



UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL
DOUTORADO EM CIÊNCIA ANIMAL

LEONARDO VITORINO COSTA DE AQUINO

**CULTIVO, CRIOPRESERVAÇÃO E SINCRONIZAÇÃO DO CICLO CELULAR DE
FIBROBLASTOS E PRODUÇÃO *IN VITRO* DE EMBRIÕES COMO
FERRAMENTAS PARA A CONSERVAÇÃO DO PREÁ-DA-CAATINGA (*Galea spixii*
WAGLER, 1831)**

MOSSORÓ

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

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal.

Orientadora: Profa. Dra. Alexsandra Fernandes Pereira

MOSSORÓ

2026

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A657c Aquino, Leonardo Vitorino Costa de.
Cultivo, criopreservação e sincronização do
ciclo celular de fibroblastos e produção in vitro
de embriões como ferramentas para a conservação do
preá-da-caatinga (*Galea spixii* Wagler, 1831) /
Leonardo Vitorino Costa de Aquino. - 2026.
275 f. : il.

Orientadora: Alexsandra Fernandes Pereira.
Tese (Doutorado) - Universidade Federal Rural
do Semi-árido, Programa de Pós-graduação em
Ciência Animal, 2026.

1. Roedores neotropicais. 2. Estratégias de
conservação. 3. Biotecnologias da reprodução. 4.
Células somáticas. 5. Oócitos. I. Pereira,
Alexsandra Fernandes, orient. II. Título.

Ficha catalográfica elaborada por sistema gerador automático em conformidade
com AACR2 e os dados fornecidos pelo(a) autor(a).
Biblioteca Campus Mossoró / Setor de Informação e Referência
Bibliotecária: Keina Cristina Santos Sousa e Silva
CRB: 15/120

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Defendida em: 27/01/2026.



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M. Sc. Leonardo Vitorino Costa de Aquino (UFERSA)
Doutorando

Dedico esse importante título acadêmico a Deus, aos meus pais, Reginaldo Vitorino da Costa e Maria de Fátima Aquino Costa, à minha orientadora, Alexsandra Fernandes Pereira, e ao meu respeito pela ciência e pelos preás.

AGRADECIMENTOS

A Deus, agradeço por me conceder saúde, força, resiliência e discernimento ao longo de toda esta trajetória. Em cada desafio, em cada dúvida e nos momentos de maior cansaço, encontrei amparo na fé, que me sustentou e me conduziu até a conclusão deste trabalho. No dia 21 de dezembro de 2025, ouvi palavras que marcaram profundamente minha caminhada: a importância de despertarmos do nosso sono sem jamais esquecermos dos nossos sonhos, para que possamos realizá-los. Essa mensagem me tocou de forma especial, pois nos momentos de fragilidade, compreendi que o medo muitas vezes caminha lado a lado com a fé. Fé necessária para viver um projeto que só foi possível pela sua permissão. Assim, reconheço que promessas e propósitos se cumpriram, e só me resta agradecer por todas as oportunidades vividas até aqui.

Aos preás da caatinga (*Galea spixii*), razão científica e ética desta tese, registro meu respeito. Reconheço que este estudo só foi possível graças a contribuição desses animais, cuja importância transcende os resultados aqui apresentados. Que este trabalho possa representar um avanço real no conhecimento, na conservação e no uso responsável de biotecnologias aplicadas às espécies silvestres.

Aos meus pais, Reginaldo Vitorino da Costa e Maria de Fátima Aquino Costa, expresso minha mais profunda gratidão. Vocês sempre foram meu alicerce e meu porto seguro, especialmente nos momentos de conversas, reflexões e risadas compartilhadas em nosso fundo de quintal, enquanto meu pai apreciava sua cerveja e minha mãe seu tradicional vinho. Ao longo de toda a minha trajetória acadêmica, estiveram presentes com amor, paciência e incentivo, celebrando conquistas, acolhendo fragilidades e acreditando em mim em todos os momentos. Nada do que construí seria possível sem a base sólida que vocês me ofereceram. Amo profundamente vocês e agradeço por todos os ensinamentos que levarei por toda a vida.

À minha família, em especial a todas as minhas tias e primos(as), agradeço pelo carinho constante e pela torcida sincera ao longo de toda a minha trajetória. O apoio de vocês sempre me fez sentir confiante e motivado, fortalecendo minha caminhada acadêmica e pessoal. Sou grato pela disponibilidade em ajudar sempre que necessário, mesmo à distância, e pelo incentivo presente em todos os momentos. Obrigado pelo apoio e pela presença de sempre.

