectants (30). For example, cryopreservation of collared peccary fibroblast using 10% DMSO plus 50% FBS and 0.2 M sucrose enhanced the mitochondrial membrane potential compared to that achieved using 10% DMSO alone or 10% DMSO plus 50% FBS or 10% DMSO plus 0.2 M sucrose (4). This is explained by the fact that the combination of FBS and sucrose can help reduce DMSO cytotoxicity and prevent possible chemical injuries (30). Such results also corroborate the fact that the slow freezing method is highly recommended for somatic cell cryopreservation.

Nonetheless, other factors might also be involved in those results since the concentration of 20% FBS without sucrose in this study also did not significantly alter the metabolic activity. This can be attributed to the concentration of FBS plus the quality of the 10% DMSO slow-freezing technique, which is commonly associated with better results for somatic cells derived from wild mammals (4). The same cryoprotectants and concentrations (10% DMSO and 20% FBS) were successfully used for cryopreservation of fibroblast cell line from the critically endangered double-humped camel, with 89% viability estimated by trypan blue dye method (*Camelus bactrianus*, 31). Moreover, in feline species, other studies demonstrated it possible to cryopreserve fibroblast cells efficiently without adding sucrose, where no difference was observed between cells cryopreserved in a medium supplemented with sucrose (24, 27).

In the meantime, regardless of the cryopreservation solution used, all the cryopreserved groups had a disadvantageous result for decreasing the proliferative activity of cells compared to non-cryopreserved cells, showing slower PDT. Unlike our results, Praxedes et al. (9), cryopreserving fibroblast from a wild rodent and evaluating 10%, 40%, and 90% FBS with and without sucrose, observed that the PDT value did not change after cryopreservation. We can assume that this difference might be related to species cell biology, meaning that six-banded armadillo fibroblast requires longer recovery time after cryopreservation. Even so, all groups presented exponential expansion. After the 24–48 h, the cell population number doubled and reached confluence at the end of 168 h. In comparison, other studies reported higher PDT values after cryopreservation, such as in Asian elephants (25 h) (20), Sumatran rhinoceros (20 h) (5), and Pumas (43–93 h) (27). It is worth saying that armadillos are closely related only to sloths and anteaters. Still, since there is a considerable lack of information on this matter, our comparison was made based on other wild mammals with which they share some similarities.

Moreover, it was possible to demonstrate that among the solutions, sucrose had an effect in cell recovery and the concentration of FBS. Good rates were obtained when the cells were

cryopreserved with 20% FBS with sucrose (20FBS + sucrose group) compared with the non-cryopreserved group and other FBS concentrations. Although the higher concentration of 40% FBS with sucrose also maintained the same result as non-cryopreserved cells in most quality parameters, it failed to maintain the mitochondrial membrane permeability. The 10% FBS with sucrose group also yielded lower $\Delta\Psi_m$ than the control group, showing that, according to this parameter, 20% FBS was the only optimal concentration when using sucrose. Several other studies used 20% FBS as a non-permeating cryoprotectant in somatic cell cryopreservation with great outcomes (8, 31, 32).

It is known that cryopreservation can cause cellular stress and change cell quality parameters (9). We showed that using DMSO, FBS, and sucrose as cryoprotectants could protect the cells and maintain most parameters unaltered. Given the complexity of FBS composition, the interaction between other cryoprotectants is challenging to predict or even explain. Nonetheless, FBS constitutes a viable supplement to be added to the cryopreservation medium since it contains a combination of elements, such as albumin, with cryoprotective impact, which can remove free radicals and show antioxidant capability (33). However, as well as DMSO, using FBS might represent a risk of toxicity and zoonotic infection (34). Therefore, the optimal concentration must be determined for establishing somatic resource biobanks, and it is suggested that a combination of cryoprotectants reduces their possible toxic effects (18). So, overall, the results suggest greater cellular protection when using 20% FBS with sucrose compared with 10% and 40% FBS with and without sucrose. Thus, using FBS combined with sucrose is advantageous and increases the cell recovery rate after cryopreservation.

In conclusion, we found optimal conditions for cryopreservation of six-banded armadillo skin-derived fibroblast lines by slow-freezing. We showed a higher success rate when using 10% DMSO supplemented with 20% SFB and 0.2 M sucrose as cryoprotectants compared with other groups, being able to promote the maintenance of cell morphology, viability, metabolic

activity, mitochondrial membrane permeability, and apoptosis levels. This protocol can be used for future genetics and conservation studies for armadillo species and other Xenarthra species.

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**CAPÍTULO 6 – TIME-DEPENDENT MODULATION OF FIBROBLAST CYCLE
ARREST IN G₀/G₁ PHASE BY DIFFERENT SYNCHRONIZATION METHODS IN
SIX-BANDED ARMADILLOS (Artigo experimental)**

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3589 **Time-dependent modulation of fibroblast cycle arrest in G₀/G₁ phase by different**
 3590 **synchronization methods in six-banded armadillos**

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Abstract

Several studies have evaluated different cell cycle synchronization methods to improve reprogramming efficiency aiming at wild species conservation. The six-banded armadillo is one of the wild mammals with significant ecological and biomedical interests but has not yet been evaluated for reprogramming purposes. Therefore, as a step towards these studies, we investigated the effects in a time-dependent manner of serum starvation (SS; 0.5% serum), contact inhibition (CI), and roscovitine (RSV 15 and 30 μ M) on the cycle synchronization at G_0/G_1 phase of six-banded armadillo fibroblasts by flow cytometric analysis. The morphology, viability, and apoptosis levels were determined by microscopic analysis. The results showed that SS significantly increased the percentage of cells arrested in the G_0/G_1 phase in a time-dependent manner, where SS for 72 h ($90.5\% \pm 0.8$) and 120 h ($90.1\% \pm 0.9$) showed the highest value compared to SS for 24 h ($86.1\% \pm 0.9$) and control group ($69.7\% \pm 0.8$; $P < 0.05$). However, the effect of CI and RSV exposure on arresting fibroblasts at the G_0/G_1 phase was not noted under any of the conditions evaluated ($P > 0.05$). In contrast with SS and RSV, CI induced necrosis as dead cells increased ($P < 0.05$) after the evaluated times. All methods presented similar morphology and maintained high viability rates (~ 72 – 97%). These results indicated SS was the most suitable method for cell cycle modulation in G_0/G_1 of six-banded armadillo fibroblasts, especially after 72–120 h of treatment.

Keywords: Xenarthra, Cell cycle arrest, Flow cytometry, Serum starvation, Contact inhibition, Roscovitine.

Introduction

The six-banded armadillo (*Euphractus sexcinctus* Linnaeus, 1758) is a wild mammalian species with a wide distribution throughout South America (Abba and Superina 2014), where they

provide key ecosystem services (Rodrigues et al. 2019), significant socio-economic (Barboza et al. 2011) and biomedical contributions as an experimental model (Oliveira et al. 2019). It is the only species of the *Euphractus* genus and, unfortunately, it has been affected by persisting threats like habitat loss, hunting, and roadkills (Barboza et al. 2011; Ribeiro et al. 2017; Teixeira et al. 2020). Therefore, there is a need to conserve the species' biodiversity. One way is to develop and adapt assisted reproductive techniques, especially considering that such data is valuable to conserve other xenarthrans (Fernandes et al. 2023; Sousa et al. 2023).

Studies on somatic cell reprogramming technologies, such as cloning by somatic cell nuclear transfer (SCNT; Iqbal et al. 2021) and induced pluripotent stem cells (iPSC; Swegen et al. 2023), open the way to a variety of research for reproductive and therapeutic purposes relevant to wild species conservation, as in the case of the six-banded armadillos. In general, these techniques are still challenging due to low efficiency and high cost, but they are seen as a hope to help preserve biodiversity (Malin et al. 2022). To this purpose, cell cycle synchronization is an essential step to effectively reprogram somatic cells (e.g., fibroblasts from skin biopsies) to pluri- or totipotency. Specifically, arresting fibroblasts in the G_0/G_1 is required to succeed because it is when the cells are likely to exit the growth phase and become quiescent. Therefore, the nucleus chromatin is more amenable to reprogramming (Campbell et al. 1996).

Serum starvation (SS), contact inhibition (CI), and cell cycle regulatory inhibitors are the prevalent methods used to arrest mammalian fibroblast in G_0/G_1 (Selokar et al. 2012; Borges et al. 2021; Xu et al. 2021). In SS (from 5–0.5%), the non-dividing state is accomplished by exposing the cells to environmental stress and nutrient-limiting conditions (Samiec et al. 2013), while CI causes cells to stop dividing due to the cell-to-cell contact through cell high-density conditions (around 100% confluency; Młodawska et al. 2022). As an alternative, roscovitine

(RSV) is a highly potent inhibitor for the kinase activity of cyclin-dependent kinases (CDKs) that also regulates the cell cycle and has been applied in many wild species (Wittayarat et al. 2012; Rodrigues et al. 2023).

Despite already being applied in wild mammals, these methods present divergent results depending on the time evaluated and the species studied (Rodrigues et al. 2023; Yagcioglu et al. 2023). Młodawska et al. (2022) evaluated SS and CI conditions for 24, 48, and 72 h in three species of felids. They concluded that the response of cells to the treatments was species-specific, depending also on culture duration, but SS was still pointed as the more efficient method. Rodrigues et al. (2023) revealed that puma fibroblasts under a CI condition for 24, 48, and 72 h significantly increased the proportion of fibroblasts in G_0/G_1 . In contrast, this increase was observed only after 96 h of SS compared to the control group. Additionally, studies revealed that longer periods of 72 h and 120 h under SS have resulted in a higher percentage of fibroblast arrested in the G_0/G_1 phase in some wild species (Veraguas et al. 2012; Młodawska et al. 2022).

Regarding RSV, the ideal concentration and incubation time varies between cell lines from different species (Wittayarat et al. 2012; Yagcioglu et al. 2023). For example, its effects were investigated in four feline species at different concentrations (7.5, 15, and 30 μM) and observed that the rates of G_0/G_1 cells differed between species (Wittayarat et al. 2012), where, overall, 15 μM showed better results. Later, 15 μM RSV of the long-tailed macaque (*Macaca fascicularis*) ear-derived fibroblasts resulted in a higher percentage in G_0/G_1 and better blastocyst formation rate than that under SS and CI (Xu et al. 2014). Meanwhile, Selokar et al. (2012) verified that 30 μM RSV is optimal for harvesting G_0/G_1 phase cells and producing high-quality cloned embryos, better than 10 μM RSV, SS, or CI methods.

We can note that the best cell cycle synchronization remains unknown and significantly ambiguous, especially depending on cell type and species (Praxedes et al. 2024; Wittayarat et al. 2012; Veraguas et al. 2017; Bordhan et al. 2022). Therefore, to select the least harmful and best synchrony method, checking the efficiency for every cell line may be necessary. Unfortunately, there is no record of synchronization methods tested in armadillo species or any other xenarthran. Therefore, for the first time, we aimed to compare the response of SS, CI, and RSV on synchronization in G_0/G_1 of six-banded armadillo fibroblasts, as well as the effects on cell survival rate.

Material and methods

This study was approved by the Animal Ethics Committee of the Rural Federal University of Semi-Arid (CEUA/UFERSA, No. 36/2020) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, No. 76655-1). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA), and the fluorescent probes were purchased from Invitrogen (Carlsbad, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation, culture, and evaluation of fibroblast lines

Fibroblast lines derived from skin of five adult six-banded armadillo were established according to Fernandes et al. (2024). Briefly, cell lines were established, frozen at the 4th passage in 10% dimethyl sulfoxide (DMSO), 10% FBS, and 0.2 M sucrose, and then thawed for use in this study. Before initiating cell cycle synchronization methods, fibroblasts cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution (38.5 °C and 5% CO₂) were evaluated for their morphology using an inverted microscope and proliferative activity by

obtaining the growth curve and determining the population doubling time (PDT). Cells were cultured in 24-well dishes at a concentration of 1.0×10^4 cells/mL, counted daily for up to seven days, and finally, the cell growth curve was generated, and the PDT was estimated based on these measurements (Mehrabani et al. 2016).

Experimental design and cell treatments

We evaluated the effect of four culture conditions on cell cycle synchronization in G_0/G_1 : serum starvation (SS), contact inhibition (CI), and the effect of roscovitine (RSV) inhibitor at 15 μ M (RSV15) and 30 μ M (RSV30) concentrations after different incubation times. For SS and RSV, cells without any treatment and at 60–70% confluence (Day 0) were used as a control group. Cells with full confluency (100%) at Day 0 (CI 0 h) were considered a control group for CI. Cell cycle analysis was estimated for each treatment by flow cytometry at Day 0 and after the respective incubation time (Młodawska et al. 2022). At least two trials were performed for the cells from each cell line, producing ten repetitions for each treatment and each incubation time. We also evaluated cell morphology, viability, and apoptosis levels.

Evaluation of serum starvation at different times

For SS, growing cells at 60–70% confluency (control, Day 0) were cultured in DMEM containing 2% antibiotic-antimycotic solution and 0.5% FBS. The influence of SS treatment was monitored by harvesting cells after 24, 72, and 120 h in this low serum medium (Borges et al. 2021; Młodawska et al. 2022). The culture medium was changed every two days.

Evaluation of contact inhibition at different times

To monitor the effects of CI on cell cycle synchronization, after reaching full confluency (control, Day 0), cells were cultured in a regular culture medium for an additional 24, 72, and

120 h (Młodawska et al. 2022). The culture medium with DMEM, 2% antibiotic-antimycotic solution, and 10% FBS was changed every two days during the treatment.

Evaluation of roscovitine at different times and concentrations

In this experiment, after the cells reached the appropriate confluence (60–70%, control), they were incubated in a culture medium with RSV at 15 or 30 μ M concentration. After starting the treatment, the stage of fibroblasts from each animal was analyzed after 12 h and 24 h of exposure to RSV (Selokar et al. 2012; Wittayarat et al. 2012).

Cell fixation and cycle analysis by flow cytometry

After the treatment's incubation time, the medium and any floating detached cells were removed before trypsinization, and the cells were harvested, centrifuged at 600 $\times$ g for 10 min, and slowly resuspended in 1.0 mL of cold 70% ethanol for fixation (Borges et al. 2021). Cells were maintained at -20 °C for five days. Fixed cells were washed in phosphate-buffered saline (PBS) to removal ethanol, and each sample was centrifuged at 400 $\times$ g for 10 min. The cells were then stained with 5 μ g/mL propidium iodide (PI), 50 μ g/mL RNase was added, and samples were incubated at 4 °C for 50 min. After that, the samples were analyzed using a Guava EasyCyte flow cytometer (Guava Technologies, Stamford, Lincolnshire, United Kingdom). Data were obtained from 15,000 events from each sample. Histograms of PI fluorescence vs. counts were generated to evaluate the percentages of cells for each cell cycle phase (G_0/G_1 , S, G_2/M) and the levels of sub- G_0/G_1 (cell death indicator). The proportions of cells in each cell cycle phase and sub- G_0/G_1 levels were assessed using MODFIT software version 5.0 (Verity, <https://www.vsh.com/products/mflt/index.asp>).

Morphological analysis and viability by cell membrane integrity

Cells were evaluated under an inverted microscope (Nikon TS100, Tokyo, Japan) for cell forms and cytoplasmic extensions. Moreover, cell viability was evaluated using the trypan blue assay (Fernandes et al. 2023). Briefly, cells were stained with 0.4% trypan blue in PBS and counted using a hemocytometer. Unstained cells were considered viable; non-viable cells became blue due to the cell's ruptured membrane and consequent dye penetration. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

Apoptosis activity

An aliquot of cells was resuspended in 2 µg/mL acridine orange and 10 µg/mL ethidium bromide. Subsequently, cells were evaluated at 480 nm using a fluorescence microscope, where 300 cells were counted at 20× magnification. The cells were classified into (i) viable cells, with a uniform light green nucleus; (ii) cells in initial apoptosis, with a non-uniform green nucleus; (iii) cells in late apoptosis, with a non-uniform bright orange nucleus; and (iv) necrotic cells, with a uniform orange nucleus (Fernandes et al. 2023). A fluorescence microscope was used to observe apoptotic changes in the stained cells, which were quantified using ImageJ software (National Institutes of Health, Bethesda, USA).

Statistical analysis

Data were expressed as mean ± standard error and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, values were transformed in arcsine and analyzed by ANOVA followed by the Tukey test. Significance was set at $P < 0.05$.

Results

Initially, post-thawed fibroblast cells presented a normal fibroblast-like morphology, with a typical flat and elongated spindle-shaped profile with an oval nucleus (Fig. 1a). The cells took approximately eight days to reach 80–90% confluence and presented high cellular viability before treatments. The growth curve was plotted, and the cells showed an S-shaped curve (Fig. 1b) with a PDT of 25.2 ± 2.4 h. After the lag phase in the first 24 h, an exponential growth phase from 24–96 h and a decreasing phase at 96–168 h was observed (Fig. 1b).

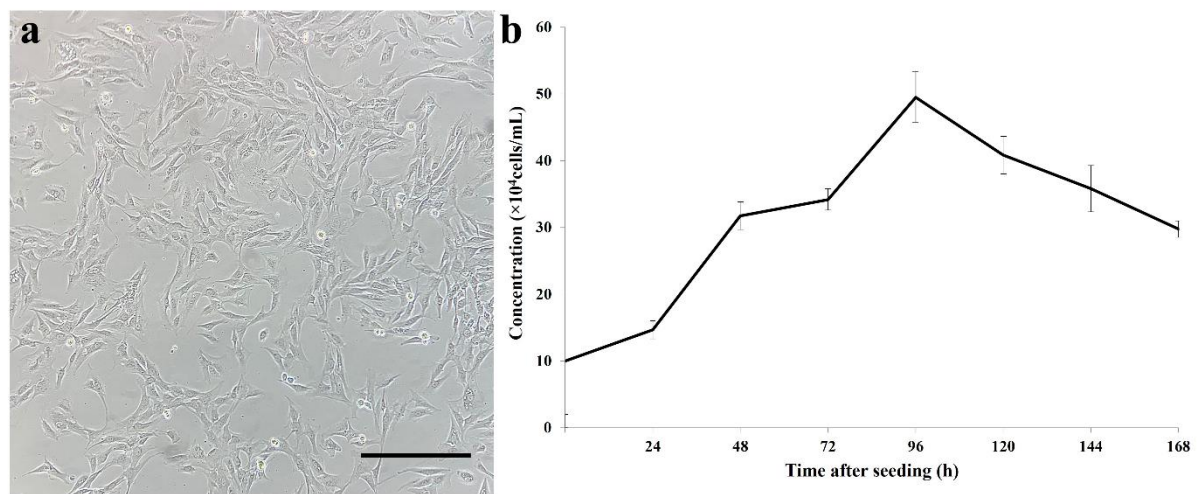


Fig. 1. Post-thawed fibroblast cells from six-banded armadillo before cell treatment for synchronization in the G_0/G_1 phase. (a) Fibroblasts showing normal morphology. (b) Growth curve of fibroblasts. Scale bar: 100 μm.

Evaluation of serum starvation at different times

The SS method, regardless of the incubation time, significantly increased the proportion of cells arrested at the G_0/G_1 phase ($>86\%$) compared to the control cells ($69.7\% \pm 0.8$; Table 1, Fig. 2a-2d). In addition, the proportion of cells in the G_0/G_1 phase was further increased by prolonging SS to 72 h ($90.5\% \pm 0.8$) and 120 h ($90.1\% \pm 0.9$). Consequently, fewer cells were arrested in the S and G_2/M cell cycle stages, but only S-phase cells were significantly lower in all SS groups compared with the control group. The level of sub- G_0/G_1 (apoptosis) and G_2/M phase arrest did not change after SS treatment (Table 1). Moreover, after the SS treatment, cells

remained showing normal morphology (Fig. 3a-3d) and high viability rates at the three times evaluated (24 h: $91.2\% \pm 6.1$; 72 h: $72.2\% \pm 14.7$; 120 h: $96.8\% \pm 1.1$), as compared with the control group ($96.3\% \pm 2.9$, Fig. 3e). Similarly, the percentage of apoptotic cells was not significantly different after treatment (Fig. 3f).

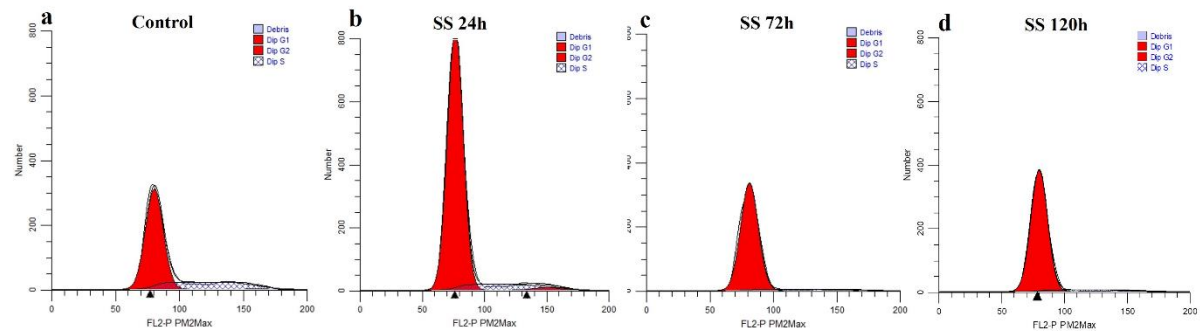


Fig. 2. Representative cell cycle histograms of the flow cytometry analysis of six-banded armadillo fibroblast culture in different conditions: (a) control (before synchronization), (b) serum starvation (SS) after 24 h, (c) 72 h, and (d) 120 h.

Table 1. Cell cycle distribution of six-banded armadillo skin fibroblasts cultured under different serum starvation conditions.

Groups	Cell cycle (%)			
	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁
Control	69.7 ± 0.8^c	27.7 ± 0.8^a	2.5 ± 0.4	0.0 ± 0.0
SS 24 h	86.1 ± 0.9^b	9.5 ± 1.2^b	4.4 ± 2.0	0.2 ± 0.0
SS 72 h	90.5 ± 0.8^a	8.4 ± 0.7^b	1.1 ± 0.2	0.2 ± 0.1
SS 120 h	90.1 ± 0.9^a	9.4 ± 1.1^b	1.7 ± 0.4	0.2 ± 0.1

SS: serum starvation; Values are expressed as mean $\pm$ standard error; Within a column, values with different letters differ ($P < 0.05$).

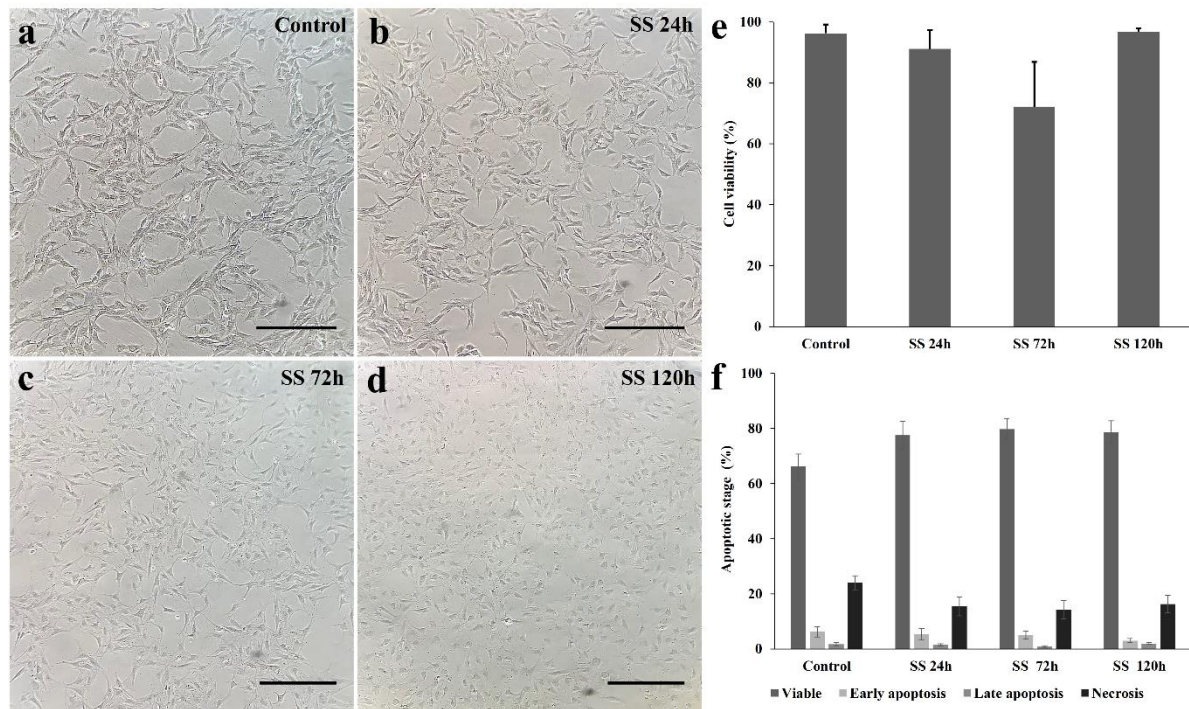


Fig. 3. Cell morphology (a, b, c, and d), viability (e), and apoptosis levels (e) of six-banded armadillo fibroblasts before synchronization (control) and after synchronization by serum starvation (SS). Scale bar: 100 μm. Values are expressed as mean ± standard error. $P > 0.05$.

Evaluation of contact inhibition at different times

The CI treatment had no apparent effect on the proportion of cells arrested at the G_0/G_1 stage, as no difference was observed compared to control cells, even in other phases of the cell cycle, including the sub- G_0/G_1 levels (Table 2; Fig. 4a-4d). Cell morphology is shown in Fig. 5a-5d. In terms of the viability rate, cells exhibited high survival rates, and there was no difference between the treated (24 h: 96.6% ± 0.8; 72 h: 88.4% ± 4.9; 120 h: 97.8% ± 0.8) and control group (95.5% ± 1.5; Fig. 5e). Although the number of live and apoptotic cells did not show significant changes, an increase in the number of necrotic cells was observed in at all evaluated times for the CI groups (24 h: 10.3% ± 3.4; 72 h: 6.7% ± 1.3; 120 h: 9.4% ± 3.2) when compared to control cells (0.4% ± 0.3; Fig. 5f).

