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LUANNA LORENNA VIEIRA RODRIGUES

COMPARAÇÃO DAS SOLUÇÕES DE CRIOPRESERVAÇÃO E MÉTODOS DE SINCRONIZAÇÃO DO CICLO EM G_0/G_1 DE FIBROBLASTOS DE ONÇAS-PARDAS, *Puma concolor* (LINNAEUS, 1771)

MOSSORÓ–UFERSA
2023

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BANCA EXAMINADORA

Profa. Dra. Alexsandra Fernandes Pereira (UFERSA)
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Profa. Dra. Cristiane Schilbach Pizzutto
Membro examinador (USP)

Profa. Dra. Fabiana Fernandes Bressan
Membro examinador (USP)

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LUANNA LORENNA VIEIRA RODRIGUES – Nascida em Mossoró, RN, no dia 11 de maio de 1998, filha de Maria Vera Lúcia Vieira Diniz e Alfredo Rodrigues Neto. Graduou-se em Biotecnologia pela Universidade Federal Rural do Semi-Árido (UFERSA, 2015–2019). Estagiou no Laboratório de Biotecnologia Animal (LBA/UFERSA), participando do Programa Institucional Voluntário de Iniciação Científica (UFERSA, 04/2017–07/2017) e do Programa Institucional de Bolsas de Iniciação Científica (UFERSA, 03/2019–07/2019 e 08/2019–02/2020). Em dezembro de 2020, foi aprovada no processo seletivo para o mestrado acadêmico em Ciência Animal (PPGCA/UFERSA), iniciando em março de 2021 as atividades no Laboratório de Biotecnologia Animal (LBA/UFERSA) com bolsa de auxílio financeiro pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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" Mas a ciência e a vida cotidiana não podem e não devem ser separadas. A ciência, para mim, dá uma explicação parcial para a vida. Na medida em que vai, é baseada no fato, experiência e experimento."

(Rosalind Franklin)

COMPARAÇÃO DAS SOLUÇÕES DE CRIOPRESERVAÇÃO E MÉTODOS DE SINCRONIZAÇÃO DO CICLO EM G_0/G_1 DE FIBROBLASTOS DE ONÇAS-PARDAS, *Puma concolor* (LINNAEUS, 1771)

Rodrigues, Luanna Lorenna Vieira. COMPARAÇÃO DAS SOLUÇÕES DE CRIOPRESERVAÇÃO E MÉTODOS DE SINCRONIZAÇÃO DO CICLO EM G_0/G_1 DE FIBROBLASTOS DE ONÇAS-PARDAS, *Puma concolor* (LINNAEUS, 1771). 2023. (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2023.

RESUMO: A ameaça ao quantitativo populacional de onças-pardas, atrelada a sua importância ecológica, resultam no desenvolvimento de estratégias como os bancos de recursos somáticos. Esses bancos atuam na preservação de células somáticas, as quais podem ser empregadas em estudos de produção de células pluripotentes e crias clones. Neste contexto, o estabelecimento de condições de criopreservação e sincronização do ciclo celular são etapas importantes na qualidade destes bancos. Portanto, o objetivo foi comparar soluções de criopreservação em células somáticas e avaliar metodologias de sincronização do ciclo em G_0/G_1. Para tanto, fibroblastos derivados da pele de três animais foram cultivados até a 3ª passagem e submetidos aos experimentos. No primeiro experimento, células foram criopreservadas utilizando diferentes soluções compostas por etilenoglicol e dimetilsulfóxido em duas concentrações (2,5% e 10%), na ausência e presença de 0,2 M de sacarose e avaliadas quanto à morfologia, viabilidade, atividade metabólica, análise proliferativa e níveis de apoptose. Células não criopreservadas foram usadas como controle. No segundo experimento, células foram submetidas a três técnicas de sincronização em diferentes tempos: inibição por contato (IC) (24, 48 e 72 h), privação de soro (PS) (24, 48, 72 e 96 h) e roscovitina (RO) (12 e 24 h). Células não sincronizadas foram usadas como controle. Neste experimento, células foram avaliadas quanto ao ciclo por citometria de fluxo e viabilidade e níveis de apoptose por ensaios microscópicos. No primeiro experimento, nenhuma diferença foi observada entre as diferentes soluções de criopreservação para nenhum dos parâmetros. Os valores de viabilidade e atividade metabólica variaram de 79,1% ± 8,3 a 91,5% ± 0,6 e 87,2% ± 5,4% a 99,9 ± 0,1, respectivamente. Adicionalmente, para a análise proliferativa, os valores variaram de 43,3% ± 9,7 a 92,7% ± 28,2. Quanto aos níveis de apoptose, os resultados variaram de 68,0% ± 7,4 a 80,0% ± 3,8 em células viáveis, 10,0% ± 2,5 a 18,0% ± 4,9 em células em apoptose inicial, 2,0% ± 0,8 a 8,0% ± 1,6 de células em apoptose tardia e 3,0% ± 0,7 a 9,0% ± 3,4 de células em necrose. No segundo experimento, fibroblastos submetidos à IC por 24 h (84,0% ± 1,8) e 48 h (84,6% ± 0,6) apresentaram um maior percentual de G_0/G_1, quando comparados ao controle (73,9% ± 3,0). Já aqueles submetidos a PS, somente o tempo de 96 h foi hábil para a sincronização em G_0/G_1 (85,4% ± 3,4) quando comparado ao controle (73,9% ± 3,0). Além disso, RO por 12 h (78,6% ± 3,5) e 24 h (82,1% ± 4,5) não foi capaz de sincronizar células. As taxas de viabilidade em todos os grupos variaram de 76,8% ± 6,7 a 96,0% ± 2,8. Para os níveis de apoptose, foi observado que IC e RO não afetaram esses parâmetros ($P>0,05$). Contudo, PS causou diferenças quanto as células viáveis e necróticas no tempo de 96 h ($P<0,05$). Em conclusão, fibroblastos de onças-pardas podem ser criopreservados utilizando ambos os crioprotetores intracelulares, em concentrações reduzidas, e na presença ou ausência de sacarose. Ainda, a IC se mostrou mais eficiente para a síncronização em G_0/G_1, sendo este estudo uma importante etapa elucidada visando o emprego dessas células para a conservação da onça-parda.

Palavras-chave: felídeos silvestres, gênero *Puma*, bancos de células somáticas, congelação lenta, sincronização em G_0/G_1.

COMPARISON OF CRYOPRESERVATION SOLUTIONS AND SYNCHRONIZATION METHODS OF THE G_0/G_1 CYCLE OF FIBROBLASTS FROM PUMA, *Puma concolor* (LINNAEUS, 1771)

Rodrigues, Luanna Lorenna Vieira COMPARISON OF CRYOPRESERVATION SOLUTIONS AND SYNCHRONIZATION METHODS OF THE G_0/G_1 CYCLE OF FIBROBLASTS FROM PUMA, *Puma concolor* (LINNAEUS, 1771). Dissertation (Master's in Animal Science*:* Morphophysiology and Animal Biotechnology) – Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, 2023.

ABSTRACT: The threat to the puma population, linked to its ecological importance, resulted in the development of strategies such as somatic resource banks. These banks act in the preservation of somatic cells, which can be used in studies of pluripotent cell production and clone offspring. In this context, the establishment of cryopreservation and cell cycle synchronization conditions are important steps in the quality of these banks. Therefore, the aim was to compare cryopreservation solutions in cells and evaluate cycle synchronization methodologies in G_0/G_1. Then, fibroblasts derived from the skin of three animals were cultured until the 3rd pass and submitted to the experiments. In the first experiment, cells were cryopreserved using different solutions composed of ethylene glycol and dimethyl sulfoxide at two concentrations (2.5% and 10%), in the absence and presence of 0.2 M sucrose and evaluated for morphology, viability, metabolic activity, proliferative analysis and levels of apoptosis. Non-cryopreserved cells were used as a control. In the second experiment, cells were subjected to three synchronization techniques at different treatment times: contact inhibition (CI) (24, 48 and 72 h), serum deprivation (SP) (24, 48, 72 and 96 h) and roscovitine (RO) (12 and 24 h). Non-synchronized cells were used as controls. In this experiment, cells were evaluated for cycle stage by flow cytometry and viability and apoptosis levels by microscopic assays. In the first experiment, no difference was observed between the different cryopreservation solutions for any of the evaluated parameters. Viability and metabolic activity values ranged from 79.1% ± 8.3 to 91.5% ± 0.6 and 87.2% ± 5.4% to 99.9 ± 0.1, respectively. Additionally, for proliferative analysis, values ranged from 43.3% ± 9.7 to 92.7% ± 28.2. As for the levels of apoptosis, the results ranged from 68.0% ± 7.4 to 80.0% ± 3.8 in viable cells, 10.0% ± 2.5 to 18.0% ± 4.9 in cells in early apoptosis, 2.0% ± 0.8 to 8.0% ± 1.6 of cells in late apoptosis and 3.0% ± 0.7 to 9.0% ± 3.4 of cells in necrosis. In the second experiment, fibroblasts submitted to CI for 24 h (84.0% ± 1.8) and 48 h (84.6% ± 0.6) showed a higher percentage of G_0/G_1, when compared to the control (73.9% ± 3.0). As for those submitted to SP, only the time of 96 h was able to synchronize in G_0/G_1 (85.4% ± 3.4) when compared to the control (73.9% ± 3.0). Furthermore, RO for 12 h (78.6% ± 3.5) and 24 h (82.1% ± 4.5) was not able to synchronize cells. Viability rates in all groups ranged from 76.8% ± 6.7 to 96.0% ± 2.8. For apoptosis levels, it was observed that CI and RO did not affect these parameters ($P>0.05$). However, PS caused differences in viable and necrotic cells at 96 h ($P<0.05$). In conclusion, puma fibroblasts can be cryopreserved using both intracellular cryoprotectants, at reduced concentrations, and in the presence or absence of sucrose. Still, the CI proved to be more efficient for the synchronization in G_0/G_1, being this study, an important elucidated step aiming at the use of these cells for the conservation of the puma.

Keywords: wild felids, *Puma* genus, somatic cell banking, slow freezing, synchronization in G_0/G_1.

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LISTA DE SÍMBOLOS E SIGLAS

%	Porcentagem
®	Marca registrada
±	Mais ou menos
<	Menor que
>	Maior que
°C	Graus Celsius
x	Vezes
CE	Ceará
cm	Centímetro
CEUA	Comissão de Ética em Uso de Animais
CO_2	Dióxido de carbono
DMEM	Meio Essencial Mínimo Modificado por Dulbecco (*Dulbecco's Modified Eagle Medium*)
DMSO	Dimetilsufóxido
EG	Etilenoglicol
EROs	Espécies Reativas de Oxigênio
FC	Full confluence
GC	Growing cells
g/L	Grama por litro
h	Hora
I	Primeiro
II	Segundo
III	Terceiro
IC	Inibição por contato
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
iPS	Células induzidas a pluripotência
IUCN	União Internacional para Conservação da Natureza (*International Union for Conservation of Nature*)
LBA	Laboratório de Biotecnologia Animal

MTT	Ensaio de 3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
mg/Kg	Miligrama por kilograma
mL	Mililitro
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
n°	Número
PDT	Tempo de Duplicação da População (*Population Doubling Time*)
PS	Privação de Soro
ROS	Roscovitina
SFB	Soro Fetal Bovino
TNCS	Transferência Nuclear de Células Somáticas
UFERSA	Universidade Federal Rural do Semi-Árido

SUMÁRIO

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1. INTRODUÇÃO

A onça-parda, também conhecida como suçuarana ou puma, apresenta ampla distribuição geográfica no continente americano, habitando desde áreas desérticas até regiões montanhosas, desempenhando a função de superpredador, e atuando no controle populacional de mesopredadores (NÁJERA et al., 2018; GUERISOLI et al., 2019). A espécie é classificada como pouco preocupante quanto ao risco de extinção; contudo, ela já se encontra extinta em parte dos Estados Unidos e algumas áreas da América do Sul, principalmente no bioma Caatinga (NIELSEN et al., 2015). Entre as razões para essa redução populacional podem ser citadas as ações antropogênicas, especialmente aquelas de atividades agrícolas e pecuárias (AZEVEDO et al., 2013).

Neste cenário, estratégias de conservação têm sido propostas. Tais estratégias em onça-parda têm sido desenvolvidas por meio de ações englobadas no Plano de Ação Nacional para a Conservação da onça-parda (PAN Onça-Parda; Portaria no. 316/2009), o qual se encontra inserido no Plano de Ação para a Conservação dos Grandes Felinos (PAN Grandes Felinos: Portaria no. 612/2018), elaborado pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). Entre as recomendações desse plano de ação tem-se o desenvolvimento de biotecnologias, como a formação de bancos de recursos biológicos e implementação de biotécnicas reprodutivas.

Especificamente, os bancos de recursos somáticos têm sido uma ferramenta interessante para salvaguardar material genético de indivíduos, independente do sexo e idade (PRAXEDES et al., 2018). Os fibroblastos recuperados destes bancos são fontes essenciais no desenvolvimento da clonagem por transferência nuclear de célula somática (TNCS, MOULAVI et al., 2017) e obtenção de células induzidas à pluripotência (VERMA et al., 2013). Para que estas células possam ser eficientemente empregadas nas finalidades citadas, é necessário que elas estejam armazenadas adequadamente (PEREIRA et al., 2018), sendo a escolha da solução de crioprotetores uma ação-chave para a manutenção da viabilidade e funcionalidade celular após a descongelação (LEÓN-QUINTO et al., 2014; OLIVEIRA et al., 2021). Além disso, a sincronização do ciclo em G_0/G_1 representa, além de um ensaio de funcionalidade, uma etapa para o sucesso da clonagem por TNCS, uma vez que a fase do ciclo em que se encontram estas células pode afetar a integridade dos embriões (VERAGUAS et al., 2017).

Portanto, considerando que existe uma variabilidade de resposta a essas metodologias entre as espécies, faz-se necessária a implementação de protocolos específicos para a onça-parda. Assim, o estudo teve como objetivo comparar soluções de criopreservação e métodos de sincronização do ciclo em G_0/G_1 de fibroblastos de onças-pardas, visando a otimização de bancos de recursos somáticos para a espécie.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. ASPECTOS ECOLÓGICOS E QUANTITATIVO POPULACIONAL DAS ONÇAS-PARDAS

A onça-parda (*Puma concolor* Linnaeus, 1771, **Figura 1**), pertence à ordem Carnívora e família Felídea. Ela é considerada o felídeo silvestre mais amplamente distribuído nas Américas, habitando uma grande variedade de ecossistemas devido aos seus amplos padrões de movimento (CEPEDA-DUQUE et al., 2021).

Figura 1. Onça-parda mantida no Zoológico Municipal Sargento Prata, em Fortaleza, Ceará. Fonte: Diário do Nordeste

Esse animal é um dos grandes carnívoros dos ecossistemas Neotropicais, desempenhando um papel fundamental na manutenção da biodiversidade, bem como dos processos ecossistêmicos (GALLO et al., 2021). Ainda, é a segunda maior espécie de felídeo das Américas, sendo menor somente que a onça-pintada (*Panthera onca*, SANTOS et al., 2022). Esses animais apresentam distribuição desde o norte do Canadá ao sul da Argentina e Chile, onde dentro desta faixa ocorrem em quase todos os habitats e em altitudes que variam desde o nível do mar até 4.000 m acima (OLIVER; ECKERLIN, 2022) (**Figura 2**).

Figura 2. Distribuição geográfica da onça-parda. Fonte: Azevedo et al. (2013).

O peso médio do macho adulto varia entre 40 e 72 kg, enquanto que na fêmea varia de 34 a 48 kg (NOVACK, 2005). No Brasil, a dieta da onça-parda é composta principalmente por capivaras (*Hydrochoerus hydrochaeris*), cervídeos (*Mazama gouazoubira* e *M. americana*), catetos (*Pecari tajacu*), pacas (*Agouti paca*) e tatus (*Dasypus novemcinctus*) (FOSTER et al., 2010; HARMSEN et al., 2011). Ainda, presas menores, como pequenos mamíferos, aves, répteis, peixes e invertebrados são consumidas (EMMONS, 1987; ROCHA-MENDES et al., 2010). Presente em todos os biomas brasileiros, a onça-parda possui grande plasticidade na

adaptação a diversos tipos de ambientes com diferentes graus de perturbação (ALMEIDA et al., 2020).

Contudo, a fragmentação do habitat e os conflitos entre o homem e a vida selvagem, causados pelo aumento da conversão do habitat em áreas de cultivo, agropecuária e habitação, tem resultado em mudanças nos padrões das comunidades das presas (ALMEIDA et al., 2020). Assim, a crescente modificação de ambientes naturais pelo homem tem interferido na estrutura das populações de carnívoros e, consequentemente, influenciado tanto na dinâmica do ecossistema, como nos seus hábitos alimentares (GHELER-COSTA et al., 2018). Além disso, o acesso humano ao habitat das onças-pardas aumenta de acordo com o avanço do desmatamento, e com isso a espécie é perseguida, seja por retaliação ao abate de criações domésticas ou por motivo cultural, geralmente associado ao medo (UBIALI et al., 2018).

No Brasil, existem poucos trabalhos que focam em estimativas populacionais, tendo sido o trabalho de Azevedo et al. (2013) o último trabalho acerca do quantitativo populacional publicado, onde foi denotada que a população efetiva de onças-pardas no Brasil era de cerca de 3.489 indivíduos. Esses autores destacaram a dificuldade na realização desse tipo de estudo em virtude da dificuldade de se individualizar a espécie usando técnicas não invasivas e ao alto custo envolvido na captura e no monitoramento desses animais. Nesse sentido, de acordo com a União Internacional para Conservação da Natureza (IUCN), a onça-parda é classificada como pouco preocupante quanto ao risco de extinção; contudo, de maneira geral, é considerada em declínio (NIELSEN et al., 2015). No Brasil, essa espécie é classificada como vulnerável ao risco de extinção (AZEVEDO et al., 2013).

Dada a importância ecológica da espécie e o declínio de sua população, estratégias de conservação *in situ* e *ex situ* têm sido desenvolvidas visando a proteção da mesma.

2.2. ESTRATÉGIAS DE CONSERVAÇÃO *IN SITU* APLICADAS EM ONÇAS-PARDAS

A conservação *in situ* consiste em estratégias de conservação de ecossistemas e habitats naturais e na manutenção e recuperação de espécies viáveis por meios naturais, promovendo a proteção de seus habitats e diminuindo a remoção de indivíduos da natureza, dependendo principalmente da compreensão e eliminação dos fatores que causam o declínio das populações silvestres (ARAÚJO et al., 2021; PIZZUTTO et al., 2021). Dentre essas estratégias desenvolvidas em onça-parda, tem-se o desenvolvimento de ações de conservação como o Plano de Ação Nacional para a conservação da onça-parda (PAN Onça Parda, Portaria MMA

nº 76, de 27 de junho de 2014) elaborado pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) em parceria com pesquisadores especialistas na área; e o monitoramento e observação do comportamento de espécies por meio de armadilhas fotográficas (COSTA et al., 2010; LAGOS et al., 2020).

Para fins de monitoramento, ao longo dos anos, registros fotográficos de onças-pardas têm sido obtidos. Entre os anos de 2004 e 2005, essa espécie foi registrada por meio de pegadas na região Centro-Oeste do Brasil (COSTA et al., 2010). Em 2011, Borges et al. (2012) registraram um indivíduo em área de queimada e denotaram que essa espécie costuma habitar locais devidamente fragmentados. Mais recentemente, entre os anos de 2017 e 2018, foi registrado por armadilhamento fotográfico um indivíduo em uma área remanescente de floresta próximo a um local turístico (MATHIAS et al., 2019). Ainda, Santos et al. (2021) constataram a presença de um animal adulto com musculatura e pelagem saudáveis em local adjacente a uma área que foi queimada recentemente.

Para fins de observação comportamental, Benson et al. (2012) relataram pseudocio (estro de fêmeas por outras razões que não a reprodução) de onça-parda, sugerindo que as fêmeas desta espécie se associam com machos enquanto amamentam crias, buscando manter relações amigáveis e evitar o infanticídio. Ainda, Lagos et al. (2020) reportaram a cópula entre fêmeas, um comportamento relacionado à dominância e hierarquia entre animais do mesmo sexo.

Apesar de sua importância, estudos para a conservação *in situ* requerem recursos financeiros e humanos mais significativos (JORGE-NETO et al., 2021). Assim, de maneira complementar a esses estudos, tem-se o aprimoramento das estratégias de conservação *ex situ*, as quais atuam de maneira alternativa visando a conservação das espécies.

2.3. ESTRATÉGIAS DE CONSERVAÇÃO *EX SITU* APLICADAS EM ONÇAS-PARDAS

As estratégias de conservação *ex situ* têm como foco a preservação e recuperação de espécies por meio de populações mantidas em cativeiro, uma vez que o ambiente *ex situ* é tido como um local mais fácil para obtenção de dados e se torna uma fonte importante de informações (JORGE-NETO et al., 2021; PIZZUTTO et al., 2021). Apesar de ser alvo de críticas em diversos países do mundo, a manutenção *ex situ* da fauna silvestre sob cuidados humanos pode ser a única alternativa para a sobrevivência de muitas espécies (PIZZUTTO et al., 2021).

Esse tipo de abordagem atua a partir do desenvolvimento de programas de educação ambiental, bem como de estudos voltados para as técnicas de reprodução assistida, a qual tem papel fundamental na conservação de espécies, auxiliando na manutenção de uma população geneticamente viável (ARAÚJO et al., 2021). Assim, estudos voltados para o estabelecimento de biotécnicas reprodutivas têm sido realizados em onças-pardas; contudo, os dados são limitados para essa espécie, pois há uma dificuldade no desenvolvimento desses estudos, sendo observada uma escassez de trabalhos para a onça-parda (ARAÚJO et al., 2021).

Em machos, quanto à avaliação do sistema reprodutor masculino, um estudo foi realizado com a subespécie pantera-da-Flórida (*Puma concolor coryi*), onde foi relatada alta incidência de criptorquidismo (BARONE et al., 1994; MANSFIELD; LAND, 2002). Quanto à colheita de sêmen, alguns estudos têm sido desenvolvidos com diferentes técnicas. Inicialmente Deco-Souza et al. (2010, 2013) utilizaram a eletroejaculação com sucesso como método de colheita. Ainda, tem sido relatada a recuperação de espermatozoides epididimários de animais *post-mortem* usando técnicas de lavagem epididimária (CARELLI et al., 2017), *slicing* (CUCHO et al., 2016) e *squeezing* (BENTO et al., 2019). Mais recentemente, Araújo et al. (2020a) realizaram a colheita de sêmen usando cateter urinário, onde relataram sucesso na colheita e obtenção desse material em onças-pardas de cativeiros. Quanto aos estudos relacionados à criopreservação de sêmen, até o presente momento, dois trabalhos foram desenvolvidos com essa finalidade utilizando diferentes meios de criopreservação: TRIS gema (20%) e glicerol (5% ou 7,5%) (DECO-SOUZA et al., 2013) e TRIS gema (20%) e glicerol (6%) (ARAÚJO et al., 2020b).

Com relação aos estudos voltados para as fêmeas, diferentes abordagens têm sido desenvolvidas ao longo dos anos. Inicialmente, autores relataram a aspiração folicular por laparoscopia (LOPU) em onças-pardas, sendo uma técnica confiável e eficiente para obtenção de oócitos de alta qualidade para uso destes oócitos na produção *in vitro* de embriões (PIVE) e clonagem por TNCS em felídeos (BALDASSARRE et al., 2015; 2017; JORGE-NETO et al., 2018; JORGE-NETO et al., 2021). Quanto à maturação oocitária e PIVE, Jorge-Neto (2019) comparou a quantidade e qualidade de oócitos maturados *in vivo* e *in vitro* de onças-pardas, as quais responderam à estimulação hormonal e esses oócitos foram submetidos à PIVE sem sucesso, não tendo sido observadas clivagens durante o cultivo. Ainda, este mesmo autor relatou a vitrificação de oócitos desta espécie. Em continuidade, oócitos vitrificados de onças-pardas foram utilizados em estudos de fertilização *in vitro* (CARELLI et al., 2017; JORGE-NETO, 2019).

Adicionalmente, estudos relacionados à formação de bancos de recursos somáticos foram desenvolvidos. Em geral, diferentes etapas estão envolvidas na formação de bancos de recursos somáticos, tais como (i) formação de bancos de tecidos somáticos, (ii) estabelecimento de linhagens celulares e (iii) avaliação de protocolos de criopreservação e funcionalidade celular. Recentemente, o Laboratório de Biotecnologia Animal (LBA), da Universidade Federal Rural do Semi-Árido (UFERSA) demonstrou a formação de bancos de tecidos derivados da pele auricular de onça-pada (LIRA et al., 2021), bem como relatou a obtenção de linhagens celulares, avaliando as condições de cultivo por período prolongado e os dados causados pela criopreservação em um protocolo contendo 10% de dimetilsulfóxido (DMSO), 10% de soro fetal bovino (SFB) e 0,2 M de sacarose (LIRA et al., 2022). Neste último estudo, os autores observaram que células criopreservadas nesta solução apresentavam uma redução na qualidade após descongelação.