À minha segunda família, expresso um agradecimento cheio de afeto e reconhecimento. Em nome de Jean Carlos Souza Silva, minha gratidão pelas conversas profundas, pelos conselhos sinceros e pelos momentos de escuta que contribuíram de forma decisiva para meu amadurecimento pessoal e profissional. As trocas ao longo dessa caminhada foram fundamentais para fortalecer minhas escolhas, ampliar minha visão de mundo e enfrentar os

desafios com mais serenidade e consciência. Estendo esse agradecimento à sua família, que sempre me acolheu com carinho, respeito e generosidade, fazendo-me sentir parte de um espaço de cuidado, afeto e pertencimento. Essa convivência, marcada por apoio mútuo e aprendizados constantes, foi essencial para tornar essa trajetória mais leve e humana. Levo comigo cada conversa, cada gesto de apoio e cada ensinamento compartilhado. Sou imensamente grato por essa segunda família, que tanto contribuiu para quem me tornei ao longo desse percurso. Amo todos vocês!

Aos meus amigos Bruno Alves da Costa e José Ítalo da Silva Oliveira, agradeço pela amizade sincera, pelo companheirismo e pelo apoio constante ao longo dessa caminhada. A presença de vocês com conversas, risadas e palavras de incentivo, tornou os momentos mais leves e fortaleceu minha trajetória acadêmica e pessoal. Obrigado por estarem ao meu lado, compartilhando desafios, conquistas e aprendizados, sempre com lealdade e amizade.

À minha orientadora, Profa. Alexsandra Fernandes Pereira, deixo registrado um agradecimento que transcende as palavras formais. Desde o quarto semestre da graduação, você me acolheu com generosidade, sensibilidade e confiança, abrindo portas e criando oportunidades em todos os momentos da minha trajetória acadêmica e profissional. Seu cuidado sempre esteve presente não apenas na orientação científica, mas também na amizade construída ao longo dos anos, marcada pelo respeito, escuta atenta e apoio constante. Esses elementos fizeram toda diferença nos períodos de incerteza, crescimento e amadurecimento. Sua postura ética, crítica e profundamente científica tem o poder de transformar pessoas e formar pesquisadores comprometidos com a excelência. Com você aprendi que o rigor científico pode e deve caminhar lado a lado com a delicadeza, a empatia e a responsabilidade. Você ensinou com firmeza, cobrou com compromisso e orientou caminhos acadêmicos sem jamais deixar de cuidar das pessoas que caminhavam ao seu lado. Essa combinação rara entre excelência científica e sensibilidade humana constitui, sem dúvida, uma das maiores heranças que levo desta trajetória. Sou imensamente grato pela confiança, pelo incentivo constante a pensar criticamente, pela valorização da autonomia intelectual e pela presença firme em todos os momentos da minha vida profissional. Mais do que uma orientadora, você se tornou uma referência ética e científica, uma parceira de caminhada e uma amiga. Este trabalho carrega muito do que aprendi com você na ciência, no estágio a docência e na forma de estar no mundo acadêmico e na vida.

Ao Prof. Dr. Alexandre Rodrigues Silva e ao Prof. Dr. Moacir Franco de Oliveira, expresso minha sincera gratidão por todos os ensinamentos, pelas valiosas contribuições científicas e pelo apoio ao longo desta trajetória. As orientações, discussões e experiências

compartilhadas foram fundamentais não apenas para o desenvolvimento deste trabalho, mas também para a minha formação como pesquisador e profissional. A convivência acadêmica com vocês contribuiu de maneira significativa para o amadurecimento científico, o fortalecimento do senso crítico e a consolidação de valores éticos que levarei por toda a minha carreira. Sou profundamente grato por tudo o que aprendi com vocês.

Ao Laboratório de Biotecnologia Animal (LBA/UFERSA), minha segunda casa, expresso minha sincera gratidão por todo o aprendizado compartilhado ao longo dessa trajetória. Foi nesse espaço que construí, de forma sólida e cotidiana, minha identidade científica, aprendendo não apenas técnicas, mas, sobretudo, a responsabilidade, o rigor e a ética que sustentam o fazer científico. Cada colaborador do laboratório teve papel fundamental nesse processo, contribuindo com conhecimento, dedicação, parceria e apoio nos diferentes momentos da pesquisa. O trabalho coletivo, a troca constante de experiências e o apoio mútuo foram essenciais para o meu crescimento profissional, que não se encerra com a conclusão do doutorado, mas se projeta para uma nova etapa como pesquisador em nível de pós-doutorado. Levo comigo tudo o que foi construído nesse laboratório, incluindo resultados, aprendizados e relações científicas que auxiliarão em meu crescimento pessoal e profissional.

Aos colegas da pós-graduação, Érika Almeida Praxedes, Lhara Ricarliany Medeiros de Oliveira, Luanna Lorena Vieira Rodrigues e Maria Valéria de Oliveira Santos, agradeço pelo companheirismo, pelas trocas acadêmicas e pela convivência ao longo dessa trajetória. A caminhada compartilhada, marcada por diálogos, apoio mútuo durante a realização dos experimentos e aprendizado coletivo, contribuiu de forma significativa para meu crescimento científico e pessoal. Dividir desafios, conquistas e reflexões com vocês tornou o percurso da pós-graduação mais leve, enriquecedor e humano. Levo comigo a certeza de que essas experiências foram fundamentais para a construção do pesquisador que sigo me tornando.