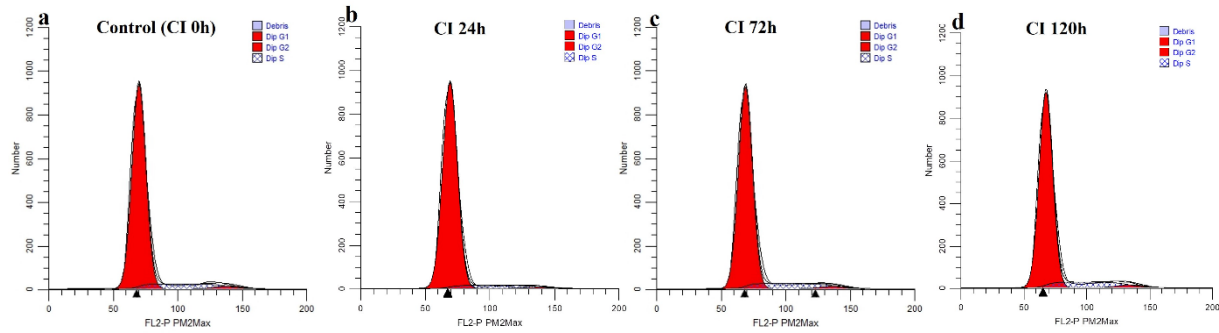


Fig. 4. Representative cell cycle histograms of the flow cytometry analysis of six-banded armadillo fibroblast culture in different conditions: (a) control (before synchronization), (b) contact inhibition (CI) after 24 h, (c) 72 h, and (D) 120 h.

Table 2. Cell cycle distribution of six-banded armadillo skin fibroblasts cultured under different contact inhibition conditions.

Treatment	Cell cycle (%)			
	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁
Control (CI 0 h)	83.3 ± 1.6	13.9 ± 1.0	3.1 ± 0.4	0.3 ± 0.0
CI 24 h	78.6 ± 2.1	16.4 ± 1.4	5.0 ± 0.6	0.1 ± 0.0
CI 72 h	76.8 ± 2.0	17.1 ± 1.3	6.1 ± 0.7	0.2 ± 0.1
CI 120 h	75.7 ± 2.0	16.8 ± 1.0	7.4 ± 1.2	0.2 ± 0.0

CI: contact inhibition; Values are expressed as mean ± standard error. *P* > 0.05.

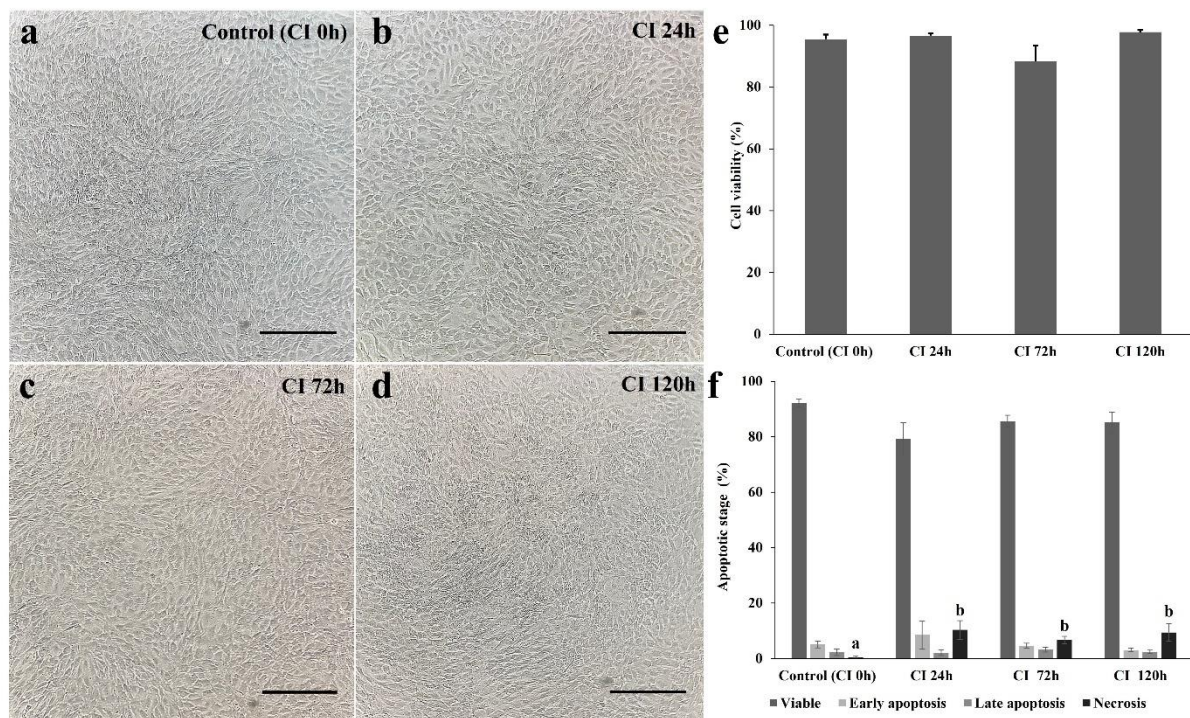
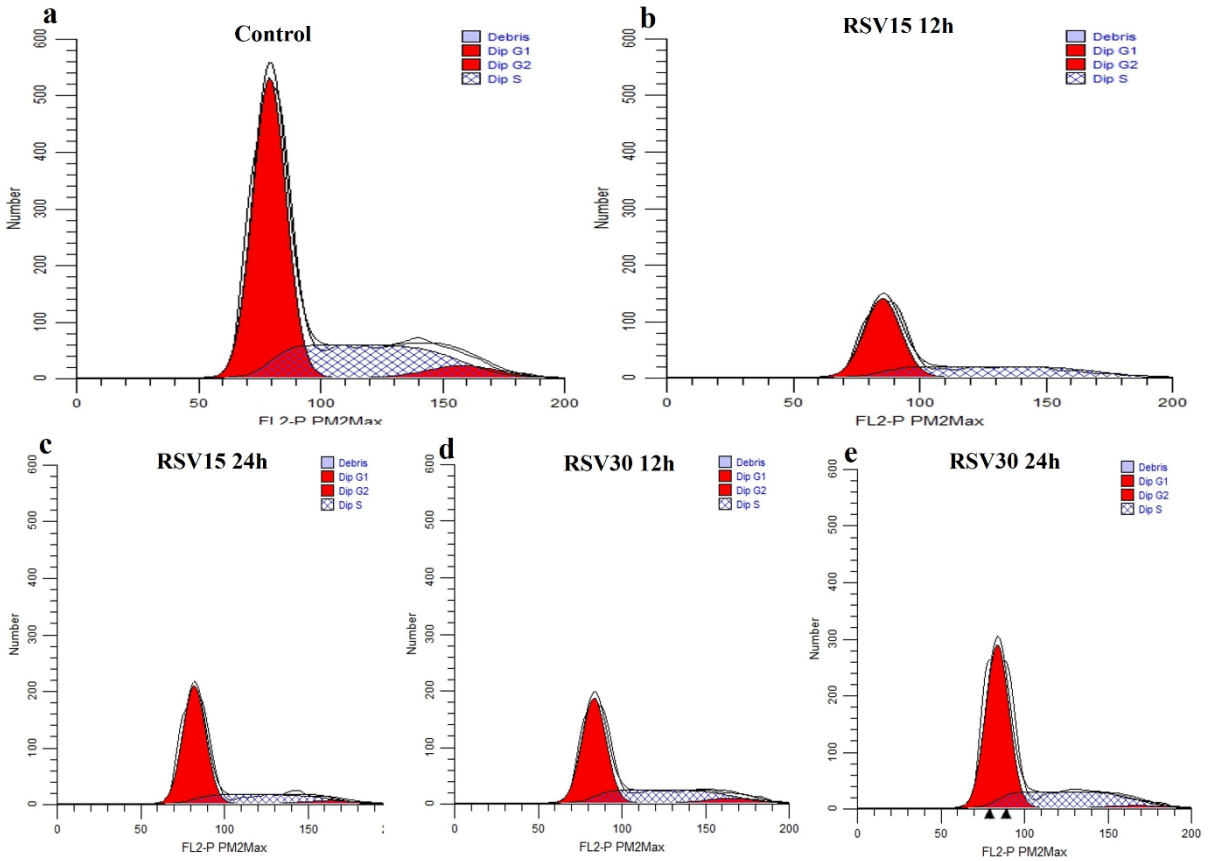


Fig. 5. Cell morphology (a, b, c, and d), viability (e), and apoptosis levels (f) of six-banded armadillo fibroblasts before synchronization (control) and after synchronization by contact inhibition (CI). Scale bar: 100 μ m. Values are expressed as mean $\pm$ standard error. Bars with different letters differ significantly ($P < 0.05$).

Evaluation of roscovitine at different times and concentrations

The RSV treatment did not promote cell synchronization in G_0/G_1 at the time and concentrations tested when compared with control cells (Table 3; Fig. 6a-6e). The proportion of cells in S phase and levels of sub G_0/G_1 also did not change with the treatment. However, the cell cycle inhibitor reduced the percentage of cells in G_2/M phase after 24 h exposure time in both concentrations of RSV (15 μ M: $3.5\% \pm 1.0$; 30 μ M: $1.9\% \pm 0.6$) compared to control cells ($7.0\% \pm 0.5$; Table 3). The cells morphology is shown in Fig. 7a-e. Moreover, the cell viability rate did not show significant changes among groups (RSV15 12 h: $86.9\% \pm 5.4$; RSV15 24 h: $88.7\% \pm 5.2$; RSV30 12 h: $75.7\% \pm 9.4$; RSV30 24 h: $95.2\% \pm 2.6$; Control: $97.8\% \pm 1.5$; Figure 7f). The apoptotic stage analysis indicated that only the RSV15 24 h and RSV30 12 h groups

3857 resulted in a higher percentage of live cells ($86.0\% \pm 2.6$ and $86.6\% \pm 3.3$, respectively) and a
3858 lower percentage of necrotic cells ($3.2\% \pm 0.6$ and $4.0\% \pm 2.5$, respectively), compared to the
3859 other groups (Fig.7g). No further significant changes were observed.



3860
3861 **Fig. 6.** Representative cell cycle histograms of the flow cytometry analysis of six-banded
3862 armadillo fibroblast culture in different conditions: (a) control (before synchronization), cells
3863 treated with (b) roscovitine (RSV) 15 μ M for 12 h and (c) 24 h, and RSV 30 μ M for (d) 12 h
3864 and (e) 24 h.

3865
3866 **Table 3.** Cell cycle distribution of six-banded armadillo skin fibroblasts cultured in different
3867 roscovitine concentrations.

Treatment	Cell cycle (%)			
	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁

Control	60.6 ± 0.7	32.4 ± 0.5	7.0 ± 0.5 ^a	0.1 ± 0.0
RSV15 12 h	57.4 ± 1.4	38.1 ± 1.4	4.5 ± 0.9 ^{ab}	0.2 ± 0.1
RSV15 24 h	57.8 ± 2.4	38.6 ± 2.5	3.5 ± 1.0 ^b	0.3 ± 0.0
RSV30 12 h	61.2 ± 0.9	33.4 ± 1.4	5.4 ± 0.6 ^a	0.2 ± 0.1
RSV30 24 h	62.5 ± 1.2	35.6 ± 1.3	1.9 ± 0.6 ^b	0.3 ± 0.0

RSV: roscovitine (15 and 30 μM); Values are expressed as mean ± standard error. Within a column, values with different letters differ ($P < 0.05$).

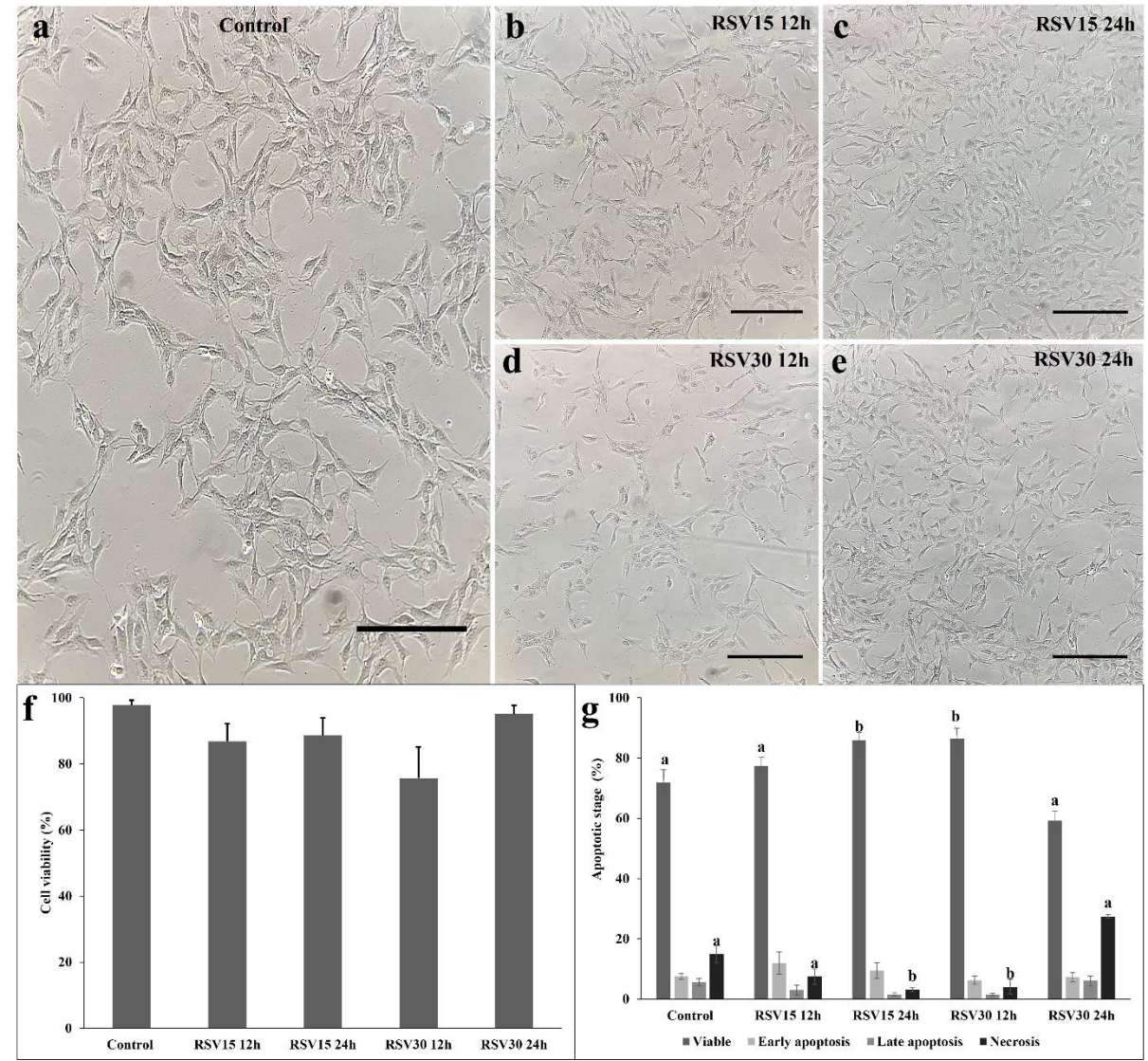


Fig. 7. Cell morphology (a, b, c, d and e), viability (f), and apoptosis levels (g) of six-banded armadillo fibroblasts before synchronization (control) and after synchronization by roscovitine

(RSV) exposure. Scale bar: 100 μ m. Values are expressed as mean $\pm$ standard error. Bars with different letters differ significantly ($P < 0.05$).

Discussion

Studies have indicated that arresting somatic cells in the G_0/G_1 phase is the best cell cycle stage to maintain the correct ploidy in nuclear transfer embryos (Campbell et al. 1996; Selokar et al. 2012; Xu et al. 2021). Since then, there has been a need to find an ideal synchronization protocol to induce most cells to be in the G_0/G_1 phase without compromising cell survival (Wittayarat et al. 2012; Ligasová and Koberna 2021). In this study, for the first time, we have developed a suitable protocol for fibroblast synchronization at the G_0/G_1 phase in six-banded armadillos. Our results demonstrated that the SS (0.5% FBS) method resulted in the highest proportion of fibroblast cells in the G_0/G_1 phase than CI and RSV under the evaluated conditions, and it maintained cell viability and apoptotic levels despite treatment duration.

Moreover, the synchrony happened in a time-dependent manner since the SS 72 h and SS 120 h groups had the highest proportion (90.5% and 90.1%) with significant differences compared to lower exposure time (SS 24 h: 86.1%). This improvement in the G_0/G_1 phase cell level can be attributed to the fact that cells under SS conditions are deprived of mitotic signals. Many essential growth factors are unavailable; therefore, they rapidly exit the cell cycle and enter the metabolically inactive G_0 state (Kues et al. 2000; Mahesh et al. 2011). In this sense, the asynchronously dividing cell population (our SS control group) will start synchronizing at a specific cell cycle stage. Upon serum withdrawal, cells that have not reached the restriction point of the G_1 phase (R point) exit the cell cycle and arrest in G_0 . In contrast, cells that have already passed the R point and those in G_2/M or S phases of the cell cycle continue cycling until the R point and are then arrested at G_0 (Memili et al. 2004). Therefore, in the case of six-banded

3898 armadillo fibroblast, the longer the cells remained in these conditions, the higher percentage
3899 were arrested in the quiescent stage, as was the case of 72 h and 120 h of serum withdrawal.

3900

3901 Other studies have also determined SS as the more effective method compared to different cell
3902 cycle synchronization methods, achieving better outcomes toward longer duration of treatment
3903 (Veraguas et al. 2017; Młodawska et al. 2022). Values over 90% of G₀/G₁ cells were obtained
3904 from SS treatment after 3 and 5 days of culture in jaguarundi (*Puma yagouaroundi*) and pallas's
3905 cat (*Otocolobus manul*; Młodawska et al. 2022). Although the extended period of SS showed
3906 better results, we also observed a rapid effect of a short time (24 h) of SS in successfully
3907 arresting a high percentage of cells in the G₀/G₁ phase, compared to the control group. This
3908 significant increase in the first 24 h of treatment was reported in other wild species, such as
3909 giant panda (*Ailuropoda melanoleuca*; 87.8%; Han et al. 2003), bison (*Bos Gaurus*; 84.1%;
3910 Mahesh et al. 2011), and felid species (74.6–91.3%; Wittayarad et al. 2012).

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3912 Additionally, we found that the serum withdrawal did not affect quality-related parameters of
3913 six-banded armadillo fibroblasts, where cell morphology and viability based on membrane
3914 integrity or apoptotic assay were maintained at all evaluated times. These results are outstanding
3915 compared to other studies. For example, in collared peccary (*Pecari tajacu*), extending the SS
3916 period by a few days (3–5 days) resulted in poor outcomes in terms of the proportion of
3917 quiescent cells and/or cellular viability (Borges et al. 2021); this was linked to the prolonged
3918 period of nutritional deprivation or simply because blocking cells from cycling could also result
3919 in cell death (Jackman and O'Connor, 1998). However, particularly in wild feline species, this
3920 response was reported to be species-specific. An increase in apoptosis levels was observed after
3921 4–5 days of SS in fibroblast cells of marbled cats (*Pardofelis marmorata*; Wittayarat et al. 2012)
3922 and puma (Rodrigues et al. 2023), but this was not the case for leopard (*Panthera pardus*;

Wittayarat et al. 2012) or Asian golden cat (Wittayarat et al. 2012) cells, where the apoptosis levels were maintained over time while generating a higher proportion of skin fibroblasts in the G_0/G_1 phase compared with control. The explanation is that cell lines of some species or individuals can be more sensitive than others to the withdrawal of nutrients (Młodawska et al. 2022), which was not the case for six-banded armadillo fibroblast cells.

For CI, no evaluated time induced the arrest of more cells in the G_0/G_1 cell cycle phase compared to the control group (CI 0 h). The Day 0 of cells reaching full confluency was considered as the control group rather than growing cells at 60–70% confluence (used as control in SS and RSV treatment) because it is the equivalent Day 0 for CI treatment, therefore precisely comparing the cells by the effect of time. As a result, a high percentage (83%) of fibroblasts was already in the G_0/G_1 cell cycle phase on Day 0. This finding was also previously reported in other studies that evaluated Day 0 of CI treatment, with values over 86% of fibroblasts in the G_0/G_1 phase (Han et al. 2003; Młodawska et al. 2022). It is well known that under high-density conditions, cells tend to be quiescent due to contact inhibition naturally, and this effect generally induces cell cycle arrest at the early G_1 phase (Davis et al. 2001; Młodawska et al. 2022). In addition, the CI 0 h group resulted in a high survival rate and a lower percentage of necrotic cells among all groups; we also observed that this group presented a very low percentage of sub- G_0/G_1 , as well as observed in the SS and RSV groups, representing a positive effect. Therefore, we can also suggest CI at Day 0 as a valuable method to be considered for obtaining a high proportion of skin fibroblasts in the G_0/G_1 phase.

However, the 24, 72, and 120 h of treatment was unsuccessful in CI groups, where less than 79% of cells were considered in the G_0/G_1 phase, although this same condition has been used with high success in other studies (~ 85%, Veraguas et al. 2017; Rodrigues et al. 2023). The

difference between previous studies and ours could be due to animal species or cell types being studied since different cell lines are affected differently (Młodawska et al. 2022). In addition, regarding apoptotic activity, CI incubation times negatively affected the treated cells by significantly increasing the proportions of necrotic cells. Therefore, it can be inferred that CI at the three evaluated times was unsuitable for somatic cell synchronization at the G₀/G₁ phase in six-banded armadillo.

Furthermore, different concentrations of RSV, ranging from 5 to 30 µM, have been tested to obtain a significantly higher percentage of cells arrested in the G₀/G₁ (Choresca et al. 2009; Yagcioglu et al. 2023; Rodrigues et al. 2023). In this study, 15 and 30 µM RSV treatment after 12 or 24 h did not increase fibroblast synchrony; the groups resulted in the lowest percentage (<63%) of cells in a G₀/G₁ phase among synchrony methods. Our findings differ from what Selokar et al. (2012) observed using buffalo skin fibroblasts, where RSV at 30 µM improved the synchronization of 96% of cells in the G₀/G₁ stage after 24 h. However, the inhibitor had a negative impact on cell viability. Moreover, different concentrations of RSV (5, 10, 15 µM) induced cell cycle arrest at the G₀/G₁ phase (>81%) in goldfish fibroblasts (*Carassius auratus*; Choresca et al. 2009), while in sheep fibroblasts, concentrations at 10, 15, 20, and 30 µM did not improve cell cycle arrest (49–63%; Yagcioglu et al. 2023). The latter results were similar to this study. The authors linked this lack of success to the high level of p53 observed in sheep fibroblast, a tumor suppressor protein that regulates cell division. Indeed, RSV is one of the most frequently studied and used CDK inhibitors. At the same time, results have shown that the drug is rather selective for CDKs. For instance, most kinases were found to be insensitive to RSV in wild-type mouse embryonic fibroblasts (Bach 2005). For armadillo species, further research is needed to support this thought.

Even though RSV groups failed to promote G₀/G₁ phase arrest, their influence on cell apoptotic levels and membrane viability was positive. The concentrations and exposure times evaluated either maintained the percentage of viable, apoptotic, and necrotic cells or resulted in a higher percentage of live cells and consequently fewer necrotic cells, as observed in the RSV15 24 h and RSV30 12 h groups compared to other groups. Furthermore, the viability rates (>86%) were maintained high despite concentration and time variations, comparable with that of previous reports (~80–90%, Selokar et al. 2012; Yagcioglu et al. 2023). Such outcomes are essential and show that the effect of this chemical compound on synchronizing fibroblasts and causing damage are unrelated and depend on the species evaluated, as pointed out before (Wittayarat et al. 2012; Rodrigues et al. 2023).

In conclusion, we described the optimal conditions for achieving highly efficient cell cycle synchronization at the G₀/G₁ phase using six-banded armadillo skin fibroblast lines under treatments. SS was shown to be the most suitable method for cell cycle synchrony in a time-dependent manner, in which the cells were kept viable and arrested at the desired cell cycle stage for 72 and 120 h, optimizing their use in routine reprogramming experiments. To our knowledge, this is the first report to characterize these cell lines for synchronization in the G₀/G₁ phase. Our data should be useful for studies on somatic cell reprogramming and increase the potential of cloning by SCNT and generating iPSC lines for armadillo species.

Author contributions

Designed the methodology and aims of the study: DP Fernandes and AF Pereira. Collected the data: DP Fernandes, JVS Viana, LLV Rodrigues, LRM Oliveira, SLA Sales, MCS Luciano, C Pessoa and CIA Freitas. Analyzed the data: AF Pereira. Writing of the manuscript: DP

3997 Fernandes, and AF Pereira. All authors contributed critically to the drafts and gave final
3998 approval for publication.

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4007 **Author Disclosure Statement**

4008 The authors declare they have no financial interests.

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6 CONSIDERAÇÕES FINAIS

Os resultados obtidos nessa tese contribuem para o estabelecimento adequado de biobancos de células e tecidos somáticos em *E. sexcintus*, além de preparar células somáticas para o estudo de reprogramação nuclear.

ETAPA 1- Biobanco de tecidos somáticos: Inicialmente, destaque-se a VSS como a técnica de escolha para conservar o tecido somático do tatu-peba, mantendo as características histológicas do tecido quando comparado a tecidos não criopreservados e possibilitando a recuperação de células somáticas sem alteração da viabilidade, atividade metabólica, atividade proliferativa, $\Delta\Psi_m$ e níveis de apoptose. Contudo, a eficiência da técnica depende do uso de menores concentrações de crioprotetores intracelulares, como no caso do grupo 3 M EG-DMSO.