Agora, com o intuito de consolidar a formação destes bancos de células somáticas e otimizar os protocolos de criopreservação de células somáticas de onças-pardas, nós hipotetizamos que a redução dos crioprotetores intracelulares poderia garantir uma maior qualidade destas células após a descongelação. Isso porque estudos sugerem que as concentrações de crioprotetores intracelulares empregadas devem ser as mais baixas possíveis, desde que sejam suficientes para promover um efeito positivo (OTSUKI et al., 2002; ARANTES et al., 2021). Adicionalmente, embora o DMSO seja o crioprotetor intracelular amplamente empregado em células somáticas de felídeos, os mecanismos envolvidos em seu metabolismo ainda não estão totalmente elucidados. Assim, considerando que o etilenoglicol possui também um menor peso molecular, nós consideramos que ele poderia ser um componente interessante a ser avaliado na criopreservação de células somáticas de onça-parda.

Além disso, a sincronização do ciclo em G_0/G_1 representa além de um ensaio de funcionalidade, uma etapa importante para o uso destas células em outras biotecnologias. De acordo com Veraguas et al. (2017), protocolos de sincronização do ciclo em G_0/G_1 possuem resposta espécie-específica, especialmente em felídeos silvestres. Portanto, considerando três metodologias atualmente desenvolvidas em felídeos silvestres (inibição por contato, privação de SFB e roscovitina), nós hipotetizamos que métodos mais simples (inibição por contato) poderiam ser empregados na sincronização do ciclo de células de onça-parda.

Portanto, como continuidade na conservação de onça-parda, esta proposta visou contribuir para estudos de criopreservação de amostras somáticas, bem como o emprego destas em estudos visando o estabelecimento de uma etapa para a aplicação dessas amostras em outras biotecnologias.

3. JUSTIFICATIVA

Em virtude de ações antrópicas, o quantitativo populacional de onças-pardas tem sofrido decréscimo ao longo dos anos e esses animais têm se tornado alvos de estudos *in situ* e *ex situ* visando a sua conservação. Quanto às estratégias *in situ*, estas apresentam algumas limitações, ao passo que as estratégias de conservação *ex situ* têm demonstrado grande relevância e eficiência no meio científico. Dentre as estratégias *ex situ* desenvolvidas para esse fim, a formação de bancos de recursos somáticos é considerada eficiente, tornando-se uma ferramenta alternativa e complementar a outras biotecnologias.

Nesse sentido, a criopreservação consiste em uma etapa crucial para o sucesso desses bancos, sendo o crioprotetor intracelular utilizado, bem como suas concentrações empregadas, pontos-chaves responsáveis pela qualidade e viabilidade das amostras após a descongelação. Ainda, para a aplicação dessas amostras em outras biotecnologias é necessária a realização da sincronização do ciclo em G_0/G_1, a qual consiste em uma etapa a ser elucidada, especificamente quanto ao método utilizado, uma vez que os protocolos desenvolvidos até o presente momento demonstram-se espécie-específicos.

Portanto, avaliar a influência de diferentes crioprotetores utilizados na criopreservação de fibroblastos de onças-pardas, bem como avaliar diferentes métodos de sincronização do ciclo em G_0/G_1 para o emprego dessas células em biotecnologias são etapas essenciais para fins de conservação e multiplicação da espécie.

4. HIPÓTESES CIENTÍFICAS

I – Ambos os crioprotetores intracelulares (dimetilsulfóxido e etilenoglicol) na concentração de 2,5% são eficientes na criopreservação de fibroblastos de onças-pardas;

II – A inibição por contato e privação de soro fetal bovino sincronizam células somáticas de onças-pardas, com taxas de sincronização superiores a 80%.

5. OBJETIVOS

5.1. OBJETIVO GERAL

Comparar diferentes condições de criopreservação e sincronização do ciclo em G_0/G_1 de fibroblastos de onças-pardas, visando contribuir para a conservação da espécie.

5.2. OBJETIVOS ESPÉCIFICOS

- Avaliar os efeitos dos crioprotetores intracelulares (2,5% e 10% dimetilsulfóxido [DMSO] e 2,5% e 10% etilenoglicol [EG]) na presença ou ausência de crioprotetores extracelulares (0,2 M sacarose [SAC] e 10% soro fetal bovino [SFB]) sobre a qualidade de fibroblastos derivados de onças-pardas;

- Comparar diferentes métodos [inibição por contato, privação de soro e roscovitina] de sincronização do ciclo em G_0/G_1 de fibroblastos de onças-pardas.

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CAPÍTULO 2 – POTENTIAL AND REALITY OF CRYOPRESERVATION OF SOMATIC CELLS FOR CONSERVATION IN WILD FELIDS

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POTENTIAL AND REALITY OF CRYOPRESERVATION OF SOMATIC CELLS FOR CONSERVATION IN WILD FELIDS

Luanna Lorenna Vieira Rodrigues[1] and Alexsandra Fernandes Pereira[1*]

[1]Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil.
*Corresponding author e-mail: alexsandra.pereira@ufersa.edu.br

Abstract

The loss of biodiversity caused by anthropogenic actions is also a reality for the members of the Felidae family. Except for the domestic cat, all felid species have some degree of threat of extinction in their natural habitat. For this reason, felids have been included in conservation-related studies. This scenario has aroused increasing interest in the formation of somatic cell banks, which when efficiently conserved can be used in preservation strategies for the species. Nevertheless, one of the important steps in the formation of these banks is the understanding of the technical principles and variations involved in cryopreservation techniques, especially because the cryopreservation increases the possibilities for ART (Assisted Reproduction Technologies) by making the use of biological materials independent of time and space. In wild felids, several species already have promising results in the formation of somatic cell banks, and studies aimed at better viability rates have been constantly proposed, as well as new species have been studied. Therefore, the aim was to present the main parameters involved in the elaboration of a somatic cell cryopreservation protocol and its effects, as well as to address the main results developed in different wild felids.

Keywords: biological banks; conservation tools; slow freezing; cryoprotectants.

INTRODUCTION

Felids are the top predators in many ecosystems (1) and their disappearance could affect community structure through meso-predator release, resulting in an increase in the abundance of small predators, a decline in prey populations, and species extinctions (2). The Felidae family is divided into two groups: small cats and big cats; this division is due to the ability of these animals to roar or not, and the large felids are those that have the ability to roar, being the entire genus *Panthera*; and the group of small felids represents all other genus that do not have the ability to roar (3).

The Felidae family consists of 40 species, among which 28 are in a decreasing population, most of which belong to the genus *Leopardus* and *Panthera*. Among the eight species of the genus *Leopardus*, seven are with their population decreasing. For the genus *Panthera*, of the five existing species, all are in population decline, mainly because of illegal hunting, habitat loss and degradation (5). This fact has aroused interest in public policies aimed at the conservation of endangered species. As an example, the leopard (*Panthera pardus*) is legally protected in South Africa; however, they continue to be persecuted, along with meso-predators, such as caracal (*Caracal caracal*). These conflicts can have negative impacts on biodiversity, and the continual and rapid decline of biodiversity at local and global scales requires informed and effective responses by policy makers, conservationists, and society to change the course of survival for species (5).

In this context, somatic cell banks provide a viable and expandable source of genetic material and living cells that offer multiple possibilities for molecular and basic research (6), such as somatic cells are used as a source of cloning by somatic cell nuclear transfer (SCNT, 7) and in obtaining of cells induced to pluripotency (8). For these banks to be functional, cell cryopreservation is a crucial step for their success, being a key point responsible for the quality and viability of the samples after thawing (9).

In general, slow freezing, at programmed or un-programmed cooling rates, is the most

commonly used technique for somatic cell cryopreservation, which the temperature is reduced in a gradual and controlled manner (10) and, in addition to the technique employed, the quality of the samples after thawing depends on the cryoprotectants (CPAs) used, which varies between intracellular, extracellular and their combinations and concentration (11, 12, 13). The slow freezing consists of the following steps: cells are resuspended and transferred to cryovials in the presence of cryoprotectant solution. These cryovials are transferred to the freezing container, which is subsequently taken to a programmable freezer at −80 ºC for a period of 12 h. After this period, the cryovials are deposited in liquid nitrogen cylinders (9).

Knowing that cryobanks of endangered animal species represent an extremely valuable backup of current biodiversity (6), over the years, somatic cells from small and big wild felids have been established and evaluated under different culture and cryopreservation conditions. Therefore, our aim was to analyze the state of the art of cryopreservation in somatic cells, as well as the use of these cells in different conservation strategies. Additionally, the preservation of wildlife genetic material is mainly derived from zoos and includes many endangered, extinct, or completely extinct species (6), and we also analyze the biobanks already formed around the world.

SLOW FREEZING OF SOMATIC CELLS AND ITS APPLICATION IN WILD FELIDS

The controlled rate or slow freezing has been developed over the past 40 years, establishing protocols to preserve different types of samples. These samples are cooled in a controlled way (for mammalian cells −1 °C/min) using lower concentrations of CPAs, and thus, producing ice crystals (14). However, the intracellular ice formation is reduced with the use of slow cooling rates and the dehydration of cells, and even if ice crystals are nucleated in the samples, cell viability and function are preserved in different cells (15). The steps that should take in consideration to achieve a successful slow freezing cryopreservation are: (I) choice of CPA solution, (II) sample preparation for freezing, (III) controlled rate cooling protocol, (IV) storage, (V) thawing and (VI) CPA removal (14).

Cell based systems must be prepared to undergo freezing, and an appropriate CPA solution must be chosen avoiding the mechanism that could impair the cell-based products function and integrity. Moreover, samples should be preserved at the adequate temperatures and thawing conditions (14).

In this sense, cells from wild felids have been cryopreserved through slow freezing aiming its applications in different ARTs (Table 1). Initially, in studies with small felids, Gómez et al. (16, 17) cryopreserved African wild cat (*Felis silva lybica*) cells with the aim of performing SCNT of synchronized somatic cells into enucleated oocytes of domestic cats. Thongphakdee et al. (4) established the culture and cryopreservation of fibroblasts from the marbled cat (*Pardofelis marmorata*). Gómez et al. (18) and Tovar et al. (19) carried out cryopreservation of cells from desert cat (*Felis margarita*) and Chilean cat (*Leopardus guigna*), respectively, both obtaining high rates (94% and 90.7%) of viability after thawing. Moro et al. (20) cryopreserved and cultured Cheetah cells producing blastocysts using domestic cat oocytes by SCNT. Moulavi et al. (7) cryopreserved and cultured Asiatic Cheetah cells. Veraguas et al. (21) used cryopreserved domestic cat and kodkod cells by slow freezing in cell cycle synchronization studies and defined fetal bovine serum deprivation as the method of choice for fibroblasts of both species, having been obtained embryos by SCNT. More recently, a study was carried out aiming at the

Table 1. Use of slow freezing in cell cryopreservation in small and big felids.

Specie	Threat degree	Main result	Authors
Small felids			
African wild cat (*Felis silvestres lybica*)	Endangered	Obtained SCNT embryos/Birth of cloned kittens born from domestic cats	Gómez et al (2003, 2004)
Sand cat (*Felis margarita*)	Near threatened	Birth of cloned kittens born from domestic cats	Gómez et al. (2008)
Kodkod (*Leopardus guigna*)	Vulnerable	Method of cell culture	Tovar et al. (2008)

Marbled car (*Pardofelis marmorata*)	Near threatened	Obtained SCNT embryos up to the blastocyst	Thongphakdee et al. (2010)
Cheetah (*Acinonyx jubatus*)	Vulnerable	Blastocysts produced using domestic cat oocytes	Moro et al (2015)
Asian Cheetah (*Acinonyx jubatus venaticus*)	Critically endangered	First report of iSCNT in Cheetah using non-viable frozen cells	Moulavi et al. (2017)
Domestic cat (*Felis silvestris catus*), Kodkod (*Leopardus guigna*)	Domestic, vulnerable	Synchronization of cell cycle	Veraguas et al. (2017)
Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*)	Vulnerable, near threatened	Establishment of cryoprotectant solution	Arantes et al. (2021)
Fishing cat (*Prionailurus viverrinus*)	Vulnerable	Cryopreservation of somatic cells from living and *post-mortem* samples	Sukparangsi et al. (2022)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	Least concern	Synchronization of cell cycle	Młodawska et al. (2022)
Big felids			
Siberian tiger (*Panthera tigres altaica*)	Endangered	Synchronization of cell cycle	Song et al (2007)
Bengal tiger (*Panthera tigris tigris*)	Endangered	Establishment and cryopreservation of a cell line	Guan et al. (2010)
Siberian tiger (*Panthera tigris altaica*)	Endangered	Establishment, characterization, and cryopreservation of a cell line	Liu et al. (2010)
Iberian Lynx (*Lynx pardinus*)	Endangered	Cryobanking from skin biopsies, cryopreservation and culture of explants and cells	León-Quinto et al. (2011)
Snow leopard (*Panthera uncia*)	Vulnerable	Inducing pluripotency in somatic cells	Verma et al. (2012)
Jaguar (*Panthera onca*)	Near threatened	Establishment, isolation, and cryopreservation of primary fibroblast culture	Mestre-Citrinovitz et al. (2016)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	Arantes et al. (2021)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	Oliveira et al. (2021)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	Silva et al. (2021)
Puma (*Puma concolor*)	Least concern	Isolation, characterization, and cryopreservation	Lira et al. (2022)
Jaguarundi (*Puma yagouaroundi*; *Harpailurus yagouaroundi*).	Least concern	Synchronization of cell cycle	Młodawska et al. (2022)

cryopreservation of cells by slow freezing and comparing the efficiency of different cryopreservation solutions (2.5% and 10% dimethylsulfoxide [DMSO]) in Northern tiger cat (*Leopardus tigrinus*) and pampas cat (*Leopardus colocolo*) and obtained rates above 80% of viability (11). Also, in Fishing cat (*Prionailurus viverrinus*), Sukaparangsi et al. (22) realized slow freezing to cryopreservation of cells using DMSO and a commercially used medium (Recovery™ Cell Culture Freezing Medium (ThermoFisher) to form a cryobank.

Regarding to big cats, studies have also been developed. Song et al. (23) cultured and cryopreserved cells from Siberian tiger (*Panthera tigres altaica*). Guan et al. (24) and Liu et al. (25) performed the establishment and cryopreservation of a line of fibroblasts derived from the Bengal tiger (*Panthera tigris tigris*) and the Siberian tiger by slow freezing, respectively. León-Quinto et al. (26) established somatic tissue

banks in the Iberian lynx (*Lynx pardinus*), the most endangered felid in the world. In 2012, Verma et al. (27), performed the cryopreservation of snow leopard *(Panthera uncia*) fibroblasts for pluripotency induction studies. Subsequently, Mestre-Citrinovitz et al. (28), described a protocol for obtaining and cryopreservation of fibroblasts from ear samples of jaguar (*Panthera onca*). More recently, studies carried out with the same species compared the efficiency of different cryoprotectant solutions in the cryopreservation of fibroblasts from these animals (9, 11). Another large felid that has been studied is the puma, where data on somatic cell cryopreservation and establishment of fibroblast lines are already available (29). In all the studies cited, both in small and big cats, slow freezing was the cryopreservation technique used, demonstrating its wide use due to its advantages for application in cells, and that over the years, has established itself as the technique of choice for cell cryopreservation. Still, most of the time, when there are variations between the protocols, it occurs in terms of the composition of the cryoprotectant solution.

VARIABLES INVOLVED IN THE EFFICIENCY OF SLOW FREEZING IN SOMATIC CELLS

Regardless of cell type, the success of any cryopreservation protocol is dictated by careful selection of a few common variables: type of CPA including permeant and non-permeant agents or a combination of both, as well as appropriate cooling and thawing rates (30). In this context, the two main challenges of slow freezing are the cooling curve and the consequent formation of ice crystals. Slow cooling rates (<1 °C/min) allow cells ample time to dehydrate and prevent excessive intracellular ice formation.

However, cells are exposed to high concentrations of solute as well as any CPAs that have been added for a long period of time. Still, disproportionate dehydration can be irreversible and is one of the main causes of damage induced by the cryopreservation of biological materials, because as the ice crystals continue to grow in the extracellular medium, the solutes that were previously dispersed in the volume of the solution become concentrated in the wastewater channels between the ice crystals, leading to osmotic shock and increased toxicity (31). In this sense, the optimal cooling rate for cell survival is outlined by the hypothesis that the highest cell viability will be achieved by an intermediate cooling rate, which will provide a balance between these two scenarios, and it is important to mention that different cell types will have different optimal cooling rates (32).

Cooling devices

The choice of equipment to deliver the chosen cooling rate in cryopreservation rests between passive cooling devices (PCDs) and controlled rate freezers (CRFs). PCDs, in which cooling is achieved using an external cold source (often a −80 °C freezer) and an insulated container to hold the samples, are the simplest and cheapest solution. Thus, varying the insulating material and the temperature of the cold source will allow control of the cooling rate and the temperature range over which cooling may be reasonably linear (33). Among the commercially available PCDs is Mr. Frosty® (Nalgene, Rochester, NY) that's been designed to offer a cooling rate of approximately 1 °C/min between about −10 °C and −40 °C, in which the conductive medium is isopropanol (33).

In this sense, different studies have used Mr. Frosty® as a cooling device on slow freezing of cells. Gómez et al. (16, 17) resuspended African wild cat cells in the CPA solution and cooled at 1.0 ºC/min to −80 ºC before storage in liquid nitrogen. Tovar et al. (19) performed cryopreservation of kodkod cells using this device. In this work, the authors refer to this system as a mechanical freezer, where the freezing rate was around 1° C/min and then its placed inside an −80° C freezer. In 2014, León-Quinto et al. (34) used the same filling system in the cryopreservation of fetal and adult Iberian lynx fibroblasts. For this, the cryovials containing 1.0 mL of each cryopreservation solution were cooled in a Mr. Frosty® freezing container at a cooling rate of 1°C/min. Subsequently, when they reached 70 °C, they were plunged into liquid nitrogen. Subsequently, Veraguas et al (21) employed Mr. Frosty® in the cryopreservation of kodkod and domestic cat cells, for this the pelleted fibroblasts were resuspended in frozen medium and placed in cryogenic vials. The vials were frozen at 1°C/min using a freezing container placed inside a −80 °C freezer for 3 days and then were transferred to liquid nitrogen. More recently, two works used Mr. Frosty® as a system for controlled temperature reduction in puma fibroblasts. For this, cell suspension in cryovials were maintained at 4 °C for 10 min, and transferred to a -80 °C freezer in this PCD system

for 12 h using a cooling rate of 1 °C/min. Subsequently, all cryovials were stored in liquid nitrogen (9, 35). More recently, the same methodology has been employed in puma fibroblasts (29).

In addition to using Mr. Frosty® as a slow freezing system, there are works that use other systems, such as the work by Arantes et al. (11) with cells from three different wild felids: Northern tiger cat, pampas cat and puma. In this work, the authors use straws. Six straws were frozen for each concentration of cryoprotectant tested. The 0.25 mL straws were submitted to freezing at -80 °C for 24 h before submersion in liquid nitrogen and remained there until further evaluation. In addition to being a device for slow freezing, straws are devices for filling samples (11).

From these data, it is possible to observe the importance of cooling devices as a parameter to success of slow freezing. Mr. Frosty® appears to be the most used due to its low cost and efficiency in gradually reducing temperature.

Cryoprotective solutions

Cryopreservation processes inflict damage to the material in several ways and to moderate the damage induced by cryopreservation, CPAs are employed (31). In general, the cryoprotective solution acts from a combination of factors, including modulation of hydrogen bonding, effects on cell membrane properties, effects of diluted solute and increase in viscosity of the solution at low temperatures, among others (31). Number of permeating agents (PAs), also knowns as intracellular CPAs, exist currently such as glycerol (the first agent discovered), DMSO, ethylene glycol (EG), and propanediol (propylene glycol) and the ability of each of these compounds to protect a cell from mechanical and osmotic effects of freezing depends on several properties (30). One of these mechanisms is because permeating CPAs interact strongly with water through hydrogen bonding, the freezing point of water is depressed, and less water molecules are available to interact with themselves to form critical nucleation sites required for crystal formation (36).

Some PAs like DMSO, the gold standard CPA, are thought to increase cellular permeability by affecting membrane dynamics in a concentration dependent manner. At low concentrations (5%), evidence suggests DMSO decreases membrane thickness and, in turn, increases membrane permeability. At commonly used concentrations (10%), water pore formation in biological membranes is induced. Formation of pores can be advantageous as intracellular water can be more readily replaced by CPAs (30). In order to reduce cell injury, the proper use of intracellular CPAs plays a significant role in the cryobank formation. For these reasons, it is necessary to evaluate and compare the effects of these intracellular cryoprotectants on the viability of the cells of interest (Table 2).

In this sense, in felids, Silva et al. (35) reported obtaining a 73.2% viability rate of jaguar (*Panthera onca*) fibroblasts when using 10% DMSO in cryoprotectant solution, demonstrating the efficiency of this compound. Furthermore, when evaluating different concentrations of DMSO (2.5% and 10%) in the cryopreservation of fibroblasts from Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*) and jaguar, Arantes et al. (11) reported no differences in cell viability of the three species after thawing, obtaining rates ranging from 82.2–98% viable cells. Both works corroborate previous studies in fibroblasts from different wild felids, which showed good rates of cell viability after thawing when using 10% DMSO as cryoprotectant, which obtained rates above 80% of viability (9, 26, 27).

Table 2. Cryoprotectant solutions on slow freezing of somatic cells derived from wild felids.

Species	Cryoprotective solution employed	Main result	Authors
Small felids			
African wild cat (*Felis silvestres lybica*)	10% DMSO + 10% FBS	85-95% of cell viability / 1.0-3.5% of embryo survival	Gómez et al. (2003, 2004)
Marbled car (*Pardofelis marmorata*)	10% DMSO + FBS	Reprogramming fibroblast cells in domestic cat and rabbit oocyte/ Obtained SCNT embryos up to the blastocyst	Thongphakdee et al. (2006, 2010)

Sand cat (*Felis margarita*)	10% DMSO + 10% FBS	94% of cell viability	Gómez et al. (2008)
Kodkod (*Leopardus guigna*)	8% DMSO + 22% FBS	79.6% of cell viability	Tovar et al. (2008)
Cheetah (*Acinonyx jubatus*)	10% DMSO + 10% FBS	47.7% of rate blastocyst	Moro et al (2015)
Asian cheetah (*Acinonyx jubatus venaticus*)	10% DMSO + 50% FBS	First report of iSCNT in Cheetah using non-viable frozen cells	Moulavi et al. (2017)
Domestic cat (*Felis silvestris catus*), Kodkod (*Leopardus guigna*)	8% DMSO + 22% FBS	74.7-95.9%/ 83.5-97.3% of cell viability	Veraguas et al. (2017)
Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*)	10% DMSO + 10% FBS, 2,5% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	82.2%-98% of cell survival	Arantes et al. (2021)
Fishing cat (*Prionailurus viverrinus*)	10% DMSO, Recovery™	Succeed in preserving somatic cells from living and *post-mortem* samples	Sukparangsi et al. (2022)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	10% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	Above 80% of cell viability	Młodawska et al. (2022)
Big felids			
Siberian tiger (*Panthera tigres altaica*)	10% DMSO + 40% FBS	95% of cell viability	Song et al (2007)
Bengal tiger (*Panthera tigris tigris*)	10% DMSO + 90% FBS	Above 90% of cell viability	Guan et al. (2010)
Siberian tiger (*Panthera tigris altaica*)	10% DMSO + 50% FBS	Above 90% of cell viability	Liu et al. (2010)
Iberian Lynx (*Lynx pardinus*)	5-15% DMSO + 0.1-0.2 M sucrose + 35% FBS	90% of cell viability	León-Quinto et al. (2011)
Snow leopard (*Panthera uncia*)	10% DMSO + 90% FBS	Inducing pluripotency in somatic cells	Verma et al. (2012)
Jaguar (*Panthera onca*)	10% DMSO + 10% FBS	Establishment of efficient culture and cryopreservation protocols	Mestre-Citrinovitz et al. (2016)
Jaguar (*Panthera onca*)	10% DMSO + 10% FBS, 2,5% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	61.9-84.4% of cell viability	Arantes et al. (2021)

Jaguar (*Panthera onca*)	10% DMSO, 10% DMSO + sucrose, 10% EG, 10% EG + sucrose	45.8-58.6% of cell viability	Oliveira et al. (2021)
Jaguar (*Panthera onca*)	1.5 M DMSO + 10% FBS + 0.2 M sucrose	Above 95.7% of cell viability	Silva et al. (2021)
Puma (*Puma concolor*)	10% DMSO + 10% FBS + 0.2 M sucrose	Above 92% of cell viability	Lira et al. (2022)
Jaguarundi (*Puma yagouaroundi*; *Harpailurus yagouaroundi*)	10% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	Above 80% of cell viability	Młodawska et al. (2022)

Specifically, regarding the use of EG in wild felids, 10% EG was evaluated as an intracellular CPAs in the cryopreservation of jaguar somatic cells in the presence or absence of sucrose, where it was observed that, when used alone, resulted in a lower rate of cell viability (45.8%), when compared to the group plus sucrose (52.4%) (9).