Aos discentes de iniciação científica, deixo meu sincero agradecimento pelo empenho, dedicação e compromisso ao longo da realização dos experimentos. Em especial, agradeço a Samara Lima Olindo e Yara Letícia Frutuoso e Silva, cujo apoio foi fundamental em etapas decisivas deste trabalho. Samara esteve presente de forma incansável, contribuindo ativamente na preparação de meios, na execução dos protocolos e em tudo o que fosse necessário para garantir o bom andamento dos experimentos, muitas vezes estendendo-se para além do horário previsto, em longas jornadas de trabalho. Esse comprometimento, aliado à responsabilidade e ao espírito colaborativo, foi essencial para a concretização dos resultados apresentados nesta tese. Sou profundamente grato pelo apoio, pela disponibilidade e pela seriedade com que ambas conduziram suas atividades, demonstrando maturidade científica e dedicação à pesquisa.

À Universidade Federal Rural do Semi-Árido (UFERSA) e ao Programa de Pós-Graduação em Ciência Animal (PPGCA), expresso minha profunda gratidão por ter sido o espaço onde minha formação acadêmica, científica e humana se construiu ao longo dos anos. Foi na universidade que aprendi que a educação não se limita à aquisição de conhecimentos, mas representa um instrumento de transformação pessoal, social e profissional. Cada sala de aula, laboratório, diálogo e desafio enfrentado contribuiu para ampliar meu olhar crítico, fortalecer minha autonomia intelectual e consolidar meu compromisso com a ciência e com a sociedade. A universidade e a pós-graduação foi, e continua sendo, um ambiente de descobertas, questionamentos e amadurecimento, no qual compreendi que o saber se constrói coletivamente e exige responsabilidade, ética e sensibilidade. Levo comigo a certeza de que a educação é o caminho que possibilita não apenas a formação de profissionais, mas a construção de sujeitos conscientes de seu papel no mundo. Sou profundamente grato por tudo o que aprendi e por tudo o que sigo aprendendo neste espaço que transforma minha vida.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), agradeço pela concessão de bolsas e pelo apoio financeiro que viabilizaram o desenvolvimento desta pesquisa. O incentivo dessas instituições foi essencial para a dedicação às atividades acadêmicas, para a execução dos experimentos e para a consolidação da formação científica ao longo do doutorado. Reconheço a relevância do investimento público em ciência, tecnologia e educação como fundamentos indispensáveis para o avanço do conhecimento e para a formação de pesquisadores comprometidos com o desenvolvimento do país.

Ao Laboratório de Conservação de Germoplasma Animal (LCGA) e ao Laboratório de Morfofisiologia Animal Aplicada (LABMORFA), expresso minha sincera gratidão pelo suporte técnico, científico e institucional ao longo do desenvolvimento deste trabalho. A disponibilização de infraestrutura, equipamentos, reagentes e, sobretudo, o apoio humano e intelectual foram fundamentais para a execução das etapas experimentais e para a qualidade dos resultados obtidos. As contribuições desses laboratórios fortaleceram áreas do conhecimento e ampliaram minha formação científica, refletindo diretamente no amadurecimento profissional e na consolidação desta pesquisa.

Ao Centro de Multiplicação de Animais Silvestres (CEMAS), expresso minha profunda gratidão pela disponibilização dos preás utilizados neste estudo e por tudo o que ele representa para a ciência, a conservação e o uso ético da fauna silvestre. O CEMAS desempenha um papel fundamental na produção de conhecimento científico responsável, aliando pesquisa, manejo adequado e compromisso com a conservação da biodiversidade. Reconheço que este trabalho

só foi possível graças à seriedade, ao cuidado e à excelência científica que norteiam as atividades desenvolvidas nesse centro. Que os resultados aqui apresentados contribuam para fortalecer ainda mais a importância do CEMAS como referência na pesquisa com animais silvestres e na conservação das espécies.

Aos membros da banca examinadora, composta pelos doutores Daniele dos Santos Martins, Sheyla Farhayldes Souza Domingues, José Roberto Viana Silva, Vicente José Figueirêdo Freitas, Cristiana Libardi Miranda Furtado, e Moacir Franco de Oliveira, expresso minha sincera gratidão pela disponibilidade, atenção e valiosas contribuições ao longo do processo de avaliação desta tese. Mesmo em período de férias, no mês de janeiro, aceitaram prontamente o convite e dedicaram tempo e conhecimento à leitura criteriosa do trabalho, oferecendo considerações fundamentais para o aprimoramento científico e acadêmico desta pesquisa. Sou profundamente grato pela generosidade intelectual, pelo rigor científico e pelo compromisso com a formação acadêmica.

“[...] Por seres tão inventivo e pareceres contínuo, tempo, tempo, tempo, tempo, és um dos deuses mais lindos, tempo, tempo, tempo, tempo [...]. Peço-te o prazer legítimo e o movimento preciso, tempo, tempo, tempo, tempo.”