ETAPA 2- Biobanco de células somáticas: Objetivou-se cultivar *in vitro*, isolar e caracterizar cinco linhagens fibroblásticas, as quais foram mantidas em cultivo ao longo de três diferentes passagens (quarta, sétima e décima) e criopreservadas sem afetar a sua morfologia e seus níveis de viabilidade, crescimento, metabolismo e apoptose. Além disso, foram analisadas as condições de criopreservação das células somáticas em diferentes soluções e foi observado que a combinação de 10% de DMSO, 20% de SFB e 0,2 M de sacarose como crioprotetores foi a mais adequada em conservar os fibroblastos quanto a sua morfologia, viabilidade, atividade metabólica, $\Delta\Psi_m$ e níveis de apoptose.

ETAPA 3- Sincronização na fase G₀/G₁: Quanto aos métodos de sincronização celular em G₀/G₁, foi observado que o método de privação de soro foi o mais eficiente em obter maiores percentuais de células sincronizadas em G₀/G₁, especialmente após 72 h e 120 h (>90%), não afetando padrões de viabilidade celular e nem níveis de apoptose.

Portanto, os resultados gerados constituem um passo importante para a conservação genética do *E. sexcintus* e pode possibilitar futuras pesquisas e aplicações científicas, como a clonagem e iPSCs a partir de células somáticas. Além disso, mediante sua importância como modelo experimental, tais informações auxiliam na manutenção da espécie em cativeiro e podem servir de base para outros tatus ameaçados ou outras espécies filogeneticamente próximas.

7 PERSPECTIVAS

Com os resultados obtidos nesta tese, foram geradas informações relevantes para a formação de biobancos para a espécie e seu uso futuro para diferentes finalidades biotecnológicas. Contudo, é sugerida a investigação de novas concentrações de soluções de criopreservação celular para entender melhor o seu efeito sobre a atividade proliferativa das amostras, como também adaptar o uso do método de privação de soro na sincronização celular em G₀/G₁ a fim de se obter taxas maiores de células sincronizadas, testando principalmente a combinação com outros métodos já relatados na literatura.

Ressalta-se ainda como próximo passo, a necessidade de usar técnicas que avaliem alterações genéticas, a fim de garantir a estabilidade genética das linhagens celulares da espécie e adequando o seu uso como doadoras de núcleo. Nesse contexto, estudos podem ser direcionados para a obtenção e preparação de carioplastos (oócitos) do *E. sexcintus*, complementando a formação dos biobancos, para aplicação em biotécnicas que visam à reprogramação nuclear.

Finalmente, vale ressaltar que além do intuito conservacionista, essas amostras podem ser utilizadas para aprimorar conhecimentos básicos em estudos genéticos, farmacológicos e em pesquisas biomédicas.

**ANEXO A – CERTIFICADO DE AUTORIZAÇÃO DO PROJETO PELA COMISSÃO
DE ÉTICA NO USO DE ANIMAIS – CEUA**

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UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Mossoró, 10 de novembro de 2020.

PARECER 36/2020

*Certificamos que o projeto intitulado “Estabelecimento das condições de cultivo in vitro, criopreservação, sincronização do ciclo celular e reprogramação nuclear em amostras somáticas de tatu-peba, *Euphractus sexcinctus* (Linnaeus, 1758)”, sob a responsabilidade de *Alexsandra Fernandes Pereira*, o qual envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da lei 11.794 de 8 de outubro de 2009 e com as normas editadas pelo Conselho Nacional de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal Rural do Semi-Árido –UFERSA em reunião no dia 03 de novembro de 2020.*

<i>Vigência do Projeto</i>	<i>Janeiro a julho de 2021</i>
<i>Espécie/linhagem</i>	<i>Euphractus sexcinctus</i>
<i>N. de Animais</i>	<i>07</i>
<i>Peso/idade</i>	<i>Adultos</i>
<i>Sexo</i>	<i>Machos e fêmeas</i>
<i>Origem</i>	<i>LEIAS/UFERSA</i>

Emanuelle Fontenele Rabelo
Emanuelle Fontenele Rabelo
Coordenadora CEUA-UFERSA

**ANEXO B – AUTORIZAÇÃO DO PROJETO PELO SISTEMA DE AUTORIZAÇÃO
E INFORMAÇÃO EM BIODIVERSIDADE – SISBIO**

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Dados do titular

Nome: Alexandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: Estabelecimento das condições de cultivo in vitro, criopreservação, sincronização do ciclo celular e reprogramação nuclear em amostras somáticas de tatu-peba, <i>Euphractus sexcinctus</i> (Linnaeus, 1758)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Colheita do material biológico	01/2021	06/2021
2	Realização dos experimentos da Etapa 2	01/2022	12/2022
3	Realização dos experimentos da Etapa 1	01/2021	12/2021
4	Realização dos experimentos da Etapa 3	01/2023	12/2025

Equipe

#	Nome	Função	CPF	Nacionalidade
1	MOACIR FRANCO DE OLIVEIRA	Colaborador	325.949.504-59	Brasileira

Observações e ressalvas

1	O pesquisador somente poderá realizar atividade de campo após o término do estado de emergência devido à COVID-19, assim declarado por ato da autoridade competente.
2	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
3	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
4	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio nº 03/2014 ou na Instrução Normativa ICMBio nº 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
5	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
6	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
7	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/gen .

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Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Outras ressalvas

1	Considerando a pandemia de COVID-19, o CPB recomenda que as atividades de pesquisa com primatas e xenartras, em vida livre ou cativo, dentro ou fora de UCs federais devem adotar as medidas recomendadas nos comunicados disponíveis nos links: https://www.icmbio.gov.br/cpb/index.php/component/content/article?id=0 https://www.icmbio.gov.br/cpb/index.php/component/content/article?id=24	CPB João Pessoa-PB
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Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Universidade Federal Rural do Semi-Árido	Mossoró-RN	Caatinga	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas ex situ	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de amostras biológicas ex situ	Euphractus sexcinctus	-

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Xenarthra)	Outras amostras biológicas (Pele auricular)

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	Laboratório

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Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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**ANEXO C – VERSÃO DO ARTIGO PUBLICADO NA BIOPRESERVATION AND
BIOBANKING**

**CAPÍTULO 2 – *EX SITU* APPROACHES FOR THE CONSERVATION OF GENETIC
RESOURCES IN THE SUPERORDER XENARTHRA**

Qualis 2017–2020: A4

Fator de impacto: 1.6

Doi: <https://doi.org/10.1089/bio.2021.0182>



Ex Situ Approaches for the Conservation of Genetic Resources in the Superorder Xenarthra

Denilsa Pires Fernandes,¹ Érika Almeida Praxedes,¹
Carlos Iberê Alves Freitas,² and Alessandra Fernandes Pereira¹

Xenarthra—a superorder of placental mammals endemic to the Neotropics—is represented by armadillos, anteaters, and sloths. Considering their long history in the Americas, extant xenarthrans represent an important group for understanding the impact of past environmental changes on species diversification and serve key ecological functions as ecosystem engineers. Unfortunately, most wild xenarthran populations are at risk, due primarily to anthropogenic activities, necessitating urgent conservation efforts. Moreover, the paucity of information on some species has rendered population estimation and, consequently, conservation management challenging. In addition, relatively few groups are researching this superorder, perhaps because fieldwork with armadillos, anteaters, or sloths and their captive care are challenging tasks. Nevertheless, dedicated research and efforts to ensure the long-term conservation of these animals are deemed essential. In this context, cryobanks are a practical approach for breeding and maintaining genetic diversity in wildlife, and they are important tools for assisting and improving both *ex situ* and *in situ* conservation strategies. Therefore, cryopreservation of biological resources may be a promising strategy for conserving xenarthrans. Specifically, semen cryopreservation, which has already been applied in some species, may be the most effective strategy for this group. The present article provides an overview of *ex situ* conservation of xenarthrans, which will contribute to the development and implementation of additional strategies for protecting these unique mammals.

Keywords: wildlife conservation, Cingulata, genome resource banking, Pilosa, threatened species

Introduction

STORAGE OF GENETIC material in cryobanks may assist and improve the long-term conservation for several species.¹ Cryobanks, or genome resource banks (GRBs), constitute reserves of gametes, embryos, and gonadal or somatic tissues, representing an important long-term biodiversity conservation strategy through the preservation of genetic material of species for several purposes, such as the development of artificial reproductive technologies (ARTs) and application in basic biological, toxicological, genetic, and epidemiological studies.²

Typically, *ex situ* conservation involves the maintenance of live populations outside of their natural habitat (*ex situ in vivo*

conservation) or preservation of genetic material in cryobanks (*ex situ in vitro* conservation). The consensus among conservationists is that habitat preservation is the most effective measure for preserving biodiversity and saving wild populations. However, these *in situ* approaches are not practical for certain species; thus, *ex situ* actions are worthwhile and, at times, the last resort to establish viable populations and preserve genetic material.² Unfortunately, although cryopreservation is common in *ex situ* programs for some species, it remains a major challenge for xenarthrans.

In xenarthrans—a unique group of placental mammals—certain conservation strategies have been implemented, including political actions, practical interventions, and educational campaigns.^{3,4} Specifically, to establish GRBs for

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this group, semen cryopreservation may be the most effective approach. However, cryopreservation has been applied only in some xenarthran species. In addition, this group has lost substantial genetic information, as many species are already extinct, and some extant ones are on the brink of extinction.

Xenarthra—a superorder of mammals endemic to the Neotropics—includes both extinct and extant species of armadillos, anteaters, and sloths. These mammals are further divided into 2 monophyletic orders, namely Cingulata (armadillos) and Pilosa (anteaters and sloths),⁵ together comprising 37 described extant species, all endemic to the Americas.^{6,7} The name Xenarthra is derived from the characteristics of these animals, such as xenarthric joints, fused pelvic bones,⁸ slow metabolic rate, and low and variable body temperature, which differentiate them from other mammals.⁹

Xenarthrans predominantly inhabit South America, whereas a few species are present in Central and North America. Some species, such as the nine-banded armadillo (*Dasypus novemcinctus*), occur throughout the Americas.⁵ Owing to their evolutionary history in the American continent (~60–100 million years ago [Mya]),¹⁰ xenarthrans constitute a promising model for studies on the impact of past environmental changes on species diversification.^{10,11} Yet, compared with other mammalian species, xenarthrans remain understudied.^{12–14}

The majority of the published studies on xenarthrans have focused on the fundamental knowledge of their ecology, diet, habitat, distribution, activity pattern, evolution, and genetics,^{4,15} mostly aimed at developing effective conservation strategies. To this end, the present review seeks to provide an overview of *ex situ* conservation of xenarthrans and emphasize the need for further research to establish GRBs, particularly for species threatened with extinction—the reality of some xenarthran species whose populations continue to dwindle.

Why Preserve Xenarthrans?

Xenarthrans are essential components of their ecosystems. By virtue of their unique dietary habits, they aid insect control and nutrient cycling. In addition, these mammals, particularly armadillos, serve as ecosystem engineers and,

thus, as indicators of environmental quality.¹⁶ Furthermore, the fossorial activities of xenarthrans for foraging and constructing burrows benefit other animals, including other xenarthrans, by serving as stable shelters and possible food sources.¹⁷ In addition to being specialized predators, xenarthrans are prey for other species, such as jaguars and wolves.¹⁸

Xenarthrans have attracted attention because of their importance as experimental models in biomedical research. For instance, armadillos are natural reservoirs of *Mycobacterium leprae*, which is the primary causative agent of leprosy. *M. leprae* cannot grow in artificial media, and armadillos and primates are the only mammals other than humans known to be natural hosts of this bacterium.¹⁹ At present, *D. novemcinctus* is considered the most suitable model species for research on leprosy.¹⁹ In addition, sloths and anteaters serve as pathological models in studies on leishmaniasis.²⁰ Meanwhile, in many regions, such as northeastern Brazil, meat and other products from xenarthrans, particularly armadillos, are consumed, indicating the close relationship between humans and these animals.²¹

In terms of the global conservation status, some xenarthran species are facing some risk of extinction.^{22,23} Currently, >20% of xenarthran species are threatened by extinction.²⁴ In particular, the pygmy three-toed sloth (*Bradypus pygmaeus*) is a critically endangered (CR) species (3%), whereas the giant anteater (*Myrmecophaga tridactyla*), giant armadillo (*Priodontes maximus*), maned three-toed sloth (*Bradypus torquatus*), and Brazilian three-banded armadillo (*Tolypeutes tricinctus*) are vulnerable (VU) species. The maned three-toed sloth and Brazilian three-banded armadillo are endemic to Brazil, whereas the pygmy three-toed sloth is endemic to Panamá. Five species (14%) are classified as data deficient (DD), and their conservation status remains unassessed. Furthermore, nine species (24%) are classified as not evaluated (NE), as these are considered new species. Overall, the current conservation status of nearly 40% of extant xenarthrans remains unknown, and the population trend for most species is either decreasing or unknown (Fig. 1).

Furthermore, the xenarthran species that are considered threatened globally are also endangered locally in countries where they are present.⁴ Even species that are listed globally as of least concern (LC) are threatened in at least one country in which they are present. For instance,

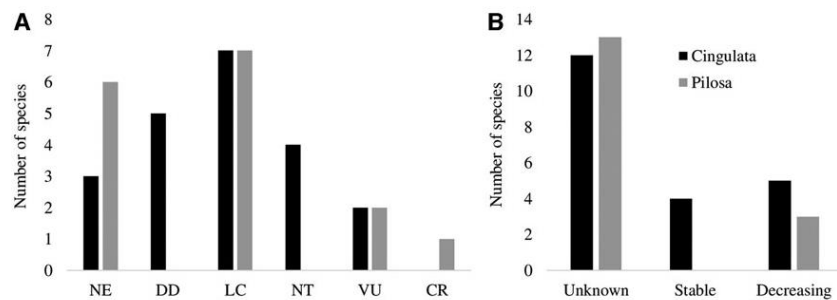


FIG. 1. Conservation status and population trend of xenarthrans. (A) Conservation status according to the division of xenarthrans in Cingulata and Pilosa. Conservation categories listed are CR, critically endangered; DD, data deficient; LC, least concern; NE, not evaluated; NT, near threatened; VU, vulnerable. (B) Population trend of Cingulata and Pilosa species.^{4,6,7,24}

EX SITU CONSERVATION OF XENARTHTRANS

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northern tamandua (*Tamandua mexicana*) is threatened in 42% of the countries in which it is present.⁴ This inconsistency may be reflected in taxonomic issues⁴ and may consequently delay the formulation of conservation strategies. Other factors attributed to the current conservation status of these species include habitat loss,²⁵ illegal trafficking, and roadkills.⁴

Ex Situ Conservation of Xenarthras

In situ conservation aims at maintaining the animals alive in their natural environment or an environment close to their natural habitats.²⁶ Therefore, this is undoubtedly the most commonly used strategy for conserving xenarthras. Much information on the various xenarthran species has been collected through *in situ* conservation.¹⁵ Table 1 lists several projects and programs developed and implemented in American countries with the aim of collecting information for the conservation of these mammals.

Although habitat preservation is, according to conservationists, the most appropriate strategy to conserve wildlife diversity, this approach alone does not suffice to prevent species extinction in the face of the current global reality and uncertain future.^{1,27} In light of this, *ex situ* conservation strategies offer essential knowledge and techniques for immediate intervention, primarily considering the instability of species at the risk of extinction. In addition, zoos and other facilities around the Americas support most of the conservation projects for xenarthras, playing a key role in their preservation by maintaining the last individuals of most threatened species and collecting genetic samples to establish GRBs.

The establishment of GRBs benefits wildlife²⁸ and is, therefore, a promising strategy that should be pursued in future studies on xenarthras. In this study, we curated studies reporting the establishment of cryobanks for the conservation of xenarthras (Table 2). All studies used male gametes of the six-banded armadillo (*Euphractus sexcinctus*)^{29,30} or giant anteater.^{31,32}

Over the years, interest in the preservation of somatic samples in GRBs as part of species conservation programs has increased.³³ This can be attributed to the advantages somatic samples offer over gametes. As such, somatic samples can be recovered regardless of sex, age, and seasonality from both live and dead animals as well as from different tissue sources.³⁴ Moreover, somatic samples are typically collected through minimally invasive procedures, such as skin biopsy. A successful long-term *in vitro* culture of skin cells from a six-banded armadillo was documented in a recent study.³⁵ In addition, the rate of cell recovery and viability over long-term culture were high. This proved an important step toward establishing cryobanks and using these cells in ARTs.³⁵ Unfortunately, cryopreservation of somatic resources from xenarthras has not been reported yet, highlighting the importance of testing protocols for preserving somatic tissues from these species.

Although all xenarthras are assigned to a single super group, they differ in terms of morphological and ecological aspects. Examples of morphological and ecological differences among xenarthras include digging as the primary method of acquiring food in anteaters and armadillos,⁵ arboreal or suspensory locomotion in sloths, and the unique protective body armor of armadillos.³⁶ In addition, these

TABLE 1. EXAMPLES OF ORGANIZATIONS AND ONGOING PROJECTS COMMITTED TO THE CONSERVATION OF XENARTHTRANS

Institution/program/project	Country	Started	General goals
Anteater, Sloth and Armadillo Specialist Group	International alliance	1980	Evaluation of the conservation status of xenarthran species for the IUCN Red List of Threatened Species
Fundación AIUNAU	Colombia	1996	Study and conservation of sloths
Green Heritage Fund	Suriname	2005	Shelter and Rehabilitation of Xenarthra; Education and Information; Habitat protection
Suriname—The Xenarthra Program			
Tamandua Institute	Brazil	2005	<i>In situ</i> and <i>ex situ</i> conservation of xenarthras
Projeto Tatu canastra—The Giant Armadillo Project	Brazil	2010	Conservation of <i>Prionomys maximus</i>
Projeto Bandeiras e Rodovias—The Anteaters and Highways Project	Brazil	Not informed	Assessment of the impact of roads on <i>Myrmecophaga tridactyla</i> populations in the Brazilian Cerrado
Tamandua Conservation Program in Northeast Brazil	Brazil	2011	Identify priority areas for the conservation of the species, restore degraded mangrove areas
Armadillo Conservation Programme	Colombia	2012	Conservation and management of armadillos in the Orinoco Llanos of Colombia
Saving the Pygmy Sloth from Extinction Project	Panama	2012	Improve the understanding of <i>Bradypus pygmaeus</i> population and threats to the species
Programa Tatu-bola—Armadillo program	Brazil	2013	Research and conservation of <i>Tolypeutes tricinctus</i> and protect its habitat
Armadillos de Chile—Chile Armadillos	Chile	2016	Promote research and initiate strategies for the three armadillo species (<i>ChaetophRACTUS vellerosus</i> , <i>Zaedyus pichiy</i> , and <i>ChaetophRACTUS villosus</i>)
Projeto Preguiças—Sloth Project	Brazil	2019	Conservation of <i>Bradypus variegatus</i> on Bahia coast

IUCN, International Union for Conservation of Nature.

TABLE 2. PROTOCOLS EMPLOYED FOR SEMEN CRYOPRESERVATION AMONG XENARTHANS

Species	Technical approach	Used solutions	Main results	Authors
<i>Euphractus sexcinctus</i>	Short-term preservation at 5°C	Tris vs powdered coconut water (ACP®) extender	No immediate effect on sperm motility or viability; nevertheless, sperm motility was reduced after 3 hours of storage.	Amorim et al. ²⁹
<i>E. sexcinctus</i>	Fast freezing and storage in N ₂	Tris, 20% of egg yolk and 3% of glycerol	Significant decline in all semen parameters, in which 6.1% motile sperm was found; a variable resistance to cryoinjuries was observed.	Sousa et al. ³⁰
<i>Myrmecophaga tridactyla</i>	Cooling and storage at 5°C	BotuCrio extender without cryoprotectant	Sperm motility index decreased from 0 to 24 hours, and vigor did not change in the first 6 hours and decreased after 18 hours.	Luba et al. ³¹
<i>M. tridactyla</i>	Fast freezing and storage in N ₂	OptiXcell® medium	There was a decrease in vigor and sperm motility in post-thawing sample (3% and 30%, respectively) compared with fresh semen (4% and 70%, respectively).	Silva et al. ³²

species have received differential scientific attention, with armadillos being the most studied xenarthran species, perhaps due to their biomedical importance.^{12,15} To better guide xenarthran conservation efforts, we have divided the discussion hereunder by their groups.

Conservation Perspectives on Armadillos

Armadillos represent the most diverse group of xenarthrans. *Dasypus* is the only extant genus characterized by the monozygotic polyembryonic form of reproduction. Unlike other members of the superorder, armadillos possess a hard and flexible carapace composed of hundreds of osteoderms.³⁶ These are semi-fossorial animals,³⁷ and their diet is one of the most diverse among xenarthrans. Armadillos are essential for the ecosystem due to their roles as predators, ecosystem engineers, seed dispersers, invertebrate vectors, and nutrient reserves.^{38,39} Within Xenarthra, armadillos are the most studied species,¹⁵ although species-specific studies are scarce.¹²

A review on armadillos by Superina et al. identified a large gap in the conservation of these species.¹² According to the authors, between 1660 and 2011, 3117 articles on armadillos were published, and the most frequently cited research topic was health care, including studies on leprosy, parasitology, and pathology. Of the 21 extant armadillo species, four species, namely *D. novemcinctus*, *Chaetophractus villosus*, *Chaetophractus vellerosus*, and *E. sexcinctus*, account for two-thirds of all publications. In addition, most studies were conducted in the United States, Brazil, Argentina, and Bolivia and were mostly performed in the laboratory, or field. The authors proposed that future research prioritize studies on the ecology, conservation, population genetics, reproduction, and threats of armadillos, particularly hairy long-nosed armadillo (*Dasypus pilosus*), Brazilian three-banded armadillo, and pink fairy armadillo (*Chlamyphorus truncatus*).

A good example of armadillo conservation efforts is the Armadillo Conservation Program initiated in 2012 in Orinoco Llanos, Colombia³ (Table 1). The program developed multiple strategies to raise awareness regarding five armadillo species, namely the southern naked-tailed armadillo (*Cabassous unicinctus*), nine-banded armadillo, greater long-nosed armadillo (*Dasypus kappleri*), northern long-nosed armadillo (*Dasypus*

sabanicola), and giant armadillo. The program achieved excellent engagement of local communities and encouraged restaurants to avoid selling wild armadillo meat. Moreover, the program addressed the topic of armadillo conservation in various educational settings, published pamphlets and books, and established breeding and rehabilitation facilities for maintaining confiscated armadillos.

Regardless of the limited information available, armadillos are used as experimental models for developing ARTs considering their adaptability to captive environments.³⁰ Although the reproductive biology of armadillos has not been completely elucidated, some studies have offered deeper insights. Semen collection using electroejaculation from the Brazilian three-banded armadillo, a species listed as near threatened (NT), and subsequent ejaculate analysis³⁹ determined that the semen samples contained abundant morphologically normal spermatozoa. However, the sperm concentration and motility were low, and urine contamination was a persistent problem. In addition, Howell-Stephens et al. assessed the gonadal hormone activity of the Brazilian three-banded armadillo in a zoo, and the results of the analysis may aid with management decisions in such facilities.⁴⁰

The giant armadillo is the largest extant representative of the order Cingulata^{41,42} and is classified as VU.⁴³ A study monitored and collected reproductive behavior data, such as gestation, inter-birth interval, litter size, and parental care, of male and female giant armadillos in Brazilian Pantanal.⁴¹ The data indicated a low population rate, underscoring the high risk of local extinction. Therefore, further studies on this species are imperative.

Moreover, the sperm cells of a giant armadillo collected *postmortem* from the epididymis were morphologically similar to those of most mammals. In particular, these sperm possessed a large oval head, similar to those in other members of the order Cingulata,⁴⁴ including the six-banded armadillo⁴⁵ and large hairy armadillo.⁴⁶ In a recent study by Luba et al.,⁴⁷ the age of sexual maturity of giant armadillos was determined, and the characteristics of the captured sexually mature males were described, providing information that can assist studies on *in situ* populations.

Semen collection using electroejaculation has been reported in captive six-banded armadillos.⁴⁸ Other parameters, such as sperm morphology, morphometry, ultrastructure,⁴⁵

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and viability, in different semen extenders have been evaluated in this species.⁴⁹ Moreover, semen conservation has been studied in some species. The first attempt to preserve the male gametes of armadillos was reported by Amorim et al. in six-banded armadillos²⁹ (Table 2). The authors observed that the sperm were sensitive to refrigeration at 5°C. Subsequently, a sperm cryopreservation protocol using a solution containing 20% egg yolk and 3% glycerol was established.³⁰ However, except for this pioneering study, cryopreservation protocols for the sperm of other armadillo species have not been established to date.

For females, *ex situ in vitro* conservation approaches are even scarcer, and no studies on the cryopreservation of ovarian tissue or oocyte recovery have been conducted in armadillos. However, studies on female armadillo reproduction, including reproductive anatomy, have been conducted.⁵⁰ The estrous cycle of six-banded armadillos has been monitored based on vaginal cytology, ultrasound examination, and hormonal measurements.⁵¹ In general, the reproduction of armadillos is seasonal, with most species producing a single litter annually. Except for pichi (*Zaedyus pichi*), which is known to hibernate during winters, other armadillo species remain active throughout the year.¹³

In addition, blood and somatic tissue samples have been collected from armadillos for taxonomic⁷ and molecular phylogenetic analyses¹³ as well as biomedical studies;⁵² however, such samples have not been used for *ex situ* species conservation through cryopreservation as yet.