The second category of CPAs is non-permeating agents (NPAs). As the name suggests, they do not permeate intracellularly and exert their protective influence outside of the cell. They are typically larger, and covalently linked as either polymers, dimers, or trimers. One of some commonly-used agents in this class are the sucrose and fetal bovine serum (FBS) (30). Several studies point to the efficiency of sucrose in association with intracellular cryoprotectants for the cryopreservation of cells in wild felids, as demonstrated by León-Quinto et al. (26), where it was observed that the combination of 10% DMSO associated with 0.1 M or 0.2 M sucrose, in both concentrations, was more efficient in cryopreservation when compared to the absence of sucrose. Subsequently, León-Quinto et al. (34) observed that sucrose has a positive effect on the conservation of cell viability, as it acted by promoting a decrease in osmotic pressure through cell dehydration, resulting in an increase in cell viability after thawing (36, 37).

Recently, Silva et al. (35) suggested that the efficiency of maintaining the cell viability of jaguar fibroblasts after cryopreservation is linked to the addition of intracellular cryoprotectant (10% DMSO) to 0.25 M sucrose.

In addition, these authors report the use of FBS in their cryoprotective solution, corroborating Oliveira et al. (9), who observed an increase in cell protection capacity when an extracellular cryoprotectant is used in the cryopreservation solution.

In this context, several studies in wild felids successfully approach the cryopreservation of cells using FBS, in different concentrations, as an extracellular CPA. Song et al. (23) used 10% DMSO in conjunction with 40% FBS, obtaining 95% viability after warming in Siberian tiger cells. Subsequently, Liu et al. (25) used 50% FBS in association with 10% DMSO to cryopreserve Siberian tiger cells and reported that the cells had a viability greater than 90% after freezing. Verma et al. (27), when using 10% DMSO and 90% FBS, they obtained an 80% survival rate in snow leopard cells. According to Moulavi et al. (7), 50% FBS, when used in association with 10% DMSO in the cryopreservation of Asiatic Cheetah cells, was efficient in maintaining these cells, of which 85% were viable. Lower concentrations of FBS have also been successfully reported, as in the work by Silva et al. (35), who used 10% FBS in association with DMSO and sucrose and obtained a cell viability rate of 73.2%.

In this sense, these works demonstrate the variability of CPA concentrations and combinations that can be successfully used in different felids, denoting the need to compare different concentrations to define an optimized protocol according to the interest species.

BIOBANKS AS A SOURCE OF SAMPLES AND THE MAIN RESULTS OBTAINED FROM THE USE OF CRYOPRESERVED CELLS

The establishment and use of wildlife biobanks has been crucial to the development of basic and applied scientific research and is indispensable for the long-term storage of somatic cells (10, 38). Zoos and zoological research institutions are key players in the conservation of genetic variability and provide

reliable access to valuable material. Sampling is optimally implemented in routine zoo veterinary work (6). Studies for the conservation and recovery of cells from endangered wild felids from several continents were carried out in cooperation with zoos and cryobanks around the world (Table 3). Thus, in studies with small felids in North America, Gómez et al. (18) used samples from animals (*Felis margarita*) from the Birmingham Zoo to obtain cells for use in SCNT cloning and three kittens from frozen/thawed cells were born from reconstructed embryos. Thongphakdee et al. (4) used somatic cells from epithelial and muscular tissues of male and female marbled cats and flat-headed cats (*Prionailurus planiceps*) in SCNT and demonstrated that individual donor cell line affects the developmental success up to the morula stage of this embryos. The authors reported that the genomes of both species have been preserved since 2003 in the Genome Resource Bank, which was jointly developed by the Zoological Park Organization of Thailand, in Asian continent. Another source of somatic resources on the same continent is the Khao Kheow Open Zoo, from where Wittayarat et al. (39) recovered skin samples from Asian golden cat (*Catopuma temminckii*), Marbled cat (*Pardofelis marmorata*), Siamese cat (*Felis catus*) and established fibroblasts which were used in different methodologies for cell cycle synchronization in G_0/G_1, an essential step for SCNT cloning.

Regarding to big cats, Song et al. (23) performed the cell culture and cell cycle synchronization of Siberian tiger fibroblasts where ear tissue was used to generate cell lines from a 9-month-old male animal. In Europe, León-Quinto et al. (26) carried out culture and cryopreservation of Iberian lynx cells obtained from a somatic bank previously established by the same group in 2009, where somatic tissues from different regions of the body (muscle, oral mucosa, bone marrow, spinal cord, and intestines) where recovered of Iberian lynx to cryobanking.

In Oceania, Verma et al. (27) used snow leopard tissue samples allocated at Mogo Wildlife Park, Australia, to obtain fibroblasts, which were used in pluripotency induction studies. Another work carried out by Mestre-Citrinovitz et al. (28) described the collection, isolation, and culture of jaguar somatic tissues by the Biobank of the Buenos Aires Zoo. The Biobank has a collection of 570 samples of 45 autochthonous and endangered species, including the jaguar. The fibroblasts generated were part of 6,700 samples, including tissues such as muscle, ovary, testis, blood, fibroblast, sperm, hair and fluids, and cells from 450 individuals from 87 different species.

In India, Yeliseti et al. (40) performed interspecies nuclear transfer using fibroblasts, derived from ear skin that was collected post-mortem from three large felids (leopard, tiger, and lion), which were successfully synchronized and used for the development of blastocysts using rabbit oocytes as recipient cytoplasm. In South America, different studies about collect, establishment and cryopreservation have been successfully carried out based on obtaining samples of big felids from zoos in the northeast region (9, 29, 35) and central west from Brazil (11).

More recently, in Poland, skin biopsies were obtained from jaguarundi and Pallas's cat that were sourced from the Zoological Garden in Kraków aiming to compare the effects of serum starvation and contact inhibition on cell cycle synchronization and survival of dermal fibroblasts from these cats and this research may find application in preparing donor karyoplasts for SCNT in felids (41). Also, Sukparangsi et al. (22) provide current conservation plan using cell technology for

Table 3. Wild felids somatic resource biobanks around the world.

Species	Threat degree	Biobank	Sample type	Country	Authors
Small felids					
Sand cat (*Felis margarita*)	Near threatened	Birmingham Zoo	Tissue	USA	Gómez et al. (2008)
Marbled cat (*Pardofelis marmorata*), flat-headed cats (*Prionailurus planiceps*)	Near threatened, Endangered	Genome Resource Bank	Somatic cell and tissue	Thailand	Thongphakdee et al. (2010)

Asian golden cat (*Catopuma temminckii*), Marbled cat (*Pardofelis marmorata*), Siamese cat (*Felis catus*)	Near threatened, near threatened, domestic	Khao Kheow Open Zoo	Tissue	Thailand	Wittayarat et al. (2013)
Fishing cat (*Prionailurus viverrinus*)	Vulnerable	Biobank Liquid Nitrogen Tank Facility	Cell	Thailand	Sukpangsi et al. (2022)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	Least concern	Zoological Garden	Cells	Poland	Młodawska et al. (2022)
Big felids					
Siberian tiger (*Panthera tigres altaica*)	Endangered	Xuzhou Zoo	Tissue	China	Song et al. (2007)
Iberian Lynx (*Lynx pardinus*)	Endangered	Iberian lynx Biological Resource Bank	Tissue	Spain	León-Quinto et al. (2011)
Snow leopard (*Panthera uncia*)	Vulnerable	Mogo Wildlife Park	Tissue	Australia	Verma et al. (2012)
Leopard (*Panthera pardus*)	Vulnerable	Khao Kheow Open Zoo	Tissue	Thailand	Wittayarat et al. (2013)
Jaguar (*Panthera onca*)	Near threatened	Biobank Buenos Aires Zoo	Tissue	Argentine	Mestre-Citrinovitz et al. (2016)
Leopard (*Panthera pardus*), tiger (*Panthera tigris*), lion (*Panthera leo*)	Vulnerable, endangered, vulnerable	Nehru Zoological Park	Tissue	India	Yeliseti et al. (2016)
Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*) and jaguar (*Panthera onca*)	Vulnerable, near threatened, near threatened	Brasília Zoo	Cells	Brazil	Arantes et al. (2021)
Jaguarundi (*Puma yagouaroundi*; *Harpailurus yagouaroundi*).	Least concern	Zoological Garden	Cells	Poland	Młodawska et al. (2022)

fishing cats and also recommendation of tissue collection and culture procedures for zoo research to facilitate the preservation of cells from *post-mortem* animals and living animals. All cells in this study were currently stored in Biobank Liquid Nitrogen Tank Facility under Zoological Park Organization (ZPO) of Thailand, from which tissue samples were also collected to obtain these cells.

Through these intensively managed breeding programs, zoos strive to maintain genetically diverse populations over time, but with very few exceptions, these populations are not currently sustainable with natural breeding alone (42). From the different works cited, it is possible to observe the great variety of somatic resource biobanks spread around the world, in addition, it is evident the different possibilities of using samples from biobanks in different works aimed at reproduction, cloning and conservation.

FINAL CONSIDERATIONS

In view of the results of the work that has been carried out with wild felids over the years, the importance of forming somatic banks in these species is evident, mainly with the aim of conserving them using ARTs that are in increasing expansion. Moreover, it is evident that knowledge and definition of all technical tools related to cell cryopreservation are necessary for the efficient use of these somatic banks, being these tools: filling devices and cryoprotectants used and the cryopreservation technique used in the material to be stored. In this sense, it is observed that in some species these aspects are

already defined, and slow freezing associated with cryoprotectant solutions composed of intra and extracellular cryoprotectants is the most used methodology. Therefore, the next step is the improvement of techniques such as cloning and inducing cells to pluripotency.

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CAPÍTULO 3 – COMPARISON BETWEEN CONCENTRATION AND TYPE OF INTRACELLULAR CRYOPROTECTANTS AND THE PRESENCE OF SUCROSE FOR CRYOBANKS OF SOMATIC CELLS DERIVED FROM CAPTIVE PUMAS

Artigo Experimental: Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas

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Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas

Luanna L. V. Rodrigues[1], Yasmin B. F. Moura[1], João V. S. Viana[1], Érika A. Praxedes[1], Lhara R. M. Oliveira[1], Herlon V. R. Silva[2], Alexsandra F. Pereira[1*]

[1]Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil. [2]Laboratory of Reproduction of Carnivorous, Ceara State University, Fortaleza, CE, Brazil.

[*]**Correspondence:** Alexsandra F. Pereira, PhD, Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró, RN, 59625900, Brazil, Phone: +55 84 3317 8361, Email address: alexsandra.pereira@ufersa.edu.br

Running head: Slow freezing of Puma somatic cells

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ABSTRACT

The loss of wild biodiversity has prompted the development of cryobanks, such as those of somatic cells. This is the reality of Pumas, wild felids of ecological importance that suffer from anthropogenic actions, population decline, and subsequent loss of genetic diversity. Somatic cell banks are a strategy for conserving population diversity. We compared different concentrations and types of intracellular cryoprotectants (dimethyl sulfoxide, DMSO; ethylene glycol, EG) associated with 0.2 M of sucrose in the cryopreservation of the somatic cells of captive Pumas. The cells were cryopreserved by slow freezing with different solutions containing Dulbecco's modified Eagle medium with 10% fetal bovine serum and varying concentrations of DMSO and EG in the absence or presence of sucrose. The cells were analyzed for morphological characteristics, viability, proliferative activity, metabolic activity, and apoptosis levels. Cells maintained similar fusiform morphology before and after cryopreservation. There was no difference in viability, regardless of the reduction in the concentration and type of intracellular cryoprotectants and sucrose. Similarly, proliferative activity, metabolic activity, and apoptosis levels were not altered by the composition of the cryoprotectants. In summary, we demonstrate that reducing the concentration of DMSO or EG ensures adequate cryopreservation of Puma somatic cells, regardless of the presence of sucrose.

KEYWORDS: Cell recovery, *ex-situ* conservation, *Puma* genus, wildlife biodiversity

1. INTRODUCTION

In recent years, zoos have become institutions for the maintenance and exhibition of wild species collections and spaces for elaborating and developing conservation strategies (Stadtländer, 2022). This is due to the loss of wildlife biodiversity, which has resulted in the establishment of different conservation tools linked to *ex-situ* and *in-situ* proposals (Pizzutto, Colachini, & Jorge-Neto, 2021). Among these propositions, the formation of cryobanks, especially of somatic cells, can be an interesting strategy for conserving the population diversity of endangered species (Ryder, & Onuma, 2022), not requiring maintaining many animals in a reduced space (Praxedes, Borges, Santos, & Pereira, 2018).

Additionally, these cells can be used both in the reproductive (Moulavi et al., 2017) and regenerative perspectives (Echeverry et al., 2020). An example of this occurred recently in 2020, when the partnership between Frozen Zoo at San Diego Zoo Wildlife Alliance, Revive and Restore and ViaGen Pets and Equine, reported the birth of Przewalski's first horse (*Equus ferus przewalskii*, Revive, & Restore, 2020), an endangered species that belongs to the same genus as horses, zebras and donkeys. In 2021, the birth of a black-footed ferret (*Mustela nigripes*) produced by the interspecific somatic cell nuclear transfer (iSCNT), was reported from cells preserved for 30 years (US Fish, & Wildlife Service, 2021). These are clear examples of how iSCNT and cryobanks of somatic cells are currently being used to preserve endangered genetics, increasing genetic variability in animal populations, and restoring genomes lost years ago (Gambini, Briski, & Kanel, 2022).

According to data from the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species, among the 138,300 species assessed, more than 38,500 species are threatened with extinction, including 26% of mammals (IUCN, 2022). This loss of wildlife biodiversity is a significant threat to ecosystem (Stadtländer, 2022). Among the mammals, all wild felids are threatened with extinction, especially those belonging to the *Felis* genus. Two species are representative of the *Puma* genus: the Puma (*Puma concolor* Linnaeus, 1771) and the Jaguarundi (*Puma yagouaroundi* Geoffroy, 1803). *P. yagouaroundi* is classified as Least Concern regarding the risk of extinction. Nevertheless, they have a low population density and a growing threat caused especially by agricultural expansion, which causes habitat loss and fragmentation, directly affecting the survival of individuals (Caso, Oliveira, & Carvajal, 2015). Specifically, the Puma has a wide geographic distribution in the American continent (Guerisoli, Caruso, Vidal, & Lucherini, 2019), being a predator of mammal species, such as collared peccary (*Pecari tajacu*), deer (*Mazzama ssp.*), and paca (*Cuniculus paca*, Guerisoli et al., 2019; Nájera, Palomares, Chávez, Tigar, & Mendoza, 2018). However, according to Nielsen, Thompson, Kelly, & Lopez-Gonzalez (2015), this species is considered extinct in part of the United States and some areas of South America, especially in the Caatinga biome.

In this scenario, we have sought to develop somatic resource banks for Pumas. In 2021, we established somatic tissue banks for the species (Lira et al., 2021), and recently, we described a protocol for cryopreservation of Puma somatic cells using 10% dimethyl sulfoxide (DMSO), 10% fetal bovine serum (FBS), and 0.2 M sucrose (SUC). In this study, cells after cryopreservation showed 79.8% viability and 68.1 h population doubling time (Lira et al., 2021). In general, the success of somatic cell cryobanks depends on the tolerance to the cryopreservation method, the appropriate cryoprotectant, and the ideal concentrations for optimizing cell viability after thawing (Arantes, Tonelli, Martins, & Báo, 2021).

The toxicity of intracellular cryoprotectants could be reduced when considering lower concentrations (Arantes et al., 2021) and the presence of extracellular cryoprotectants, such as SUC (León-Quinto, Simón, Sánchez, Martín, & Soria, 2011). Moreover, the protectant ability of DMSO and ethylene glycol (EG) depends on the species, cell type (Li, Lu, Luo, Pang, & Yang, 2006), and concentration (Arantes et al., 2021). The low molecular weight of EG (62.1 g/mol) and DMSO (78.0 g/mol) ensures the high permeability in the cell. However, such permeability may cause osmotic stress (Schneider, & Mazur, 1984), provoking cell injury and, therefore, the use of cryoprotectants must be optimized for type cell and the species of interest (Oliveira et al., 2021). The pathways used by DMSO in its metabolism are not yet fully elucidated. The same is true for the effects they cause in biological systems. However, there are toxic effects associated with its use, and it is recommended that the concentrations are as low as possible but enough to promote a positive effect (Arantes et al., 2021; Otsuki, Quitina, Ishihara, & Kae, 2002). When metabolized by the endoplasmic reticulum, EG can generate toxic components, including glycolic acid and oxaloacetate, which are harmful to cellular functioning (Castro et al., 2011).

The choice of an appropriate cryoprotectant is essential for generating somatic resource biobanks that allow the development of studies for biodiversity conservation (Dua et al., 2021; Pereira, Borges, Praxedes, & Silva, 2018). Therefore, the present study aimed to establish whether the reduction in concentration and the type of intracellular cryoprotectants associated or not with the presence of SUC alters the conservation of Puma somatic cells.

2. MATERIALS AND METHODS

2.1. Chemicals, solutions, and media

All the reagents, solutions, and media were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Carlsbad, CA, USA).

2.2. Ethics statement and animals

The Ethics Committees of the Rural Federal University of Semi-Arid (process nº 23091.010755/2019-32) and Chico Mendes Institute for Biodiversity Conservation (process nº 71804-1) approved all animal experimentation and the care of animals under study. Three healthy Pumas, maintained in zoos in northeastern Brazil (Fortaleza, CE, Brazil), were used. Data on age, sex, and recovery location are presented in Table 1.

TABLE 1. Details of the main biological aspects of Pumas used in this study.

Animal	Estimated age (years)	Sex	Location
P1	5	Female	Ecologic Park Ecopoint
P2	2	Male	Sargento Prata Municipal Zoo
P3	5	Male	Ecologic Park Ecopoint

2.3. Skin biopsy, establishment, and *in vitro* culture of somatic cells

Peripheral ear skin samples (1.0–2.0 cm^2) were collected after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, SP, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, São Paulo, SP, Brazil) and mechanical containment (Lira et al., 2021). Subsequently, the somatic tissues were transported to the laboratory in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 4 °C for 4 h.

148 The tissues were fragmented (9.0 mm^3) and cultured in the laboratory, according to Lira et al. (2022).
149 The samples were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic
150 solution at 38.5 °C and 5% CO_2. The culture medium was changed every 24 h. When they reached 70%
151 confluency, the cells were harvested and cultured until the third passage, then subjected to
152 cryopreservation.
153
154 **2.4. Study design and cell cryopreservation**
155 The cells were cryopreserved according to eight groups to identify the appropriate cryoprotectant
156 solution. They were diluted in DMEM containing 10% FBS and (i) 2.5% DMSO, (ii) 2.5% DMSO-0.2
157 M SUC, (iii) 10% DMSO, (iv) 10% DMSO-0.2 M SUC, (v) 2.5% EG, (vi) 2.5% EG-0.2 M SUC, (vii)
158 10% EG, and (viii) 10% EG-0.2 M SUC. Non-cryopreserved and cryopreserved cells were evaluated
159 for morphological characteristics, viability, proliferative activity, metabolic activity, and apoptosis
160 levels.
161
162 Cells were resuspended at a final concentration of 1.0×10^5 cells/mL in cryopreservation solution for
163 cryopreservation. The cells remained in contact with the cryopreservation solution for 15 minutes at 4
164 °C. For the groups cryopreserved with SUC in the medium, cells were in contact with the
165 cryopreservation solution without SUC for 15 minutes at 4 °C, and then for more 15 minutes at 4 °C
166 with SUC. Subsequently, the cell suspension was stored in cryotubes and allocated to a freezing
167 container (Mr. Frosty, Thermo Scientific Nalgene, Rochester, NY, USA), which was transferred to a -
168 80 °C freezer, maintaining a cooling rate of 1 °C/min for 12 h until reaching -80 °C. Then, the samples
169 were stored in liquid nitrogen (Lira et al., 2022).
170
171 **2.5. Thawing**
172 After two weeks, the cryovials were kept at 25 °C for 1 minute and immersed in a water bath at 37 °C
173 for 4 minutes for thawing. Cell suspensions derived from groups containing SUC were added in DMEM
174 plus 10% FBS and 0.2 M SUC and maintained at 4 °C for 15 minutes, followed by centrifugation at

1300*g* for 10 minutes to remove the cryoprotectants. Subsequently, the supernatant was removed, and cellular content was suspended in DMEM with 10% FBS, maintained at 25 °C for 15 minutes, followed by another centrifugation at 1300*g* for 10 minutes. Finally, cell suspensions from the groups without SUC were centrifuged twice, as previously described, using DMEM plus 10% FBS. After the first and second medium addition, cells were maintained at 4 °C for 15 minutes and 25 °C for 15 minutes, respectively (Oliveira et al., 2021).

2.6. Morphological analysis and cell membrane integrity

The morphological characteristics were observed during *in vitro* culture under an inverted microscope (Nikon TS100, Tokyo, Japan) for cell forms and cytoplasmic extensions (Lira et al., 2021). Cell membrane integrity was determined using the trypan blue assay. An aliquot of suspended cells was stained with 0.4% trypan blue (in PBS) in a 1:1 ratio and counted using a Neubauer chamber. Unstained cells were considered living due to the intact membrane, whereas cells stained with trypan blue were considered dead due to the penetration of the dye. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

2.7. Analysis of proliferative and metabolism activity

The proliferative activity of cells was quantified according to the growth curve and determination of population doubling time (PDT). The cells (1.0×10^4 cells/mL) were plated in 24-well dishes, trypsinized, and counted. The readings were recorded every 24 h for a period of 168 h. The average counts at regular intervals of 24 h were used to elaborate the growth curve and estimate PDT (Roth, 2006), according to the equation: PDT=T ln2/ln (Xe/Xb), where PDT is the time of culture (in h), T is the incubation time, Xb is the number of cells at the beginning of the incubation time, Xe is the number of cells at the end of the incubation time, and ln is the Napierian logarithm.

Metabolic activity was determined by 5.0×10^4 cells/mL grown in 12-well dishes. After five days, 1.5 mL [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium] (MTT) (5 mg/mL in DMEM) was added and

the dishes, which were incubated for 3 h. The MTT solution was removed, and 1.0 mL DMSO was added for 5 minutes under a stirring process to solubilize the MTT crystals. After the total dissolution of the crystals, the samples were analyzed in a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) using an absorbance wavelength of 595 nm. The values obtained from reading the groups were transformed into percentages by dividing them by the mean value obtained with the non-cryopreserved cells (Silva et al., 2021).

2.8. Analysis of apoptosis levels

The cells were stained with the fluorescent combination of 2 μg/mL acridine orange and 10 μg/mL ethidium bromide to assess apoptosis levels (Lira et al., 2021). Subsequently, 300 cells/animal/group were analyzed by fluorescence microscopy (Olympus BX51TF, Tokyo, Japan) at 480 nm with 200× magnification and classified into (i) viable cells with a uniform light green nucleus; (ii) early apoptotic cells with a nonuniform green nucleus; (iii) late apoptotic cells with a nonuniform bright orange nucleus, and (iv) necrotic cells with a uniform orange nucleus. A fluorescence microscope was used to observe apoptotic changes in the stained cells, quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9. Statistical analysis

All data were expressed as the mean ± standard error and analyzed using the Stat View software (Graph-Pad Software Incorporation, La Jolla, CA, USA). Normality of all results was verified using the Shapiro-Wilk test, and homoscedasticity was verified using Levene's test. Viability, metabolism, and apoptosis levels were altered with arcsine and analyzed using analysis of variance (ANOVA) followed by the Tukey test. Proliferative activity was compared using ANOVA followed by the unpaired t-test. Statistical significance was set at a *p*-value less than 0.05.

3. RESULTS

Before cryopreservation, the cells from three animals showed viability by the trypan blue assay of 84.2% ± 6.7, metabolic activity of 100.0% ± 0.0, PDT values of 49.1 h ± 8.2, and 68.7% ± 22.0 of viable cells for the apoptosis levels, with the remaining values of 14.5% ± 7.5 for early apoptosis, 15.2% ± 13.5 for late apoptosis, and 1.7% ± 1.0 for necrosis.