Oração ao Tempo, Caetano Veloso

RESUMO

A conservação de espécies silvestres requer a integração de estratégias celulares e reprodutivas que ampliem a preservação do material genético e o avanço das biotecnologias aplicadas. Nesse contexto, os fibroblastos são utilizados em estudos de cultivo, criopreservação, e sincronização do ciclo visando aplicações em biotecnologia reprodutiva. Paralelamente, a maturação e ativação *in vitro* de oócitos constitui uma ferramenta essencial para espécies não convencionais, ainda carentes de protocolos padronizados. O preá-da-caatinga (*Galea spixii*), um roedor silvestre neotropical de interesse científico e conservacionista, apresenta lacunas metodológicas que justificam o desenvolvimento de abordagens integradas para sua conservação. Portanto, este estudo objetivou avaliar estratégias celulares e reprodutivas envolvendo a obtenção, criopreservação e sincronização do ciclo em G₀/G₁ de fibroblastos, bem como as condições de maturação e ativação de oócitos em *G. spixii*. Para tanto, a pesquisa foi conduzida em quatro etapas. Na **primeira etapa**, linhagens de fibroblastos foram avaliadas nas 2^a, 5^a, 8^a e 10^a passagem, bem como quanto à criotolerância após congelação lenta usando 10% DMSO e 0,2 M sacarose. Nesta etapa, observou-se que, embora células viáveis tenham sido identificadas até a 10^a passagem, fibroblastos na 5^a passagem apresentaram maior estabilidade morfológica, genética e fisiológica. Contudo, a criopreservação induziu alterações ultraestruturais e fisiológicas, evidenciando a necessidade de otimização dos protocolos. Na **segunda etapa**, objetivou-se otimizar a eficiência da criopreservação por meio da adição de L-prolina (LP), um aminoácido com ação osmoprotetora, em substituição parcial aos crioprotetores convencionais. Para tanto, fibroblastos foram criopreservados em quatro condições: Grupo A (Controle, não submetidos à criopreservação), Grupo B (10% DMSO e 0,2 M sacarose), Grupo C (10% DMSO e 5 mM LP) e Grupo D (10% DMSO, 0,2 M sacarose e 5 mM LP). A adição de LP (Grupos C e D) mostrou-se mais eficaz que o uso isolado de sacarose (Grupos A e B), resultando em maior viabilidade celular, menor dano fisiológico e redução de espécies reativas de oxigênio, com melhor atividade mitocondrial, evidenciando menor estresse celular. Na **terceira etapa**, fibroblastos foram submetidos à diferentes condições de sincronização do ciclo em G₀/G₁: roscovitina (RSV; 7,5; 10; e 15 µM por 6–24 h) ou privação de soro fetal bovino (PSFB; por 24–96 h). Embora 15 µM de RSV por 12 h tenha gerado 91,55% ± 1,20 das células em G₀/G₁, as concentrações 7,5 µM (89,87% ± 1,70; 96,17% ± 1,27) e 10 µM (90,01% ± 0,84; 92,22% ± 1,92) alcançaram melhor equilíbrio entre sincronização e apoptose em relação às células não submetidas a nenhum protocolo de sincronização (86,33% ± 0,73; 97,89% ± 0,34). A PSFB manteve 95,3% ± 0,7 de viabilidade após 24 h e 90,3% ± 1,1 das células em G₀/G₁, embora apresentasse perda de colônia, aderência e aumento de apoptose após 48 h. Na **quarta etapa**, oócitos foram avaliados quanto às condições do meio de maturação (primeiro experimento) e ativação artificial (segundo experimento). Nesse contexto, oócitos maturados na presença de 10 ng/mL EGF apresentaram maior viabilidade das células do *cumulus* e melhor qualidade morfolétrica quando comparados àqueles expostos a 50 ng/mL. Adicionalmente, observaram-se taxas de maturação nuclear entre 55% e 64%, com extrusão do primeiro corpúsculo polar, e de 50% a 63% de oócitos em metáfase II. Posteriormente, embora não tenham sido observadas diferenças nas taxas de mórulas, a ativação de oócitos com 10 mM SrCl₂ por 2,5 h, seguida da exposição a 5 µg/mL citocalasina B e 2 mM 6-DMAP por 3,5 h, resultou na maior taxa de clivagem (69,3% ± 5,0%) quando comparada à ativação realizada na ausência de 6-DMAP (53,5% ± 3,5). Por fim, fibroblastos foram isolados, criopreservados e sincronizados eficientemente, enquanto a produção *in vitro* de embriões apresentaram parâmetros favoráveis, reforçando o potencial da espécie como modelo biológico.

Palavra-chave: roedores neotropicais; estratégias de conservação; biotecnologias da reprodução; células somáticas; oócitos.