Conservation Perspectives on Anteaters

Anteaters belong to the suborder Vermilingua of the order Pilosa, and although they are not the most abundant or diverse xenarthrans, these mammals exhibit a characteristic set of morphological adaptations.⁵³ Anteaters are the only toothless xenarthrans, despite the nomenclature “Edentata” (meaning toothless) formerly used to refer to xenarthrans. Anteaters possess a long tongue that they use to capture ants, termites, and other insects.⁴

The number of known anteater species has increased after the description of six newly recognized species of the genus *Cyclopes* in the family Cyclopedidae,⁶ which previously had only one representative—silky anteater (*Cyclopes didactylus*). These recent discoveries indicate that anteater populations warrant further research, particularly for establishing conservation and management practices, as these mammals occur in highly fragmented habitats, such as the Atlantic Forest.^{6,53} The other anteater family Myrmecophagidae, represented by three species, namely *M. tridactyla*, *T. mexicana*, and *Tamandua tetradactyla*, is a group requiring cryobank establishment since the giant anteater is categorized as VU.⁵⁴

Regarding male reproductive traits, anteaters (similar to all xenarthrans) possess internal testes and disproportionate sperm heads.⁵⁵ Rossi et al.⁵⁵ described the histology of the male reproductive tract and the process of spermatogenesis in lesser anteaters. After complete spermatogenesis, two individuals were azoospermic and showed considerable differences in the size of their genital tract organs, although some other characteristics were similar to those in other xenarthrans. The authors recommended further studies with more individuals from their natural environment to establish whether lesser anteaters exhibit reproductive seasonality.

Investigation of female reproductive traits, such as the estrus cycle, can allow for the implementation of ARTs in captive animals. Related research in southern tamandua revealed bloody vaginal secretion ~3 weeks before estrus, which may be a useful signal to monitor the ovarian cycle of the species.⁵⁶ Major advances in the noninvasive tracking of progesterone and estrogen metabolites in fecal samples have allowed for monitoring the estrous cycle in this species.⁵⁷

There are no records of an ovarian cycle in northern tamandua. Meanwhile, the ovarian cycle (51.4 days) of the giant anteater has been monitored through the analysis of hormones in the feces of captive females.⁵⁸ Nonetheless, knowledge of the ovarian cycle and semen and sperm of anteaters remains limited.

Regarding semen conservation in giant anteaters, Mendonça et al.⁵⁹ described the physical, chemical, morphological, and functional characteristics of semen collected using electroejaculation from captive animals. Semen was successfully collected from 62.2% of the analyzed males, and although the results varied among individuals, these differences were attributed to age, captive stress, and nutritional and health management. In another study, Luba et al.³¹ reported a rapid decline in the sperm quality of giant anteaters after dilution with a commercial semen extender (Botucrio®; Botupharma Ltd., Botucatu, Brazil) and storage at 5°C for 18 hours. However, low resistance to cold temperatures may be characteristic of xenarthrans.³⁰ Similar results were observed by Silva et al.,³² who reported a reduction in sperm vigor and motility after semen cryopreservation in giant anteaters (Table 2). Nonetheless, semen collection with medetomidine performed in that study was efficient in terms of sperm concentration, motility, and vigor.

Ultimately, efforts for the conservation of anteaters, focusing on their reproduction and germplasm preservation, are imperative to ensure their multiplication both in captivity and the wild. Such interventions may decelerate the loss of valuable genes in these species. Threatened and endangered species, such as the giant panda (*Ailuropoda melanoleuca*), have directly benefited from biobanking programs, highlighting the importance of systematic biobanking in species conservation.⁶⁰

Conservation Perspectives on Sloths

Sloths, similar to anteaters, belong to the order Pilosa and are divided into two families, namely Bradypodidae (genus *Bradypus*), represented by three-toed sloths,⁶¹ and Megalonychidae (genus *Choloepus*), represented by two-toed sloths.⁶² Sloths are arboreal and adapted for climbing.⁶³ All sloth species are herbivorous. *Bradypus* species are obligate folivores, whereas *Choloepus* species consume a more diverse diet, including fruits, flower buds, young stems, and some animal materials.⁴ Their global range is restricted to forested areas ranging from Honduras to Central Bolivia and Brazil.⁴ Sloths are known for their slow movements due to their restrictive diet.⁶⁴ Whereas three-toed sloths are exclusively folivorous, two-toed sloths feed on leaves and fruits and, occasionally, animal matter. They are characterized as being solitary with parsimonious movements.⁶⁵ In addition, sloths exhibit a very charismatic behavior, garnering much interest internationally.⁶⁴

In terms of conservation, sloths are the xenarthrans with the most critical conservation status. Of the six extant

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species, two are categorized as threatened.^{66,67} Nevertheless, all sloth species are directly affected by the destruction and fragmentation of forests, particularly since their population growth rate is low⁶³ and they are strictly arboreal species depending on trees throughout their life. Research on sloths is scarce, even among xenarthrans. For instance, the vertebral processes common to all members of the group, which allow these animals to stand upright on the tripod formed by their hind limbs and tail, were only recently described in sloths (*Bradypus variegatus*),⁶⁸ as opposed to their relatively early descriptions in armadillos (*D. novemcinctus*)⁶⁹ and anteaters (*M. tridactyla*).⁷⁰

Moreover, it is difficult to locate sloths in the forest, and even more so for untrained or inexperienced people,⁶³ rendering studies on these mammals in their natural habitats challenging. Another challenge is the low adaptability of *Bradypus* species to captive environments,⁶³ as they cannot survive on substitute diets that seem to lack certain trace elements, are very susceptible to cold, and are not immune to even common diseases.⁷¹

In this context, improving knowledge of sloth species through fundamental research on their reproductive parameters is an important step toward improving and understanding the reproductive functioning of this group to develop ARTs.^{63,65} In an attempt to gather deeper basic information on sloths, semen of free-living *Bradypus tridactylus* was successfully collected using electroejaculation, and the seminal characteristics of this species were described.⁶³ However, the low volume of the ejaculate limited the possibility of seminal analysis. The samples showed high viscosity and low motility and vigor. Satisfactory results were obtained by monitoring the ovarian cycle of female *B. variegatus* through the analysis of hormones present in fecal samples.⁷² Nevertheless, further studies are warranted to characterize the hormonal cycle, spermatogenesis, estrus cycle, and gestational period of sloth species.

B. pygmaeus, recognized as a distinct species in 2001,⁶¹ is the smallest sloth species; unfortunately, it is the world's most endangered xenarthran and, hence, a global conservation priority. Further research on this species is urgently required to establish conservation measures for the following reasons: (1) *B. pygmaeus* is classified as CR; (2) its range is extremely limited, as it is endemic to a single island, Escudo de Veraguas, in Panamá; (3) its population trend is declining; (4) it is directly affected by habitat loss; (5) it exhibits lower genetic diversity than its putative population; and (6) there is growing interest in its collection for captive rearing.⁶⁴ In a similar situation is *B. torquatus*,⁶⁶ a species also at high risk of extinction for almost the same reasons cited for *Bradypus pygmaeus*. Silva et al.⁷³ have highlighted the need for protecting *Bradypus* populations, which inhabit areas with high fragmentation and exhibit low genetic diversity.

Future Perspectives and Conclusions

Challenges arising from the limited knowledge of xenarthrans and the lack of attention in terms of research have hindered the conservation of these unique mammals, particularly with regard to the development of *ex situ* strategies. Semen cryopreservation in armadillos and anteaters may represent the first step toward conserving biological resources from xenarthrans. Nevertheless, these protocols are

only initial attempts, and further optimization is warranted, particularly considering the low cryotolerance reported for xenarthran sperm.

Furthermore, to overcome the limitations encountered when working with sperm and oocytes, the use of somatic cells may be an optimal alternative. In this regard, somatic tissues and cell banks must be explored as potential resources for the conservation of xenarthrans. These genetic resources are important for the development and application of advanced technologies, such as somatic cell nuclear transfer and induced pluripotent stem cell culture, as well as for the generation of information for fundamental and applied research on understudied species. To the best of our knowledge, comprehensive somatic cell banks for xenarthrans are lacking, although fibroblast lines have been established from skin biopsies of anteaters (*T. tetradactyla*) and sloths (*C. didactylus*).⁷⁴ Nevertheless, previous studies focused on the phylogeny of these mammals through genome mapping rather than their *ex situ in vitro* conservation.

Much remains unknown regarding the reproductive physiology of xenarthrans. Long-term storage of somatic cells as a potential conservation strategy can aid conservation by contributing to fundamental scientific research and ART development. Therefore, cryopreservation of genetic resources from xenarthrans holds immense potential in *ex situ* conservation programs and can serve as an invaluable tool in conservation, assisted reproduction, evolutionary biology, and wildlife medicine. To preserve the remaining genetic diversity of Xenarthra, threatened species, including pygmy sloth, maned sloth, giant armadillo, Brazilian three-banded armadillo, and giant anteater, must be prioritized in conservation studies, although this important strategy must cover all xenarthran species.

Authors' Confirmation Statement

All persons who meet the authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in this study to take public responsibility for the contents of this article. Furthermore, each author certifies that this material or similar material has not been submitted to or published elsewhere.

Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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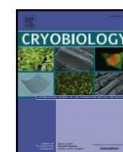
ANEXO D – VERSÃO DO ARTIGO PUBLICADO NA CRYOBIOLOGY

**CAPÍTULO 3 – INFLUENCE OF CRYOPRESERVATION TECHNIQUES AND LOW
CONCENTRATIONS OF PERMEATING CRYOPROTECTANTS ON THE
CONSERVATION OF EAR CARTILAGE AND SKIN DERIVED FROM SIX-
BANDED ARMADILLOS (*Euphractus sexcinctus* LINNAEUS, 1758)**

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Influence of cryopreservation techniques and low concentrations of permeating cryoprotectants on the conservation of ear cartilage and skin derived from six-banded armadillos (*Euphractus sexcinctus* Linnaeus, 1758)

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ABSTRACT

Considering the importance of efficiently obtaining somatic resource banks as an *ex-situ* conservation strategy for wild mammals, we evaluated two techniques (slow freezing - SF and solid-surface vitrification - SSV) for the cryopreservation of ear cartilage and skin from six-banded armadillos. Additionally, we analyzed the effects of two combinations of intracellular cryoprotectants (3.0 M or 6.0 M ethylene glycol - EG and dimethyl sulfoxide - Me₂SO) on SSV. Tissues not subjected to cryopreservation were used as controls. All samples were evaluated for morphological analysis and cell ability during culture. The thickness of the basal layer was similar to the control only for tissues derived from SF, while SSV ensured the preservation of the cartilage thickness. Moreover, fragments derived from SF and SSV, especially in the 3.0 M EG-Me₂SO group, resulted in dermis thickness and total skin similar to the control. All cryopreserved techniques maintained normal patterns of the fibroblasts, epidermal cells, and melanocytes. While only SF-derived fragments maintained the number of degenerated chondrocytes similar to the control, no difference was observed between groups for normal chondrocytes and lacunae. Moreover, SSV maintained the collagen fibers percentage of the tissues even after warming. After culture, SF and SSV techniques were efficient for the recovery of the somatic cells in all parameters evaluated, except the day of subconfluence, which was greater for the SSV group with 6.0 M EG-Me₂SO. In summary, SSV, especially with 3 M EG-Me₂SO, was as efficient as SF in preserving ear skin and cartilage derived from six-banded armadillos.

1. Introduction

The six-banded armadillo, *Euphractus sexcinctus* (Linnaeus, 1758), is one of the wild mammals representing the superorder Xenarthra with significant socio-economic and biomedical interest [1–4]. In 2014, the species was globally listed as Least Concern (LC) by the International Union for Conservation of Nature (IUCN) [1]. Nevertheless, particularly in Brazil, the species faces several threats driven by the high hunting pressure [1,5,6], habitat destruction and roadkills [7,8], which has contributed to the reduction of the species in its habitat. In this scenario, species conservation is essential for maintaining its biodiversity,

especially considering the lack of knowledge concerning armadillos' species which represents a challenge to successfully implement conservation strategies [2].

One of the conservation strategies that can be employed involves using biological sample banks through cryopreservation [2,9,10], including cartilage and skin cryobanks. During cryopreservation, cryoinjuries can occur but are also reduced by proper choice of cryopreservation technique (e.g., slow freezing - SF and solid-surface vitrification - SSV), use of cryoprotectants, and temperature control equipment (for SF) [11]. The SF is a well-documented technique used for the cryopreservation of mammalian tissues [12,13] and one benefit of it is the

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need of lower concentrations of cryoprotectants to minimize ice crystal formation comparing to SSV [14]. However, vitrification techniques are preferable to SF [12,15] for not requiring equipment or being more time-consuming; it is easier to perform in field research and reduces intracellular ice formation by using higher concentrations of cryoprotectants followed by a short exposure [16].

In general, cryopreservation techniques and the various cryoprotectants, their concentration, and the combination added can have different outcomes depending on the types of biological samples and species studied [12,17,18,13]. Combining these cryoprotective additives is advantageous since the toxic properties of individual cryoprotectants can be nullified, and at the same time, they might act synergistically in the system [19]. The use of ethylene glycol (EG) and dimethyl sulfoxide (Me₂SO) as permeating cryoprotectants, and sucrose and fetal bovine serum (FBS) as non-permeating cryoprotectants [20] is prevailing for many cryopreservation protocols of wild species tissues [21,22,23]. The combination of 3 M EG, 3 M Me₂SO, 0.25 M sucrose, and 10% FBS resulted in good outcomes on red-rumped agouti (*Dasyprocta leporina*) ear skin cryopreservation by SSV technique compared to other vitrification techniques [23]. Later, we evaluated the SSV technique following exposure to different combinations of intracellular cryoprotectants (3.0 M EG, 3.0 M Me₂SO or 1.5 M EG + 1.5 M Me₂SO) and 0.25 M sucrose and found that the 3.0 M EG-Me₂SO plus sucrose in combination is better than Me₂SO or EG alone in preserving the somatic tissue characteristics of this species [24]. Moreover, the combination of 6.0 M EG-Me₂SO was beneficial in terms of maintaining the tissue ultrastructure characteristic, dermis thickness, and proliferative activity in Antillean manatees (*Trichechus manatus manatus*) species compared to other vitrification solutions after SSV. However, none of the cryopreserved tissues could outgrowth cells *in vitro* [12].

The variable responsiveness to the cryopreservation technique and cryoprotectant concentration is species and tissue-specific and must be considered in order to develop a successful cryopreservation protocol [14]. Therefore, we aimed to evaluate the cryopreservation of ear cartilage and skin from six-banded armadillos comparing (1) conventional slow freezing versus solid-surface vitrification and (2) two concentrations of intracellular cryoprotectants (3.0 M or 6.0 M EG-Me₂SO) during solid-surface vitrification.

2. Material and methods

2.1. Chemicals and media

Sigma provided all the chemicals, except when indicated otherwise (Sigma Chemical Co., St. Louis, USA).

2.2. Bioethics and animals

The experiment was conducted with the ethical approval of the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 36/2020) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Five six-banded armadillos were used, consisting of two males and three females, and each individual was considered a replicate. These animals belonged to the Laboratory of Studies in Immunology and Wild Animals (LEIAS/UFERSA). They were kept in individual enclosures with daily feeding of pet food (Pedigree®, MARS, Campinas, Brazil), vegetables, and water *ad libitum*.

2.3. Skin and cartilage collection and experimental design

For skin and cartilage biopsies, the animals were previously anesthetized with xylazine (1 mg/kg; Sedomin®, König S.A., Buenos Aires, Argentina), ketamine (7 mg/kg; Injectable Ketamine, Vetnil®, São Paulo, Brazil) intramuscularly, and propofol (5 mg/kg; Propovan®, Cristalia, Fortaleza, Brazil) intravenously [25]. Subsequently, samples (1–1.5 cm²) from the ear region (one of each ear) were collected with

surgical scissors, washed rapidly with 70% alcohol, and deposited in tubes containing Dulbecco's Modified Minimal Essential Medium (DMEM; Gibco-BRL, Carlsbad, USA) plus 10% FBS (Gibco-BRL, Carlsbad, USA) and 2% antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B; Gibco-BRL, Carlsbad, USA) and transported within 1 h to the laboratory.

In the laboratory, the skin tissues were sectioned into 6.0 mm³ using a scalpel blade and tweezers, and 32 fragments per animal were randomly assigned among the non-cryopreserved and cryopreserved groups (8 fragments to each group per animal; Fig. 1). The fragments were cryopreserved by slow freezing (SF group) and solid-surface vitrification (SSV) testing two cryoprotectant solutions; 1.5 M EG + 1.5 M Me₂SO (SSV-3M group) and 3.0 M EG + 3.0 M Me₂SO (SSV-6M group), with a base solution composed of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, and 0.25 M sucrose. After thawing, the tissues were evaluated by histological analysis and their capacity for *in vitro* culture.

2.4. Cryopreservation by slow freezing

For SF, fragments were stored in cryovials containing 2.0 mL of the freezing solution composed of DMEM supplemented with 10% FBS, 0.75 M EG, 0.75 M Me₂SO, and 0.25 M sucrose [15], and transferred within 30 min to a freezer at −80 °C in Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, USA) for 12 h, using a cooling rate of 1 °C/min. Subsequently, all cryovials were kept in liquid nitrogen (−196 °C).

2.5. Cryopreservation by solid-surface vitrification

For SSV, a vitrification solution consisting of DMEM supplemented with 10% FBS and 0.25 M sucrose was used, containing different concentrations of intracellular cryoprotectants: 1.5 M EG + 1.5 M Me₂SO (SSV-3M group) and 3.0 M EG + 3.0 M Me₂SO (SSV-6M group) [21]. Two fragments at a time were exposed to 1.8 mL solution for 5 min and dried on absorbent paper. Subsequently, they were placed individually on a thin rectangular aluminum recipient (9 × 12 × 3 cm) kept directly over the liquid nitrogen cooled to around −150 °C to −180 °C, transferred to cryovials, and stored in liquid nitrogen (−196 °C).

2.6. Thawing of cryopreserved tissues

The cryopreserved fragments were thawed after two weeks. Initially, they were exposed for 1 min at room temperature and then immersed in a water bath at 37 °C for 30 s. To remove cryoprotectants, fragments were washed three times for 5 min in DMEM supplemented with 10% FBS and sucrose in decreasing concentrations (0.50 M, 0.25 M, and 0.0 M). Finally, the fragments were destined for the evaluations described below.

2.7. Evaluation of the skin and cartilage by histological analysis

The fragments were conditioned in a 4% paraformaldehyde solution for up to 2 weeks, followed by histological processing [26], sectioned at 5.0 µm using a microtome (Leica RM2125 RT, Leica Microsystems, Wetzlar, Germany), and stained with hematoxylin-eosin, Gomori's trichrome, or the silver-stained nucleolar organizer region (AgNOR) for analysis of morphometric aspects, collagen matrix, and tissue proliferative activity, respectively. To obtain the images, all samples were visualized using microscopy (Leica DM500, Leica Microsystems, Wetzlar, Germany) with an attached camera (Leica ICC50 HD, Leica Microsystems, Wetzlar, Germany).

For morphometric analysis and cell quantification and distribution, the following parameters were evaluated: the thickness of the epidermis, dermis, cartilage, total skin, and epidermal layers (in µm), number of epidermal cells, fibroblasts, keratinocytes, melanocytes, perinuclear

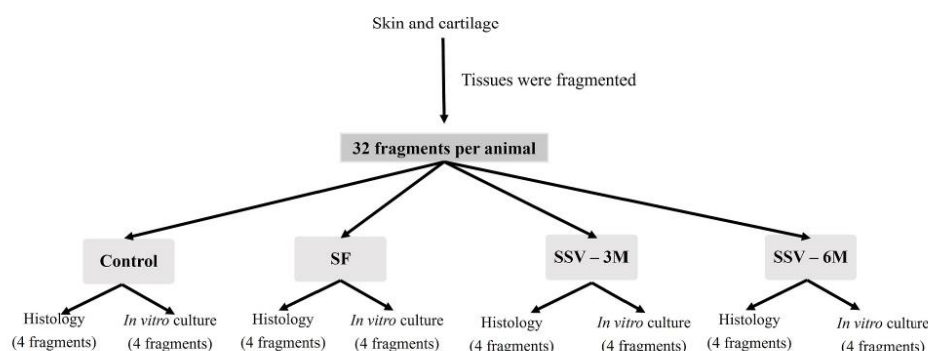


Fig. 1. Distribution of tissue fragments to assess the cryopreservation of ear cartilage and skin from six-banded amadillos comparing conventional slow freezing versus solid-surface vitrification and two concentrations of intracellular cryoprotectants (3.0 M or 6.0 M EG-Me₂SO) during solid-surface vitrification.

halos, normal and degenerate chondrocytes, and number of filled and empty lacunae [24]. Additionally, fibers in the dermis were quantified and presented in percentage to analyze the collagen matrix, dividing the area of collagen deposits by the total area of the analyzed image [24].

To analyze tissue proliferative activity, dark spots marked by silver nitrate bound to nuclear proteins were counted according to cell location [26]. The AgNORs (discrete black dots) were measured in 100 nuclei of stained and randomly selected fibroblast cells in the dermis using the ImageJ software, and the AgNOR number/cell and AgNOR area/cell were quantified by the Image Pro Plus software [15].

2.8. Evaluation of the skin and cartilage by in vitro culture

Cryopreserved and non-cryopreserved tissues were placed in 60 × 15 mm culture dishes and cultured in DMEM containing 10% FBS and 2% antibiotic-antimycotic solution at 38.5 °C in a humidified atmosphere of 5% CO₂ [27]. The dishes were monitored daily under an inverted microscope (Nikon TS100, Tokyo, Japan), and the culture medium was replaced every 1–2 days to verify cell outgrowth and any contamination. The tissues were evaluated for their morphology, number of attached explants, number of subconfluent explants, the day on which all explants were attached, the day at which explants reached subconfluence, the total time to reach subconfluence, and total culture duration [15].

After the outgrowth cells reached 70–80% confluence, after approximately 30 days, they were detached with 0.25% Trypsin-EDTA solution and evaluated for their morphology, viability, proliferative activity, metabolism, mitochondrial membrane potential, and apoptosis levels.

2.8.1. Assessment of cell viability

Live cells were estimated using 0.04% trypan blue dye, applying the traditional cell counting method [28]. For each animal per group, two replicates were performed, where dead cells were identified (stained in blue) by membrane rupture that allowed the permeabilization of the dye (non-viable cells) and those alive as colorless, without membrane rupture (viable cells).

2.8.2. Assessment of proliferative and metabolic activity

To determine the proliferative capability, the cells were cultured at a concentration of 1.0×10^4 cells/mL into 24-well culture plates, and the count was taken every 24 h for seven days. The population doubling times (PDT) were estimated according to the following formula [29]:

$$PDT = T \ln 2 / \ln (X_t / X_i)$$

where T is the incubation time (in hours), X_i is the initial cell count, X_t is the final cell count, and ln is the Napierian logarithm. The growth curve for seven days was plotted using cell numbers only.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoline bromide (MTT) assay was performed to evaluate the metabolic functionality. For this, 5.0×10^4 cells/mL was incubated at 38.5 °C and 5% CO₂. After five days, the MTT solution (5 mg/mL in DMEM) was added, and the plates were incubated for 3 h at 38.5 °C. Subsequently, Me₂SO was added as an MTT solubilization solution, and the reading was performed at an absorbance of 595 nm [21].

2.8.3. Assessment of mitochondrial membrane potential and apoptosis level

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined using the MitoTracker Red probe (CMXRos) diluted in Me₂SO for 30 min at 38.5 °C, 5% CO₂ [28]. Cells were washed with PBS and incubated with 500 nM CMXRos for 30 min at 38.5 °C, 5% CO₂, visualized under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and the intensity of the fluorescence signal (pixels) was quantified. Images were evaluated using the ImageJ software.

The apoptotic cell death was analyzed according to Lima et al. [30], where cells were stained with acridine orange (AO, 1.0 µg/mL) and ethidium bromide (EB, 1.0 µg/mL) and evaluated under a fluorescence microscope. Images obtained were analyzed using the Image J software (National Institutes of Health, NIH, USA) to quantify the fluorescence signal intensity (pixels). The viable cell had a uniform light green nucleus, an early apoptosis cell had a non-uniform green nucleus, a late apoptotic cell had bright orange areas, and a necrotic cell had a uniform orange nucleus.

2.9. Statistical analyses

Data were expressed as mean ± standard error and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at $p < 0.05$.

3. Results

3.1. Effects of skin and cartilage cryopreservation on histomorphological analysis

The morphological characteristics of non-cryopreserved (control) and cryopreserved (SF, SSV-3M, SSV-6M) skin explants obtained by

hematoxylin-eosin stain are shown in Fig. 2. Considering the epidermis layers, all cryopreservation techniques, SF ($96.5 \pm 16.8 \mu\text{m}$), SSV-3M ($98.1 \pm 23.6 \mu\text{m}$), SSV-6M ($93.4 \pm 18.3 \mu\text{m}$), reduced the thickness of the spinosum compared to the control group ($127.3 \pm 26.2 \mu\text{m}$), while only the thickness of the basal layer for tissues derived from SF ($50.0 \pm 16.9 \mu\text{m}$) remained similar to the control ($54.8 \pm 11.4 \mu\text{m}$; Fig. 3a). No difference was observed among cryopreserved (SF: $316.5 \pm 45.4 \mu\text{m}$, SSV-3M: $352.8 \pm 74.0 \mu\text{m}$, and SSV-6M: $334.8 \pm 58.8 \mu\text{m}$) and non-cryopreserved tissues ($363.8 \pm 56.0 \mu\text{m}$) for epidermis thickness. The dermis and total skin thickness were similar to the control ($1339.2 \pm 114.1 \mu\text{m}$ and $1702.9 \pm 125.8 \mu\text{m}$) for the SF ($1339.3 \pm 125.3 \mu\text{m}$ and $1655.8 \pm 136.3 \mu\text{m}$) and SSV-3M groups ($1408.8 \pm 185.3 \mu\text{m}$, and $1761.6 \pm 218.4 \mu\text{m}$), while the SSV-6M group ($1588.9 \pm 109.7 \mu\text{m}$, and $1923.6 \pm 109.6 \mu\text{m}$) presented an increase in both parameters ($P < 0.05$). Moreover, the cartilage thickness was preserved only after the evaluated vitrification techniques (SSV-3M: $273.5 \pm 28.6 \mu\text{m}$ and SSV-6M: $276.5 \pm 31.5 \mu\text{m}$), compared to the control ($286.4 \pm 26.3 \mu\text{m}$).