After cryopreservation in eight different cryoprotectants and thawing, Puma cells cultured for 10 days under identical conditions, showed similar morphology with a confluence of 60–100% (Figure 1). Therefore, cell morphology and confluence were not altered after cryopreservation, and the morphological characteristics of these cells included a fusiform shape with cytoplasmic extensions and a central nucleus, like fibroblasts.

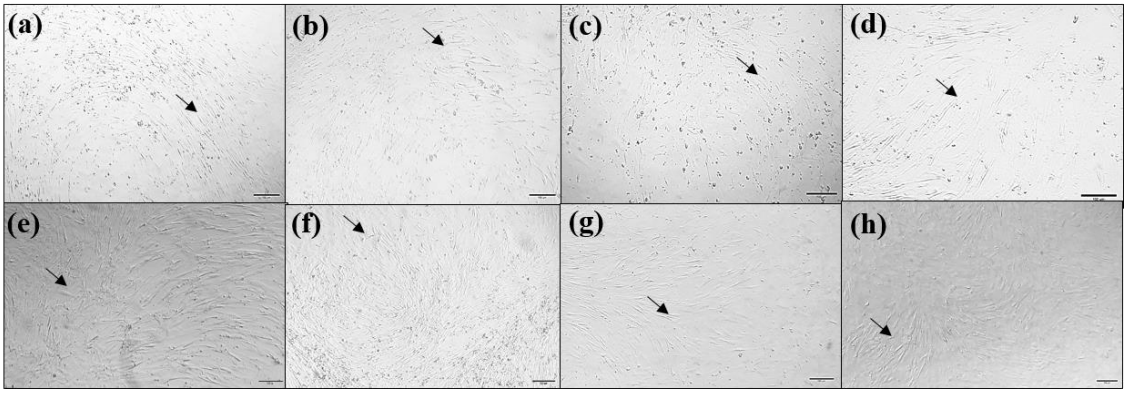

FIGURE 1. Cultures of somatic cells derived from ear skin samples of Pumas. Cells after cryopreservation in DMEM containing 10% FBS and **(a)** 2.5% DMSO, **(b)** 2.5% DMSO-SUC, **(c)** 10% DMSO, **(d)** 10% DMSO-SUC, **(e)** 2.5% EG, **(f)** 2.5% EG-SUC, **(g)** 10% EG, and **(h)** 10% EG-SUC. Arrows indicate fibroblasts. Scale bar: 100 µm, magnification: 40×.

Moreover, no difference was observed for viability, regardless of the reduction in the concentration and type of intracellular cryoprotectants and presence of SUC, ranging from 79.1% ± 8.3 to 91.5% ± 0.6 (Table 2, $p > 0.05$). Similarly, metabolic activity was not altered by the composition of the cryoprotectants (Table 2, $p > 0.05$).

TABLE 2. Viability and metabolic activity of Puma cryopreserved somatic cells using different combinations of intracellular cryoprotectants in the presence of sucrose.

Concentration of DMSO or EG	Intracellular cryoprotectant	0.2 M sucrose (SUC)	Viability (%)	Metabolic activity (%)
2.5%	DMSO	-	85.5 ± 1.8	87.6 ± 10.1
	DMSO	+	91.5 ± 0.6	99.9 ± 0.1
10%	DMSO	-	86.7 ± 4.8	99.8 ± 0.1
	DMSO	+	87.4 ± 1.8	94.6 ± 4.3
2.5%	EG	-	89.9 ± 2.6	99.8 ± 0.1
	EG	+	79.1 ± 8.3	87.3 ± 5.4
10%	EG	-	83.5 ± 8.6	94.4 ± 4.5
	EG	+	90.9 ± 2.2	87.2 ± 5.4

Mean ± standard error. $p > 0.05$.

All groups showed a sigmoid growth curve for proliferative activity, with cells entering the adaptation phase on day 1, followed by the exponential phase (Figure 2A). Additionally, an evident phase of decline was observed in all cells of most experimental groups. Also, the cryoprotectant solution did not affect the proliferative activity after thawing ($p > 0.05$), with PDT values ranging from 43.3 to 92.7 h (Figure 2B).

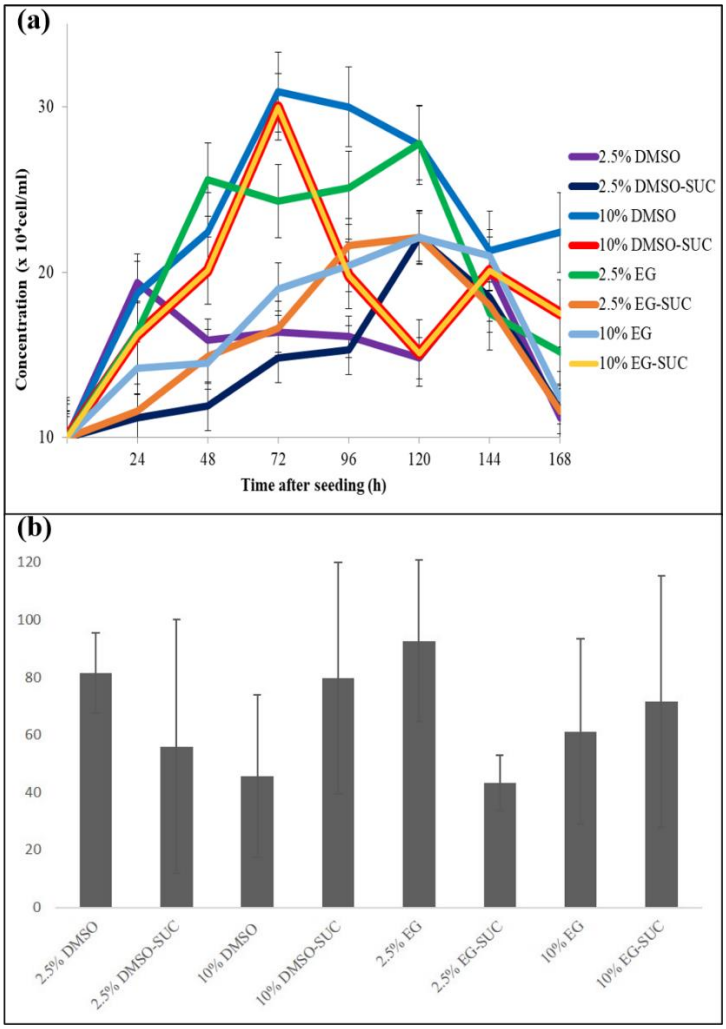

FIGURE 2. Growth dynamics **(a)** and population doubling time (PDT), **(b)** after culture for seven days, of Puma cryopreserved somatic cells using DMEM containing 10% FBS and 2.5% DMSO, 2.5% DMSO-SUC, 10% DMSO, 10% DMSO-SUC, 2.5% EG, 2.5% EG-SUC, 10% EG, and 10% EG-SUC. Bars indicate standard error. $p > 0.05$.

Finally, the apoptosis levels evaluated for viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells were not altered by the composition of the cryoprotectants ($p > 0.05$, Figure 3, Table 3).

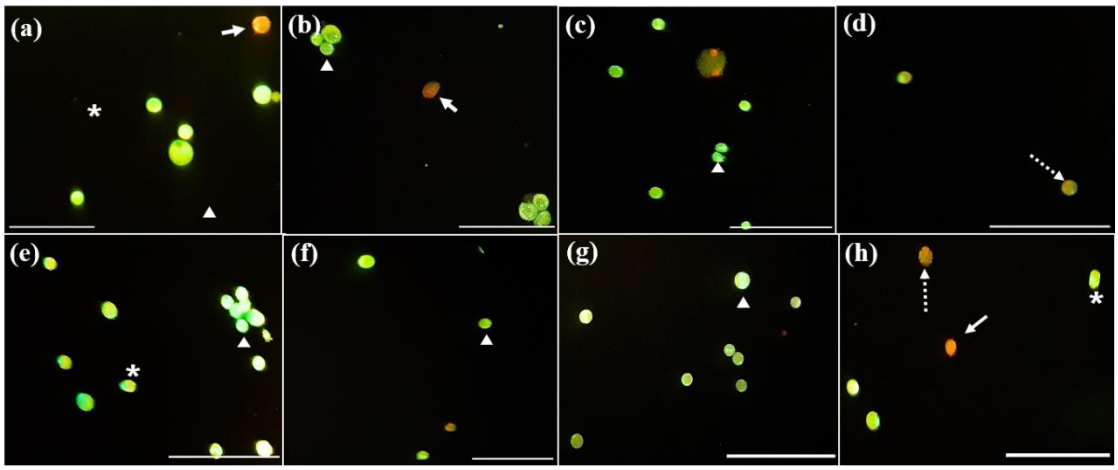

FIGURE 3. Apoptosis levels in Puma somatic cells cryopreserved in **(a)** 2.5% DMSO, **(b)** 2.5% DMSO-SUC, **(c)** 10% DMSO, **(d)** 10% DMSO-SUC, **(e)** 2.5% EG, **(f)** 2.5% EG-SUC, **(g)** 10% EG, **(h)** 10% EG-SUC. Viable cells (triangles). Initial apoptotic cells (asterisk). Late apoptotic cells (dotted arrow). Necrotic cells (arrow). Scale bar: 50 µm, magnification: 40×.

TABLE 3. Evaluation of apoptosis levels in Puma cryopreserved somatic cells.

Concentration of DMSO or EG	Intracellular cryoprotectant	0.2 M sucrose (SUC)	Cells			
			Viability (%)	Initial apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
2.5%	DMSO	-	80.0 ± 3.8	12.0 ± 3.6	2.0 ± 0.6	5.0 ± 0.4
	DMSO	+	69.0 ± 1.1	16.0 ± 2.6	7.0 ± 0.7	8.0 ± 2.7
10%	DMSO	-	76.0 ± 3.4	17.0 ± 5.0	2.0 ± 0.3	5.0 ± 1.5
	DMSO	+	68.0 ± 7.4	18.0 ± 4.9	8.0 ± 1.6	7.0 ± 0.9
2.5%	EG	-	82.0 ± 1.0	13.0 ± 2.1	2.0 ± 0.8	3.0 ± 0.7
	EG	+	78.0 ± 3.4	11.0 ± 2.1	2.0 ± 0.4	9.0 ± 3.4
10%	EG	-	80.0 ± 3.4	10.0 ± 2.5	2.0 ± 0.1	8.0 ± 3.9

	EG	+	80.0 ± 1.3	12.0 ± 0.7	6.0 ± 1.1	3.0 ± 0.4

Mean ± standard error. $p > 0.05$.

4. DISCUSSION

A fundamental step to establishing somatic cell banks of wild felids is the definition of optimized cryopreservation conditions, especially regarding the cryoprotectants, since implementing an ideal cryoprotective solution allows the reduction of cellular cryoinjuries, resulting in the maintenance of cellular parameters (Oliveira et al., 2021). In this study, we demonstrated the efficiency of intracellular (DMSO, EG) and extracellular (SUC) cryoprotectants in maintaining morphology, viability, proliferative activity, metabolic activity, and apoptosis levels of Puma cells after thawing. In this sense, we observed that reducing the concentration of intracellular cryoprotectants, regardless of the presence of SUC, can be useful for the cryopreservation of Puma somatic cells.

Our results corroborate the findings of León-Quinto et al. (2011), who compared three different freezing solutions in Iberian lynx (*Lynx pardinus*) cells (DMSO alone and in combination with 0.1 or 0.2 M SUC). The authors observed viability rates after thawing around 90% for the three freezing solutions used. Cells cryopreserved in the three freezing media presented after thawing metabolic activity values around 85%, with no significant difference between the three groups, demonstrating that intracellular cryoprotectants alone or in conjunction with 0.1 or 0.2 M SUC appear to be very suitable for cryopreserving isolated somatic cells from wild felids. Furthermore, Oliveira et al. (2021) observed that the addition of SUC in the cryoprotectant solution did not influence the viability rate or the metabolic activity of jaguar (*Panthera onca*) cells, with data similar to the cryopreserved cells without SUC and non-cryopreserved cells. Therefore, our result demonstrates that it is possible to cryopreserve cells efficiently without the addition of SUC, as observed in this study.

In the fact, the conservation of Puma somatic cells may be due to the cryoprotectants employed. DMSO has been reported as the most explored cryoprotectant in cryopreservation due to its efficiency in reducing the freezing point of cells and its low cost, in addition to being easily miscible in water (Costa et al., 2020; Weng, & Beauchesne, 2020). DMSO at 10% has been efficient in the cryopreservation of

somatic cells from different wild felids, such as cheetah (*Acinonyx jubatus*, Moro et al., 2015) and jaguar (*P. onca*, Silva et al., 2021). In jaguar, 10% DMSO was efficient for conserving the viability de somatic cells, with values of 73.2% (Silva et al., 2021). Arantes et al. (2021) reported no difference between 2.5% and 10% DMSO in the cryopreservation solution for somatic cells derived from Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*), and jaguar. These works demonstrate that both concentrations act beneficially on the cells of these wild felids. Our findings show that the reduction of the cryoprotectant concentration did not affect the cryoprotectant solution, which maintained its protective function.

In studies with wild felids, 10% EG was evaluated as an intracellular cryoprotectant in the cryopreservation of jaguar somatic cells in the presence or absence of SUC, showing viability rates above 50% after thawing and above 95% after *in vitro* culture of these cells (Oliveira et al., 2021). The EG has also been successfully used in fibroblasts and somatic tissues from other mammals, such as porcine (Li et al., 2006) and collared peccaries (Borges et al., 2018). Also, EG has been successfully used in the cryopreservation of epididymal spermatozoa from domestic cats (Buranaamnuay, 2020) and the vitrification of the epididymal tail (Lima, Soares, Stalker, Santos, & Domingues, 2021). Our data demonstrate the possibility of using both intracellular cryoprotectants (DMSO and EG) in different concentrations in the presence or absence of SUC in the cryopreservation of felid cells. Additionally, our study is the first to evaluate the efficiency of this cryoprotectant (EG) in Puma cells.

Another important aspect is the use of FBS as an extracellular cryoprotectant in all cryopreservation solutions evaluated. In all solutions, the addition of FBS showed a significant impact on cell growth (Oliveira et al., 2021). This stimulatory effect can be attributed to growth factors, collagen, proteins, vitamins, antioxidant properties, trace elements, hormones, and fibronectin supplied by the serum, which collectively play an important role in cell growth, cell adhesion, and cell expansion and maintenance (Lira et al., 2021; Silva., et al., 2021; Hosokawa, Fijisawa, Bing-Hua, Jujo, & Higuchi, 1997). These

aspects show that the presence of FBS in all solutions increased the efficiency of the cryopreservation used in this study.

Puma somatic cells exhibited normal morphology, showing a fusiform shape with cytoplasmic extensions and a large oval-shaped nucleus, and fast growth patterns, corroborating what had been observed in somatic cells derived from jaguar (Oliveira et al., 2021). It is possible to associate the maintenance of these morphological characteristics with the combination of intra and extracellular cryoprotectants since they act by preventing the formation of intracellular ice crystals (León-Quinto, Simón, Cadenas, Martinéz, & Serna, 2014). Nevertheless, cells were used up to the third passage in our study, which may have positively influenced the maintenance of cell growth since, according to Mestre-Citrinovitz, Sestelo, Ceballos, Barañao, & Saragueta (2016), cells after the fourth passage will have their growth level reduced and take longer to reach the desired confluence, besides dying due to cellular senescence (Oliveira et al., 2021).

The trypan blue assay was used to assess cell viability, which is based on evaluating the integrity of the plasma membrane. This assay showed that all cryoprotectant solutions in our study used promoted the maintenance of the cell membrane, with viability rates that range from 85.5 to 91.5%. The reduction of osmotic pressure by cryoprotectants during freezing prevents cell rupture. Therefore, higher viability rates are directly related to the ability of cryoprotectants (DMSO, EG, SUC, and FBS) to protect cell membranes from injuries caused by freezing (Oliveira et al., 2021). Similar rates have already been observed in cryopreserved cells from other wild felids, such as Siberian tiger (*Panthera tigris altaica*, 98.5%) (Liu et al., 2010), Bengal tiger (*Panthera tigris tigris*, 97.6%) (Guan et al., 2010), and jaguar (87.6% to 91.2%, Silva et al., 2021). Evaluating the viability through the membrane integrity is essential for optimizing slow freezing protocols since cells with ruptured membranes are not viable for future applications because they present initial apoptosis (Park et al., 2017).

In the present study, metabolic activity rates ranged between 87.2 and 99.9%, demonstrating that cells remained metabolically active after cryopreservation and thawing. Our results are similar to those observed in jaguars by Oliveira et al. (2021), who obtained rates above 70% after MTT assay in somatic cells from these animals. Silva et al. (2021) obtained metabolic activity rates above 90%, using the same assay, in jaguar fibroblasts cultured until the third passage. Furthermore, these data corroborate those found by León-Quinto et al. (2011), who, after cryopreserving Iberian Lynx fibroblasts, found a metabolic activity rate above 80%. The number of passages can explain our good metabolic activity rates. Cells up to the third passage were used in our study, which is considered a low number of passages. It was previously observed that, after several passages, the cells' genetic characteristics could be modified by the culture conditions (Lira et al., 2022). Additionally, a high number of passages could reduce the rate of cellular metabolism, and a minimum number of passages is the most recommended to preserve cellular characteristics (Li et al., 2009; Mehrabani et al., 2014).

The cellular growth curve was also evaluated as a parameter in analyzing the proliferative activity. It was observed that the PDT value (43.3–92.7 h) for the Puma was slightly above the values previously observed in other wild felids, such as leopard (*Panthera pardus*, 26.7 h), lion (*Panthera leo*, 27.2 h) (Yellisetti, Komjeti, Katari, Sisinthy, & Brahmasani, 2016), Siberian tiger (24.0 h (Liu et al., 2010), and jaguar (22.8 h) (Silva et al., 2021). The experimental groups evaluated in our study showed no difference concerning PDT values, demonstrating that all combinations of cryoprotectants were efficient, and the cells showed high resistance to slow freezing, not affecting the doubling time of their population.

The levels of apoptosis of the cells after cryopreservation was another parameter evaluated. The rate of viable cells that varies between 68.0–80.0% was observed between groups. These values are above those obtained by Silva et al. (2021) in jaguar fibroblasts until the third passage (59.3%) using a cryopreservation solution composed of DMEM with 1.5 M DMSO, 0.2 M SUC, and 10% FBS. Furthermore, our values for early apoptosis (10.0–18.0%) and late apoptosis (2.0–8.0%) were very low when compared to the values obtained by these authors for the jaguar (29.3%) and (10.7%), respectively.

Only the necrosis rate was higher in our study (3.0–9.0%) when compared to the value observed in jaguars (0.7%), but still a low rate compared to the rate of viable cells obtained in our study.

In conclusion, we demonstrate that reducing the concentration of DMSO/EG ensures adequate cryopreservation of Puma somatic cells regardless of the presence of SUC. Therefore, all combinations of DMSO or EG in different concentrations (2.5% and 10%), with or without 0.2 M SUC, were efficient for the cryopreservation of Puma somatic cells and maintained the cells with good rates of viability, metabolic activity, and proliferative activity. These results represent an advance for establishing somatic resource banks in zoos, aiming mainly at conserving Pumas or phylogenetically close individuals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Alexsandra F Pereira: https://orcid.org/0000-0003-2137-854X

AUTHOR CONTRIBUTIONS

L.L.V. Rodrigues has designed the study, acquired and analyzed the data, and drafted the paper, Y.B.F. Moura, J.V.S. Viana, É.A. Praxedes, and L.R.M. Oliveira contributed to the experiments, H.V.R. Silva, contributed to somatic tissue recovery, A.F. Pereira designed and guided the experimental work, acquired and analyzed data, drafted the paper, and revised it critically.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding authors upon reasonable request.

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CAPÍTULO 4 – FULL CONFLUENCY, SERUM STARVATION AND ROSCOVITINE FOR INDUCING ARREST IN THE G0/G1 PHASE OF CELL CYCLE IN PUMA SKYN-DERIVED FIBROBLAST LINES

Artigo Experimental: Full confluency, serum starvation and roscovitine for inducing arrest in the G_0/G_1 phase of cell cycle in puma skin-derived fibroblast lines

Periódico de submissão: Animal Reproduction

Fator de impacto: 1,81

Data de submissão: 28 de janeiro de 2023

Full confluency, serum starvation, and roscovitine for inducing arrest in the G_0/G_1 phase of the cell cycle in puma skin-derived fibroblast lines

Cell cycle synchronization of puma fibroblasts

Luanna Lorenna Vieira Rodrigues[1] (https://orcid.org/0000-0002-1764-1205), Yasmin Beatriz França Moura[1] (https://orcid.org/0000-0002-7115-482X), João Vitor da Silva Viana[1] (https://orcid.org/0000-0001-9712-0313), Lhara Ricarliany Medeiros de Oliveira[1] (https://orcid.org/0000-0002-7292-6908), Érika Almeida Praxedes[1] (https://orcid.org/0000-0002-6406-6346), José de Brito Vieira Neto[2] (https://orcid.org/0000-0002-2881-4220), Sarah Leyenne Alves Sales[2] (https://orcid.org/0000-0001-9379-2817), Herlon Victor Rodrigues Silva[3] (https://orcid.org/0000-0003-1327-9613), Maria Claudia dos Santos Luciano[2] (https://orcid.org/0000-0002-7871-2513), Claudia Pessoa[2] (https://orcid.org/0000-0002-4344-4336), Alexsandra Fernandes Pereira[1*] (https://orcid.org/0000-0003-2137-854X)

[1]Laboratório de Biotecnologia Animal, Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró-RN, Brasil.

[2]Laboratório de Oncologia Experimental, Universidade Estadual do Ceará (UECE), Fortaleza-CE, Brasil.

[3]Laboratório de Reprodução de Carnívoros, Universidade Estadual do Ceará (UECE), Fortaleza-CE, Brasil.

*__Corresponding author__: alexsandra.pereira@ufersa.edu.br

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Author contributions: LLVR: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft & review; YBFM: Data curation, Methodology; JVSV: Data curation, Methodology; LRMO: Data curation, Methodology; EAP: Data curation; Methodology; JBVN: Data curation, Methodology; SLAS: Data curation, Methodology; HVRS: Methodology; MCSL: Methodology, Writing – original draft; CP: Methodology, Writing – original draft; AFP: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

ABSTRACT

The puma population is constantly decreasing, and cloning by somatic cell nuclear transfer can be used to conserve the species. One of the factors determining the success of the development of cloned embryos is the cell cycle stage of the donor cells. We evaluated the effects of full confluency (~100%), serum starvation (0.5% serum), and roscovitine (15 µM) treatments on the cell cycle synchronization in G_0/G_1 of puma skin-derived fibroblasts by flow cytometric analysis. Also, we assessed the effects of these synchronization methods on morphology, viability, and apoptosis levels using microscopy tools. The results showed that culturing the cells to confluence for 24 h (84.0%), 48 h (84.6%), and 72 h (84.2%) and serum starvation for 96 h (85.4%) yielded a significantly higher percentage of cells arrested in the G_0/G_1 ($P < 0.05$) phase than cells not subjected to any cell cycle synchronization method (73.9%). Nevertheless, while serum starvation reduced the percentage of viable cells, no difference was observed for

the full confluence and roscovitine treatments (P > 0.05). Moreover, roscovitine for 12 h (78.6%) and 24 h (82.1%) was unable to synchronize cells in G_0/G_1 (P > 0.05). In summary, full confluency induces puma fibroblast cell cycle synchronization at the G_0/G_1 stage without affecting cell viability. These outcomes may be valuable for planning donor cells for somatic cell nuclear transfer in pumas.

Keywords:

Felids, cell cycle synchronization, culture to confluence, somatic cell nuclear transfer.

Introduction

Cloning by somatic cell nuclear transfer (SCNT) has been used as an essential tool in the generation of identical individuals (Gómez et al., 2008), a model for understanding the cellular and molecular aspects involved in nuclear reprogramming (Moro et al., 2015), production of stem cells for use in regenerative medicine (Jun et al., 2019), and conservation strategy for endangered species (Moulavi et al., 2017). In this last application, the success of this technique can already be observed in different groups of species, such as gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), African wild cats (Gómez et al., 2004) and grey wolf (Kim et al., 2009). In large felids, establishing SCNT for the birth of offspring is still challenging, especially in species of the genus *Puma* and *Panthera*. On the other hand, in some individuals, SCNT has allowed the development of somatic resource banks (Praxedes et al., 2019) and the production of cells induced to pluripotency (Verma et al., 2013).