ABSTRACT

The conservation of wild species requires the integration of cellular and reproductive strategies that enhance genetic material preservation and advance applied biotechnologies. In this context, fibroblasts are widely used in studies involving culture, cryopreservation, cell cycle synchronization, and aiming applications in reproductive biotechnology. In parallel, *in vitro* oocyte maturation and activation constitute essential tools for non-conventional species, which still lack standardized protocols. *Galea spixii*, a neotropical wild rodent of scientific and conservation interest, presents methodological gaps that justify the development of integrated approaches for its conservation. Therefore, this study aimed to evaluate cellular and reproductive strategies involving the establishment, cryopreservation, and G₀/G₁ cell cycle synchronization of fibroblasts, as well as oocyte maturation and activation conditions in *Galea spixii*. The research was conducted in four stages. In the **first stage**, fibroblast lines were evaluated at the 2nd, 5th, 8th, and 10th passages, as well as for cryotolerance after slow freezing using 10% DMSO and 0.2 M sucrose. Although viable cells were identified up to the 10th passage, fibroblasts at the 5th passage exhibited greater morphological, genetic, and physiological stability. However, cryopreservation induced ultrastructural and physiological alterations, highlighting the need for protocol optimization. In the **second stage**, the efficiency of cryopreservation was optimized through the addition of L-proline (LP), an amino acid with osmoprotective properties, as a partial substitute for conventional cryoprotectants. Fibroblasts were cryopreserved under four conditions: Group A (Control, non-cryopreserved), Group B (10% DMSO and 0.2 M sucrose), Group C (10% DMSO and 5 mM LP), and Group D (10% DMSO, 0.2 M sucrose and 5 mM LP). The addition of LP (Groups C and D) was more effective than sucrose alone (Groups A and B), resulting in higher cell viability, reduced physiological damage, lower reactive oxygen species levels, and improved mitochondrial activity, indicating reduced cellular stress. In the **third stage**, fibroblasts were subjected to different G₀/G₁ cell cycle synchronization conditions: roscovitine (RSV; 7.5, 10, and 15 μ M for 6–24 h) or fetal bovine serum starvation (SFBS; for 24–96 h). Although 15 μ M RSV for 12 h yielded 91.55% $\pm$ 1.20 of cells in G₀/G₁, the concentrations of 7.5 μ M (89.87% $\pm$ 1.70; 96.17% $\pm$ 1.27) and 10 μ M (90.01% $\pm$ 0.84; 92.22% $\pm$ 1.92) provided a better balance between synchronization efficiency and apoptosis compared to non-synchronized cells (86.33% $\pm$ 0.73; 97.89% $\pm$ 0.34). SFBS maintained 95.3% $\pm$ 0.7 viability after 24 h and 90.3% $\pm$ 1.1 of cells in G₀/G₁; however, colony loss, reduced adhesion, and increased apoptosis were observed after 48 h. In the **fourth stage**, oocytes were evaluated under different maturation medium conditions (first experiment) and artificial activation protocols (second experiment). Oocytes matured in the presence of 10 ng/mL EGF showed greater *cumulus* cell viability and improved morphometric quality compared to those exposed to 50 ng/mL EGF. Nuclear maturation rates ranged from 55% to 64%, with extrusion of the first polar body, and 50% to 63% of oocytes reached metaphase II. Subsequently, although no differences were observed in morula rates, oocyte activation using 10 mM SrCl₂ for 2.5 h followed by exposure to 5 μ g/mL cytochalasin B and 2 mM 6-DMAP for 3.5 h resulted in the highest cleavage rate (69.3% $\pm$ 5.0) compared to activation in the absence of 6-DMAP (53.5% $\pm$ 3.5). Finally, fibroblasts were efficiently isolated, cryopreserved, and synchronized, while *in vitro* embryo production was carried out in an advanced manner, reinforcing the species' potential as a biological model.

Keywords: neotropical rodents; conservation strategies; reproductive biotechnologies; somatic cells; oocytes.

LISTA DE FIGURAS

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

- Figura 1** – Exemplos de espécies de *G. spixii*. (A) Animal adulto. (B) Filhotes..... 30
- Figura 2** – Registros geográficos de exemplares de *G. spixii* no território sul-americano (A) e sua maior ocorrência no bioma da Caatinga (B)..... 32
- Figura 3** – Distribuição evolutiva do *G. spixii* ao longo dos anos..... 33
- Figura 4** – Estratégias de conservação desenvolvidas na conservação da biodiversidade animal..... 39

CAPÍTULO 2 – CRYOPRESERVATION AND PASSAGING OPTIMIZATION FOR *Galea spixii* (WAGLER, 1831) ADULT SKIN FIBROBLAST LINES: A STEP FORWARD IN SPECIES MANAGEMENT AND GENETIC STUDIES