As a result of main skin cells quantification, the epidermal cells and melanocytes number alone were similar among evaluated groups, but the number of keratinocytes was lower in cryopreserved groups (SF: 87.0 ± 13.1 ; SSV-3M: 82.4 ± 14.8 ; SSV-6M: 79.5 ± 13.5) when compared to the control (103.5 ± 19.7 ; Fig. 3b). Moreover, comparing the result with the control group (51.6 ± 7.9), the fibroblast number remained through cryopreservation (SF: 46.9 ± 6.3 ; SSV-3M: 57.0 ± 11.0 , SSV-6M: 58.3 ± 9.5). Although only the SF group showed a similar value of degenerated chondrocytes when compared to the control, we found no difference in normal chondrocytes and filled, and empty lacunae count between groups (Fig. 3c). Nevertheless, the tissues from both SSV groups (SSV-3M: 0.8 ± 0.8 , SSV-6M: 1.5 ± 1.2) presented fewer perinuclear halos when compared to the SF group (3.2 ± 1.7), but these results did not differ from the non-cryopreserved tissues (2.6 ± 1.5 ; Fig. 3d).

Furthermore, we evaluated the collagen matrix from non-cryopreserved (control) or cryopreserved tissues by Gomori trichrome stain (Fig. 4a-d). The vitrified groups (SSV-3M: $72 \pm 0.03\%$; SSV-6M: 73

$\pm 0.03\%$) showed collagen matrix percentage similar to the control group ($74 \pm 0.03\%$); however, the percentage from the SF group ($78 \pm 0.04\%$) was significantly higher (Fig. 4e). Although the AgNOR number per fibroblast cell did not alter after cryopreservation and thawing, the cryopreserved groups showed an increase in AgNOR area per cell (Fig. 5).

3.2. Effect of skin and cartilage cryopreservation on primary culture and subculture

According to the *in vitro* culture evaluation, both cryopreserved and non-cryopreserved tissues were able to adhere and detach somatic cells until they reached subconfluence (70–80% of confluence; Fig. 6). As shown in Fig. 6, the cells presented a star or spindle-shape with prolonged cytoplasmic projections like fibroblasts. All explants were attached within 1–7 days of culture, and the migration of primary cells was seen in the first days. Still, the monolayer of epithelial-like and fibroblast-like cells around all explants was observed at 12.6, 9.8, 15.0, and 14.5 days in the non-cryopreserved, SF, SSV-3M, SSV-6M groups, respectively (Table 1). Additionally, all explants showed subconfluence, but the day they reached subconfluence was longer in SSV-6M compared to the control and SF group, yet not differing from the same vitrification technique with a higher concentration of intracellular cryoprotectants (SSV-3M). The duration of culture was similar between groups (Table 1).

After the primary culture reached subconfluence, the cells were detached and submitted to quality analysis. The cell viability, metabolic and proliferation activity, and growth curve results are shown in Fig. 7. The viability and metabolic activity were high (over 85% and 70%, respectively) in all groups and remained similar after cryopreservation, regardless of the technique or solution (Fig. 7a–b). The population doubling time did not change between cryopreserved and control groups (Fig. 7c). Additionally, the graph to observe the cell growth pattern was plotted (Fig. 7d). The non-cryopreserved group showed an increase in the number of cells, increasing exponentially after one day of culture, and decreasing on the seventh day. In general, the cryopreserved groups

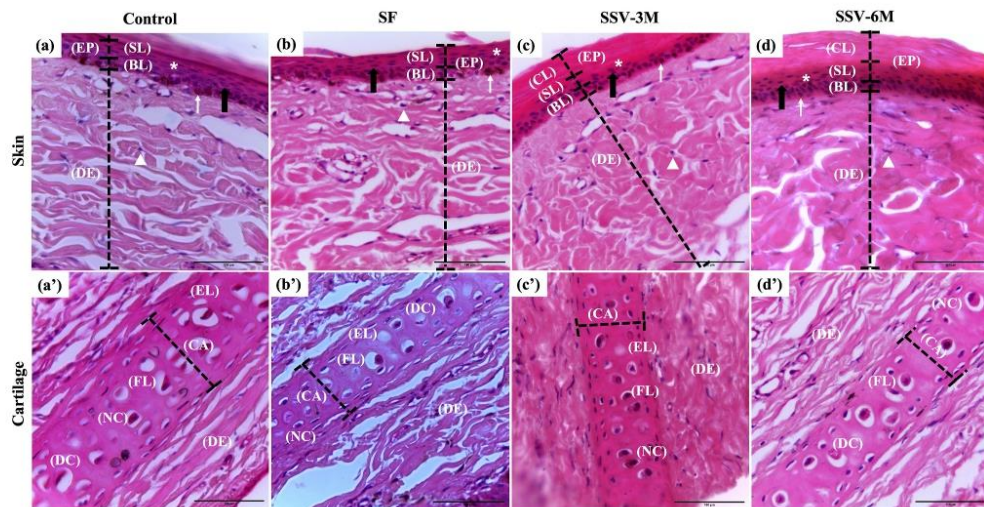


Fig. 2. Histological sections of ear skin layers (a-d) and cartilage (a'-d') of non-cryopreserved and cryopreserved tissues derived from six-banded armadillos using hematoxylin-eosin. (a, a') non-cryopreserved (control); and cryopreserved tissues by (b, b') slow freezing, (c, c') solid-surface vitrification at 3.0 M concentration of intracellular cryoprotectants, and (d, d') solid-surface vitrification at 6.0 M concentration of intracellular cryoprotectants. BL, basal layer; CA, cartilage; CL, corneum layer; DC, degenerated chondrocytes; DE, dermis; EL, empty lacunae; EP, epidermis; FL, filled lacunae; NC, normal chondrocytes; SL, spinosum layer. Epidermal cells (*), melanocyte (white arrow), keratinocyte (black arrow), and fibroblast (triangle). Scale bar: 100 μm . 40 $\times$ magnification.

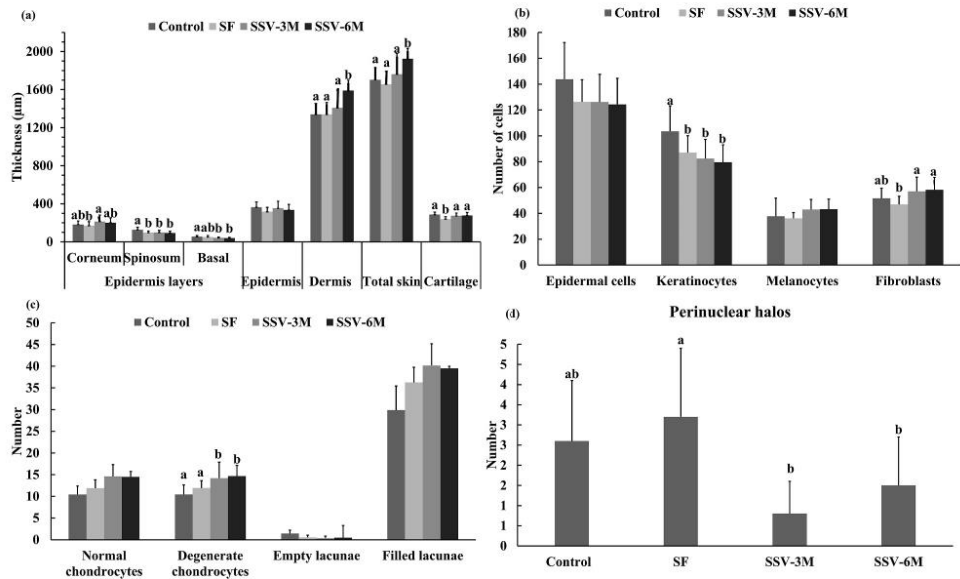


Fig. 3. Thickness of skin layers and cartilage and main cell quantification of non-cryopreserved and cryopreserved tissues derived from six-banded armadillos. (a) Thickness of the epidermis layers, epidermis, dermis, total skin, and cartilage; (b) Mean number of epidermal cells, keratinocytes, melanocytes, and fibroblasts; (c) Mean number of normal chondrocytes, degenerate chondrocytes, filled lacunae and empty lacunae in cartilage; (d) Mean number of perinuclear halos in the epidermis.

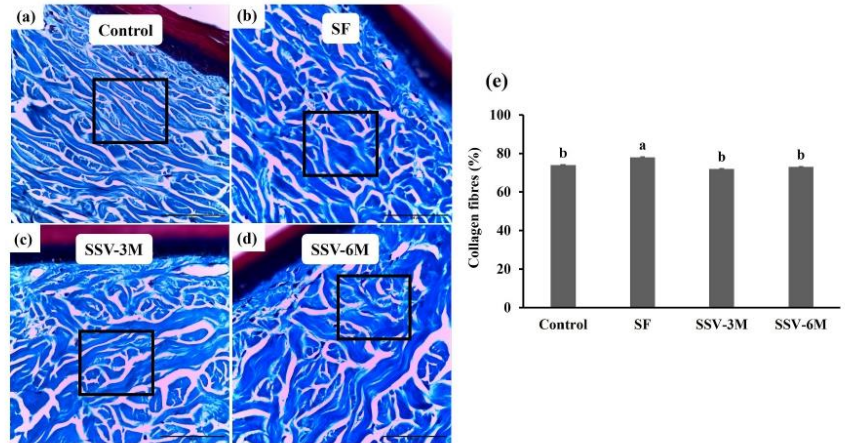


Fig. 4. Evaluation of collagen density from ear skin of six-banded armadillos using Gomori trichrome (a) Staining of collagen fibers in the dermis from non-cryopreserved tissues; and cryopreserved tissues by (b) slow freezing (SF), (c) solid-surface vitrification at 3 M concentration of intracellular cryoprotectants (SSV 3 M), (d) solid-surface vitrification at double concentration of intracellular cryoprotectants (SSV 6 M). (e) Percentage of collagen fibers in the dermis from non-cryopreserved and cryopreserved tissues. Square exemplifies the dermal area of evaluation of collagen matrix. Bars represent standard error. Scale bar: 100 μm 40 × magnification.

showed lower cell concentration than the control group but similar growth patterns between them, except in the SSV-6M group at Day 7, where cell growth continued and did not decrease.

Our results showed that the intercellular $\Delta\Psi_m$ level remained similar in cryopreserved and non-cryopreserved groups (Fig. 7e). The signs of

apoptosis were evaluated using AO/EB dual staining. The results revealed a high incidence of viable cells and a low rate of cells in the apoptotic stage. The necrotic cells were rarely observed in all groups, and all types of cells, such as viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells, showed similar percentages among

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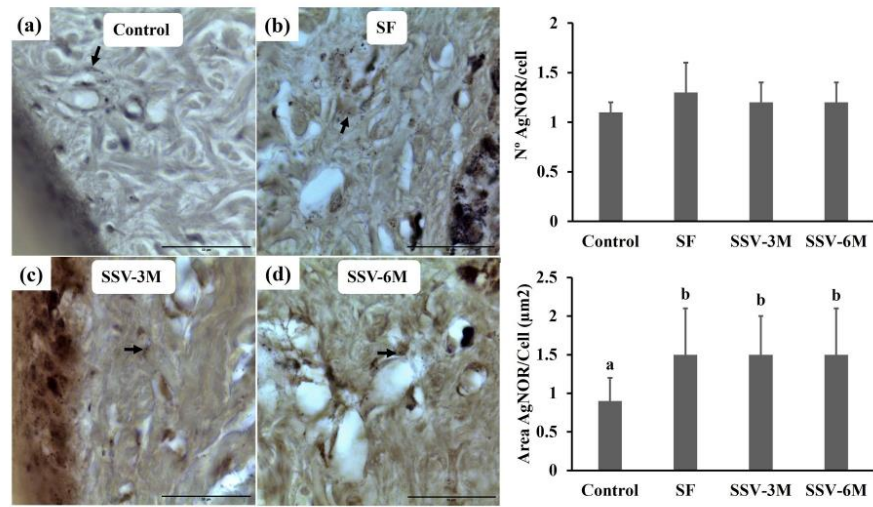


Fig. 5. Proliferative activity of non-cryopreserved and cryopreserved skin derived from six-banded armadillos submitted to different techniques. (a) Staining of AgNOR in the fibroblasts from fresh skin (control); (b) slow freezing, (c) solid-surface vitrification at 3.0 M concentration of intracellular cryoprotectants, and (d) solid-surface vitrification at 6.0 M concentration of intracellular cryoprotectants. (e) Quantification of AgNOR number/cell. (f) Quantification of AgNOR area/cell. SF: slow freezing. SSV: solid-surface vitrification. Bars represent standard error. Arrow indicates fibroblast nuclei stained with AgNOR. Scale bar: 50 µm 100 × magnification.

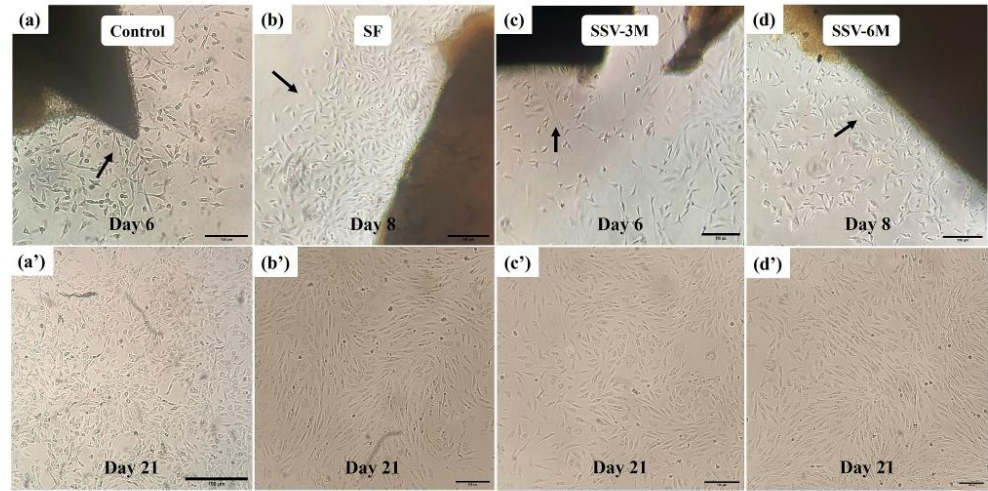


Fig. 6. Examples of cell outgrowth from six-banded armadillos skin tissue explants. (a) and (a') cells from non-cryopreserved tissues; and cryopreserved tissues by (b) and (b') slow freezing (SF), (c) and (c') solid-surface vitrification at 3.0 M concentration of intracellular cryoprotectants (SSV-3M), (d) and (d') solid-surface vitrification at higher concentration of intracellular cryoprotectants (SSV-6M). Arrow indicates the beginning of cell detachment in primary cultures. Scale bar: 100 µm. Magnification 10 ×.

the groups (Fig. 7f).

4. Discussion

In this study, we report the first attempt to preserve ear skin and cartilage of six-banded armadillos by assessing three cryopreservation

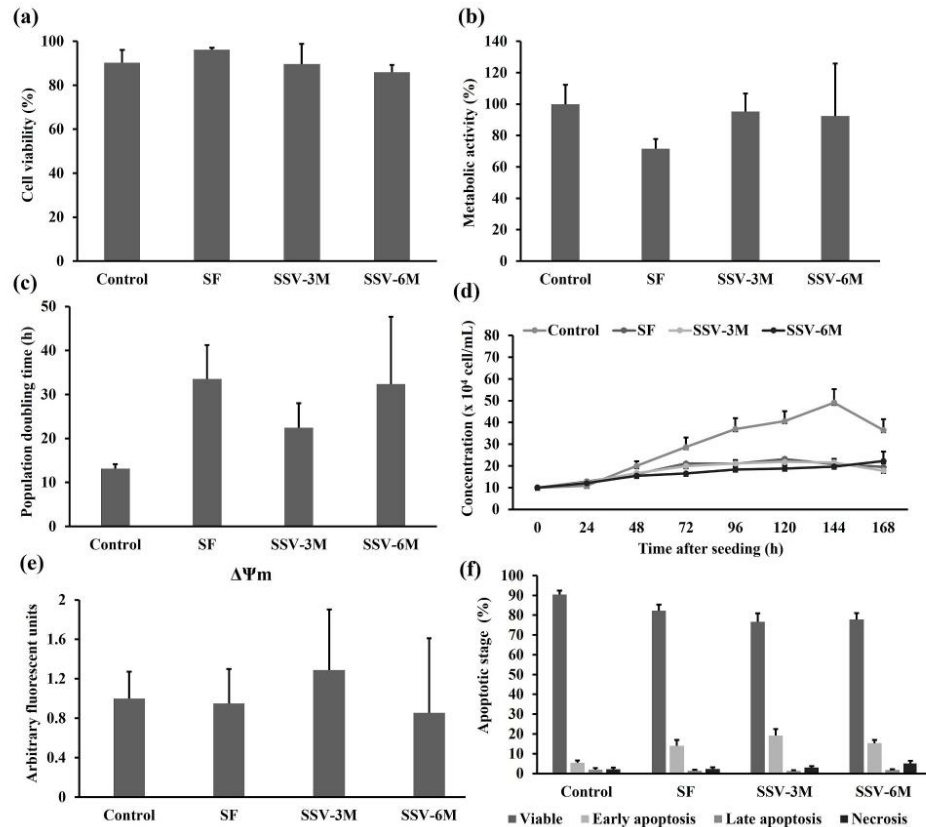
protocols and evaluating the post-thawed tissue histological aspects and *in vitro* culture for cell recovery. The results demonstrated that the SSV cryopreservation technique, especially with 3 M EG-Me₂SO, was as effective as SF in conserving the tissue (skin and cartilage) in terms of histological characteristics, highlighting both groups as an ideal cryopreservation method and solution in maintaining tissue and cell

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Table 1

Effect of cryopreservation techniques and solutions on primary cultures of skin tissues derived from six-banded armadillos.

Groups	Attached explants (%)	Day of all attached explants $\pm$ S.E.	Day of all explants with cell growth $\pm$ S.E.	Grow to subconfluence (%)	Subconfluence day $\pm$ S.E.	Duration of culture (days) $\pm$ S.E.
Control	20/20 (100) ^a	1.4 $\pm$ 0.4 ^a	12.6 $\pm$ 1.0 ^a	23/24 (96) ^a	15.6 $\pm$ 1.2 ^a	36.8 $\pm$ 1.2 ^a
SF	20/20 (100) ^a	6.0 $\pm$ 1.8 ^a	9.8 $\pm$ 1.2 ^a	24/24 (100) ^a	14.8 $\pm$ 1.2 ^a	28.0 $\pm$ 2.1 ^a
SSV-	19/20 (95) ^a	6.8 $\pm$ 2.6 ^a	15.0 $\pm$ 1.5 ^a	13/16 (81) ^a	20.0 $\pm$ 1.0 ^{ab}	31.8 $\pm$ 1.7 ^a
3M						
SSV-	20/20 (100) ^a	3.5 $\pm$ 2.5 ^a	14.5 $\pm$ 2.5 ^a	7/8 (88) ^a	24.0 $\pm$ 5.0 ^b	34.0 $\pm$ 6.0 ^a
6M						

SF: slow freezing. SSV: solid-surface vitrification. S.E.: standard error. ^{a,b}: P < 0.05.Fig. 7. Survival rate, functionality, grow dynamics, oxidative stress and apoptosis levels of six-banded armadillos fibroblast-like cells from non-cryopreserved and cryopreserved tissues. (a) viability, (b) metabolic activity, (c) population double time and (d) growth curve, (e) measurement of intracellular mitochondrial membrane potential ($\Delta\Psi_m$) and (f) apoptosis levels. Bars represent standard error. P > 0.05.

functionality unchanged after culture. Nevertheless, the SSV is a fast, cheap, and practical cryopreservation technique [31], outstanding as a more suitable alternative to be performed in field work compared to SF, and therefore recommended for routine use in assisted reproduction for wildlife species conservation.

The armadillo skin is thick [32]. In this study, the average thickness of the total skin in the non-cryopreserved group was 1702.9 μm , and the cryopreservation process did not shrink the total skin. Moreover, the epidermis layers (comeum, spinosum, and basal layer), epidermis,

dermis, and cartilage were identified and measured separately in skin samples from six-banded armadillos. The thickness of the basal and spinosum layers decreased only after vitrification. This change can be explained by the thermal stress effect due to nonuniform changes in tissue temperature that might occur during cryopreservation since it has been reported that the tissue layers tend to shrink, in this case, more in the inside layers than the outside [33]. The same alteration was seen in ocelot (*Leopardus pardalis*) skin basal and spinosum layers after SSV [34]. Further, no retraction was observed in the epidermis, dermis, and

total skin after cryopreservation, possibly because of the presence and concentration of intra- and extracellular cryoprotectants that reduce cryoinjuries and prevents tissue alterations. However, even briefly, 6 M of permeating cryoprotectants in the SSV-6M group were not adequate for preserving the tissues since the dermis and total skin thickness were greater than in the non-cryopreserved fragments. This result is explained by using a higher concentration of cryoprotectants, triggering a greater risk of cryoprotectant toxicity during the addition or removal of cryoprotectants and causing a change in the tissue due to excessive osmotic forces [35,36].

On the other hand, only the SSV groups did not differ from the control group in cartilage thickness. The shorter cartilage thickness in the SF group can be attributed to the cooling rate used since the intracellular water is effluxed from the cell in the slow cooling process, reducing the intracellular water amount, which does prevent interior ice formation and further damage. Still, there is a high risk of extracellular ice formation in SF [16,19], which could alter the tissue histomorphology compared to vitrification and non-cryopreserved tissues. In SSV, due to the rapid cooling, the intracellular water has less time to move to extracellular space and gets supercooled, which may cause intracellular ice formation instead [19]. We used higher concentrations of cryoprotectants to prevent this injury. Accordingly, cryoprotective additives and the cooling rate adjustment are critical for successful vitrification [19]. A similar response was observed in the jaguar, in which the ear skin tissue was better conserved in the SSV group after histological evaluation compared to SF [15]. Our data demonstrate that the SSV-3M group ensured the conservation of tissue integrity.

Additionally, the cryopreservation by SSV positively affected the dermis and the cartilage with greater fibroblast when evaluating the histological quantification of cells. In the dermis, the fibroblasts are the primary cell type. They are desired for genetic material conservation and research studies, being the most frequently used somatic cells in cloning by nuclear transfer [37] and favorable for wild species [23]. The vitrification groups (SSV-3M and SSV-6M) showed lesser perinuclear halos than SF but no difference between cryopreserved and non-cryopreserved tissues. The perinuclear halos were measured as a number of apoptotic cells and are visualized in the epidermal as retractions of the nucleus from the cytoplasm [38]. Our result showed that the cryopreservation techniques preserved the tissues from this damage.

Nevertheless, while the SSV caused an increase in degenerated chondrocytes, the SF maintained a similar number compared to the control, indicating low cell degeneration. The slow cooling rate may explain this result, along with using a lower concentration of permeating cryoprotectants since the intracellular ice formation is reduced during slow freezing, preventing cell degeneration [39]. Alternately, the percentage of collagen fibers was only unchanged in the SSV groups, according to the knowledge that vitrification promotes better maintenance of structural and extracellular matrix of tissues due to the rapid vitreous state, causing less risk of freeze injury [16,34].

Further, evidencing the efficiency of the techniques and the combination of cryoprotectants, the tissue proliferative activity was maintained through cryopreservation, proved by the measure of AgNORs. AgNOR has been used as histochemical pointers of cell multiplication, a good marker of proliferative action, and is moderately inexpensive and direct in understanding [40]. Moreover, during the *in vitro* culture, we observed that the primary cell morphology was both epithelial-like and fibroblast-like cell types at the first migration. The fibroblasts present a different behavior in *in vitro* environment; they detach faster after trypsin digestion and can attach more easily and readily than epithelial cells [41,37]. Thus, after a few days of culture, both cryopreserved and non-cryopreserved groups showed a predominant cell type, fibroblast-like cells, growing as monolayers. In general, these cells showed rapid proliferation and good *in vitro* cell performance, with no difference observed among the control, SF, or SSV groups.

The combination of both intra and extracellular cryoprotectants may be the cause of that since the use of Me₂SO and EG as intracellular

cryoprotectants and sucrose and FBS as extracellular cryoprotectants had proved to be effective in reducing the possible damages in the cryopreservation process and maintained the tissues and cells viable [22,15]. Me₂SO is the most used cryoprotectant; it penetrates the cells and prevents ice crystal formation and membrane rupture [39]. EG is considerably less toxic, but both cryoprotectants have been proven reliable for vitrification with tolerable toxicity at high concentrations. Moreover, vitrification immediately in liquid nitrogen can reduce the cytotoxic effect.

Accordingly, the cell quality was preserved by comparing the standard slow freezing technique and vitrification (by SSV) and the vitrification solutions set to 3.0 M or 6.0 M of intracellular cryoprotectants. Cell viability was high in control tissues (90.2%) and maintained in non-cryopreserved tissues, corroborating other studies [30,15]. The PDT value did not alter, with a maximum of 34 h in the SF group. These cells' PDT rates in armadillos deferred from other wild mammals after cryopreservation, considering the maximum value, 66 h in collared peccaries [22], 26 h in jaguar [15], and 46 h in puma (*Puma concolor*) [30]. The growth curve from the control group showed high exponential growth reaching a maximum cell concentration of 49×10^4 cells/mL at 144 h of culture. The cryopreserved groups presented a very similar profile, showing lower concentrations of cells during culture, but still with normal proliferation capacity regardless of the cryopreservation process.