In general, it is well known that the success of this technique in any species is initially identified by the studies of all aspects related to cells donor from nuclei or karyoplasts (Borges and Pereira, 2019). These cells, which in most cases have been fibroblasts derived from the skin of animals (Praxedes et al., 2018), need to be established *in vitro* before being used in SCNT. Three steps are involved in the establishment of nucleus donor cells: (i) isolation and characterization of somatic cells, (ii) establishment of cell lines, and (iii) development of cell cycle synchronization methods in the G_0/G_1 phase (Pereira et al., 2019).

We have developed the first two steps in puma (*Puma concolor* Linnaeus, 1781). Recently, we have established somatic tissue banks for the species (Lira et al., 2021), as well as we have established cell lines for their use as nucleus donor cells for cloning (Lira et al., 2022). Considering that there are variations between the synchronization methods of the cell cycle in G_0/G_1 among species (Młodawska et al., 2022), including even among felids (Veraguas

et al., 2017), we propose to evaluate different culture conditions for puma fibroblasts, aiming to use these cells in G_0/G_1.

Three methodologies have been proposed for cell synchronization in G_0/G_1. Full confluency is a method that allows cell high-density conditions. The contact surface between adjacent cells gradually increases, leading to contact inhibition causing most cells to stop dividing and remain in the G_0/G_1 phase despite the availability of nutrients and growth factors (Curto et al., 2007). Additionally, high-density conditions favor the regulation of reactive oxygen species (ROS) as well as activates coactivator-1α (PGC1α), which functions as a key regulator of energy expenditure, involved in ROS reduction and protection of cells against oxidative stress (Yang et al., 2018). Regarding serum starvation, this methodology act on the checkpoints by depriving the cells of adequate environmental or nutritional conditions (Kues et al., 2000), more specifically acting due to the response to the absence or presence of mitogens to continue the cell cycle during the onset of the G_0/G_1 phase, so when these cells are in the absence of mitochondrial growth factors that the serum would offer, they accumulate in a state of a 2n DNA content (Coller et al., 2007). Finally, roscovitine is a potent aminopurine inhibitor of cyclin-dependent kinase 1 (CDK1/cyclin B), cyclin-dependent kinase 2 (CDK2), and cyclin-dependent kinase 5 (CDK5), thereby synchronizing the cells in G_0/G_1 (Gómez et al., 2018).

In this sense, non-activated cytoplasts are high in maturation-promoting factor (MPF) activity, a complex of cyclin B and CDK 1 or $p34^{cdc2}$. When an interphase donor nucleus is introduced into a high MPF milieu, it undergoes nuclear envelope breakdown and premature chromosome condensation (Hashem et al., 2006). Therefore, MPF levels decline following activation, chromatin decondenses, and a nuclear envelope is formed. All nuclei that have undergone nuclear envelope breakdown will then undergo DNA synthesis. Hence, donor nuclei must be in G_0 or G_1 when transferred to metaphase II recipient oocytes with high levels of MPF to condense chromosomes normally and maintain the correct ploidy of reconstructed embryos

660 at the end of the first cell cycle (Han et al., 2003). The coordination of the cell cycle between
661 the nucleus of the donor cell and the cytoplasm of the recipient has been widely recognized as
662 a critical factor for the adequate maintenance of integrity and ploidy in SCNT embryos, being
663 necessary for the definition of an adequate method of synchronization of the cell cycle to SCNT
664 success (Nguyen et al., 2021).

665 The puma is one of seven large felid species in the world and the only one native to the
666 non-tropical regions of the New World (Karandikar et al., 2022), being the fourth largest wild
667 felid and the most widespread native terrestrial mammal of the Americas (LaBarge et al., 2022).
668 These animals are found in diverse habitats and environments, from mountainous temperate
669 regions to tropical areas and from wilderness to areas with high levels of human use (Benson
670 et al., 2020). In this sense, most large carnivore species worldwide have seen significant
671 population declines and range contractions (Wolf and Ripple, 2017), being one of the most
672 common species in zoos and rehabilitation centers (Echeverry et al., 2020).

673 Despite being considered of Least Concern by the International Union for Conservation
674 of Nature's Red List of Threatened Species (Nielsen et al., 2015), the puma is considered to be
675 declining in some areas. As a large carnivore intricately linked to other wildlife and habitat
676 associations, from a social and political perspective, its conservation and management present
677 numerous challenges (Nielsen et al., 2015). Most of these species are now legally protected and
678 the focus of worldwide conservation actions (Ripple et al., 2014). Therefore, we aim to compare
679 different cell cycle synchronization methods in G_0/G_1 of puma's fibroblasts to establish this
680 step of the SCNT for the conservation of these animals.

681

682 **Material and Methods**

683

684 The experiments were conducted following the Animal Ethics Committee
685 (CEUA/UFERSA, No. 23091.010755/2019-32) and Chico Mendes Institute for Biodiversity
686 Conservation (ICMBio, No. 71804-1). Unless otherwise stated, the reagents used in this study
687 were obtained from Sigma-Aldrich (St. Louis, USA). Dulbecco's modified Eagle medium
688 (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Carlsbad, USA).
689
690 *Establishment of cell lines*
691 Skin tissue samples were obtained from the ear notch of three healthy adult puma, one female
692 and two males, between 2 to 5 years old, at the Ecologic Park Ecopoint (Fortaleza, CE, Brazil)
693 and Sargento Prata Municipal Zoo (Fortaleza, CE, Brazil). The skin samples were cultured, and
694 three fibroblast lines were previously established (Lira et al., 2022). Subsequently, cells from
695 three lines frozen in 10% dimethyl sulfoxide (DMSO), 10% FBS, and 0.2 M sucrose were
696 thawed, and 4^{th} and 5^{th} passage cells were used for this study.
697 Then, cells were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-
698 antimycotic solution in a humid atmosphere containing 6.5% CO_2 at 38.5 °C. Before initiating
699 cell cycle synchronization protocols, cells were evaluated for their proliferative activity by
700 obtaining the growth curve and determining the population doubling time (PDT). Cells were
701 seeded in 12-well plates at a 1.0×10^4 cells/mL concentration. Cells from each well were
702 counted at 24 h intervals for up to 216 h of culture. After each interval, the mean cell count was
703 recorded; finally, the cell growth curve was generated, and the PDT was estimated based on
704 these measurements.
705
706 *Cell treatments and experimental design*
707 In a series of three experiments, we examined the effect of various culture conditions such as
708 full confluency (FC), serum starvation (SS), and the effect of roscovitine (RO) on the cycle

synchrony of puma skin fibroblasts in different incubation times. In each treatment group, cells without any treatment and with 70% confluence were used as a control (growing cells, GC). All treatments were performed in duplicate for each animal, producing six repetitions for each treatment and each incubation time.

In the first experiment, cells were harvested at 90–100% confluency (full confluency) to monitor the effects of confluency on synchronization. The effects of contact inhibition were monitored after 24, 48, and 72 h of an extended culture of full confluency cells. During the treatment of FC, the culture medium composed of DMEM and 10% FBS was changed every 2 days (Gómez et al., 2018).

In the second experiment, for SS, cells at 70% confluency in DMEM with 10% FBS were cultured in DMEM supplemented with 0.5% FBS for 24, 48, 72, and 96 h. The culture medium was changed every 2 days (Wittayarat et al., 2013).

In the third experiment, treatment with 15 μM roscovitine was performed for 12 and 24 h of cell culture after the cell confluence reached 70%. After starting treatment (day = 0), the stage of fibroblasts from each animal was analyzed after 12 h and 24 h of exposure to roscovitine (Wittayarat et al., 2013).

Cell fixation, staining, and cell cycle analysis

After the treatments, cells were trypsinized, centrifuged at 600×g for 10 min, and resuspended in 1.0 mL of cold 70% ethanol for fixation. The cells were then maintained at –20°C for 5 days. The fixed cells were washed in PBS for ethanol removal, and each sample was centrifuged at 400×g for 10 min. Subsequently, cells were stained with 5 μg/mL propidium iodide (PI), 50 μg/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).

734 Data were obtained from 15,000 events from each sample. Histograms of PI
735 fluorescence vs. counts were generated to evaluate the percentages of cells for each cell cycle
736 phase (G_0/G_1, S, G_2/M) as well as the levels of sub-G_0/G_1. The proportion of cells in each cell
737 cycle phase and sub-G_0/G_1 levels was assessed using MODFIT software version 5.0 (Verity,
738 https://www.vsh.com/products/mflt/index.asp).
739
740 *Morphological analysis and viability by cell membrane integrity*
741 Cells were evaluated under an inverted microscope (Nikon TS100, Tokyo, Japan) for cell forms
742 and cytoplasmic extensions (Lira et al., 2022). Moreover, trypan blue exclusion assay
743 determined the percentage of living cells (Lira et al., 2022). Briefly, the cells were stained with
744 0.4% trypan blue in phosphate-buffered saline (PBS) and counted using a hemocytometer.
745
746 *Apoptosis level assessment*
747 Twelve microlitres of dye mixture [2 μg/mL acridine orange and 10 μg/mL ethidium bromide,
748 diluted in 8 μL PBS (pH 7.4)], was mixed gently with 50 μL of cell suspension and put onto a
749 clean microscope slide. The suspension was immediately (dye uptake is very fast) examined in
750 a fluorescence microscope (Olympus BX51TF) under 200× magnification at 480 nm (Leite et
751 al., 1999). A minimum of 300 cells was counted using ImageJ software (National Institutes of
752 Health, Bethesda, Maryland, USA) in every sample, and the percentage of cells recorded in
753 four different groups: viable (V), early apoptotic (EA), late apoptotic (LA), and necrotic cells
754 (N). With V: cells with a uniform light green nucleus; EA: cells in initial apoptosis, with a non-
755 uniform green nucleus; LA: cells in late apoptosis, with a non-uniform bright orange nucleus,
756 and N: necrotic cells, with a uniform orange nucleus (Lira et al., 2022).
757
758 *Statistical analysis*

Data were expressed as mean ± standard error (one fibroblast line/one repetition) and analyzed using the Graph-Pad 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

Prior to initiating cell cycle synchronization treatments, thawed cells showed normal morphology with PDT of 53.1 ± 4.1 h. Moreover, cells demonstrated a sigmoidal curve (Fig. 1) with the lag phase of adaptation up to day 1 followed by exponential and stationary growth, indicating that these cells were going through various growth phases. Additionally, the decreasing phase was observed.

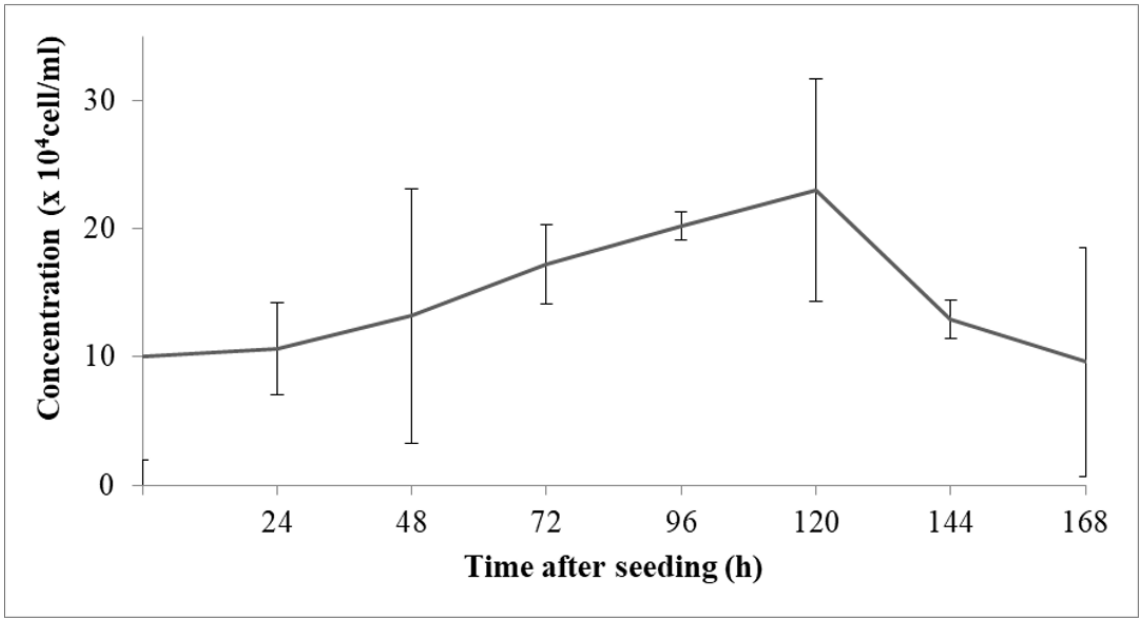
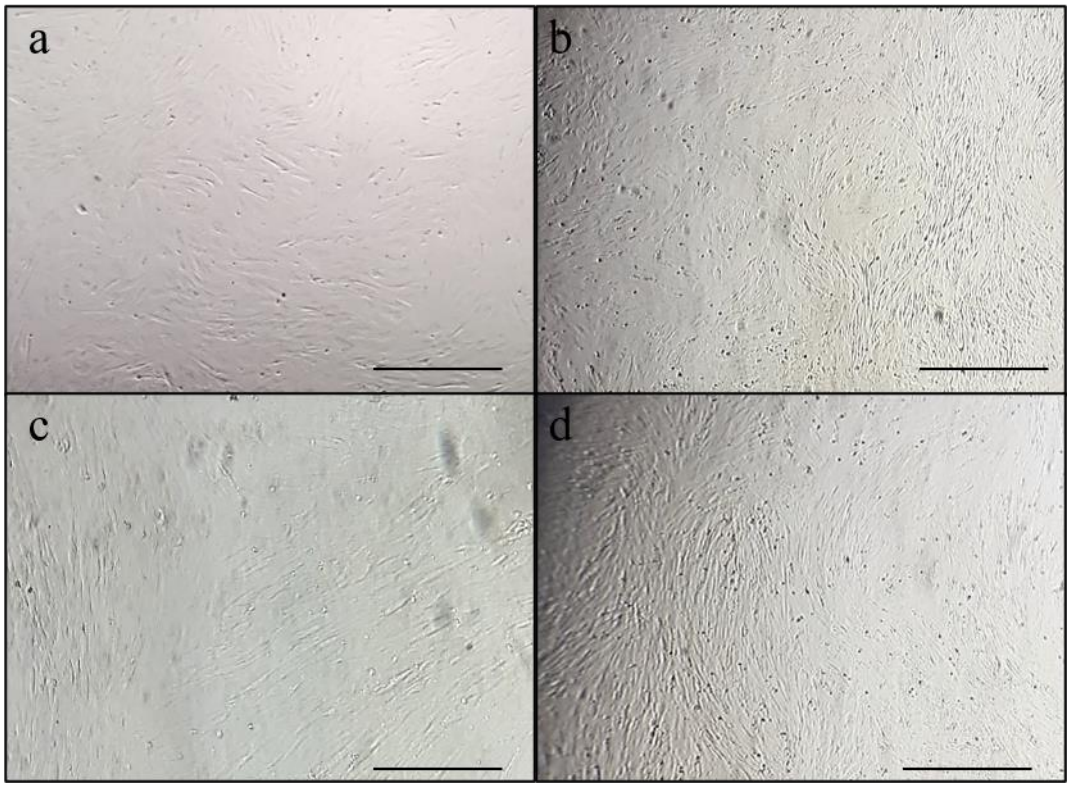


Figure 1. Population double time of puma fibroblasts before cell treatment for synchronization of cells in G0/G1. Values presented in mean ± standard error.

776 *Effects of the synchronization methods on cell morphology*

777 After the three experiments of cell cycle synchronization, cells remained showing
778 normal morphology with a fusiform shape, elongated nucleus, and its extensions, maintaining
779 their characteristics (Fig. 2).



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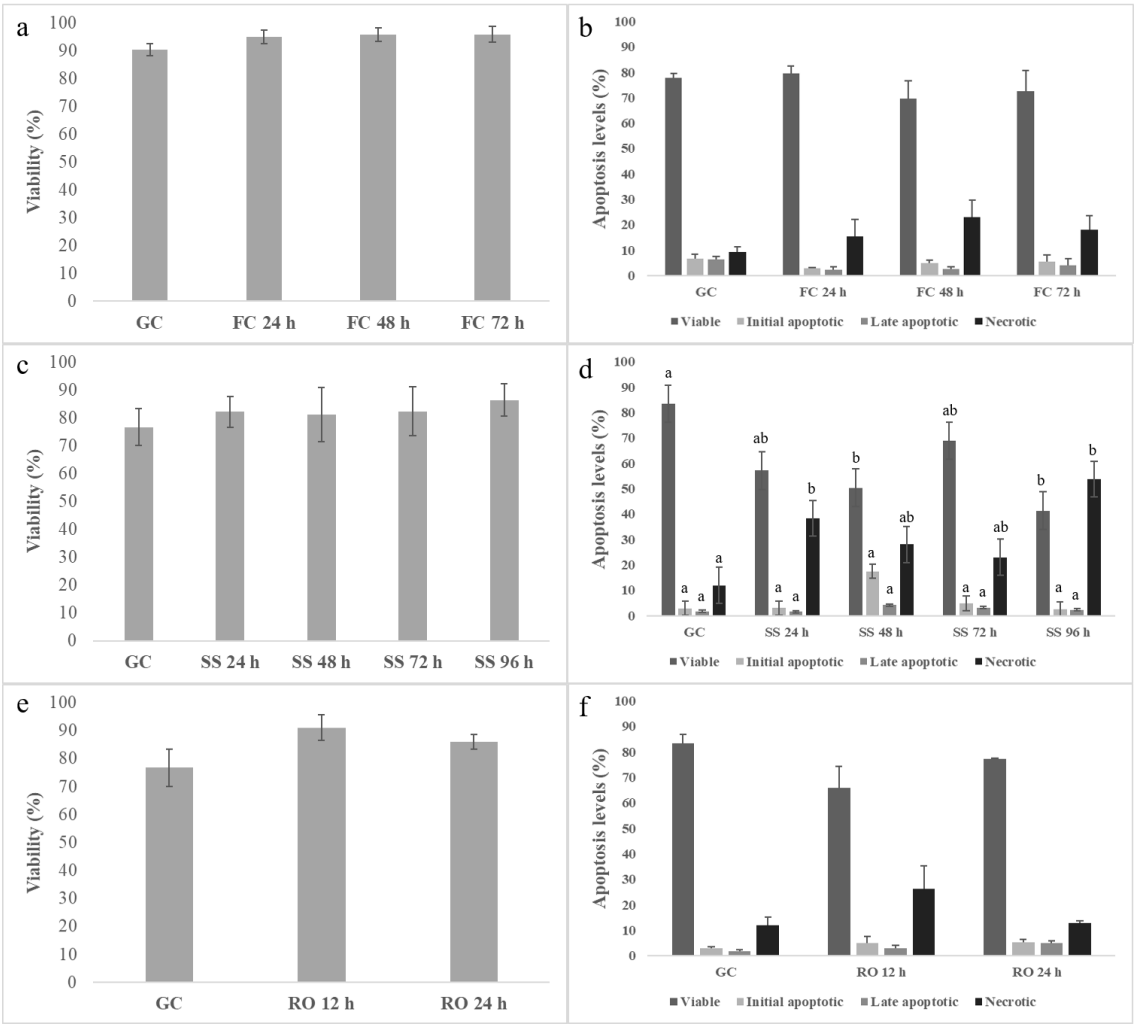
781 Figure 2. Cell morphology of puma fibroblasts before synchronization (a) and after
782 synchronization by different methods, full confluency (b), serum starvation (c) and exposure to
783 roscovitine (d). Scale bar: 100 µm.

784

785 *Effects of the synchronization methods on cell viability*

786 Regarding viability by the trypan blue assay, the FC group was similar to the CG group
787 (90.5% ± 2.2) ($P > 0.05$) at the three times evaluated 24 h (95.1% ± 2.5), 48 h (95.9% 2.4), and
788 72 h (96.0% ± 2.8) (Fig. 3a). The SS group was also similar to its control group (76.8% ± 6.7)
789 ($P > 0.05$) in the four evaluated times: 24 h (82.3% ± 5.5), 48 h (81.2% ± 9.7), 72 h (82.4% ±
790 8.8), and 96 h (86.5% ± 5.8) (Fig. 3c). Finally, regarding the RO group, there was also no

791 difference (P>0.05) between the two evaluated times (12 h - 91.1% ± 4.5) and (24 h - 86.1% ±
792 2.6) compared to the CG group (76.8% ± 6.7). (Figure 3e).
793
794 *Effects of the synchronization methods on apoptosis levels*
795 In the first experiment, FC for 24, 48 and 72 h did not affect apoptosis levels (V, EA,
796 LA, N) at any of the analyzed intervals (P > 0.05, Fig. 3b). Regarding to SS, when the apoptosis
797 levels were evaluated by differential staining, SS caused cell damage (P < 0.05, Fig. 3d). In the
798 third experiment, RO did not affect apoptosis levels at any of the analyzed intervals (P > 0.05,
799 Fig. 3f).



800

Figure 3. Cell viability (a, c, and e) and apoptosis levels (b, d, and f) in puma fibroblasts subjected to different cell cycle synchronization methods. GC: growing cells, FC: full confluency, SS: serum starvation, and RO: roscovitine. a,b indicates differences among parameters (viable, initial apoptosis, late apoptosis, and necrosis) in each treatment (24, 48, 72, 96 h) ($P < 0.05$).

Effects of the synchronization methods on cell cycle stages

The fibroblasts under the FC treatment for 24, 48, and 72 h significantly increased the proportion of fibroblasts in G_0/G_1 phase compared to CG, and the 72-h group decreased the proportion of cells in the S phase. Regarding the G_2/M phase, FC 24 h decreased the proportion of cells when compared to CG ($P < 0.05$) (Table 1). Also, the three-time intervals evaluated promoted modifications in levels of sub-G_0/G_1 ($P < 0.05$). The group of cells on SS for 96 h displayed an increase in the percentage of cells in G_0/G_1 ($P < 0.05$) when compared to CG (Table 2). Additionally, the time of 96 h significantly increased the percentage of G_2/M and sub-G_0/G_1 cells ($P < 0.05$). In the third experiment, it was observed that after 12 and 24 h of RO treatment, the percentage of G_0/G_1, S, G_2/M, and sub-G_0/G_1 was not significantly higher ($P > 0.05$) compared to CG (Table 3).

Table 1 – Effect of full confluency (FC) on the percentage of puma fibroblasts in the G0/G1, S and G2/M phases of the cycle.

Conditions	**Cell cycle phase (%)**			
	G_0/G_1	**S**	**G_2/M**	**Sub G_0/G_1**
GC	73.9 ± 3.0^{a}	10.4 ± 2.3^{a}	15.7 ± 1.2^{a}	0.5 ± 0.0^{a}
FC 24 h	84.0 ± 1.8^{b}	7.8 ± 2.3^{ab}	8.3 ± 1.1^{b}	5.4 ± 1.1^{b}

FC 48 h	84.6 ± 0.6[b]	3.9 ± 1.2[ab]	11.5 ± 1.2[ab]	12.6 ± 1.6[c]
FC 72 h	84.2 ± 1.0[b]	2.3 ± 1.1[b]	13.5 ± 1.1[a]	17.8 ± 1.7[c]

Table 2 – Effect of serum starvation (SS) on the percentage of puma fibroblasts in the G0/G1, S and G2/M phases of the cycle.

Conditions	**Cycle cell phase (%)**			
	G_0/G_1	**S**	**G_2/M**	**Sub G_0/G_1**
GC	73.9 ± 3.0[a]	10.4 ± 2.3[a]	15.7 ± 1.2[a]	0.5 ± 0.0[a]
SS 24 h	81.3 ± 1.0[a]	6.0 ± 1.0[a]	12.7 ± 0.6[ab]	0.7 ± 0.1[a]
SS 48 h	80.4 ± 3.0[a]	6.1 ± 1.4[a]	13.5 ± 1.7[ab]	0.9 ± 0.4[a]
SS 72 h	78.4 ± 4.3[a]	6.5 ± 2.6[a]	14.3 ± 1.7[ab]	1.0 ± 0.2[a]
SS 96 h	85.4 ± 3.4[b]	6.3 ± 1.5[a]	8.3 ± 2.4[b]	2.3 ± 0.3[b]

Table 3 – Effect of roscovitine on the percentage of puma fibroblasts in the G0/G1, S and G2/M phases of the cycle.