- Figure 1** – Establishment of *in vitro* culture of *G. spixii* fibroblasts and their morphological characteristics in different passages. (A) Primary culture of *G. spixii* skin explant with cell shedding after 6 days of *in vitro* culture. (B) Primary culture of skin explants in cell growth after 8 days of *in vitro* culture. (C) Culture of *G. spixii* skin fibroblasts in cell growth after the removal of somatic skin explants. (D) *G. spixii* skin fibroblasts after reaching 100% subconfluence after 14 days of cell culture. (E) *In vitro* culture of *G. spixii* skin fibroblasts in the second, (F) fifth, (G) eighth, and (H) tenth passages. Bar scale = 50 µm, mag. 4× (B); 50 µm, mag. 10× (A, C, D, E, F, G, H)..... 74
- Figure 2** – Representation of the fibroblastic lineage of *G. spixii* after morphological analysis, immunostaining, and karyotyping. (A) Morphological evaluation by inverted microscope. Bar scale = 50 µm, mag. 40×. (B) Immunostaining of cell cytoplasm by vimentin antibody. Bar scale = 50 µm, mag. 40×. (C) Nucleus of cells stained by Hoechst 33342. Bar scale = 50 µm, mag. 40×. (D) Composition of the cytoplasm and nucleus of *G. spixii* fibroblasts after cell type identification. Bar scale = 50 µm, mag. 40×. (E-H) Karyotype of fibroblasts stained by Giemsa in the second (E), fifth (F), eighth (G), and tenth passages (H). Mag. 100×..... 76

Figure 3 – Evaluation of viability and physiological rates of fibroblastic strains of *G. spixii* after the second, fifth, eighth, and tenth passages. **(A)** Percentage of cell viability. **(B)** Quantification of metabolic activity. **(C)** Analysis of the proliferative curve. **(D)** Population doubling time of fibroblasts in the second, fifth, eighth, and tenth passages. $P < 0.05$ 77

Figure 4 – Evaluation of reactive oxygen species (ROS) and mitochondrial activity of *G. spixii* adult skin fibroblasts after the second, fifth, eighth, and tenth passages of *in vitro* culture. **(A-D)** Representation of cells stained by the H₂DCFDA probe. Bar scale = 50 μ m, mag. 20 $\times$. **(E-H)** Representation of cells stained by the MitoTracker Red[®] probe (CMXRos). **(I)** Quantification of oxidative stress after assessment of intracellular levels of ROS. **(J)** Evaluation of mitochondrial activity ($\Delta\Psi$ m) of *G. spixii* adult skin fibroblasts. $P < 0.05$ 78

Figure 5 – Evaluation of the apoptotic levels of the *G. spixii* fibroblastic lineage after the second **(A)**, fifth **(B)**, eighth **(C)**, and tenth **(D)** passages of cell culture. Bar scale = 50 μ m, mag. 20 $\times$. **(E)** Percentage of the number of viable cells in early and late apoptosis and necrotic during the ten passages of *in vitro* culture. $P > 0.05$ 79

Figure 6 – Morphological, viability, and metabolism evaluation of *G. spixii* fibroblastic strains after slow freezing. **(A-A')** Morphological analysis of cells before and after cryopreservation. Bar scale = 50 μ m, mag. 10 $\times$. **(B)** Percentage of fibroblast viability before, immediately after cryopreservation and *in vitro* culture. **(C)** Metabolic activity of cells after cell cryopreservation. $P < 0.05$ 81

Figure 7 – Ultrastructural evaluation and proliferative rate of *G. spixii* adult skin fibroblasts after cell cryopreservation. **(A-A')** Ultrastructural analysis of cells before and after cryopreservation. Bar scale = 10 μ m. **(B)** Cell concentration after evaluating the growth curve. **(C)** Population doubling time of fibroblasts before and after slow freezing. $P < 0.05$ 82

Figure 8 – Quantification of oxidative stress, mitochondrial membrane potential ($\Delta\Psi$ m), and apoptotic levels of *G. spixii* adult skin fibroblasts before and after slow freezing. **(A-A'')** Representative image and arbitrary units of fluorescence obtained in each cell evaluated after oxidative stress analysis. Bar scale = 50 μ m, mag. 20 $\times$ (A-A'). **(B-B'')** Representative image and arbitrary unit of fluorescence obtained in each cell evaluated after the $\Delta\Psi$ m evaluation. Bar scale = 50 μ m, mag. 20 $\times$ (B-B'). **(C-C'')** Representative image and percentage of the number of fibroblasts in cellular apoptosis. Bar scale = 50 μ m, mag. 20 $\times$ (C-C'). $P < 0.05$ 83

CAPÍTULO 3 – L-PROLINE AS A CRYOPROTECTIVE AGENT FOR THE PRESERVATION OF *Galea spixii* SKIN FIBROBLASTS

Figure 1 – Representative images of groups of fibroblasts isolated from *G. spixii* ear skin before and after slow freezing. **(A)** Control (non-cryopreserved). **(B)** 10% Me₂SO, 10% FBS, and 0.2 M sucrose. **(C)** 10% Me₂SO, 10% FBS, and 5 mM L-proline. **(D)** 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. Bar scale = 50 µm, magnitude 40×..... 102

Figure 2 – Viability analysis (immediately after cryopreservation and after *in vitro* culture) and physiological characteristics of *G. spixii* skin fibroblasts before and after slow freezing. **(A)** Quantification of cell viability by trypan blue assay. **(B)** Percentage of metabolic activity mediated by MTT assay. **(C)** Analysis of population doubling time. **(D)** Evaluation of the cell growth curve. ^{a,b}Subscript indicates difference between the experimental groups ($p < 0.05$). 103