The cells also showed no changes in metabolic activity through the MTT assay, and the $\Delta\Psi_m$ were not affected by the cryopreservation. The $\Delta\Psi_m$ value was evaluated as a hallmark of mitochondrial dysfunction compared with the control group. The apoptosis rate was low in all groups with no statistical difference, confirmed by the AO/EB dual staining and considering early apoptosis, late apoptosis, and necrosis stage. The EB stain can only damage cells and gives orange-red fluorescence when bound to the DNA; different color tones indicate apoptotic stages. The green fluorescent resulting from the AO stain indicates viable cells. The control group had 90% of viable cells after culture, and this parameter was also unaffected by the cryopreservation protocols.

5. Conclusions

Although both cryopreservation techniques were able to conserve the somatic tissue with minor changes compared to the control group, we highlight the SSV technique, especially using permeating cryoprotectants in combination at lower concentrations (3 M). This vitrification process is more practical in the field, less time-consuming, requires minimal equipment, and can preserve cells closer to their natural state. Regarding the vitrification solutions, the SSV-3M is preferable for less concentration of EG-Me₂SO, reducing the chances of cell toxicity. Our finding supports that the efficiency of the technique depends on the use of lower concentrations of permeating cryoprotectants at 3 M. We highlight the importance of an adequate cryopreservation protocol for the six-banded armadillo genetic resource bank that can be used in assisted reproduction techniques for the species conservation and others phylogenetically closely related species.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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**ANEXO E – VERSÃO DO ARTIGO PUBLICADO NA IN VITRO CELLULAR &
DEVELOPMENTAL BIOLOGY – ANIMAL**

**CAPÍTULO 4 – LONG-TERM PRESERVATION OF ESTABLISHED FIBROBLAST
LINES FROM SIX-BANDED ARMADILLOS (*Euphractus sexcintus*, LINNAEUS,
1758) BY EXTENDED PASSAGE AND CRYOPRESERVATION**

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Long-term preservation of established fibroblast lines from six-banded armadillos (*Euphractus sexcinctus*, Linnaeus, 1758) by extended passage and cryopreservation

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Abstract

Establishing new somatic cell cultures has raised significant attention as an effective and convenient way to preserve genetic samples for different applications. Although many lines have been established in model animals, none derived from six-banded armadillo species is currently available. We report the successful isolation and characterization of fibroblasts from six-banded armadillos, evaluating the cell quality after extended culture and cryopreservation. Initially, we collected ear skin from five captive adult individuals and identified fibroblast lines by morphology, karyotyping, and immunophenotyping assays. The isolated fibroblasts were evaluated after several passages (fourth, seventh, and tenth passages) and cryopreservation by slow freezing. Cell morphology, viability, metabolism, proliferative activity, mitochondrial membrane potential, and apoptosis levels were analyzed. The skin explants had great adhesion, and cell outgrowth could be seen after 3–6 d. The cells were verified as fibroblasts at the fourth passage by vimentin expression and normal karyotype ($2n = 58$). The viability remained high ($> 87\%$) and constant from the fourth to the tenth passage ($p > 0.05$). The passages did not change the cell morphology and metabolic and growth rates. Moreover, cryopreservation did not affect most evaluated parameters; post-thawed cells maintained their viability, growth, metabolism, and apoptosis levels. Nevertheless, cryopreservation increased mitochondrial membrane permeability and cell population doubling time compared to non-cryopreserved cells ($p < 0.05$). In summary, viable fibroblasts can be obtained from six-banded armadillo skin while conserving their quality as the number of passages increases and featuring few changes after cryopreservation.

Keywords Xenarthra · Genetic resource banks · In vitro culture · Somatic cells

Introduction

The six-banded armadillo (*Euphractus sexcinctus* Linnaeus, 1758) is a wild mammal native to the Americas and found in all biomes in Brazil (Paglia et al. 2012; Superina and Abba 2018). The species is an important ecosystem engineer,

mainly for seed dispersal, soil aeration, and pest control services (Abba et al. 2014; Rodrigues et al. 2019). Moreover, the six-banded armadillo arouses interest in studies due to their scientific classification as a natural reservoir of zoonoses and economical due to carcass, meat, and medicine value (Abba et al. 2014; Teixeira et al. 2020).

It is the armadillo species with the largest geographic distribution (Santos et al. 2019), being very diverse and not in danger of extinction like other armadillo species (Abba et al. 2014). Despite their resistance to environmental alterations, the six-banded armadillo population suffers from anthropological pressures, such as habitat degradation, hunting, and roadkill incidents (Barbosa and Oliveira 2022). Furthermore, the species is easily adapted to captivity; they are often globally transported to various zoos as ornamental animals (Guo et al. 2022) and represent an ideal experimental model for reproductive biotechnology

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for the species and other threatened members of the Xenarthra superorder (Sousa et al. 2016), which comprehends more than 20% of xenarthrans (Fernandes et al. 2023a). Such information gives strong arguments about the need to develop research on conservation strategies and expand knowledge about this species.

Cell line storage is considered an effective and promising tool for biodiversity conservation (Borges et al. 2020; Wang et al. 2020). The long-term conservation of genetic materials and their original characteristics and functions offers enormous possibilities. In this case, using fibroblast skin cells is straightforward due to their inexpensive recovery, isolation, and maintenance in culture (Praxedes et al. 2018; Dua et al. 2021). Moreover, fibroblasts are suitable as donor cells for advanced reproductive technologies, like somatic cell nuclear transfer (SCNT) and induced pluripotent stem cell (iPSC) techniques (Praxedes et al. 2018; Sharma et al. 2018). In general, three essential steps must be performed for the successful establishment of somatic cell lines: (1) identify and establish the cell line, (2) characterize the cell during extended culture, and (3) evaluate the effects of cryopreservation (Praxedes et al. 2021).

Extended culture and cryopreservation can stress the cells or even cause their senescence (Magalhães et al. 2017; Praxedes et al. 2021). Skin fibroblast cells derived from different species have been cultured in up to ten passages, maintaining their functionality (Borges et al. 2020; Wang et al. 2020). Nevertheless, earlier passages (fourth and seventh passages) demonstrated the better quality of fibroblasts from brown brocket deer (*Mazama gouazoubira*) compared to the tenth passage. This was also evidenced in cryopreserved cells within the same passage (Magalhães et al. 2017). In subsequent studies, skin fibroblast viability and metabolic and proliferative activities were not affected by cryopreservation in red-rumped agouti (*Dasyprocta leporina*; Praxedes et al. 2021) and puma (*Puma concolor*; Rodrigues et al. 2023). A similar result was obtained in skin fibroblasts taken post-mortem from the ear of an Asian elephant (*Elephas maximus*), where the cells had high viability and post-freezing recovery rates (Siengdee et al. 2018). Cryopreservation induced changes on mitochondrial membrane potential ($\Delta\Psi_m$) in fibroblasts derived from collared peccaries (*Pecari tajacu*; Borges et al. 2020) and puma (*P. concolor*; Lira et al. 2022).

We initiated a protocol for skin fibroblast cryobanks of six-banded armadillos, considering the need for developing somatic cell resource banks for this species. Therefore, we aimed to (1) isolate, identify, and establish cell lines, evaluating the effects of (2) extended culture time (fourth, seventh, and tenth passages) and (3) cryopreservation on the quality parameters of somatic cells derived from ear skin of six-banded armadillos kept in captivity.

Materials and methods

Chemicals and media The Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin solutions were obtained from Gibco-BRL (Carlsbad, CA). The fluorescent probes were purchased from Invitrogen (Carlsbad, CA), and the mouse anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor 488, Warrington, PA) were purchased from Abcam (Cambridge, MA). The other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Media were filtered using a 0.22- μm system (Corning, NY).

Bioethics and animals All experiments were approved by the Animal Ethics Committee of the Rural Federal University of Semi-Arid (CEUA/UFERSA, no. 36/2020) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655–1). Five adults, 3 females and 2 males, six-banded armadillos were selected for the tissue biopsies (Fig. 1a). They were kept in individual enclosures at the Laboratory of Studies in Immunology and Wild Animals (LEIAS/UFERSA) with daily feeding of pet food (Pedigree®, MARS, Campinas, Brazil) plus vegetables and water ad libitum.

Tissue collection and explant preparation Skin tissue biopsies (1–1.5 cm^2) from the basal region of the ear were collected using surgical scissors (Fig. 1b). After the collection, the tissues were washed rapidly with 70% alcohol, deposited in tubes containing DMEM plus 10% FBS and 2% antibiotic–antimycotic solution, and transported immediately to the laboratory (Fig. 1c).

In the laboratory, the biopsies were chopped in small pieces (Fig. 1d), approximately 6.0 mm^3 in size per piece (explants) and washed quickly in sequential baths of (1) DMEM supplemented with 10% FBS and 10% antibiotic–antimycotic solution, (2) alcohol, and (3) DMEM plus 10% FBS and 2% antibiotic–antimycotic solution (Fig. 1e). Four explants per animal (Fig. 1f) were placed in polystyrene culture dishes with a culture medium consisting of DMEM plus 10% FBS and 2% antibiotic–antimycotic solution and cultured at 38.5°C under a controlled environment with 5% CO_2 and 95% air (Borges et al. 2020).

Experimental design We divided the experiment into three steps to establish and generate cell lines derived from the tissues. At first, the cells were grown under primary culture conditions and analyzed regarding the in vitro characteristics of explant culture and cell morphology. Moreover, upon the fourth passage, the cells were subjected to an immunofluorescence assay to confirm the cell type identity and karyotype analysis to assess chromosomal stability. In the second step, the cells were cultured and evaluated on the fourth (P4),

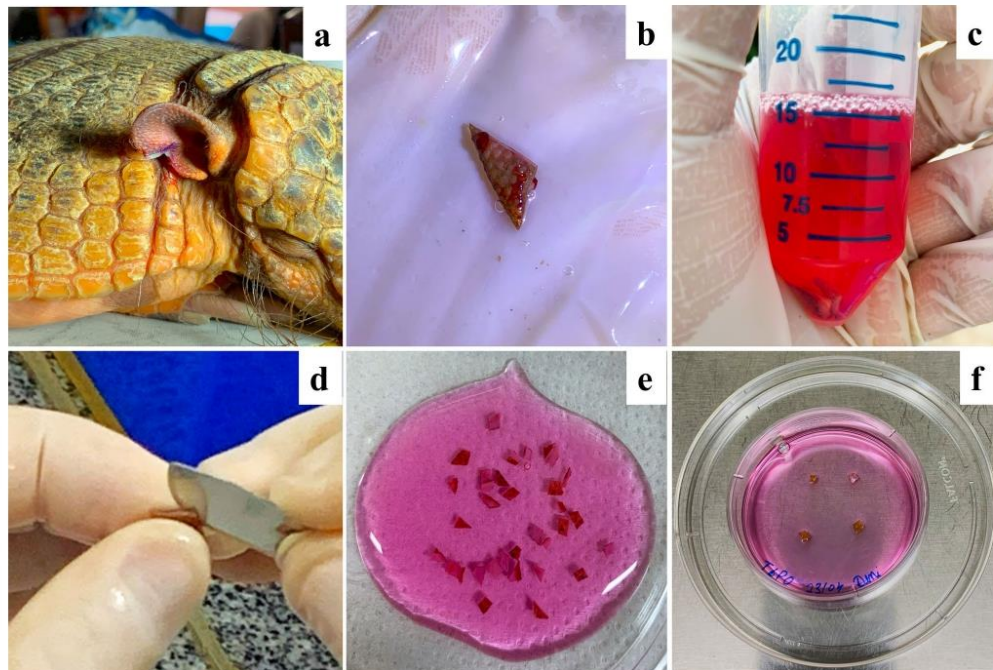


Figure 1. Ear explant collection and preparation. (a–b) Ear sample cut from a six-banded armadillo, and (c) transferred in a sterile 50 mL tube containing DMEM, 10% FBS and 2% antibiotic–antimycotic. (d) The front and back of the excised skin sample were separated, (e) cut into pieces approximately 6.0 mm³ in size, washed, and (f) four pieces were placed in a 60-mm-diameter culture dish with DMEM, 10% FBS and 2% antibiotic–antimycotic for culture.

seventh (P7), and tenth (P10) passages for in vitro culture effects on morphology, viability, proliferative activity, and metabolic activity. Finally, the cells (passage 4) were submitted to cryopreservation, evaluating the process's effect by analyzing viability, metabolism, proliferative activity, mitochondrial membrane potential ($\Delta\Psi_m$), and apoptosis levels. The cryopreserved cells' performance post-thawing was compared to those of non-cryopreserved cells.

Primary culture evaluation and subcultures The medium was changed every 24 h for the primary culture. The cultures were observed daily, distinguishing the following parameters: number of samples, number of adhered samples, day of sample adherence, day of subconfluent growth of the samples, and total time to reach 70–80% confluence (Fernandes et al. 2023b). When most of the explant area were surrounded by outgrowing cells at 70%–90% confluence, they were removed, leaving only the cells; once the cells reached 70%–80% confluence over the whole dish, they were

trypsinized and subcultured. Cell morphology and growth were also noted. For trypsinization, the cells were previously washed with phosphate-buffered saline (PBS), incubated with trypsin/EDTA solution (0.25%/0.2%) for 7 min, and centrifuged at 600×g for 10 min. The resultant supernatant was discarded, the cell pellet was resuspended in a culture medium, and the cell suspension was transferred to another dish for subculturing. They were monitored daily, and the medium was replaced with fresh medium every other day.

Moreover, the cells were cultured without using antibiotics and antimycotics to ensure the absence of contamination. Subsequently, after characterization, the influence of the number of passages (fourth, seventh, and tenth passage) and the effects of slow freezing cryopreservation were analyzed.

Characterization of cells by morphological characteristics Morphological characteristics were observed under a light microscope throughout the in vitro culture period to trace cellular and nuclear shapes and cytoplasmic extensions.

Characterization of cells by karyotype analysis For the karyotype evaluation, the cells were subjected to a protocol adapted from the methodology described by Mehrabani et al. (2014) and Praxedes et al. (2021). Initially, the cells at the fourth passage were exposed to colchicine solution at a final concentration of 0.1%, followed by incubation at 38.5°C for 2 h. The medium was removed, the cells were trypsinized, resuspended in DMEM, and transferred to a tube. After centrifugation ($600 \times g$ for 10 min), 5 mL of hypotonic solution (0.075 M KCl) was added to the pellet, mixed gently, and incubated for 10 min at 37°C. After this period, the tube was centrifuged ($600 \times g$ for 10 min), and the supernatant was discarded. Then, 5 mL of cold fixative solution (3:1 solution of methanol: acetic acid) was added, mixed gently, and centrifuged at $600 \times g$ for 10 min. The cell pellet was suspended by flicking the bottom of the tube. The fixation step was repeated three times. After the final fixation step, 1.0 mL of fixative solution was added to the suspended pellet. Two to three drops of the cytogenetic suspension were placed on a slide. Once the slides had dried, they were stained for 5 min using Giemsa, washed in distilled water, and air dried for subsequent analysis. At least ten metaphases were analyzed (Olympus BX51TF, Tokyo, Japan) at $100 \times$ magnification.

Characterization of cells by anti-vimentin immunofluorescence staining The cell line's identity was confirmed by immunofluorescence staining according to the protocol described by Selokar et al. (2018), using antibodies against vimentin (a marker of fibroblasts). After the fourth passage, the cells were cultured until 70–80% confluence, fixed in 4% paraformaldehyde for 10 min at 25°C, and washed three times with chilled PBS. Subsequently, they were incubated with antigen retrieval buffer (100 mM Tris, 5% urea, pH 9.5) for 24 h, washed three times with PBS, and permeabilized for 1 h in 0.4% Triton X-100. Afterward, the cells were incubated in a solution composed of 5% bovine serum albumin (BSA) in PBS and 0.1% Tween 20 for 1 h to block the non-specific binding of antibodies. Finally, the cells were immuno-stained with mouse anti-vimentin antibody (ab8979, 1:200) for 24 h at 4°C, followed by incubation with secondary antibody (Alexa Fluor® 488-coupled goat anti-mouse IgG, ab150113, 1:400) for 1 h at 25°C in the dark. Nuclei were counter-stained with 1.0 µg/mL Hoechst 33,342 for 1 min and observed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at $20 \times$ magnification.

Confirmation of the absence of bacterial and fungal contamination To confirm the absence of possible contamination in the cell line, cells at fourth passage were also cultured for 28 d in DMEM containing 10% FBS without an antibiotic–antimycotic solution at 38.5°C, 5% CO₂, and 95% air. The daily evaluation was performed under light microscopy to identify bacterial and fungal contamination. After this, the cells were discarded.

Passage influence on the quality of fibroblast lines Subculturing of the cells was performed up to the tenth passage (from P0 to P10). We started the evaluation on the fourth passage precisely because only fibroblasts were visualized, and immunofluorescence confirmed the cell identity. Thus, when the cells reached 70–80% of confluence, they were resuspended after trypsinization during the fourth, seventh, and tenth passages and then subjected to further analyses performed immediately after cell resuspension. These are described below.

Cryopreservation influence on cell quality After characterization, cells (at P4) were cryopreserved using a slow freezing method as previously described (Selokar et al. 2018; Praxedes et al. 2021). Briefly, 100% confluent cells were trypsinized, resuspended in DMEM to obtain a final concentration of 1.0×10^5 cells/mL, and then submitted to cryopreservation solution containing DMEM, 10% FBS, 10% dimethyl sulfoxide (Me₂SO), and 0.2 M sucrose. The cells were maintained in contact with the cryopreservation solution for 15 min at 4°C. Later, they were stored in cryotubes and transferred to the freezing container Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, NY) for 12 h, and subsequently, to -80°C freezer while maintaining a cooling rate of 1°C/min. After reaching the desired temperature, the samples were stored in liquid nitrogen (-196°C).

After two weeks, the cells were thawed for 1 min at 25°C and 4 min at 37°C, added in DMEM plus 0.2 M sucrose, and kept at 4°C for 15 min. The cell suspension was then centrifuged for 10 min at $600 \times g$, diluted in DMEM, and maintained at 25°C for up to 15 min. Subsequently, it was centrifuged again. At the end of the thawing procedure, the cells were subjected to the analyses described below.

Cell viability and metabolic activity Cell viability was evaluated using the trypan blue assay, according to the methodology described by Praxedes et al. (2021). The cells were stained with 0.4% trypan blue in PBS (1:1 ratio) and counted on a hemocytometer. Unstained cells were considered viable; non-viable cells became blue due to the cell's ruptured membrane and consequent dye penetration. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

Subsequently, the cells were analyzed for metabolic activity using the 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay, according to the method described by Borges et al. (2018). A 5.0×10^4 cells/mL concentration was grown in 12-well polystyrene plates treated for cell adhesion. After five days, 1.5 mL of the MTT solution (5 mg/mL in DMEM) was added and incubated for 3 h. The MTT solution was then removed, and 1.0 mL of Me₂SO was added for 5 min under slow stirring to solubilize the MTT. After the total dissolution of formazan crystals,

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samples were analyzed in a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) at an absorbance wavelength of 595 nm.

Proliferative activity The proliferative activity of the cells was described by growth curve modeling and by determining the population doubling time (PDT) over 168 h. Cells at 1.0×10^4 cells/mL were plated in 24-well dishes, trypsinized, counted, and recorded. Average counts at regular intervals of 24 h were used for determining the growth curve, and PDT was estimated (Mehrabani et al. 2016) according to the following equation:

$$PDT = T \ln 2 / \ln(Xe/Xb)$$

where PDT is the doubling time of the culture (in h), T is the incubation time, Xb is the number of cells at the beginning of the incubation period, and Xe is the number of cells at the end of the incubation period.

Measurements of $\Delta\Psi_m$ After having reached 70–80% confluence, the cells were exposed to 500 nM of MitoTracker Red (CMXRos) for 30 min at 5% CO₂ and 38.5°C and then analyzed under a fluorescence microscope (excitation: 579 nm and emission: 599 nm) to determine the $\Delta\Psi_m$, obtaining ten images for each type of probe at 40× magnification. Images were evaluated using Image J software (National Institute of Health, Bethesda, MD) for quantifying the fluorescence intensities of the pixels, according to Lira et al. (2022).

Apoptosis levels An aliquot of cells was resuspended in 2 µg/mL acridine orange and 10 µg/mL ethidium bromide. Subsequently, the cells were evaluated at 480 nm using a fluorescence microscope, where 300 cells were counted at 40× magnification. The cells were classified into (i) viable cells, with a uniform light green nucleus; (ii) cells in initial apoptosis, with a non-uniform green nucleus; (iii) cells in late apoptosis, with

a non-uniform bright orange nucleus; and (iv) necrotic cells, with a uniform orange nucleus (Praxedes et al. 2021). A fluorescence microscope was used to observe apoptotic changes in the stained cells, which were quantified using ImageJ software (National Institutes of Health).

Statistical analyses Data were expressed as mean ± standard error (one animal/ repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA). All results were verified for normality by the Shapiro–Wilk test and homoscedasticity by Levene’s test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at $p < 0.05$.

Results

Primary culture quality and cell morphology The total culture time reached 81 d up to the tenth passage, except for one animal that lasted 61 d of culture. The 20 skin tissue fragments (four fragments per animal) had fast adhesion in dishes, and the first cells appearance was seen on day 3. We observed that all fragments attached to the dishes detached cells and showed great proliferative capacity during in vitro culture. All the samples adhered in a couple of days (1.4 ± 0.4 d). The cells around adhered fragments reached confluence within 15.6 ± 1.2 d (Table 1), and the total primary in vitro culture time was 36.8 d. The cells had fibroblast-like morphology, a fusiform shape, and oval nuclei and could quickly replace the epithelial cells that also appeared in the first days of culture (Fig. 2a).

Karyotyping and cell type confirmation Our analysis of the six-banded armadillo karyotype was consistent with previous reports on this species; $2n = 58$ chromosomes were

Table 1. Establishment of primary culture of somatic cells derived from six-banded armadillos ear skin explants

Animals	No. samples			No. attached samples			
	Initial	Attached (%)	Day all attached explants	Day cell outgrow	Confluent* (%)	Days to reach confluence*	Total primary culture time (days)
A1	4	4/4 (100)	3	3	4/4 (100)	17	34
A2	4	4/4 (100)	1	6	4/4 (100)	17	39
A3	4	4/4 (100)	1	6	4/4 (100)	15	39
A4	4	4/4 (100)	1	3	4/4 (100)	11	38
A5	4	4/4 (100)	1	3	4/4 (100)	18	34
Mean ± S.E	4	4/4 (100)	1.4 ± 0.4	4.2 ± 0.7	4/4 (100)	15.6 ± 1.2	36.8 ± 1.2

* Confluence 70–80%. S.E.: standard error

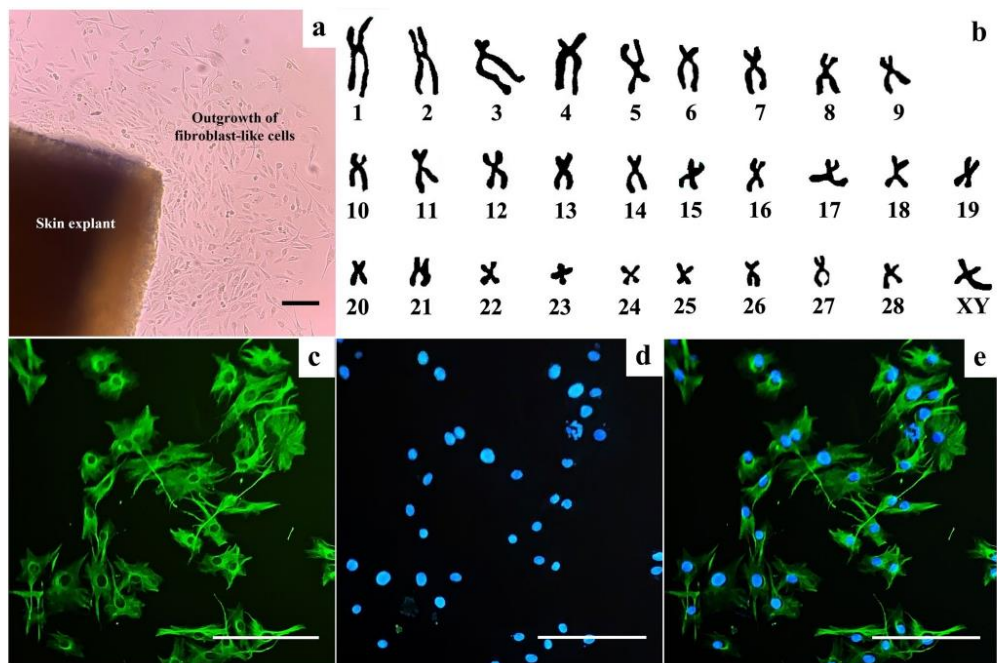


Figure 2. Primary culture of ear skin tissues of six-banded armadillos. Line identification and cell type confirmation using a primary antibody (anti-vimentin). (a) Cell detachment and morphological aspects of fibroblasts on Day 7. Scale bar 50 μ m, 10 $\times$ magnification. (b) Karyotype representation of six-banded armadillos (2n=58). (c) Cells stained with vimentin antibody (green). (d) Cells labeled with Hoechst 33,342 (blue), and (e) Image overlapping. Scale bar 50 μ m, 20 $\times$ magnification.

observed, of which 28 pair were autosomal, plus XY sex chromosomes. Moreover, the analyzed cells were of male origin, and the morphological characterization showed acrocentric, metacentric, and submetacentric stages of chromosomes (Fig. 2b). No chromosomal aberration was observed. Additionally, we confirmed the cells as fibroblasts by vimentin immunofluorescence (Fig. 2c–e). The identification was evident since the cells exhibited a high expression of vimentin that marked the cytoplasm completely (Fig. 2c), indicating the presence of intermediate filaments. The Hoechst labeling highlighted the spindle-like shape and ovoid nucleus (Fig. 2d).