Conditions	**Cycle cell phase (%)**			
	G_0/G_1	**S**	**G_2/M**	**Sub G_0/G_1**
GC	73.9 ± 3.0	10.4 ± 2.3	15.7 ± 1.2	0.5 ± 0.0
RO 12 h	78.6 ± 3.5	6.6 ± 1.7	14.8 ± 2.1	1.1 ± 0.3
RO 24 h	82.1 ± 4.5	6.5 ± 2.2	11.4 ± 2.5	1.9 ± 0.8

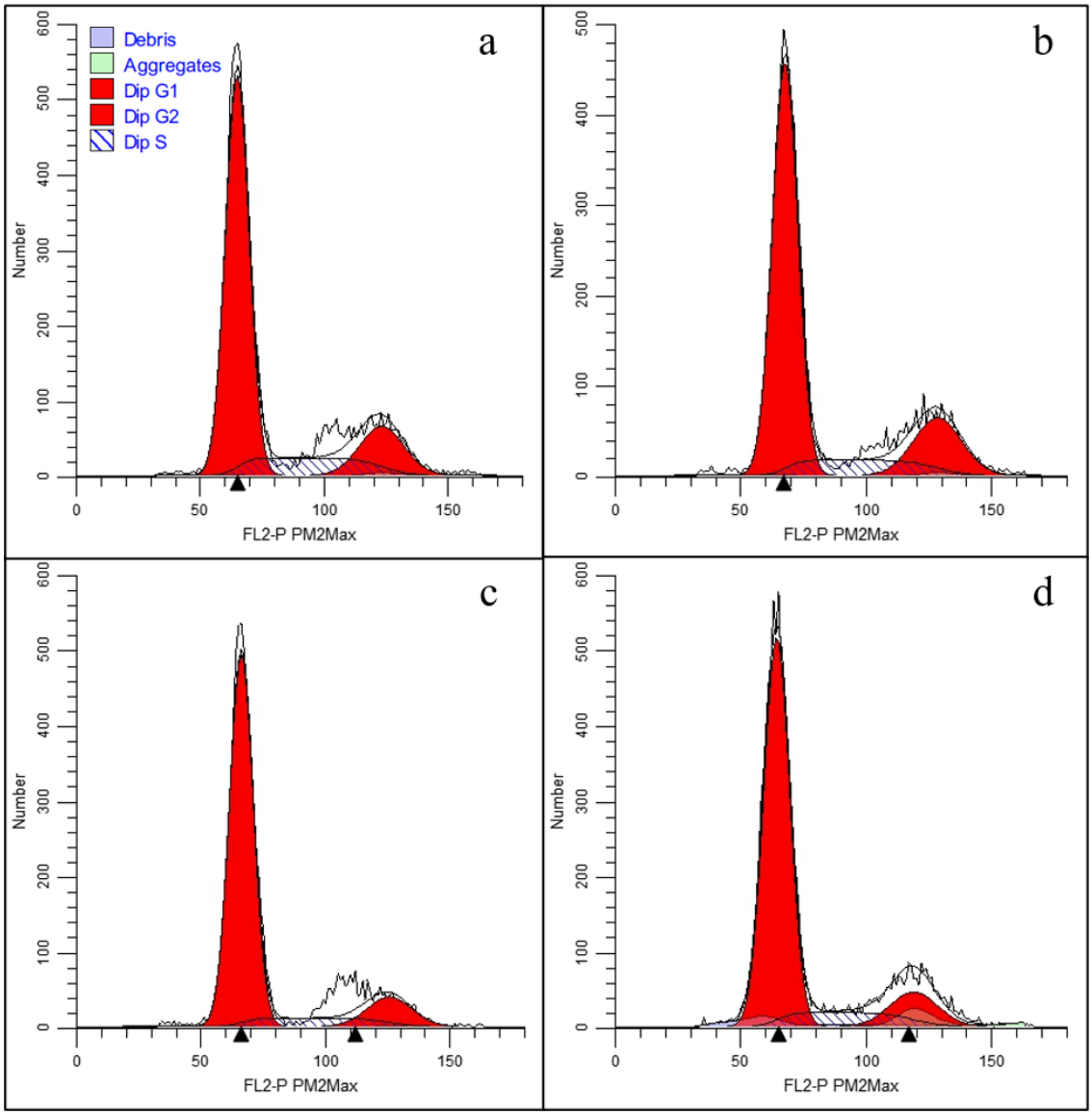


Figure 4. Representative histograms of the flow cytometry analysis of puma fibroblasts. (a) growing cells; (b) full confluency treatment; (c) serum starvation treatment, and (d) roscovitine treatment.

Discussion

The establishment of karyoplasts as a stage of SCNT is fundamental for developing this technique in a species, especially regarding the synchronization of the cell cycle since the stage of the cycle in which they are found is crucial to guarantee the maintenance of normal ploidy

838 in the embryo reconstructed (Campbell, 1996). In this study, we have developed a suitable
839 protocol for somatic cell synchronization in pumas. To our knowledge, this is the first work to
840 elucidate nuclear reprogramming in the cells of pumas.

841 Thus, it was observed that FC for 24 h was more efficient in synchronizing the cell cycle
842 in puma fibroblasts than SS and RO, as it promoted a greater arrest of cells in the G_0/G_1 phase
843 when compared to the other methods. Although no difference was observed for the three times
844 tested (24, 48, and 72 h), we defined the 24 h time as the best because it presented the lowest
845 percentage of cells in sub-G_0/G_1, indicating apoptosis, when compared to the CG group.
846 Regarding the viability tests and apoptosis levels, it was observed that the FC treatment at the
847 three evaluated times did not change these parameters, and no difference was observed between
848 the experimental groups and the CG group.

849 Our findings corroborate what was observed in different studies with wild felids and
850 domestic cats. Gómez et al. (2003) observed that 5 days of FC was more efficient in increasing
851 the percentage of fibroblasts in G_0/G_1 of domestic cats (*Felis silvestris catus*) than of African
852 wild cats (*Felis silvestris libica*) (88% vs. 61%, respectively). Song et al. (2007) defined FC
853 (80–90%) as the best methodology for tiger (*Panthera tiger*) fibroblasts. In studies with the
854 Asian golden cat (*Catopuma temminckii*), leopard (*Panthera pardus*), marbled cat (*Pardofelis*
855 *marmorata*), and Siamese cat, Wittayarat et al. (2013) observed that FC for 5 days promoted an
856 increase of more than 80% in the percentage of cells in G_0/G_1 when compared to non-
857 synchronized cells, without an increase in apoptotic cells in all species, except for the marbled
858 cat. Still, in the domestic cat, 3–5 days of FC was effective in inducing a higher percentage of
859 G_0/G_1 fibroblasts (~80–85%) compared to growing cells; however, in kodkod (*Leopardus*
860 *guigna*), FC was efficient after 1–3 days, but not after 5 days of treatment (Veraguas et al.,
861 2017).

Recently, Młodawska et al. (2022) observed that in Pallas's cat (*Otocolobus manul*; *Felis manul*) or jaguarundi (*Puma yagouaroundi*; *Harpailurus yagouaroundi*), FC alone did not cause a major change in the proportion of quiescent cells. However, in the domestic cat, FC prolonged more efficiently than the same period of SS at 40–50% confluence. These authors also point out that cell culture to total confluence in jaguarundi can be a valuable method to obtain a high proportion of skin fibroblasts in the G_0/G_1 phase without causing damage to the cells, which corroborates our work using cells from animals of the same genus.

As for SS, only the 96-h group increased the proportion of puma fibroblasts in G_0/G_1, showing a significant difference from the CG group. The viability test by exclusion by the trypan blue assay did not show significant results, with the cells remaining viable after the time intervals analyzed. This assay is based on assessing plasma membrane integrity and showed that SS does not adversely affect the cell membrane. However, regarding the intracellular levels of apoptosis, it was observed that SS affects this parameter in terms of the percentage of viable cells and necrotic cells. Additionally, we observed that this group increased the proportion of cells in sub-G_0/G_1, a negative effect.

Our results regarding SS synchronization differ from what Gómez et al. (2003) observed in the domestic cat and the African wild cat, which on SS for 5 days, generated a higher proportion of skin fibroblasts in the G_0/G_1 phase than treatment with FC and RO. However, these authors observed that SS induced higher DNA fragmentation rates in both species' fibroblasts. In other felid species, an increasing incidence of apoptosis was observed after 4–5 days of SS for Siamese cat and marbled cat fibroblasts, but not for leopard or Asian golden cat cells (Wittayarat et al., 2013).

Veraguas et al. (2017) observed that in the domestic cat, SS and FC, for both 3 and 5 days, similarly increased the proportion of fibroblasts trapped in the G_0/G_1 phase. Nevertheless, SS for 5 days significantly reduced the fibroblast viability of these animals. Still, SS for 3 and

887 5 days produced the highest proportion of kodkod fibroblasts in the G_0/G_1 phase; however, after
888 viability evaluation, only SS for 5 days significantly reduced the fibroblast viability of this wild
889 felid.

890 Recently, in manul and jaguarundi (Młodawska et al., 2022), the culture of G50
891 (growing cells at 50% of confluence) and G70 (growing cells at 70% of confluence) under SS
892 conditions resulted in a high proportion of fibroblasts in the G_0/G_1 phase, while in the domestic
893 cat, this treatment was efficient only at the G50 confluence. In manul, the fastest effect of SS
894 on the fibroblast cell cycle (after only one day of treatment) was observed at the G70
895 confluence, while in jaguarundi, at the G50 confluence, demonstrating there is a different
896 response of growing cells to the SS and that this response depends on the level of cell confluence
897 and the treatment duration. These variations between our findings and those of other authors
898 may be due to individual characteristics of the animal (species, breed, sex, and age) from which
899 the cells were obtained, as well as the cell types and culture conditions used (Młodawska et al.,
900 2022).

901 In the third experiment of this study, we evaluated the efficiency of RO in fibroblast
902 synchronization and its influence on cell membrane viability and apoptotic levels. The cell
903 membrane remained viable when using RO at both evaluated times. Furthermore, it was
904 observed that this chemical compound did not promote cell cycle synchronization in puma
905 fibroblasts, not having differed from the percentage of cells in G_0/G_1 of the CG group. For the
906 levels of apoptosis, from our data, it is possible to infer that the concentration of 15 µM does
907 not affect these parameters since these data remained similar to the control group.

908 Apparently, the effect of RO depends on the concentration used and the species under
909 study, as shown by the results observed by Wittayarat et al. (2013), where, in the Asian golden
910 cat and the Siamese cat, treatment with RO greater than 7.5 µM significantly increased the
911 proportion of G_0/G_1 phase cells compared to the CG group. In contrast, the treatment of marbled

912 cat cells with more than 15 µM of RO produced the highest percentage of cells in the G_0/G_1
913 stage compared to the control groups. However, there was no apparent effect of RO treatment
914 on the leopard (Wittayarat et al., 2013). Furthermore, it was observed that treatment with 30
915 µM roscovitine significantly increased the proportions of apoptotic cells in the Asian golden
916 cat and leopard compared to the control group (Wittayarat et al., 2013).
917
918 **Conclusion**
919 In summary, full confluency treatment successfully induces puma fibroblast cell cycle
920 synchronization at the G_0/G_1 stage without affecting cell viability. These results may be
921 valuable for planning donor cells for somatic cell nuclear transfer in pumas. Thus, we
922 established the last preparation step for using these fibroblasts as karyoplasts with potential
923 application for conservation.
924
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931
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CONSIDERAÇÕES FINAIS

O presente trabalho foi o primeiro a comparar diferentes soluções crioprotetoras na criopreservação de células somáticas de onças-pardas mantidas em cativeiro. Além disso, foi o primeiro a comparar e avaliar a eficiência de diferentes métodos de sincronização do ciclo celular em G_0/G_1, sendo então um estudo pioneiro sobre onças-pardas nesta área.

Quanto à criopreservação, as células foram avaliadas quanto à sua morfologia, viabilidade, atividade proliferativa, metabólica e indicativos de apoptose após a descongelação e, a partir dos resultados obtidos, foi possível observar que a redução da concentração dos crioprotetores intracelulares de 10% para 2,5%, bem como a presença ou ausência de sacarose nas soluções, mostraram-se efetivas para a conservação de fibroblastos na referida espécie. Ainda, o EG mostrou-se como um crioprotetor alternativo que pode ser utilizado com eficiência para esta espécie, uma vez que os resultados foram semelhantes aos resultados das soluções compostas pelo DMSO, o crioprotetor intracelular mais utilizado na criopreservação de células somáticas em felídeos.

Quanto à comparação entre metodologias de sincronização do ciclo celular, foi observado que o método de inibição por contato foi o mais eficiente para células dessa espécie. Este método induziu uma taxa que variou entre 84,0% e 84,2% de células sincronizadas, sendo a elucidação desta etapa de fundamental importância para o desenvolvimento de biotecnologias voltadas para a conservação.

Portanto, com os resultados obtidos por este trabalho, conclui-se o estabelecimento das etapas necessárias para a formação de bancos somáticos, as quais foram iniciadas com trabalhos anteriores oriundos do nosso grupo de pesquisa, sendo o próximo passo o emprego dessas células e metodologias em estudos ainda mais aprofundados, como a clonagem por TNCS e a indução de células a pluripotência. Além disso, destaca-se a possibilidade de utilização de animais mantidos em cativeiros para o desenvolvimento de estratégias de conservação *ex situ*, demonstrando que as estratégias *in situ* e *ex situ* atuam de maneira eficiente quando combinadas.

ANEXOS

ANEXO No. 01: COMPROVANTE DE SUBMISSÃO DO ARTIGO DE REVISÃO: POTENTIAL AND REALITY OF CRYOPRESERVATION OF SOMATIC CELLS FOR CONSERVATION IN WILD FELIDS

Alexsandra Fernandes Pereira <alexsandra.pereira@ufersa.edu.br>

Manuscript Submission

Alexsandra Fernandes Pereira <alexsandra.pereira@ufersa.edu.br> 24 de janeiro de 2023 às 16:54
Para: editor@cryoletters.org

Dear Editor

I would like to submit for evaluation in Cryo-Letters the manuscript titled "Potential and Reality of Cryopreservation of Somatic Cells for Conservation in Wild Felids". All material submitted for consideration is original and is being submitted exclusively to Cryo-Letters.

Yours sincerely,

Alexsandra

--
Profa. Dra. Alexsandra Fernandes Pereira
Bolsista de Produtividade 1D CNPq - Área de Medicina Veterinária
Laboratório de Biotecnologia Animal - LBA
Programa de Pós-Graduação em Ciência Animal - PPGCA
Universidade Federal Rural do Semi-Árido - UFERSA
Av. Francisco Mota, 572, Costa e Silva, 59.625-900, Mossoró-RN, Brasil
Fone: (85) 9 9903 6715

2 anexos

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ANEXO No. 02: COMPROVANTE DE SUBMISSÃO DO ARTIGO EXPERIMENTAL: COMPARISON BETWEEN CONCENTRATION AND TYPE OF INTRACELLULAR CRIOPROTECTANTS AND THE PRESENCE OF SUCROSE FOR CRYOBANKS OF SOMATIC CELLS DERIVED FORM CAPTIVE PUMAS

UFERSA mail

Alexsandra Fernandes Pereira <alexsandra.pereira@ufersa.edu.br>

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Bethany Krebs <onbehalfof@manuscriptcentral.com> 6 de dezembro de 2022 às 21:21
Responder a: zoobiology@sfzoo.org
Para: alexsandra.pereira@ufersa.edu.br, luannavieira59@gmail.com, yasminbfm8718@gmail.com, joaovitorvianajr@gmail.com, erikaalmeida-@hotmail.com, lharagirs@hotmail.com, herlonvrs@hotmail.com

06-Dec-2022

Dear Dr. Pereira,

It is a pleasure to accept your manuscript entitled "Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas" in its current form for publication in Zoo Biology. The comments of the referee(s) who reviewed your manuscript are included at the bottom of this letter.

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ANEXO No. 03: ARTIGO "COMPARISON BETWEEN CONCENTRATION AND TYPE OF INTRACELLULAR CRIOPROTECTANTS AND THE PRESENCE OF SUCROSE FOR CRYOBANKS OF SOMATIC CELLS DERIVED FORM CAPTIVE PUMAS" PUBLICADO

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RESEARCH ARTICLE

ZOOBIOLOGY WILEY

Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive *Pumas*

Luanna L. V. Rodrigues[1] | Yasmin B. F. Moura[1] | João V. S. Viana[1] | Érika A. Praxedes[1] | Lhara R. M. Oliveira[1] | Herlon V. R. Silva[2] | Alexsandra F. Pereira[1]

[1]Laboratory of Animal Biotechnology, Department of Biosciences, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil

[2]Laboratory of Reproduction of Carnivorous, Faculty of Veterinary, Ceara State University, Fortaleza, CE, Brazil

Correspondence
Alexsandra F. Pereira, Laboratory of Animal Biotechnology, Department of Biosciences, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró - RN, 59625900, Brazil.
Email: alexsandra.pereira@ufersa.edu.br

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Abstract

The loss of wild biodiversity has prompted the development of cryobanks, such as those of somatic cells. This is the reality of *Pumas*, wild felids of ecological importance that suffer from anthropogenic actions, population decline, and subsequent loss of genetic diversity. Somatic cell banks are a strategy for conserving population diversity. We compared different concentrations and types of intracellular cryoprotectants (dimethyl sulfoxide, DMSO; ethylene glycol, EG) associated with 0.2 M of sucrose (SUC) in the cryopreservation of the somatic cells of captive *Pumas*. The cells were cryopreserved by slow freezing with different solutions containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum and varying concentrations of DMSO and EG in the absence or presence of SUC. The cells were analyzed for morphological characteristics, viability, proliferative activity, metabolic activity, and apoptosis levels. Cells maintained similar fusiform morphology before and after cryopreservation. There was no difference in viability, regardless of the reduction in the concentration and type of intracellular cryoprotectants and sucrose. Similarly, proliferative activity, metabolic activity, and apoptosis levels were not altered by the composition of the cryoprotectants. In summary, we demonstrate that reducing the concentration of DMSO or EG ensures adequate cryopreservation of Puma somatic cells, regardless of the presence of SUC.

KEYWORDS
cell recovery, ex situ conservation, *Puma* genus, wildlife biodiversity

1 | INTRODUCTION

In recent years, zoos have become institutions for the maintenance and exhibition of wild species collections and spaces for elaborating and developing conservation strategies (Stadtländer, 2018). This is due to the loss of wildlife biodiversity, which has resulted in the establishment of different conservation tools linked to ex situ and in situ proposals (Pizzutto et al., 2021). Among these propositions, the formation of cryobanks, especially of somatic cells, can be an interesting strategy for conserving the population diversity of endangered species (Ryder & Onuma, 2018), not requiring maintaining many animals in a reduced space (Praxedes et al., 2018). Additionally, these cells can be used both in the reproductive (Moulavi et al., 2017) and regenerative perspectives (Echeverry

et al., 2020). An example of this occurred recently in 2020, when the partnership between Frozen Zoo at San Diego Zoo Wildlife Alliance, Revive and Restore and ViaGen Pets and Equine, reported the birth of Przewalski's first horse (*Equus ferus przewalskii*, Revive, & Restore, 2020), an endangered species that belongs to the same genus as horses, zebras, and donkeys. In 2021, the birth of a black-footed ferret (*Mustela nigripes*) produced by the interspecific somatic cell nuclear transfer (iSCNT) was reported from cells preserved for 30 years (US Fish and Wildlife Service, 2021). These are clear examples of how iSCNT and cryobanks of somatic cells are currently being used to preserve endangered genetics, increase genetic variability in animal populations, and restore genomes lost years ago (Gambini et al., 2022).

According to data from the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species, among the 138,300 species assessed, more than 38,500 species are threatened with extinction, including 26% of mammals (IUCN, 2022). This loss of wildlife biodiversity is a significant threat to the ecosystem (Stadtländer, 2018). Among the mammals, all wild felids are threatened with extinction, especially those belonging to the *Felis* genus. Two species are representative of the *Puma* genus: the *Puma* (*Puma concolor* Linnaeus, 1771) and the *Jaguarundi* (*Puma yagouaroundi* Geoffroy, 1803). *P. yagouaroundi* is classified as least concern regarding the risk of extinction. Nevertheless, they have a low population density and a growing threat caused especially by agricultural expansion, which causes habitat loss and fragmentation, directly affecting the survival of individuals (Caso et al., 2015). Specifically, the *Puma* has a wide geographic distribution in the American continent (Guerisoli et al., 2019), is a predator of mammal species, such as collared peccary (*Pecari tajacu*), deer (*Mazzama* ssp.), and paca (*Cuniculus paca*, Ávila-Nájera et al., 2018; Guerisoli et al., 2019). However, according to Nielsen et al. (2015), this species is considered extinct in part of the United States and some areas of South America, especially in the Caatinga biome.

In this scenario, we have sought to develop somatic resource banks for Pumas. In 2021, we established somatic tissue banks for the species (Lira et al., 2021), and recently, we described a protocol for cryopreservation of *Puma* somatic cells using 10% dimethyl sulfoxide (DMSO), 10% fetal bovine serum (FBS), and 0.2 M sucrose (SUC). In this study, cells after cryopreservation showed 79.8% viability and 68.1 h population doubling time (PDT; Lira et al., 2021). In general, the success of somatic cell cryobanks depends on tolerance to the cryopreservation method, the appropriate cryoprotectant, and the ideal concentrations for optimizing cell viability after thawing (Arantes et al., 2021).

The toxicity of intracellular cryoprotectants could be reduced when considering lower concentrations (Arantes et al., 2021) and the presence of extracellular cryoprotectants, such as SUC (León-Quinto et al., 2011). Moreover, the protectant ability of DMSO and ethylene glycol (EG) depends on the species, cell type (Y. Li et al., 2006), and concentration (Arantes et al., 2021). The low molecular weight of EG (62.1 g/mol) and DMSO (78.0 g/mol) ensures high permeability in the cell. However, such permeability may cause osmotic stress (Schneider & Mazur, 1984), provoking cell injury and, therefore, the use of cryoprotectants must be optimized for the type cell and the species of interest (Oliveira et al., 2021). The pathways used by DMSO in its metabolism are not yet fully elucidated. The same is true for the effects they cause in biological systems. However, there are toxic effects associated with its use, and it is recommended that the concentrations are as low as possible but enough to promote a positive effect (Arantes et al., 2021; Otsuki et al., 2002). When metabolized by the endoplasmic reticulum, EG can generate toxic components, including glycolic acid and oxaloacetate, which are harmful to cellular functioning (Castro et al., 2011).

The choice of an appropriate cryoprotectant is essential for generating somatic resource biobanks that allow the development of studies for biodiversity conservation (Dua et al., 2021; Pereira et al., 2018). Therefore, the present study aimed to establish whether the reduction in concentration and the type of intracellular cryoprotectants associated or not with the presence of SUC alters the conservation of *Puma* somatic cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals, solutions, and media

All the reagents, solutions, and media were obtained from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) and FBS were obtained from Gibco-BRL.

2.2 | Ethics statement and animals

The Ethics Committees of the Rural Federal University of Semi-Arid (process no. 23091.010755/2019-32) and Chico Mendes Institute for Biodiversity Conservation (process no. 71804-1) approved all animal experimentation and the care of animals under study. Three healthy *Pumas*, maintained in zoos in northeastern Brazil (Fortaleza, CE, Brazil), were used. Data on age, sex, and recovery location are presented in Table 1.

2.3 | Skin biopsy, establishment, and in vitro culture of somatic cells

Peripheral ear skin samples (1.0–2.0 cm^2) were collected after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®; Zoetis) combined with 5 mg/kg ketamine hydrochloride (Ketalar®; Pfizer) and mechanical containment (Lira et al., 2021). Subsequently, the somatic tissues were transported to the laboratory in DMEM supplemented with 10% FBS and 2% antibiotic–antimycotic solution at 4°C for 4 h.

The tissues were fragmented (9.0 mm^3) and cultured in the laboratory, according to Lira et al. (2022). The samples were cultured in DMEM supplemented with 10% FBS and 2% antibiotic–antimycotic

TABLE 1 Details of the main biological aspects of *Pumas* used in this study

Animal	Estimated age (years)	Sex	Location
P1	5	Female	Ecologic Park Ecopoint
P2	2	Male	Sargento Prata Municipal Zoo
P3	5	Male	Ecologic Park Ecopoint

solution at 38.5°C and 5% CO_2. The culture medium was changed every 24 h. When they reached 70% confluency, the cells were harvested and cultured until the third passage, then subjected to cryopreservation.

2.4 | Study design and cell cryopreservation

The cells were cryopreserved according to eight groups to identify the appropriate cryoprotectant solution. They were diluted in DMEM containing 10% FBS and (i) 2.5% DMSO, (ii) 2.5% DMSO–0.2 M SUC, (iii) 10% DMSO, (iv) 10% DMSO–0.2 M SUC, (v) 2.5% EG, (vi) 2.5% EG–0.2 M SUC, (vii) 10% EG, and (viii) 10% EG–0.2 M SUC. Noncryopreserved and cryopreserved cells were evaluated for morphological characteristics, viability, proliferative activity, metabolic activity, and apoptosis levels.