Figure 3 – Assessment of the percentage of apoptotic levels after *in vitro* culture of *G. spixii* skin fibroblast lines. **(A)** Viable cell. **(B)** Initial apoptosis. **(C)** Late apoptosis. **(D)** Necrotic cell. **(E)** Percentage after assessment of apoptotic levels. Bar scale = 100 µm, magnitude 100×. ^{a,b}Subscript indicates difference between the experimental groups ($p < 0.05$)..... 105

Figure 4 – Assessment of intracellular levels of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) of *G. spixii* fibroblasts before and after slow freezing. **(A-D)** Representative image of the ROS assessment of the groups (Control [Non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 µm, magnitude 20×. **(E)** Arbitrary fluorescence units obtained after evaluating cellular oxidative stress. **(F-I)** Representative image of the $\Delta\Psi_m$ assessment of the groups (Control [non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 µm, magnitude 20×. **(J)** Arbitrary fluorescence units obtained after evaluating the mitochondrial activity of fibroblasts. ^{a,b}Subscript indicates difference between the experimental groups ($p < 0.05$). 106

CAPÍTULO 4 – EFFECT OF ROSCOVITINE AND SERUM STARVATION ON THE INDUCTION OF G₀/G₁ CELL CYCLE ARREST IN SPIX'S YELLOW-TOOTHED CAVY (*Galea spixii*, WAGLER, 1831) FIBROBLASTS

Figure 1 - Physiological assessment of Spix's yellow-toothed cavy fibroblasts before synchronization treatments by growth curve (A), metabolic activity (B) PDT assay, and viability after cell cycle synchronization treatments using different concentrations and exposure times of RSV (C) and SS (D). Mean $\pm$ standard error. a,b: $P < 0.05$ 130

Figure 2 - Morphological analysis of Spix's yellow-toothed cavy fibroblasts before and after cell cycle synchronization using RSV (A–D'') and SS (E–I). (A) Control group (no treatment). (B–B'') Cells treated with 7.5 μ M RSV for 6 h, 12 h, and 24 h. (C–C'') Cells treated with 10 μ M RSV for 6 h, 12 h, and 24 h. (D–D'') Cells treated with 15 μ M RSV for 6 h, 12 h, and 24 h. (E) Control group (no treatment). (F–I) Cells subjected to SS for 24 h (F), 48 h (G), 72 h (H), and 96 h (I). Scale bar = 100 μ m; magnification = 20 $\times$ 131

Figure 3 - Apoptotic profile of Spix's yellow-toothed cavy fibroblast lines before and after cell cycle synchronization (A–D). Representative images of viable cells (A), early apoptotic (B), late apoptotic (C), and necrotic cells (D). (E–F) Graphical representations of apoptotic cell percentages after different synchronization treatments using RSV (E) and SS (F). Scale bar = 100 μ m, 40 $\times$ mag. Mean $\pm$ standard error. a,b: Values with different superscript letters within columns significantly differ ($P < 0.05$)..... 133

Figure 4 - Quantification of intracellular ROS levels in Spix's yellow-toothed cavy fibroblasts after cell cycle synchronization with RSV and SS. (A) RSV control (no treatment). (B–B'') Cells treated with 7.5 μ M RSV for 6 h (B), 12 h (B'), and 24 h (B''). (C–C'') Cells treated with 10 μ M RSV for 6 h (C), 12 h (C'), and 24 h (C''). (D–D'') Cells treated with 15 μ M RSV for 6 h (D), 12 h (D'), and 24 h (D''). (E) ROS levels after RSV treatments. (F) SS control (no treatment). (G–J) Cells subjected to SS for 24 h (G), 48 h (H), 72 h (I), and 96 h (J). (K) ROS levels after SS. Scale bar = 100 μ m; magnification = 20 $\times$. Mean $\pm$ standard error. a,b: $P < 0.05$ 135

Figure 5 - Cell cycle analysis of Spix's yellow-toothed cavy fibroblast lines before and after treatment with different concentrations and exposure times of RSV. (A) Representative histograms of cell cycle stages assessed by flow cytometry. (B) Quantitative graph showing the percentage of cells in G0/G1, S, and G2/M phases. mean $\pm$ standard error. a,b: Values with different superscript letters within columns significantly differ ($P < 0.05$)..... 137

Figure 6 - Cell cycle analysis of Spix's yellow-toothed cavy fibroblast lines before and after treatment with different SS exposure times. (A) Representative histograms of cell cycle stages assessed by flow cytometry. (B) Quantitative graph showing the percentage of cells in G0/G1, S, and G2/M phases. Mean $\pm$ standard error. a,b: Values with different superscript letters within columns significantly differ ($P < 0.05$)..... 139

CAPÍTULO 5 – UNDERSANTING OOCYTE COMPETENCE TO IMPROVE *IN VITRO* EMBRYO PRODUCTION IN CAVIIDAE RODENTS

Figure 1 - A representative overview of the main studies developed on the reproductive physiology of rodents belonging to the Caviidae family, focusing on understanding reproductive processes and developing reproductive techniques..... 152