Confirmation of the absence of bacterial and fungal contamination No sign of contamination (turbidity, colony, or hyphal growth) was observed for 28 d of culture in media without antibiotics and antifungals. Visually, the culture medium did not show any change in appearance, and the cells proliferated to confluence and still maintained a steady flow in a confluent condition, indicating an absence of contamination.

Influence of the passage number on the quality of fibroblast lines The prolonged in vitro culture time did not alter the fibroblast shape, with no changes observed after the tenth passage (Fig. 3a–c). The cells reached confluence in every passage; for the primary culture, they took approximately 37 d and only four days in the subsequent subcultures. Moreover, the cell viability in P4 was $90.6 \pm 3.3\%$, in P7 $95.1 \pm 2.3\%$, and in P10 $87.1 \pm 3.6\%$ (Fig. 4a), remaining high throughout passages and showing no significant difference ($p > 0.05$). Additionally, the metabolic activity was maintained in all evaluated passages, with high values of 100%, 99.2%, and 100% for the fourth, seventh, and tenth passages, respectively ($p > 0.05$, Fig. 4b). Overall, the cells took a short time to double their population. No differences were detected between the P4 (15.2 ± 2.0 h), P7 (14.3 ± 0.9 h), and P10 (21.5 ± 2.7 h) regarding their PDT value ($p > 0.05$, Fig. 4c). The cell growth curves of the groups were plotted by counting the total number of cells every 24 h for seven days. Accordingly, we observed the latency, exponential, and stationary phases (Fig. 4d). Cells in P7 and P10 showed exponential growth

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Figure 3. Morphological characteristic of six-banded armadillo fibroblast cells. Cells were evaluated during the (a) Fourth passage, (b) Seventh passage and (c) Tenth passage, (d) Before, and (e) After freezing, and nine days of in vitro culture. Scale bar 50 μ m, 10 $\times$ magnification (a–c) and 4 $\times$ magnification (d–e).

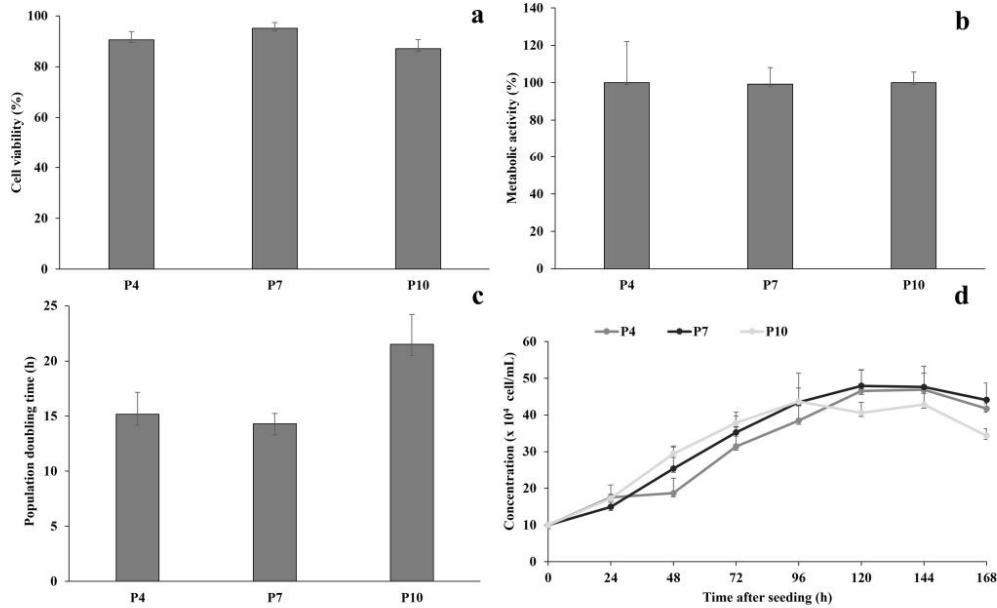
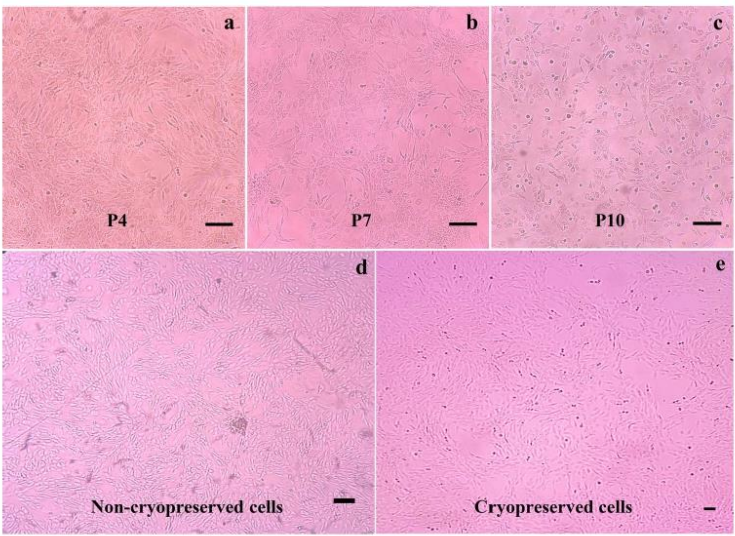


Figure 4. Survival rate, functionality, and growth dynamics of skin fibroblast cells of six-banded armadillo after prolonged in vitro culture. (a) Viability, (b) Metabolic activity, (c) Population doubling time, and (d) Growth curve. Bars represent standard error. $p > 0.05$.

in the first 24 h, which lasted until 96 h, later the cells in the fourth passage showed exponential growth after 48 h of culture up to 120 h. The stationary phase and eventual population decrease started on the sixth day of culture in all groups. Moreover, we noticed in P4 and P7 that the cells had maximum cellular growth, $47 \pm 6.4 \times 10^4$ cells/well and $48 \pm 4.3 \times 10^4$ cells/well, respectively.

Effect of cryopreservation on cell quality The cryopreservation did not change the cell morphology (Fig. 3d–e). Moreover, no difference was observed in the cell viability ($98.7 \pm 0.3\%$) when compared to the non-cryopreserved group ($90.2 \pm 5.9\%$; $p > 0.05$, Fig. 5a).

Also, no alteration of cryopreserved cells' metabolic activity was observed ($89.3 \pm 4.4\%$ vs. $100.0 \pm 12.3\%$; $p > 0.05$, Fig. 5b). Nonetheless, they took more time to double the cellular population since the PDT values were higher (16.2 ± 1.2 h) when compared to the non-cryopreserved cells (13.1 ± 1.0 h; $p < 0.05$, Fig. 5c). Moreover, we compared the growth dynamic between cryopreserved and the non-cryopreserved cells, the latter showed latency, and exponential and plateau phases. Cryopreserved cells exhibited exponential growth on the first day until the fifth day, when the population started to decrease; on the last day,

both groups diminished (plateau phase; Fig. 5d). It was also observed that maximum cellular growth was noticed in the group of cryopreserved cells ($67 \pm 5.1 \times 10^4$ cells/well vs. $49 \pm 6.2 \times 10^4$ cells/well).

Furthermore, the cryopreservation changed the $\Delta\Psi_m$ in arbitrary fluorescence units when compared to that of the non-cryopreserved cells; the value was significantly lower (0.6 ± 0.1 vs. 1.0 ± 0.3 , $p < 0.05$, Fig. 6a–c). Nevertheless, the number of viable and non-viable cells in different apoptotic stages (early apoptosis, late apoptosis, and necrosis) did not differ statistically between non- and cryopreserved cells ($p > 0.05$), and both groups presented great number of viable cells with values above 80% (Fig. 6d–f).

Discussion

In this study, we successfully isolated, characterized, and evaluated the effects of serial passages and cryopreservation processes on the quality preservation of fibroblast cells derived from the skin tissues of six-banded armadillos. The fibroblast cells maintained their viability from the fourth to tenth passages and their metabolism and growth

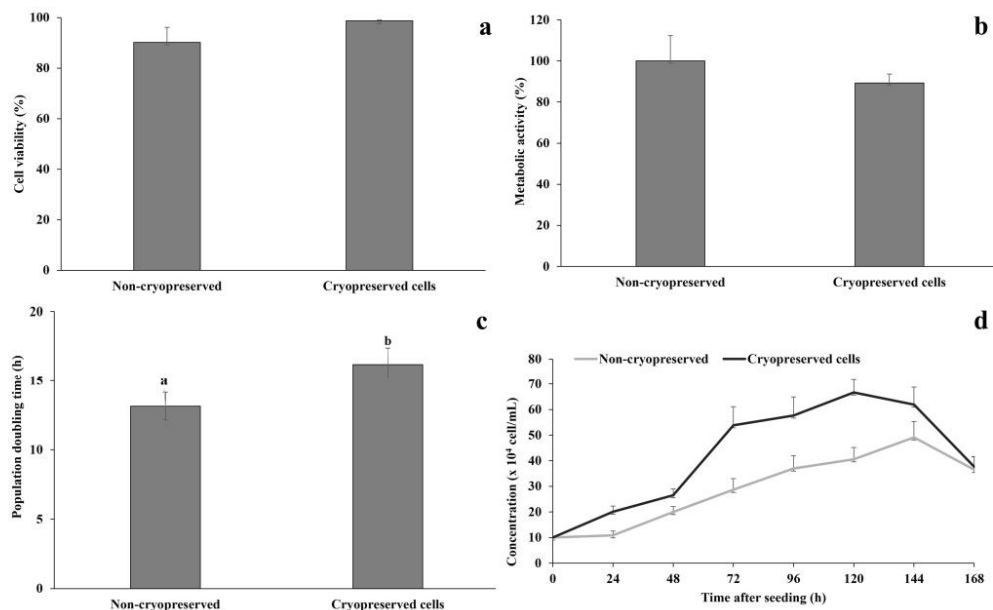


Figure 5. Survival rate, functionality, and growth dynamics of skin fibroblast cells of six-banded armadillo before and after cryopreservation and seven days of in vitro culture. (a) Viability, (b) Metabolic

activity, (c) Population doubling time, and (d) Growth curve. Bars represent standard error. ^{a,b}; $p < 0.05$.

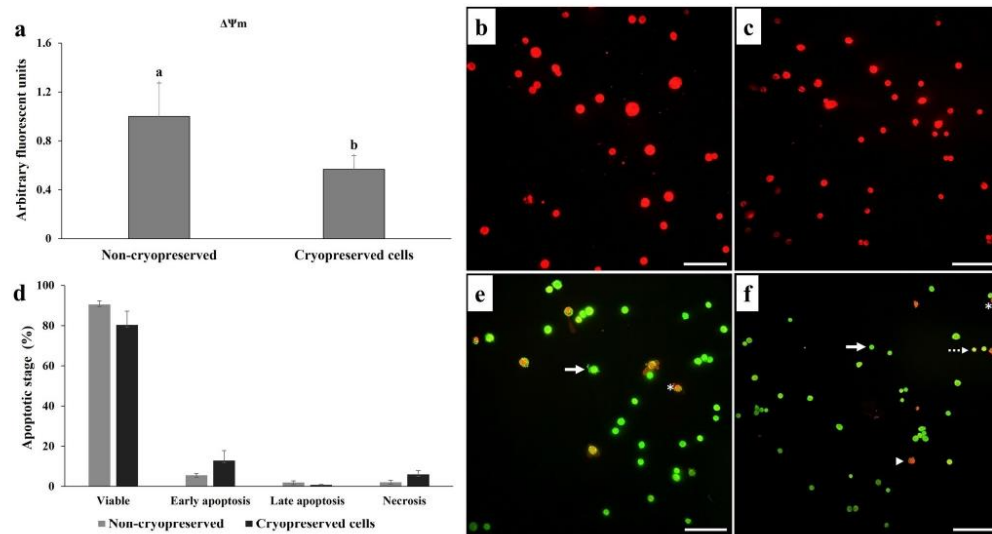


Figure 6. Influence of cryopreservation on oxidative stress and apoptosis levels in fibroblast cells of six-banded armadillos. (a) Measurement of intracellular mitochondrial membrane potential ($\Delta\Psi m$) with MitoTracker and d) Apoptosis levels. (b), (e) Before, and (c), (f) After

cryopreservation. Bars represent standard error. Viable cells (arrow); initial apoptotic cells (dotted arrow); late apoptotic cells (asterisk); necrotic cells (triangles). Scale bar 50 μm , 20 $\times$ magnification. ^{a,b}: $p < 0.05$.

patterns. They were also efficiently cryopreserved, showing few changes in their biological characteristics in vitro. Establishing the fibroblast cell line in this species is essential for future use in genetic studies, cryobanking, cloning, or another reproductive research. These results have been achieved in many wild mammals aiming to preserve materials from valuable species, including species that occur in the worldwide biomes, such as collared peccaries (*P. tajacu*, Borges et al. 2020), red-rumped agouti (*D. leporina*, Praxedes et al. 2021), jaguar (*Panthera onca*, Oliveira et al. 2021), and puma (*P. concolor*, Rodrigues et al. 2023). Studies in armadillo fibroblast culture are very scarce, and there are no reports detailing establishing this cell line from six-banded armadillo species.

The six-banded armadillo ear fragments were thick, and the tissue was separated in two (front and back) to isolate fibroblasts for culture. The adherent method for primary cell culture was preferable in this study because it is simpler and avoids tissue damage compared to other methods, such as enzyme digestion (Sharma et al. 2018). In the first's days of primary culture, the medium was carefully replaced to prevent the detachment of the explants. However, some fragments still detached after manipulation, which delayed their firm attachment. Therefore, some samples took more days to liberate cells than others. Overall, the skin explants had early and great adherence (1.4 ± 0.4 d), and the cells

of the samples quickly grew (4.2 ± 0.7 d). Similar results were observed in other wild animals, such as in collared peccary ear skin culture, where tissues adhered in 1.3 ± 0.5 d and cell growth was seen at day 6.6 ± 1.6 (Borges et al. 2017). Siengdee et al. (2018) also reported the early outgrowth around explants in skin fragments from the Asian elephant (*Elephas maximus*; 4–12 d). The Asian elephant was proved to have a closer phylogenetic relationship with the nine-banded armadillo (*Dasypus novemcinctus*; Sree-kumar et al. 2007). Therefore, the protocol and results reported by Siengdee et al. (2018) were a meaningful basis for comparison.

Moreover, in our study, the cells around explants took two weeks to reach confluence, but it is worth mentioning that some fragments reached confluence faster than others in the same dish. This observation indicates that the cells had a fast proliferation rate in the primary culture. In accordance, the primary fibroblast cells from the killer whale (*Orcinus orca*) skin tissue culture appeared in 4–7 d after biopsy, and cell confluence (90–95%) around explants was observed after 13–14 d (Bjørneset et al. 2023).

Overall, the total primary culture time was shorter than in other studies with similar conditions (Borges et al. 2020; Praxedes et al. 2021), indicating that the cells were grown in good culture conditions. Initially, epithelial and fibroblast cells grew together during explant culture. However, over

time, fibroblast cells overgrew the epithelial cells since fibroblasts grow faster, have greater adherence, require less nutritional support in vitro environment, and respond fast to trypsinization (Selokar et al. 2018; Siengdee et al. 2018; Wang et al. 2020; Praxedes et al. 2021). After the first passage, the fibroblast cells grew rapidly; within four days, they reached 80% confluence, and subsequent passages occurred.

Morphologically, it was easy to identify the fibroblast cells, especially with low confluence. At the P4, when the cells were characterized, fibroblast monolayer was predominant in culture dishes, and the purity of these cells was then confirmed by vimentin staining. The anti-vimentin antibody binds to intermediate filaments, a typical structure among fibroblasts, proving that the cells have a mesenchymal origin.

When establishing cell lines, it is essential to confirm the normal genetic makeup of the animal by karyotyping, especially while conserving animal germplasm (Sharma et al. 2018). The cells under in vitro conditions should maintain the same diploid character as those in vivo. Our results supported that the chromosome karyotyping showed normal diploid ($2n=58$) numbers, validating the stability of these cells (Liu et al. 2010). Furthermore, it is worth mentioning that we could not find many cells containing separate chromosomes, which would allow better visualization of individual chromosomes under a light microscope. This result was also observed in Chinese muntjac (*Muntiacus reevesi*) fibroblasts karyotype analysis (Wang et al. 2020) when they collected the fibroblast cells after 80 h culture; however, the cell culture collected after 68 h of seeding, when they were highly proliferative, presented separated chromosomes for the analysis. The authors pointed that is important to evaluate when to collect the cells for the karyotyping to find more cells containing separated chromosomes at metaphase of mitosis. Nevertheless, even so that the chromosomes were paired in our result, they were well condensed, visible and in correct number, allowing us to conclude that the genetic properties of six-banded armadillo were maintained.

Bacterial and fungal contaminations are detected easily with the naked eye (Sharma et al. 2018). Our study confirmed the absence of bacterial or fungal contamination by examining the cell line in culture medium without antibiotics and antifungals under daily observation with light microscope. Fortunately, the culture medium remained clear during the 28-d assay, and no other alterations (like moving granules or fungal structures) were noted, suggesting that the cells were free from microbial contaminations. However, despite best efforts, microbial contaminations are quite frequent during mammalian cell culture (Malik et al. 2023). Therefore, antibiotics and antimycotics are still recommended, and combined with good laboratory working practices, will ensure the absence of contamination (Borges et al. 2020).

Considering the prolonged culture time evaluation, adequate results were obtained, and the cells showed no changes in their morphology, viability, metabolism, and proliferative activity parameters. These findings are consistent with other studies that evaluated longer culture time up to the fourth passage for wild species, supporting the idea that the fibroblast cell line was stable. We imaged P4, P7, and P10 fibroblasts to monitor possible morphology changes due to serial passaging, but no significant difference was observed. Liu et al. (2022) reported that giant panda (*Ailuropoda melanoleuca*) fibroblasts changed their morphology (began to enlarge) and had their proliferation rate decrease dramatically from P9. Magalhães et al. (2017) also evaluated the quality of brown brocket deer (*Mazama gouazoubira*) fibroblasts after P4, P7, and P10; however, they noticed a significant decrease in cell survival rate at P10, compared with earlier passages. Here, the cell viability remained high (> 87%) in earlier and later passages. The percentage of viable cells at P10 was higher when compared with fibroblasts cultured from collared peccary (84%; Borges et al. 2020) and Chinese muntjac (85%; Wang et al. 2020), considering the same passage.

The extended culture also did not affect the cell's metabolism or proliferative activity. To evaluate the PDT, we manually counted the cell suspension for seven days daily and plotted a cell growth curve with a Napierian logarithm. The growth was similar between the serial passages, and the growth curve was noted as parallel. As shown in Wang et al. (2020) report, the cells proliferated rapidly after a short latency phase and entered the exponential growth phase. In this study, the cells at P4 had an initial lag phase of 48 h for adaptation, followed by an exponential phase between 48–120 h. As expected, cell proliferation started to slow and eventually decreased their population at six days because of contact inhibition; the same was observed in P7 and P10 groups and camel's fibroblasts at P5 (Sharma et al. 2018). The established cell line revealed PDT of 15.2 h in P4, 14.3 h in P7, and 21.5 h in P10. Similar estimates of PDT have been obtained from several studies (21.3 h at P3: Wang et al. 2020; 26.1 h at P5: Sharma et al. 2018; 25.0 h at P6: Siengdee et al. 2018; 22.4 h at P10: Jenuit et al. 2021).

On the other hand, it has been documented that wild species present a higher PDT value than domestic animals, as observed in giant pandas (33.8 h: Yu et al. 2015) and brown brocket deer (90.0 h: Magalhães et al. 2017). The latter showed that the fibroblast cells had different metabolism and proliferative activity between passages, the PDT of cells from P10 was higher, and the cell proliferation rate was lower than that from P4 and P7. In addition, the cells at P10 exhibited lower metabolic activity compared with P4 cells (Magalhães et al. 2017). Jenuit et al. (2021) also evaluated primary fibroblast cells from Sumatran rhinoceros (*Dicerorhinus sumatrensis*) throughout P5, P10, and P15, reporting that cell senescence

level increased significantly as the passage number increased. They also observed significant changes in morphology, growth, and viability with an increasing number of passages. No significant changes in our cell line throughout passages suggests that our experimental protocol was highly efficacious, providing six-banded armadillo fibroblast cells with excellent number and quality.

The cryopreservation of fibroblast cells is usually performed in early passages. This is recommended to obtain cells with conserved genetic characteristics (Borges et al. 2020). In this study, we chose the fourth passage, in which the cells were characterized, and the purity of the fibroblast cell line was confirmed. Regarding survival rate, the cryopreserved cells showed excellent viability (98.7%) after being thawed and cultured, not differing from the non-cryopreserved cells, as observed in Asian elephant (Siengdee et al. 2018) and Sumatran rhinoceros (Jenuit et al. 2021).

Meanwhile, the growth of non- and cryopreserved cells was different; according to the growth curve, the comparison showed a higher proliferation rate in cryopreserved cells, but the PDT value was higher compared to non-cryopreserved cells (16.2 h vs. 13.1 h). This delay might have been due to the freezing process, but it did not induce cell growth reduction. Furthermore, the after-effects of cryopreservation might cause mitochondrial structural abnormalities (Borges et al. 2020). Therefore, $\Delta\Psi_m$ is a global indicator of mitochondrial function. Here, we observed an effective decrease in $\Delta\Psi_m$ after cryopreservation, a sign of cellular stress that eventually leads to apoptosis.

In contrast, apoptosis levels and their metabolic rate remained constant after cryopreservation. Overall, our fibroblast cell line showed high tolerance to cryopreservation since most of the quality parameters were unchanged. This implied that the slow freezing technique and a cryopreservation medium composed of 80% DMEM, 10% FBS, 10% Me_2SO , and 0.2 M sucrose were suitable for conserving fibroblast cells of six-banded armadillo. The addition of both intra- (Me_2SO) and extracellular cryoprotectants (FBS and sucrose) protected the cells from significant damage associated with the freezing process. This result agrees with other studies (Praxedes et al. 2021; Lira et al. 2022).

Conclusions

In conclusion, we demonstrated that the isolation, characterization and cryopreservation of stable fibroblast cell lines derived from six-banded armadillos is possible. Moreover, the cell viability was maintained until the tenth passage and after cryopreservation. The cell lines were of high quality and confirmed to have no contamination, and the protocol provided useful information to extend their storage efficiently. Additionally, establishing the fibroblast cell lines

from this species offers manifold possibilities, preserving the genetic diversity of six-banded armadillo and providing excellent biological resources for future research studies, such as cloning and other somatic cell reprogramming technologies.

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Data availability Data may be made available upon reasonable request to the corresponding author.

Declarations

Conflict of interests The authors declare no conflict of interests.

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ANEXO F – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 2**CAPÍTULO 2 – *EX SITU* APPROACHES FOR THE CONSERVATION OF GENETIC
RESOURCES IN THE SUPERORDER XENARTHRA**



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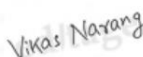
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ANEXO G – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 3

**CAPÍTULO 3 – INFLUENCE OF CRYOPRESERVATION TECHNIQUES AND LOW
CONCENTRATIONS OF PERMEATING CRYOPROTECTANTS ON THE
CONSERVATION OF EAR CARTILAGE AND SKIN DERIVED FROM SIX-
BANDED ARMADILLOS (*Euphractus sexcinctus* LINNAEUS, 1758)**

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ANEXO H – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 4**CAPÍTULO 4 – LONG-TERM PRESERVATION OF ESTABLISHED FIBROBLAST
LINES FROM SIX-BANDED ARMADILLOS (*Euphractus sexcintus*, LINNAEUS,
1758) BY EXTENDED PASSAGE AND CRYOPRESERVATION**

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ANEXO I – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 5

**CAPÍTULO 5 – NON-PERMEABLE CRYOPROTECTANTS' INFLUENCE ON
FIBROBLAST SLOW FREEZING IN SIX-BANDED ARMADILLOS (*Euphractus
sexcinctus* LINNAEUS, 1758)**

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Authors: Denilsa Pires Fernandes, Érika Almeida Praxedes, João Vitor da Silva Viana, Maria Valéria de Oliveira Santos, Carlos Iberê Alves Freitas, Alessandra Fernandes Pereira

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ANEXO J – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 6**CAPÍTULO 6 – TIME-DEPENDENT MODULATION OF FIBROBLAST CYCLE
ARREST IN G₀/G₁ PHASE BY DIFFERENT SYNCHRONIZATION METHODS IN
SIX-BANDED ARMADILLOS**

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**ANEXO K – RESUMO PUBLICADO NO ISABR
VIII INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF
REPRODUCTION 2021**

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Evaluation of extended *in vitro* culture of somatic cells from six-banded armadillos (*Euphractus sexcinctus* Linnaeus, 1758) as a step towards for cloning by nuclear transfer

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The six-banded armadillo is a wild mammal with an important ecological role because it is an intermediary link in food networks, being responsible for the balance of termite and ant colonies, as well as for the cycling of nutrients. Although its populations are considered stable, conservation strategies need to be employed, aiming at the conservation of biodiversity. An interesting conservation strategy would be one aimed at the development of cryobanks. In general, the characterization of the cells during prolonged culture represents an important step in the formation of these banks. Therefore, we aimed to evaluate the effects of culture time (fourth, seventh, and tenth passages) on the functional parameters of somatic cells derived from six-banded armadillos. All procedures were approved by the ethics committee of UFERSA (no. 36/2020) and Institute Chico Mendes for Conservation of Biodiversity (no. 76655-1). Then, ear skin samples were obtained from five adult six-banded armadillos and were transported in minimal essential medium modified by Dulbecco (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin and streptomycin solution at 37 °C. In the laboratory, fragments (9.0 mm³) were cultured under controlled atmosphere (38.5 °C, 5% CO₂) and evaluated every 24 h. The cells were harvested when they reached 70% confluency, subcultured until the fourth, seventh, and tenth passages for the analysis. All cells were evaluated for viability using trypan blue assay, metabolic activity using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay, and proliferative activity by determining the population doubling time (PDT) over 168 h. Data were expressed as mean ± standard error (one animal/ one repetition) and analyzed using StatView 5.0 software. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by Tukey test ($P < 0.05$). The viability remained high throughout the three different passages evaluated, with values above 85% (fourth: 90.6% ± 3.3 vs. seventh: 95.1% ± 2.3 vs. tenth: 87.1% ± 3.6). Additionally, high metabolic activity was maintained throughout the different passages (fourth: 100.0% ± 21.9 vs. seventh: 99.2% ± 8.9 vs. tenth: 100.0% ± 5.7). No difference was observed among the passages for proliferative activity (fourth: 15.2 h ± 2.0 vs. seventh: 14.3 h ± 0.9 vs. tenth: 21.5 h ± 2.7). In summary, we were able to isolate somatic cells derived from six-banded armadillo skin, associated with high viability rates, and high metabolic activity after extended *in vitro* culture. These results provide security for the application of these cells in reproductive biotechnologies in the species.