Cells were resuspended at a final concentration of 1.0×10^5 cells/ml in cryopreservation solution for cryopreservation. The cells remained in contact with the cryopreservation solution for 15 min at 4°C. For the groups cryopreserved with SUC in the medium, cells were in contact with the cryopreservation solution without SUC for 15 min at 4°C, and then for more 15 min at 4°C with SUC. Subsequently, the cell suspension was stored in cryotubes and allocated to a freezing container (Mr. Frosty; Thermo Scientific Nalgene), which was transferred to a −80°C freezers, maintaining a cooling rate of 1°C/min for 12 h until reaching −80°C. Then, the samples were stored in liquid nitrogen (Lira et al., 2022).

2.5 | Thawing

After 2 weeks, the cryovials were kept at 25°C for 1 min and immersed in a water bath at 37°C for 4 min for thawing. Cell suspensions derived from groups containing SUC were added in DMEM plus 10% FBS and 0.2 M SUC and maintained at 4°C for 15 min, followed by centrifugation at 1300*g* for 10 min to remove the cryoprotectants. Subsequently, the supernatant was removed, and cellular content was suspended in DMEM with 10% FBS, maintained at 25°C for 15 min, followed by another centrifugation at 1300*g* for 10 min. Finally, cell suspensions from the groups without SUC were centrifuged twice, as previously described, using DMEM plus 10% FBS. After the first and second medium addition, cells were maintained at 4°C for 15 min and 25°C for 15 min, respectively (Oliveira et al., 2021).

2.6 | Morphological analysis and cell membrane integrity

The morphological characteristics were observed during in vitro culture under an inverted microscope (Nikon TS100) for cell forms and cytoplasmic extensions (Lira et al., 2021). Cell membrane integrity was determined using the trypan blue assay. An aliquot of suspended cells was stained with 0.4% trypan blue (in phosphate-buffered saline) in a 1:1 ratio and counted using a Neubauer chamber. Unstained cells were considered living due to the intact membrane, whereas cells stained with trypan blue were considered dead due to the penetration of the dye. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

2.7 | Analysis of proliferative and metabolism activity

The proliferative activity of cells was quantified according to the growth curve and determination of PDT. The cells (1.0×10^4 cells/ml) were plated in 24-well dishes, trypsinized, and counted. The readings were recorded every 24 h for a period of 168 h. The average counts at regular intervals of 24 h were used to elaborate the growth curve and estimate PDT (Roth, 2006), according to the equation: $PDT = T \ln 2 / \ln (Xe/Xb)$, where PDT is the time of culture (in h), T is the incubation time, Xb is the number of cells at the beginning of the incubation time, Xe is the number of cells at the end of the incubation time, and ln is the Napierian logarithm.

Metabolic activity was determined by 5.0×10^4 cells/ml grown in 12-well dishes. After 5 days, 1.5 ml [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium] (MTT) (5 mg/ml in DMEM) were added and the dishes, which were incubated for 3 h. The MTT solution was removed, and 1.0 ml DMSO was added for 5 min under a stirring process to solubilize the MTT crystals. After the total dissolution of the crystals, the samples were analyzed in a spectrophotometer (Shimadzu® UVmini-1240) using an absorbance wavelength of 595 nm. The values obtained from reading the groups were transformed into percentages by dividing them by the mean value obtained with the non-cryopreserved cells (Silva et al., 2021).

2.8 | Analysis of apoptosis levels

The cells were stained with the fluorescent combination of 2 μg/ml acridine orange and 10 μg/ml ethidium bromide to assess apoptosis levels (Lira et al., 2021). Subsequently, 300 cells/animal/group were analyzed by fluorescence microscopy (Olympus BX51TF) at 480 nm with ×200 magnification and classified into (i) viable cells with a uniform light green nucleus; (ii) early apoptotic cells with a nonuniform green nucleus; (iii) late

apoptotic cells with a nonuniform bright orange nucleus, and (iv) necrotic cells with a uniform orange nucleus. A fluorescence microscope was used to observe apoptotic changes in the stained cells, quantified using the ImageJ software (National Institutes of Health).

2.9 | Statistical analysis

All data were expressed as the mean ± standard error and analyzed using the Stat View software (Graph-Pad Software Incorporation). The normality of all results was verified using the Shapiro–Wilk test, and homoscedasticity was verified using Levene's test. Viability, metabolism, and apoptosis levels were altered with arcsine and analyzed using analysis of variance (ANOVA) followed by the Tukey test. Proliferative activity was compared using ANOVA followed by the unpaired *t*-test. Statistical significance was set at a *p* value less than .05.

3 | RESULTS

Before cryopreservation, the cells from three animals showed viability by the trypan blue assay of 84.2 ± 6.7%, the metabolic activity of 100.0 ± 0.0%, PDT values of 49.1 ± 8.2 h, and 68.7 ± 22.0% of viable cells for the apoptosis levels, with the remaining values of 14.5 ± 7.5% for early apoptosis, 15.2 ± 13.5% for late apoptosis, and 1.7 ± 1.0% for necrosis.

After cryopreservation in eight different cryoprotectants and thawing, *Puma* cells cultured for 10 days under identical conditions, showed similar morphology with a confluence of 60%–100% (Figure 1). Therefore, cell morphology and confluence were not altered after cryopreservation, and the morphological characteristics of these cells included a fusiform shape with cytoplasmic extensions and a central nucleus, like fibroblasts.

Moreover, no difference was observed for viability, regardless of the reduction in the concentration and type of intracellular cryoprotectants and the presence of SUC, ranging from 79.1 ± 8.3% to 91.5 ± 0.6% (Table 2, $p > .05$). Similarly, metabolic activity was not altered by the composition of the cryoprotectants (Table 2, $p > .05$).

All groups showed a sigmoid growth curve for proliferative activity, with cells entering the adaptation phase on Day 1, followed by the exponential phase (Figure 2a). Additionally, an evident phase of decline was observed in all cells of most experimental groups. Also, the cryoprotectant solution did not affect the proliferative activity after thawing ($p > .05$), with PDT values ranging from 43.3 to 92.7 h (Figure 2b).

Finally, the apoptosis levels evaluated for viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells were not altered by the composition of the cryoprotectants ($p > .05$, Figure 3, Table 3).

4 | DISCUSSION

A fundamental step to establishing somatic cell banks of wild felids is the definition of optimized cryopreservation conditions, especially regarding the cryoprotectants, since implementing an ideal cryoprotective solution allows the reduction of cellular cryoinjuries, resulting in the maintenance of cellular parameters (Oliveira et al., 2021). In this study, we demonstrated the efficiency of intracellular (DMSO and EG) and extracellular (SUC) cryoprotectants in maintaining morphology, viability, proliferative activity, metabolic activity, and apoptosis levels of *Puma* cells after thawing. In this sense, we observed that reducing the concentration of intracellular

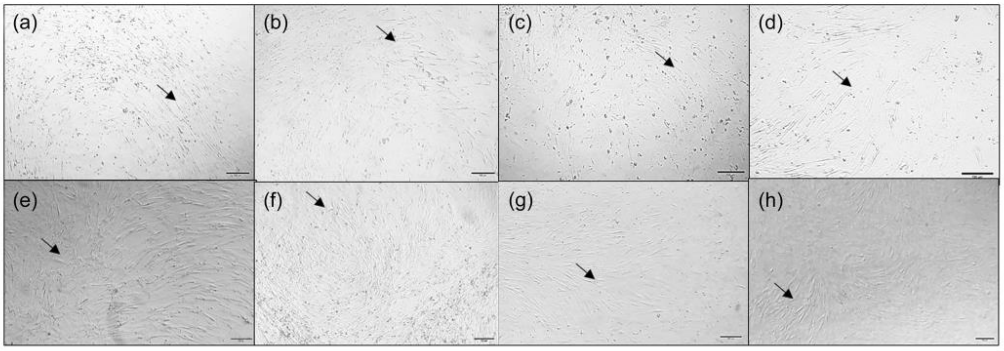

FIGURE 1 Cultures of somatic cells derived from ear skin samples of *Pumas*. Cells after cryopreservation in DMEM containing 10% FBS and (a) 2.5% DMSO, (b) 2.5% DMSO–SUC, (c) 10% DMSO, (d) 10% DMSO–SUC, (e) 2.5% EG, (f) 2.5% EG-SUC, (g) 10% EG, and (h) 10% EG–SUC. Arrows indicate fibroblasts. Scale bar: 100 μm, magnification: ×40. DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EG, ethylene glycol; FBS, fetal bovine serum; SUC, sucrose.

TABLE 2 Viability and metabolic activity of *Puma* cryopreserved somatic cells using different combinations of intracellular cryoprotectants in the presence of sucrose

The concentration of DMSO or EG	Intracellular cryoprotectant	0.2 M sucrose	Viability (%)	Metabolic activity (%)
2.5%	DMSO	–	85.5 ± 1.8	87.6 ± 10.1
	DMSO	+	91.5 ± 0.6	99.9 ± 0.1
10%	DMSO	–	86.7 ± 4.8	99.8 ± 0.1
	DMSO	+	87.4 ± 1.8	94.6 ± 4.3
2.5%	EG	–	89.9 ± 2.6	99.8 ± 0.1
	EG	+	79.1 ± 8.3	87.3 ± 5.4
10%	EG	–	83.5 ± 8.6	94.4 ± 4.5
	EG	+	90.9 ± 2.2	87.2 ± 5.4

Note: Mean ± standard error. $p > .05$.

Abbreviations: DMSO, dimethyl sulfoxide; EG, ethylene glycol.

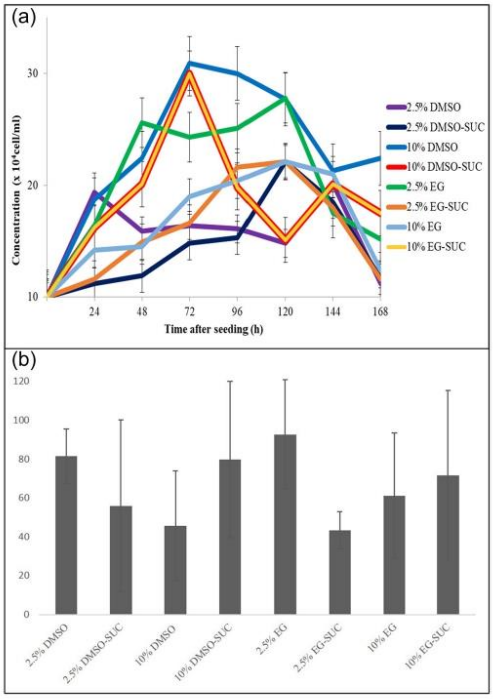

FIGURE 2 Growth dynamics (a) and population doubling time, (b) after culture for 7 days, of *Puma* cryopreserved somatic cells using DMEM containing 10% FBS and 2.5% DMSO, 2.5% DMSO–SUC, 10% DMSO, 10% DMSO–SUC, 2.5% EG, 2.5% EG–SUC, 10% EG, and 10% EG–SUC. Bars indicate standard error. DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EG, ethylene glycol; FBS, fetal bovine serum; SUC, sucrose. $p > .05$. [Color figure can be viewed at wileyonlinelibrary.com]

cryoprotectants, regardless of the presence of SUC, can be useful for the cryopreservation of Puma somatic cells.

Our results corroborate the findings of León-Quinto et al. (2011), who compared three different freezing solutions in Iberian lynx (*Lynx pardinus*) cells (DMSO alone and in combination with 0.1 or 0.2 M SUC). The authors observed viability rates after thawing around 90% for the three freezing solutions used. Cells cryopreserved in the three freezing media presented after thawing metabolic activity values around 85%, with no significant difference between the three groups, demonstrating that intracellular cryoprotectants alone or in conjunction with 0.1 or 0.2 M SUC appear to be very suitable for cryopreserving isolated somatic cells from wild felids. Furthermore, Oliveira et al. (2021) observed that the addition of SUC in the cryoprotectant solution did not influence the viability rate or the metabolic activity of jaguar (*Panthera onca*) cells, with data similar to the cryopreserved cells without SUC and non-cryopreserved cells. Therefore, our result demonstrates that it is possible to cryopreserve cells efficiently without the addition of SUC, as observed in this study.

In the fact, the conservation of *Puma* somatic cells may be due to the cryoprotectants employed. DMSO has been reported as the most explored cryoprotectant in cryopreservation due to its efficiency in reducing the freezing point of cells and its low cost, in addition to being easily miscible in water (Costa et al., 2020; Weng & Beauchesne, 2020). DMSO at 10% has been efficient in the cryopreservation of somatic cells from different wild felids, such as cheetah (*Acinonyx jubatus*, Moro et al., 2015) and jaguar (*P. onca*, Silva et al., 2021). In jaguar, 10% DMSO was efficient for conserving the viability de somatic cells, with values of 73.2% (Silva et al., 2021). Arantes et al. (2021) reported no difference between 2.5% and 10% DMSO in the cryopreservation solution for somatic cells derived from Northern tiger cats (*Leopardus tigrinus*), pampas cats (*Leopardus colocolo*), and jaguars. These works demonstrate that both concentrations act beneficially on the cells of these wild felids. Our findings

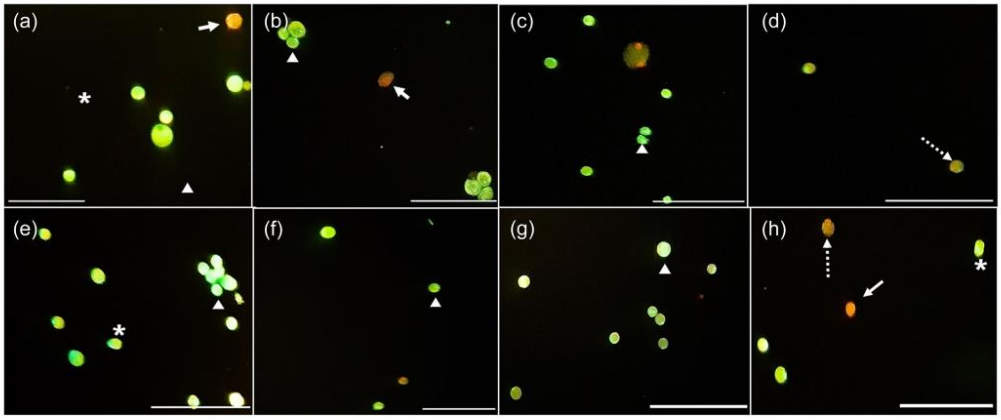

FIGURE 3 Apoptosis levels in *Puma* somatic cells cryopreserved in (a) 2.5% DMSO, (b) 2.5% DMSO–SUC, (c) 10% DMSO, (d) 10% DMSO–SUC, (e) 2.5% EG, (f) 2.5% EG–SUC, (g) 10% EG, and (h) 10% EG–SUC. Viable cells (triangles). Initial apoptotic cells (asterisk). Late apoptotic cells (dotted arrow). Necrotic cells (arrow). Scale bar: 50 μm, magnification: ×40. DMSO, dimethyl sulfoxide; EG, ethylene glycol; SUC, sucrose. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Evaluation of apoptosis levels in *Puma* cryopreserved somatic cells

The concentration of DMSO or EG	Intracellular cryoprotectant	0.2 M sucrose	Cells			
			Viability (%)	Initial apoptotic (%)	Late apoptotic (%)	Necrotic (%)
2.5%	DMSO	–	80.0 ± 3.8	12.0 ± 3.6	2.0 ± 0.6	5.0 ± 0.4
	DMSO	+	69.0 ± 1.1	16.0 ± 2.6	7.0 ± 0.7	8.0 ± 2.7
10%	DMSO	–	76.0 ± 3.4	17.0 ± 5.0	2.0 ± 0.3	5.0 ± 1.5
	DMSO	+	68.0 ± 7.4	18.0 ± 4.9	8.0 ± 1.6	7.0 ± 0.9
2.5%	EG	–	82.0 ± 1.0	13.0 ± 2.1	2.0 ± 0.8	3.0 ± 0.7
	EG	+	78.0 ± 3.4	11.0 ± 2.1	2.0 ± 0.4	9.0 ± 3.4
10%	EG	–	80.0 ± 3.4	10.0 ± 2.5	2.0 ± 0.1	8.0 ± 3.9
	EG	+	80.0 ± 1.3	12.0 ± 0.7	6.0 ± 1.1	3.0 ± 0.4

Note: Mean ± standard error. $p > .05$.

Abbreviations: DMSO, dimethyl sulfoxide; EG, ethylene glycol.

show that the reduction of the cryoprotectant concentration did not affect the cryoprotectant solution, which maintained its protective function.

In studies with wild felids, 10% EG was evaluated as an intracellular cryoprotectant in the cryopreservation of jaguar somatic cells in the presence or absence of SUC, showing viability rates above 50% after thawing and above 95% after in vitro culture of these cells (Oliveira et al., 2021). The EG has also been successfully used in fibroblasts and somatic tissues from other mammals, such as porcine (Y. Li et al., 2006) and collared peccaries (Borges et al., 2018). Also, EG has been successfully used in the cryopreservation of epididymal spermatozoa from domestic cats (Buranaamnuay, 2020) and the vitrification of the epididymal tail (Lima et al., 2021). Our data demonstrate the possibility of using both intracellular cryoprotectants (DMSO and EG) in different concentrations in the presence or absence of SUC in the cryopreservation of felid cells. Additionally, our study is the first to evaluate the efficiency of this cryoprotectant (EG) in *Puma* cells.

Another important aspect is the use of FBS as an extracellular cryoprotectant in all cryopreservation solutions evaluated. In all solutions, the addition of FBS showed a significant impact on cell growth (Oliveira et al., 2021). This stimulatory effect can be attributed to growth factors, collagen, proteins, vitamins, antioxidant properties, trace elements, hormones, and fibronectin supplied by the serum, which collectively play an important role in cell growth, cell adhesion, and cell expansion and maintenance (Hosokawa et al., 1997;

Lira et al., 2021; Silva et al., 2021). These aspects show that the presence of FBS in all solutions increased the efficiency of the cryopreservation used in this study.

Puma somatic cells exhibited normal morphology, showing a fusiform shape with cytoplasmic extensions and a large oval-shaped nucleus, and fast growth patterns, corroborating what had been observed in somatic cells derived from jaguar (Oliveira et al., 2021). It is possible to associate the maintenance of these morphological characteristics with the combination of intra and extracellular cryoprotectants since they act by preventing the formation of intracellular ice crystals (León-Quinto et al., 2014). Nevertheless, cells were used up to the third passage in our study, which may have positively influenced the maintenance of cell growth since, according to Mestre-Citrinovitz et al. (2016), cells after the fourth passage will have their growth level reduced and take longer to reach the desired confluence, besides dying due to cellular senescence (Oliveira et al., 2021).

The trypan blue assay was used to assess cell viability, which is based on evaluating the integrity of the plasma membrane. This assay showed that all cryoprotectant solutions in our study used promoted the maintenance of the cell membrane, with viability rates that range from 85.5% to 91.5%. The reduction of osmotic pressure by cryoprotectants during freezing prevents cell rupture. Therefore, higher viability rates are directly related to the ability of cryoprotectants (DMSO, EG, SUC, and FBS) to protect cell membranes from injuries caused by freezing (Oliveira et al., 2021). Similar rates have already been observed in cryopreserved cells from other wild felids, such as the Siberian tiger (*Panthera tigris altaica*, 98.5%) (Liu et al., 2010), Bengal tiger (*Panthera tigris tigris*, 97.6%) (Guan et al., 2010), and jaguar (87.6%–91.2%, Silva et al., 2021). Evaluating the viability through the membrane integrity is essential for optimizing slow-freezing protocols since cells with ruptured membranes are not viable for future applications because they present initial apoptosis (Park et al., 2017).

In the present study, metabolic activity rates ranged between 87.2% and 99.9%, demonstrating that cells remained metabolically active after cryopreservation and thawing. Our results are similar to those observed in jaguars by Oliveira et al. (2021), who obtained rates above 70% after MTT assay in somatic cells from these animals. Silva et al. (2021) obtained metabolic activity rates above 90%, using the same assay, in jaguar fibroblasts cultured until the third passage. Furthermore, these data corroborate those found by León-Quinto et al. (2011), who, after cryopreserving Iberian Lynx fibroblasts, found a metabolic activity rate above 80%. The number of passages can explain our good metabolic activity rates. Cells up to the third passage were used in our study, which is considered a low number of passages. It was previously observed that, after several passages, the cells' genetic characteristics could be modified by the culture conditions (Lira et al., 2022). Additionally, a high number of passages could reduce the rate of cellular metabolism, and a minimum number of passages is the most recommended to preserve cellular characteristics (X. C. Li et al., 2009; Mehrabani et al., 2014).

The cellular growth curve was also evaluated as a parameter in analyzing the proliferative activity. It was observed that the PDT value (43.3–92.7 h) for the *Puma* was slightly above the values previously observed in other wild felids, such as leopard (*Panthera pardus*, 26.7 h), lion (*Panthera leo*, 27.2 h) (Yellisetti et al., 2016), Siberian tiger (24.0 h (Liu et al., 2010), and jaguar (22.8 h) (Silva et al., 2021). The experimental groups evaluated in our study showed no difference concerning PDT values, demonstrating that all combinations of cryoprotectants were efficient, and the cells showed high resistance to slow freezing, not affecting the doubling time of their population.

The levels of apoptosis of the cells after cryopreservation was another parameter evaluated. The rate of viable cells that varies between 68.0% and 80.0% was observed between groups. These values are above those obtained by Silva et al. (2021) in jaguar fibroblasts until the third passage (59.3%) using a cryopreservation solution composed of DMEM with 1.5 M DMSO, 0.2 M SUC, and 10% FBS. Furthermore, our values for early apoptosis (10.0%–18.0%) and late apoptosis (2.0%–8.0%) were very low when compared to the values obtained by these authors for the jaguar (29.3%) and (10.7%), respectively. Only the necrosis rate was higher in our study (3.0%–9.0%) when compared to the value observed in jaguars (0.7%), but still, a low rate compared to the rate of viable cells obtained in our study.

In conclusion, we demonstrate that reducing the concentration of DMSO/EG ensures adequate cryopreservation of Puma somatic cells regardless of the presence of SUC. Therefore, all combinations of DMSO or EG in different concentrations (2.5% and 10%), with or without 0.2 M SUC, were efficient for the cryopreservation of *Puma* somatic cells and maintained the cells with good rates of viability, metabolic activity, and proliferative activity. These results represent an advance for establishing somatic resource banks in zoos, aiming mainly at conserving Pumas or phylogenetically close individuals.

AUTHOR CONTRIBUTIONS

Luanna L. V. Rodrigues has designed the study, acquired and analyzed the data, and drafted the paper. Yasmin B. F. Moura, João V. S. Viana, Érika A. Praxedes, and Lhara R. M. Oliveira contributed to the experiments. Herlon V. R. Silva contributed to somatic tissue recovery. Alexsandra F. Pereira designed and guided the experimental work, acquired and analyzed data, drafted the paper, and revised it critically.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding authors upon reasonable request.

ORCID

Alexsandra F. Pereira http://orcid.org/0000-0003-2137-854X

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ANEXO No. 04: COMPROVANTE DE SUBMISSÃO DO ARTIGO EXPERIMENTAL: FULL CONFLUENCY, SERUM STARVATION AND ROSCOVITINE FOR INDUCING ARREST IN THE G0/G1 PHASE OF CELL CYCLE IN PUMA SKYN-DERIVED FIBROBLAST LINES

Gmail **Luanna Rodrigues <luannavieira59@gmail.com>**

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APÊNDICES

APÊNDICE A: RESUMO CIENTÍFICO SUBMETIDO NO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL, REALIZADO REMOTAMENTE DE 17 A 19 DE NOVEMBRO DE 2022

AVALIAÇÃO DE DIFERENTES CRIOPROTETORES INTRACELULARES NA CRIOPRESERVAÇÃO DE FIBROBLASTOS DERIVADOS DE ONÇAS-PARDAS

Evaluation of different intracellular cryoprotectants in the cryopreservation of puma-derived fibroblasts

Luanna Lorenna Vieira RODRIGUES[1]*; Yasmin Beatriz França MOURA[1], João Vitor da Silva VIANA[1]; Érika Almeida PRAXEDES[1]; Lhara Ricarliany Medeiros de OLIVEIRA[1]; Herlon Victor Rodrigues SILVA[2]; Alexsandra Fernandes PEREIRA[1]

[1]Laboratório de Biotecnologia Animal, Universidade Federal Rural do Semi-Árido, Mossoró, RN, Brasil.

[2]Laboratório de Reprodução de Carnívoros, Universidade Estadual do Ceará, Fortaleza, CE, Brasil.