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**ANEXO L – RESUMO EXPANDIDO PUBLICADO NO CONERA
XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL 2022**

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EFEITO DA CRIOPRESERVAÇÃO DE CÉLULAS SOMÁTICAS DE TATU-PEBA UTILIZANDO CRIOPROTETORES INTRACELULARES E EXTRACELULARES

Effect of cryopreservation of somatic cells derived from six-banded armadillos using intracellular and extracellular cryoprotectants

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RESUMO

Euphractus sexcinctus é uma das espécies de tatus encontradas no Brasil com grande importância ecológica e econômica. Contudo, a espécie sofre redução populacional em virtude de atividades antrópicas. Metodologias voltadas para sua conservação são importantes e podem ser implementadas, como a criação de criobancos, desenvolvendo um protocolo de criopreservação eficaz. Assim, o estudo objetivou criopreservar células somáticas de tatu-peba utilizando uma combinação de 10% de dimetilsulfóxido e 0,2 M de sacarose. Para tanto, cinco animais foram anestesiados e submetidos a biópsias de pele para obtenção de fragmentos, os quais foram cultivados *in vitro* e as células recuperadas. Na quarta passagem, as células foram criopreservadas e analisadas antes e após a criopreservação quanto à viabilidade com corante azul de tripan, atividade metabólica pelo ensaio de MTT, tempo de duplicação populacional e níveis apoptóticos. Os resultados demonstraram a eficiência da solução crioprotetora e, consequentemente, do protocolo de criopreservação, onde se obteve uma viabilidade superior a 90%, alta atividade metabólica (>80%) e índice de células necróticas inferior a 6%. Portanto, este protocolo promove a conservação da qualidade de células somáticas de tatu-peba.

Palavras-chave: Xenartra, congelamento lento, recursos somáticos.

ABSTRACT

Euphractus sexcinctus is one of the armadillos species in Brazil with great ecological and economic importance. Nevertheless, the suffering species reduced due to anthropic activities. Methodologies aimed at their conservation are important and can be implemented, such as the creation of cryobanks, developing an effective cryopreservation protocol. Therefore, the study aimed to cryopreserve somatic cells of six-banded armadillos using a combination of 10% dimethyl sulfoxide and 0.2 M sucrose. Then, five animals were anesthetized and submitted to skin biopsies to obtain fragments, which were cultured *in vitro*, and the cells recovered. In the fourth passage, cells were cryopreserved and analyzed before and after cryopreservation for viability with trypan blue dye, metabolic activity by MTT assay, population doubling time and apoptotic levels. The results demonstrated the efficiency of the cryoprotectant solution and, consequently, of the cryopreservation protocol, which obtained a viability greater than 90%, high metabolic activity (>80%) and a necrotic cell index below 6%. Therefore, this protocol promotes the conservation of the quality of six-banded armadillo somatic cells.

Keywords: Xenarthra, slow freezing, somatic resources.

INTRODUÇÃO

O *Euphractus sexcinctus* é uma das espécies silvestres que ocorrem nos biomas brasileiros. Popularmente, a espécie é conhecida por tatu-peba e integra a superordem Xenarthra. Esta espécie desempenha um papel importante na dispersão de sementes, bem como tem sido empregado como um modelo experimental. Associada à sua importância, e em virtude da sua diminuição populacional, é relevante o desenvolvimento de métodos de conservação, como a formação de criobancos, visando seu uso em clonagem reprodutiva e indução de células à pluripotência.

Em geral, uma variabilidade na eficiência da criopreservação tem sido observada e justificada pelas características espécie-específicas, além dos possíveis danos decorrentes das baixas temperaturas que as amostras são submetidas (BORGES et al., 2020), sendo a escolha dos crioprotetores uma etapa essencial para a garantia da eficiência do processo. Dentre os protocolos utilizados em diferentes espécies, aqueles empregando a combinação de crioprotetores intra e extracelulares têm-se destacado em virtude da otimização da concentração dos crioprotetores e manutenção da viabilidade celular (MURRAY; GIBSON, 2022).

Portanto, o objetivo foi avaliar os efeitos da combinação de crioprotetor intracelular e extracelular na criopreservação de células somáticas de *E. sexcinctus* sobre a viabilidade, metabolismo, tempo de duplicação e níveis apoptóticos.

MATERIAL E MÉTODOS

Um total de cinco animais foram anestesiados e amostras da pele auricular foram recuperadas (CEUA/UFERSA, no. 36/2020 e ICMBio, no. 76655-1). No laboratório, após a fragmentação (9,0 mm³) das amostras, fragmentos foram cultivados em meio essencial mínimo modificado por Dulbecco (DMEM) acrescido de 10% de soro fetal bovino (SFB) e 2% de solução de antibióticos-antimicóticos, à 38,5°C e 5% de CO₂. Posteriormente, células na quarta passagem foram criopreservadas por congelamento lento, na presença da solução crioprotetora composta por 10% de dimetilsulfóxido (DMSO), 10% de SFB e 0,2 M de sacarose (SAC). Após duas semanas, amostras foram descongeladas, cultivadas *in vitro* e após atingirem 70–80% de confluência foram submetidas às análises de viabilidade, metabolismo, tempo de duplicação e níveis de apoptose.

Para a análise da viabilidade celular, células foram expostas ao corante azul de tripan a 0,04%, onde foi avaliada a integridade da membrana plasmática por meio da penetração do corante, onde células viáveis apresentaram coloração translúcida e células inviáveis, coloração

azul. Para avaliação do metabolismo celular, foi realizado o ensaio de brometo de 3-4,5-dimetiltiazol-2-il-2,5-difeniltetrazólio (MTT), onde as amostras foram incubadas com a solução de MTT (5 mg/mL em PBS) por 3 h a 38,5°C, posteriormente, foi acrescido DMSO, como solução de solubilização do MTT, e a leitura foi realizada a 595 nm. Para determinação do tempo de duplicação celular, células foram cultivadas em placas de 24 poços e realizada a contagem a cada 24 h por 7 dias, sendo o tempo de duplicação determinado por meio da fórmula $PDT = T \ln 2 / \ln (X_f/X_i)$. Para a avaliação dos níveis apoptóticos, células foram submetidas aos marcadores laranja de acridina e brometo de etídio e avaliadas sob microscópio de fluorescência, onde as células consideradas viáveis apresentaram núcleo verde claro uniforme, células em apoptose precoce ou inicial apresentaram núcleo verde não uniforme, células em apoptose tardia apresentaram áreas alaranjadas brilhantes e as células necróticas apresentaram núcleo laranja uniforme.

Finalmente, os dados foram expressos como média $\pm$ erro padrão e analisados quanto à normalidade e homocedasticidade pelo teste de Shapiro-Wilk e Levene's, respectivamente. Os dados não mostraram distribuição normal e foram transformados em arco-seno e submetidos a ANOVA seguido pelo teste de Tukey ($P < 0,05$).

RESULTADOS E DISCUSSÃO

Não foi observada diferença quanto à viabilidade das células entre os grupos criopreservados e não criopreservados, tendo $90,2\% \pm 5,9$ e $98,7\% \pm 0,3$, respectivamente (Fig. 1A).

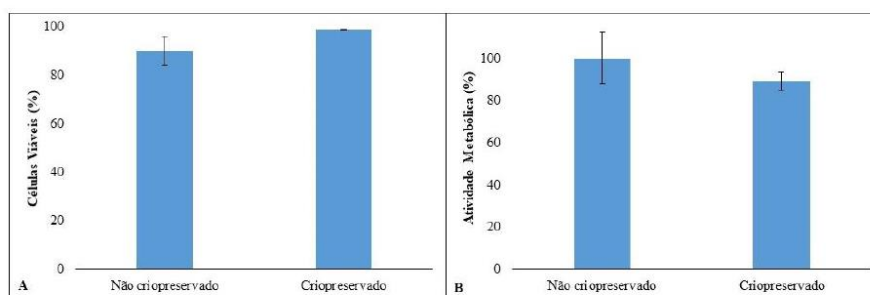


Fig. 1: Viabilidade e atividade metabólica de células de *E. sexcinctus* antes e após a criopreservação. (A) Viabilidade celular. (B) Atividade metabólica. $P > 0,05$.

Do mesmo modo, os grupos não apresentaram divergências quanto ao metabolismo celular, onde foi mantida atividade metabólica acima de 80% no grupo de células

criopreservadas ($89,3\% \pm 4,4$; Fig. 1B). Por outro lado, houve um aumento no tempo de duplicação após o processo de criopreservação ($16,2 \text{ h} \pm 1,2$; Fig. 2A) quando comparada às células não criopreservadas ($13,1 \text{ h} \pm 1,0$). A atividade proliferativa de ambos os grupos também pode ser observada a partir da curva de crescimento na Fig. 2B, onde foram observadas todas as fases do ciclo celular: adaptação, crescimento, estacionária e declínio celular.

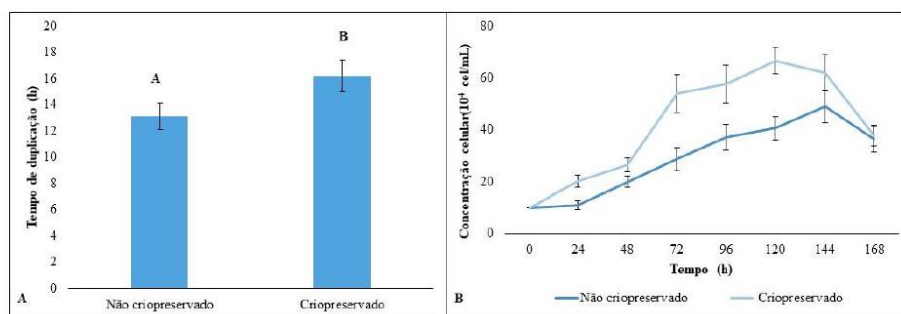


Fig. 2: Determinação do tempo de duplicação e curva de crescimento de células de *E. sexcinctus* antes e após a criopreservação. ^{A,B}: $P < 0,05$.

Quanto aos níveis apoptóticos (Fig. 3), ambos os grupos criopreservados e não criopreservados apresentaram alta porcentagem de células viáveis com valores acima de 80%, sendo observadas poucas células em apoptose, tanto inicial quanto tardia e, consequentemente, um reduzido percentual de células em necrose. Esse resultado demonstra a eficiência da técnica na manutenção da viabilidade dessas células.

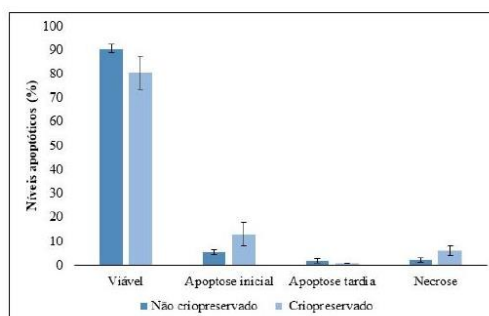


Fig. 3: Níveis apoptóticos de células de *E. sexcinctus* antes e após a criopreservação. $P > 0,05$.

Tais resultados corroboram com estudos anteriores em outras espécies [*Dasyprocta leporina*: PRAXEDES et al. (2021); *Panthera onca*: OLIVEIRA et al. (2021)] que demonstraram o efeito positivo da utilização da combinação de crioprotetores intra e

extracelulares, promovendo a manutenção da viabilidade e metabolismo celular. Além disso, a diminuição gradual da temperatura e desidratação podem afetar as células de diferentes formas, uma delas é o aumento das espécies reativas de oxigênio, tais radicais livres afetam às células negativamente (LEN et al., 2019) podendo aumentar seu tempo de duplicação da população, como foi observado no grupo das células criopreservadas.

CONCLUSÃO

O protocolo de criopreservação para células somáticas de *E. sexcinctus* permitiu a manutenção da sua viabilidade, atividade metabólica e uma baixa porcentagem de células necróticas, possibilitando que as células sejam armazenadas por longos períodos e viabilizando seu uso em biotecnologias para a espécie, tais como a clonagem por transferência nuclear.

AGRADECIMENTOS

Este estudo foi financiado pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Código Financeiro 001) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, no. 309078/2021-0).

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**ANEXO M – RESUMO PUBLICADO NO ZOOTECH
32° CONGRESSO BRASILEIRO DE ZOOTECHNIA 2023**

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AVALIAÇÃO DA ROSCOVITINA NA SINCRONIZAÇÃO DO CICLO EM G₀/G₁ DE FIBROBLASTOS DE TATU-PEBA

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O tatu-peba (*Euphractus sexcinctus*) é uma espécie exclusivamente das Américas, com importante papel ecológico e grande interesse genético. Contudo, a espécie é alvo direto da interferência humana em seu habitat. Por esta razão, o desenvolvimento da clonagem por transferência nuclear de célula somática torna-se uma ferramenta interessante para estudos na espécie. Para o sucesso da técnica, os carioplastos (fibroblastos doadores de núcleo) devem ser sincronizados na fase adequada do ciclo, permitindo a recuperação de células viáveis. Com esta finalidade, a roscovitina tem sido utilizada como inibidor químico do ciclo celular com resultados variáveis. Portanto, o objetivo foi avaliar o efeito da adição de 15 µM de roscovitina na sincronização do ciclo em G₀/G₁ em células de tatu-peba. Para tanto, fragmentos da região auricular de cinco tatus-pebas foram recuperados e submetidos ao cultivo *in vitro* em meio essencial mínimo modificado por Dulbecco (DMEM) suplementado com 10% de soro fetal bovino (SFB) e 2% de antibiótico-antimicótico a 38,5°C e 6,5% de CO₂. Posteriormente, fibroblastos foram destinados ao grupo controle (não submetido à sincronização), e grupos tratados com roscovitina por 12 h (RSV12) e 24 h (RSV24). Para análise da viabilidade, os grupos foram expostos ao corante azul de tripan e a determinação da fase do ciclo celular realizada por citometria de fluxo. Para cada amostra, 15.000 eventos foram registrados, e histogramas gerados para avaliar o percentual de células em cada fase do ciclo celular (G₀/G₁, S, G₂/M) usando o software MODFIT versão 5.0. Os dados foram expressos como média ± erro padrão e analisados por ANOVA seguido pelo teste de Tukey. Quanto à viabilidade celular, todos os grupos obtiveram valores similares e superiores a 85% de viabilidade (P > 0,05). Na análise do ciclo celular, o grupo controle obteve 60,6% ± 0,7 de células em G₀/G₁, sem apresentar diferença entre os tratamentos RSV12 (57,4% ± 1,4) e RSV24 (57,8% ± 2,4). Ainda, o grupo RSV24 teve uma menor porcentagem de células em G₂/M (3,5% ± 1,0) em comparação ao grupo controle (7,0% ± 0,5). Portanto, apesar da roscovitina não ter alterado a viabilidade dos fibroblastos, não foi constatada a sincronização do ciclo das mesmas nas condições testadas. Mais estudos são necessários para uma análise dos possíveis efeitos tóxicos do inibidor nas células de tatu-peba, visando alcançar as melhores condições para a clonagem reprodutiva na espécie.

Palavras-chave: Xenarthra, cultivo *in vitro*, transferência nuclear de célula somática.

Agradecimento: CNPq e CAPES.

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ANEXO N – RESUMO PUBLICADO NO CBRA
27º CONGRESSO BRASILEIRO DE REPRODUÇÃO ASSISTIDA 2023

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Evaluation of the serum starvation method on the cycle synchronization in G₀/G₁ phase of skin fibroblasts from six-banded armadillos

Avaliação do método de privação de soro na sincronização do ciclo em G₀/G₁ de fibroblastos oriundos da pele de tatus-pebas

João Vitor da Silva Viana^{1*}, Denilsa Pires Fernandes¹, Luanna Lorena Vieira Rodrigues¹, Lhara Ricarliany Medeiros de Oliveira¹, Carlos Iberê Alves Freitas², Sarah Leyenne Alves Sales³, Claudia Pessoa³, Alexsandra Fernandes Pereira¹

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Cloning by somatic cell nuclear transfer (SCNT) is an interesting and promising reproductive biotechnology for multiplication and conservation of wild species with great ecological and scientific value, such as six-banded armadillos. For the establishment of the technique, the somatic cells must be correctly synchronized in G₀/G₁. For this, the serum starvation had been highlighted due the efficiency; nevertheless, the results vary as does the treatment time and the impact on cell viability. Therefore, we aimed to evaluate the serum starvation method on cycle synchronization in G₀/G₁ phase of skin fibroblasts from six-banded armadillos. Initially, skin biopsies on the ear region were performed on five six-banded armadillos and the samples were cultured on minimal essential medium modified by Dulbecco (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin and streptomycin solution at 38.5 °C and 6.5% CO₂. On fourth passage and after 60–70% confluence, the isolated fibroblasts were randomly divided into groups: (i) control group (non-synchronized cells), (ii) serum starvation (0.5%) for 24 h (SS24), and (iii) serum starvation (0.5%) for 72 h (SS72). Following, the cells were submitted to trypan blue viability assay and the phase cell cycle were determined by flow cytometry. For each sample, 15,000 events were recorded, and histograms generated to evaluate the percentage of cells in each phase of the cell cycle (G₀/G₁, S, G₂/M) using MODFIT software version 5.0. Data were expressed as mean ± standard error, analyzed by ANOVA followed by Tukey test (P < 0.05). The results indicate that the cell viability did not differ among treatment by 24 h (91.2% ± 6.1) and 72 h (72.2% ± 14.7), when compared to control (96.3% ± 2.9). Interestingly, we observed a great increase in synchronized cells in G₀/G₁ after the treatment with SS, in which SS72 group showed the highest percentage of synchronized cells (90.5% ± 0.8), the SS24 group showed 86.1% ± 0.9, while the control group had 69.7% ± 0.8. Moreover, SS24 and SS72 groups decreased the number of cells in S phase of 9.5% ± 1.2 and 8.4% ± 0.7, respectively, in comparison with control (27.7% ± 0.8). No alterations were observed in the percentage of G₂/M phase between treated and non-treated groups. In conclusion, the SS method was successful in the six-banded armadillos fibroblast synchronization, and the serum starvation for 72 h were more suitable due to the highest proportion of synchronized cells in G₀/G₁ and no alterations in cell viability. These results can benefit in preparing donor karyoplasts for SCNT in this species.

Keywords: karyoplasts; somatic cell nuclear transfer; flow cytometry.

Palavras-chave: carioplastos; transferência nuclear de células somáticas; citometria de fluxo.

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**ANEXO O – RESUMO PUBLICADO NO SEMIC
XXIX SEMANA DE INICIAÇÃO CIENTÍFICA DA UNIVERSIDADE FEDERAL
RURAL DO SEMI-ÁRIDO 2023**

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Área temática: Ciências Agrárias

Avaliação da vitrificação em superfície sólida como ferramenta de conservação de tecidos somáticos de tatu-peba, *Euphractus sexcinctus* Linnaeus, 1758 (Cingulata: Clamiforídeos)

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A formação de bancos de recursos genéticos usando a criopreservação de amostras biológicas consiste em uma ferramenta fundamental para a conservação da biodiversidade de espécies silvestres, tais como o tatu-peba. Para tanto, a determinação de um protocolo eficaz torna-se uma etapa essencial para a formação destes bancos. Portanto, o objetivo foi avaliar os efeitos da vitrificação em superfície sólida (VSS) como técnica de criopreservação de tecidos somáticos de tatu-peba sobre a morfologia, viabilidade, atividade metabólica e níveis apoptóticos das células obtidas após cultivo *in vitro*. Todos os procedimentos foram aprovados pelo Comitê de Ética e Uso de Animais (CEUA/UFRSA, no. 36/2020), bem como pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, no. 76655-1). Biópsias da pele auricular foram recuperadas de cinco indivíduos adultos oriundos do Laboratório de Estudos em Imunologia e Animais Silvestres (LEIAS/UFRSA). No laboratório, amostras foram fragmentadas (6,0 mm³) e divididas nos grupos: tecidos não submetidos à criopreservação (grupo controle) e submetidos à VSS (grupo criopreservado). Para a VSS, tecidos foram vitrificados usando solução constituída por meio essencial mínimo modificado por Dulbecco (DMEM), 1,5 M de etilenoglicol, 1,5 M de dimetilsulfóxido, 10% de soro fetal bovino (SFB) e 0,25 M de sacarose. Tecidos não criopreservados e criopreservados foram cultivados *in vitro* (38,5 °C; 6,5% CO₂) em DMEM acrescido de 10% de SFB e 2% de solução de antibiótico-antimicótico. Células oriundas destes tecidos foram avaliadas quanto à morfologia usando microscópio invertido, viabilidade usando azul de tripan, atividade metabólica pelo ensaio de brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio, e níveis apoptóticos usando sondas fluorescentes. Todos os resultados foram expressos como média $\pm$ erro padrão e analisados por ANOVA e teste de Tukey ($P < 0,05$). Nenhuma diferença foi observada quanto aos aspectos morfológicos, tendo todas as células apresentado aspecto fusiforme e prolongamento citoplasmáticos semelhante a fibroblastos. Além disso, nenhuma diferença foi observada quanto à viabilidade (controle: 90,2% $\pm$ 5,9 e VSS: 89,7% $\pm$ 9,1) e atividade metabólica (controle: 90,3% $\pm$ 4,9 e VSS: 89,7% $\pm$ 9,1) das células provenientes de tecidos criopreservados e não criopreservados ($P > 0,05$). Quanto à análise da viabilidade celular pelo ensaio de apoptose, nenhuma diferença foi observada entre os grupos, com taxas de células apoptóticas $< 24\%$. Em conclusão, a vitrificação em superfície sólida é um protocolo de criopreservação eficiente para a conservação de tecidos somáticos de tatu-peba, não comprometendo a morfologia e funcionalidade das células recuperadas após cultivo *in vitro*.

Palavras-chave: Mamífero silvestre, Criopreservação, Cultivo *in vitro*, Célula somática.

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**ANEXO P – RESUMO PUBLICADO NO SIMPA
I SIMPÓSIO POTIGUAR DE CIÊNCIAS ANIMAIS 2023**

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**EFEITO DOS CRIOPROTETORES SOBRE A CONSERVAÇÃO DE CÉLULAS
SOMÁTICAS DE TATU-PEBA, *Euphractus sexcinctus* (LINNAEUS, 1758)**

*EFFECT OF CRYOPROTECTANTS ON THE CONSERVATION OF SOMATIC CELLS OF
SIX-BANDED ARMADILLO, *Euphractus sexcinctus* (LINNAEUS, 1758)*

Morfofisiologia e Biotecnologia Animal

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Objetivos: Avaliar o efeito da combinação de crioprotetores intra (10% de dimetilsulfóxido, DMSO) e extracelulares (10% de soro fetal bovino, SFB, na presença ou ausência de 0,2 M de Sacarose, SAC) sobre a criopreservação de células somáticas derivadas de tatus-peba, visando aumentar a eficiência no armazenamento deste material em bancos de recursos genéticos.

Material e Métodos: Para tanto, foram obtidos fragmentos da região auricular de cinco tatus-peba adultos, sendo três fêmeas e dois machos, e cultivados *in vitro*. As células isoladas foram criopreservadas na quarta passagem por congelamento lento e analisadas antes (grupo controle) e após a criopreservação quanto à viabilidade com corante azul de tripan, atividade metabólica pelo ensaio do brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazólio e atividade proliferativa pela determinação do tempo de duplicação da população por 168 h. Os dados foram expressos como média $\pm$ erro padrão (um animal/uma repetição) e analisados utilizando o software StatView 5.0. Como os dados não apresentaram distribuição normal, foram transformados em arco seno e analisados por ANOVA seguida de teste de Tukey ($p < 0,05$).

Resultados: As taxas de viabilidade e atividade metabólica não foram alteradas após a criopreservação, mantendo-se elevadas em ambos os grupos com valores superiores a 96% e 78%, respectivamente ($p > 0,05$). A atividade proliferativa foi significativamente menor nos grupos criopreservados comparados ao grupo controle ($p < 0,05$); contudo, não diferiram entre si (controle: $15,2 \pm 2,0$ h; 10% de DMSO e 10% de SFB: $17,8 \pm 1,4$ h; 10% de DMSO, 10% de SFB e 0,2 M SAC: $16,7 \pm 1,0$ h). **Conclusões:** Combinações de DMSO e SFB na ausência e presença de SAC apresentaram eficiência similar na criopreservação de células somáticas de tatus-peba, associadas a altas taxas de viabilidade e atividade metabólica. Esses resultados são relevantes para a criopreservação e aplicação dessas células em biotecnologias reprodutivas na espécie.

Palavras-chave: biobancos, criopreservação, congelamento lento, mamíferos silvestres.

Keywords: biobanks, cryopreservation, slow freezing, wild mammals.

Protocolo CEUA: no. 36/2020. **Protocolo ICMBio:** no. 71837-1.

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