*luannavieira59@gmail.com

RESUMO

A redução de onças-pardas associada à sua importância ecológica tem resultado no desenvolvimento de bancos de fibroblastos. Em geral, o sucesso destes bancos e sua aplicação na transferência nuclear de célula somática depende da escolha das condições de criopreservação destas células. Portanto, o objetivo foi avaliar dois tipos de crioprotetores intracelulares (dimetilsulfóxido, DMSO e etilenoglicol, EG) na criopreservação de fibroblastos derivados da pele auricular de três onças-pardas. Para tanto, fibroblastos em terceira passagem foram criopreservados por congelação lenta em meio essencial mínimo modificado por Dulbecco (DMEM) contendo 10% de soro fetal bovino e 10% de DMSO (grupo DMSO) ou 10% de EG (grupo EG). Todas as células foram avaliadas para viabilidade usando azul de tripan e atividade metabólica usando o ensaio de brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio. Os dados foram expressos como média ± erro padrão e avaliados por ANOVA seguida de teste Tukey. Nenhuma diferença foi observada para a viabilidade em células criopreservadas com 10% de DMSO (86,7% ± 4,8) e 10% de EG (83,5% ± 8,6). Além disso, nenhuma diferença foi observada para as células criopreservadas quanto à atividade metabólica com 10% de DMSO (99,8% ± 0,07) e 10% de EG (94,4% ± 4,5). Em conclusão, ambos os crioprotetores intracelulares (DMSO e EG) foram eficientes na criopreservação de fibroblastos de onças-pardas. Esses resultados representam informações importantes na formação de bancos de recursos somáticos para a espécie.

Palavras-chave: Felídeos silvestres, criobancos, congelação lenta.

ABSTRACT

The reduction of pumas associated with their ecological importance has resulted in the development of fibroblast banks. In general, the success of these banks and their application in

somatic cell nuclear transfer depends on the choice of cryopreservation conditions for these cells. Therefore, the objective was to evaluate two types of intracellular cryoprotectants (dimethylsulfoxide, DMSO and ethylene glycol, EG) in the cryopreservation of fibroblasts derived from the auricular skin of three puma. For this purpose, third passage fibroblasts were cryopreserved by slow freezing in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal bovine serum and 10% DMSO (DMSO group) or 10% EG (EG group). All cells were evaluated for viability using trypan blue and metabolic activity using the 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide assay. Data were expressed as mean ± standard error and evaluated by ANOVA followed by Tukey test. No difference was observed for viability in cells cryopreserved with 10% DMSO (86.7% ± 4.8) and 10% EG (83.5% ± 8.6). Furthermore, no difference was observed for cryopreserved cells regarding metabolic activity with 10% DMSO (99.8% ± 0.07) and 10% EG (94.4% ± 4.5). In conclusion, both intracellular cryoprotectants (DMSO and EG) were efficient in the cryopreservation of puma fibroblasts. These results represent important information in the formation of somatic resource banks for the species.
Keywords: Wild felids, cryobanks, slow freezing.

INTRODUÇÃO

A onça-parda (*Puma concolor*) é um grande felídeo que ocorre em grande parte das Américas. Esta espécie tem uma distribuição geográfica muito ampla e tolera uma maior variedade de tipos de clima do que as onças-pintadas, por exemplo (ÁVILA-NÁJERA et al., 2018). Essa espécie é listada como de menor preocupação pela IUCN (NIELSEN et al., 2015) embora não ocorram mais em algumas regiões onde antes eram comuns (NOWELL; JACKSON, 1996), como em parte dos Estados Unidos e algumas áreas da América do Sul, principalmente no bioma Caatinga. Por essa razão, a formação de biobancos para esta espécie é fundamental.

Entre os bancos de células que podem ser empregados, visando o desenvolvimento de técnicas de reprodução assistida, têm-se os bancos de células somáticas. Esses bancos quando adequadamente estabelecidos podem ser empregados na clonagem por transferência nuclear de células somáticas (MOULAVI et al., 2017) e na indução de células à pluripotência (VERMA et al., 2013). Dentre os fatores que podem garantir uma maior eficiência da criopreservação está a escolha dos crioprotetores intracelulares (ARANTES et al., 2021; OLIVEIRA et al., 2021).

Portanto, o objetivo foi avaliar dois tipos de crioprotetores intracelulares (dimetilsulfóxido, DMSO e etilenoglicol, EG) na criopreservação de fibroblastos derivados da pele auricular de onças-pardas.

MATERIAL E MÉTODOS

Para o presente estudo, três onças-pardas adultas fêmeas pertencentes à zoológicos de Fortaleza (Ceará, Brasil) foram anestesiadas com 0,04 mg/kg de cloridrato de dexmedetomidina, por via intramuscular, e com auxílio de zarabatana, para a realização das

biópsias de pele (1,0–2,0 cm^2). Após a recuperação dos tecidos e transporte por 3 h a 4 °C em meio essencial mínimo modificado por Dulbecco (DMEM) acrescido de 10% de soro fetal bovino (SFB) e 2% de solução de antibióticos e antimicóticos, tecidos foram cultivados (5% CO_2 e 38,5 °C) para a obtenção de fibroblastos.

As células em terceira passagem foram criopreservadas por congelação lenta, conforme Oliveira et al. (2021), em DMEM contendo 10% de SFB e 10% de DMSO (grupo DMSO) ou 10% de EG (grupo EG). Brevemente, células foram ressuspensas em uma concentração final de $1,0 \times 10^5$ células/mL em solução de criopreservação. As células permaneceram em contato com a solução de criopreservação por 15 min a 4 °C. Em seguida, a suspensão celular foi armazenada em criotubos, e os mesmos transferidos para o recipiente de congelação (Mr. Frosty-Thermo Scientific Nalgene), o qual foi mantido em um freezer -80 °C, a fim de alcançar uma taxa de resfriamento de 1 °C/min por 12 h. Após esse período, as amostras foram armazenadas em nitrogênio líquido (-196 °C).

Após duas semanas, as amostras foram descongeladas e analisadas para viabilidade usando azul de tripan e atividade metabólica usando o ensaio de brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio. Para a análise da viabilidade das células foi realizada a coloração por azul de tripan, onde a taxa de sobrevivência foi calculada como a razão entre o número de células vivas e o número total de células × 100. Para a análise metabólica, células foram cultivadas em placas de 12 poços. Após 5 dias, a solução de [3-(4,5-dimetiltiazol-2il)-2,5-difenil tetrazólio] (MTT) foi adicionada e as placas incubadas. Posteriormente, a solução foi removida e 1,0 mL de DMSO foi adicionado sob agitação lenta para solubilização dos cristais de MTT. Após a dissolução total dos cristais, as amostras foram analisadas em espectrofotômetro a 595 nm.

Finalmente, os dados foram expressos como média ± erro padrão e avaliados por ANOVA seguida de teste Tukey, usando o software Graphpad Instat 3.06 (GraphPad Software Inc., La Jolla, EUA).

RESULTADOS E DISCUSSÃO

Após criopreservação e cultivo *in vitro* (Fig. 1), nenhuma diferença foi observada para a viabilidade em células criopreservadas com 10% de DMSO (86,7% ± 4,8) e 10% de EG (83,5% ± 8,6, Fig 2). Este ensaio mostrou que as soluções crioprotetoras utilizadas promoveram a manutenção da membrana celular. Taxas semelhantes já foram observadas em células criopreservadas de outros felídeos silvestres, como tigre siberiano (*Panthera tigris altaica*,

98,5%) (LIU et al., 2010), tigre de bengala (*Panthera tigris tigris*, 97,6%) (GUAN et al., 2010) e onça-pintada (*Panthera onca*, 87,6% a 91,2%, SILVA et al., 2021).

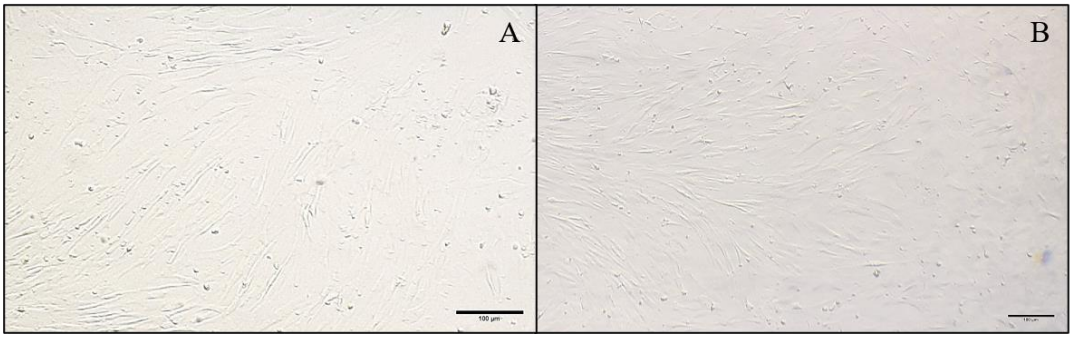

Fig. 1: Cultivo de fibroblastos derivados de amostras da pele auricular de pumas. Células após criopreservação em DMEM contendo 10% de SFB e (A) 10% de DMSO e (B) 10% de EG. Barra de escala: 100 µm, ampliação: 40×.

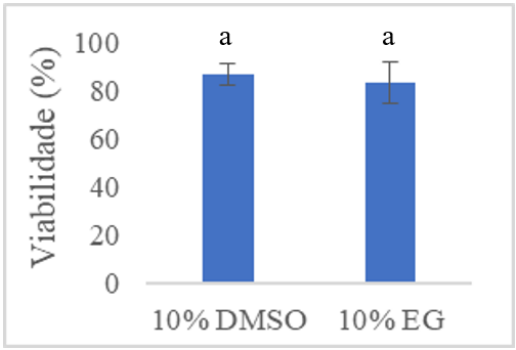

Fig. 2: Efeito da criopreservação sobre a viabilidade de fibroblastos derivados da pele auricular de pumas após a descongelação. [a,b:] letras indicam diferenças entre os grupos (P > 0,05).

Além disso, nenhuma diferença foi observada para as células criopreservadas quanto à atividade metabólica com 10% de DMSO (99,8% ± 0,07) e 10% de EG (94,4% ± 4,5, Fig. 3). Nossos resultados são semelhantes aos observados em células somáticas de outro grande felídeo silvestre. Em onça-pintada, Oliveira et al. (2021), relataram taxas acima de 70% após o ensaio de MTT em células desses animais. Ainda, Silva et al. (2021) obtiveram taxas de atividade metabólica acima de 90%, utilizando o mesmo ensaio, em fibroblastos da mesma espécie cultivados até a terceira passagem.

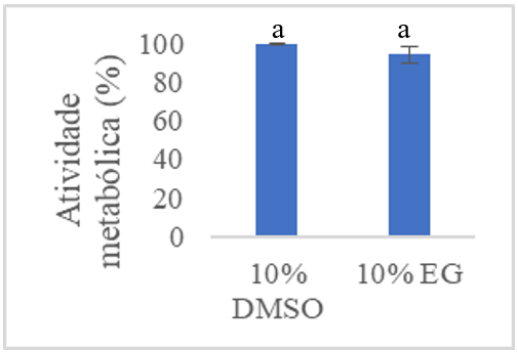

Fig. 3: Efeito da criopreservação sobre a atividade metabólica de fibroblastos derivados de pumas após a descongelação. [a,b:] letras indicam diferenças entre os grupos (P > 0,05).

CONCLUSÃO

Ambos os crioprotetores intracelulares (DMSO e EG) foram eficientes na criopreservação de fibroblastos de onças-pardas. Esses resultados representam informações importantes na formação de bancos de recursos somáticos para a espécie.

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APENDICE B: RESUMO CIENTÍFICO SUBMETIDO NO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL, REALIZADO REMOTAMENTE DE 17 A 19 DE NOVEMBRO DE 2022

EFEITO DA INIBIÇÃO POR CONTATO SOBRE A SINCRONIZAÇÃO EM G_0/G_1 DE FIBROBLASTOS DERIVADOS DE ONÇAS-PARDAS

Effect of contact inhibition on G_0/G_1 synchronization of puma-derived fibroblasts

Luanna Lorenna Vieira RODRIGUES[1*]; Yasmin Beatriz França MOURA[1], João Vitor da Silva VIANA[1]; Lhara Ricarliany Medeiros de OLIVEIRA[1]; Herlon Victor Rodrigues SILVA[2]; José de Brito VIEIRA NETO[3]; Claudia PESSOA[3]; Alexsandra Fernandes PEREIRA[1]

[1]Laboratório de Biotecnologia Animal, Universidade Federal Rural do Semi-Árido, Mossoró, RN, Brasil. [2]Laboratório de Reprodução de Carnívoros, Universidade Estadual do Ceará, Fortaleza, CE, Brasil. [3]Laboratório de Oncologia Experimental, Universidade Federal do Ceará, Fortaleza, CE, Brasil *luannavieira59@gmail.com

RESUMO

A clonagem por transferência nuclear consiste em uma alternativa para a conservação de onças-pardas. Nesse contexto, a otimização das etapas envolvidas nessa biotécnica, como a sincronização dos fibroblastos (carioplastos) em G_0/G_1, são cruciais para o seu sucesso na espécie. Portanto, o objetivo foi avaliar o efeito da inibição por contato sobre a sincronização em G_0/G_1 de fibroblastos derivados de onças-pardas. Para tanto, foram utilizados fibroblastos criopreservados da terceira passagem obtidos de fragmentos de pele de três fêmeas adultas. Após a descongelação, fibroblastos cultivados e com 90–100% de confluência foram avaliados por 24 h e 48 h quanto à viabilidade e sincronização em G_0/G_1. Fibroblastos em 70% de confluência foram empregados como o grupo controle. Para análise do ciclo celular, células ao final de cada período foram tripsinizadas, centrifugadas, fixadas em etanol e armazenadas a -4°C. Posteriormente, células foram incubadas em solução composta por iodeto de propídio (20 µg/mL) e RNAse (50 µg/mL) por 50 min. Subsequentemente, todas as células foram analisadas por citômetro 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 (G_0/G_1, S, G2/M) usando o software MODFIT. Os dados foram expressos como média ± erro padrão e analisados pelo software GraphPad. Assim, fibroblastos submetidos à inibição por contato por 24 h (84,0% ± 1,8) e 48 h (84,6% ± 0,6) apresentaram um maior percentual de G_0/G_1, quando comparados aos fibroblastos não submetidos à sincronização (73,9% ± 3,0, $P < 0,05$). Além disso, nenhuma diferença foi observada entre os fibroblastos não sincronizados (10,4% ± 2,3) e sincronizados por 24 h (7,8% ± 2,3) e 48 h (3,9% ± 1,2) para a fase S. Adicionalmente, um menor percentual em G_2/M foi observado para o grupo sincronizado com 24 h, quando comparado aos demais grupos. Portanto, inibição por contato por 24 h ou 48 h promoveu a sincronização de

fibroblastos de onças-pardas em G_0/G1. Estes resultados são relevantes para o desenvolvimento da técnica de clonagem em onça-parda.
Palavras-chave: felídeos silvestres, reprodução assistida, clonagem, carioplastos.

ABSTRACT

Cloning by nuclear transfer is an alternative for the conservation of pumas. In this context, the optimization of the steps involved in this biotechnique, such as the synchronization of fibroblasts (karyoplasts) in G0/G1, are crucial for its success in the species. Therefore, the objective was to evaluate the effect of contact inhibition on G0/G1 synchronization of fibroblasts derived from puma. For this purpose, cryopreserved third passage fibroblasts obtained from skin fragments of three adult females were used. After thawing, cultured fibroblasts with 90–100% confluence were evaluated for 24 h and 48 h for viability and synchronization in G0/G1. Fibroblasts at 70% confluence were used as the control group. For cell cycle analysis, cells at the end of each period were trypsinized, centrifuged, fixed in ethanol and stored at -4°C. Subsequently, cells were incubated in a solution composed of propidium iodide (20 μg/ml) and RNAse (50 μg/ml) for 50 min. Subsequently, all cells were analyzed by flow cytometer. For each sample, 15,000 events were recorded, and histograms generated to assess the percentage of cells in each cell cycle phase (G0/G1, S, G2/M) using MODFIT software. Data were expressed as mean ± standard error and analyzed using the GraphPad software. Thus, fibroblasts submitted to contact inhibition for 24 h (84.0% ± 1.8) and 48 h (84.6% ± 0.6) showed a higher percentage of G0/G1 when compared to fibroblasts not submitted to synchronization (73.9% ± 3.0, $P < 0.05$). Furthermore, no difference was observed between non synchronized (10.4% ± 2.3) and synchronized fibroblasts for 24 h (7.8% ± 2.3) and 48 h (3.9% ± 1.2) for the S phase. Additionally, a lower percentage in G2/M was observed for the group synchronized with 24 h, when compared to the other groups. Therefore, contact inhibition for 24 h or 48 h promoted the synchronization of puma fibroblasts in G0/G1. These results are relevant for the development of the puma cloning technique.
Keywords: Wild felids, assisted reproduction, cloning, karyoplasts.

INTRODUÇÃO

A onça-parda possui ampla distribuição geográfica no continente americano, habitando desde áreas desérticas até regiões montanhosas (GUERISOLI et al., 2019). Nestes habitats, essa espécie apresenta importantes papéis ecológicos, como a função de superpredador, atuando no controle populacional de mesopredadores (NÁJERA et al., 2018; GUERISOLI et al., 2019). De acordo com a União Internacional para a Conservação da Natureza (NIELSEN et al., 2015), a espécie é classificada como pouco preocupante quanto ao risco de extinção. Contudo, os referidos autores descreveram que a onça-parda já se encontra extinta em parte dos Estados Unidos e algumas áreas da América do Sul, principalmente no bioma Caatinga.

Neste cenário, estratégias de conservação têm sido propostas, tais como o uso da clonagem por transferência nuclear de células somáticas. Nesta técnica, o desenvolvimento da etapa de sincronização dos fibroblastos (carioplastos) em G_0/G_1 é crucial para o sucesso da clonagem na espécie (VERAGUAS et al., 2017). Entre os métodos de sincronização do ciclo

celular já empregados têm-se a inibição por contato, que embora apresente resultados promissores em diferentes espécies, sua resposta ainda é espécie-específica e depende do tempo de avaliação, sendo necessária sua avaliação em fibroblastos de onças-pardas.

Portanto, o objetivo foi avaliar o efeito da inibição por contato sobre a sincronização em G_0/G_1 de fibroblastos derivados de onças-pardas.

MATERIAL E MÉTODOS

Fibroblastos criopreservados da terceira passagem obtidos de fragmentos de pele de três fêmeas adultas foram empregados no presente trabalho. Após a descongelação, fibroblastos cultivados e com 90–100% de confluência foram avaliados por 24 h e 48 h quanto à viabilidade e sincronização em G_0/G_1. Fibroblastos em 70% de confluência foram empregados como o grupo controle.

Para análise do ciclo celular, células ao final de cada período foram tripsinizadas, centrifugadas, fixadas em etanol e armazenadas a -4°C. Posteriormente, células foram incubadas em solução composta por iodeto de propídio (20 μg/mL) e RNAse (50 μg/mL) por 50 min. Subsequentemente, todas as células foram analisadas por citômetro de fluxo (Guava Technologies, Stamford, Lincolnshire, United Kingdom). Para cada amostra, 15.000 eventos foram registrados, e histogramas de fluorescência vermelha vs. contagens foram gerados para avaliar os percentuais de células para cada fase do ciclo celular (G_0/G_1, S, G_2/M). A proporção de células em cada fase do ciclo celular foi avaliada usando o software MODFIT versão 5.0.

Finalmente, os dados foram expressos como média ± erro padrão e analisados pelo software Graphpad Instat 3.06 (GraphPad Software Inc., La Jolla, EUA). Todos os resultados foram verificados quanto à normalidade pelo teste de Shapiro-Wilk e homocedasticidade pelo teste de Levene. Como os dados não apresentaram distribuição normal, eles foram transformados em arco-seno e analisados por ANOVA seguido de teste de Tukey, a $P < 0,05$.

RESULTADOS E DISCUSSÃO

Após a análise em citometria de fluxo (Fig. 1A), os fibroblastos submetidos à inibição por contato por 24 h (84,0% ± 1,8) e 48 h (84,6% ± 0,6) apresentaram um maior percentual de G_0/G_1, quando comparados aos fibroblastos não submetidos à sincronização (73,9% ± 3,0, Fig. 1).

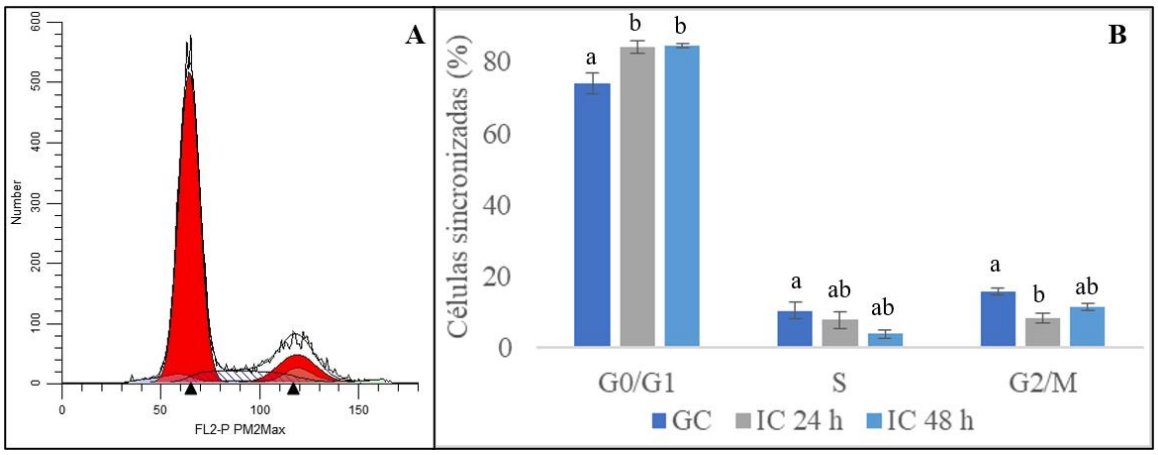

Fig. 1: Efeito da inibição por contato sobre a sincronização do ciclo de fibroblastos de onças-pardas. **(A)** Histogramas representativo de células submetidas à sincronização. **(B)** Percentuais de G_0/G_1, S e G_2/M em fibroblastos de onças-pardas submetidos à sincronização por inibição por contato. [a,b:] letras indicam diferenças entre os grupos na mesma fase ($P < 0,05$).

Além disso, nenhuma diferença foi observada entre os fibroblastos não sincronizados (10,4% ± 2,3) e sincronizados por 24 h (7,8% ± 2,3) e 48 h (3,9% ± 1,2) para a fase S. Adicionalmente, um menor percentual em G_2/M foi observado para o grupo sincronizado com 24 h (8,3% ± 1,1), quando comparado ao grupo controle (15,7% ± 1,2) e sincronizado por 48 h (11,5% ± 1,2, $P < 0,05$).

Esses resultados corroboram com aqueles observados em outros felídeos silvestres, tais como leopardo, tigre e leão (YELISETTI et al., 2016) e em gato doméstico (VERAGUAS et al., 2017). Em geral a eficiência da inibição por contato ocorre em virtude da privação das condições ideais de nutrição e ambiente, permitindo a célula permanecer em G_0/G_1 do ciclo (KUES et al., 2000).

CONCLUSÃO

Esses resultados indicam que a inibição por contato por 24 h ou 48 h promoveu a sincronização de fibroblastos de onças-pardas em G_0/G_1. Tais informações são relevantes para o desenvolvimento da técnica de clonagem em onça-parda.

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APÊNDICE C: COMPROVANTE DO CERTIFICADO DE REVISÃO E EDIÇÃO DE LINGUAGEM E GRAMÁTICA DA LÍNGUA INGLESA DO ARTIGO “COMPARISON BETWEEN CONCENTRATION AND TYPE OF INTRACELLULAR CRYOPROTECTANTS AND THE PRESENCE OF SUCROSE FOR CRYOBANKS OF SOMATIC CELLS DERIVED FROM CAPTIVE PUMAS”.

REVIEW
CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas

Authors: Luanna L. V. Rodrigues, Yasmin B. F. Moura, João V. S. Viana, Érika A. Praxedes, Lhara R. M. Oliveira, Herlon V. R. Silva, Alexsandra F. Pereira

Was reviewed into the English language and edited for being clearer, according to the scientific writing, by the professional scientific translation team from Cia das Traduções (MS Traduções Ltda.- National Registry of Legal Entities – CNPJ n. 08.966.620/0001-91).

We testify that the manuscript was carefully reviewed for being clearer and with the proper scientific language in English, including grammar, punctuation, spelling and general style. However, the study content and the authors' purposes, as understood from the original text, were not changed anyhow.

The respective author read the manuscript reviewed into English by us before the final review, and the same had the opportunity of selecting the terms and expressions suggested by the reviewer in case of doubts, so that the final version was adjusted according to his intentions and prepared for publishing.

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