Figure 2 – Schematic representation of the evaluation of immature and mature oocytes, focusing on the selection of higher quality gametes and their application in the development of ARTs..... 174

CAPÍTULO 6 – EFFECT OF EPIDERMAL GROWTH FACTOR AND 6-DIMETHYLAMINOPURINE ON *IN VITRO* MATURATION AND ARTIFICIAL ACTIVATION OF SPIX'S YELLOW-TOOTHED CAVY (*Galea spixii* WAGLER, 1831) OOCYTES

Figure 1 – Representation of Spix's yellow toothed cavy ovaries and oocytes before and after IVM with EGF as a supplement to evaluate oocyte competence acquisition. (A) Ovary containing follicles (arrow-triangle) before processing by slicing. (B-B') Immature oocytes and cumulus cell expansion after 24 h of IVM with 10 ng/mL EGF. (B''-B''') Ultrastructural analysis showing transzonal projections (arrow) of *cumulus* cells surrounding the oocyte under the zona pellucida in IVM medium with 10 ng/mL EGF. (C-C') Immature oocytes and cumulus cell expansion after 24 h of IVM with 50 ng/mL EGF. (C''-C''') Ultrastructural analysis showing transzonal projections (arrow) of *cumulus* cells surrounding the oocyte under the zona pellucida in IVM medium with 50 ng/mL EGF. Scale bar = 100 μ m (A); 50 μ m, mag. 20 $\times$ (B, B', C, C'); 20 μ m (B'', C''); 5 μ m (B''', C''')..... 194

Figure 2 – Assessment of viability and apoptotic levels of *cumulus* cells after 24 h of IVM of Spix’s yellow toothed cavy oocytes. **(A)** Percentage of cell viability by Trypan blue assay. **(B)** Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 10 ng/mL EGF. **(C)** Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 50 ng/mL EGF. **(D)** Rate of apoptotic levels of *cumulus* cells under 10 and 50 ng/mL EGF. ^{a,b}Values with different superscript letters within columns significantly differ ($P < 0.05$). Scale bar = 50 μm (B, C), mag. 20 $\times$. Three repetitions..... 196

Figure 3 – Representative image of Spix’s yellow toothed cavy oocytes before and after 24 h of IVM containing different concentrations of EGF. **(A)** Immature oocyte. **(B)** Oocyte after 1PB extrusion. **(C)** Oocyte in germinal vesicle breakdown (GVBD) configuration. **(D)** Oocyte in metaphase II. **(E)** Percentage of oocyte rate after nuclear maturation assessment. Scale bar = 100 μm , mag. 20 $\times$ (A, B, C, D). Three repetitions..... 197

Figure 4 – Representative image of cleaved zygotes after artificial activation and two days of embryonic culture. **(A)** Zygotes with 2 cleavages. **(B)** Zygotes with 3-7 cleavages. **(A')** Ultrastructure of zygotes with two cleavages. The arrow-triangle indicates remains of the degraded zona pellucida after processing for visualization in SEM. **(B')** Ultrastructure of cleavages containing three blastomeres and their microvilli. Scale bar = 100 μm , mag. 20 $\times$ (A, B); 20 μm (A', B'). Four repetitions..... 200

Figure 5 – Embryonic development rate of artificially activated *G. spixii* oocytes. **(A)** Zygotes with cleavages >8 cells after two days of embryonic culture. **(B)** Compacted morulae after five days of embryonic culture. **(C)** Percentage of cleaved zygotes after two days of *in vitro* culture. **(D)** Percentage of cleavages with more than 8 blastomeres and production of morulae. Scale bar = 100 μm , mag. 20 $\times$ (A, B). ^{a,b}Values with different superscript letters within columns significantly differ ($P < 0.05$)..... 201

LISTA DE QUADRO

Quadro 1 – Estratégias de conservação <i>ex situ in vitro</i> desenvolvidas em <i>G. spixii</i>	41
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LISTA DE TABELAS

CAPÍTULO 2 - CRYOPRESERVATION AND PASSAGING OPTIMIZATION FOR *Galea spixii* (WAGLER, 1831) ADULT SKIN FIBROBLAST LINES: A STEP FORWARD IN SPECIES MANAGEMENT AND GENETIC STUDIES

Table 1 – Establishment of the physiological characteristics of the primary culture and subculture of somatic cells of six *G. spixii*..... 73

CAPÍTULO 5 – UNDERSTANDING OOCYTE COMPETENCE TO IMPROVE *IN VITRO* EMBRYO PRODUCTION IN CAVIIDAE RODENTS

Table 1 – List of species belonging to the Caviidae family..... 154

Table 2 – Morphological characteristics of the follicular population of species belonging to the order Rodentia..... 162

CAPÍTULO 6 – EFFECT OF EPIDERMAL GROWTH FACTOR AND 6-DIMETHYLAMINOPURINE ON *IN VITRO* MATURATION AND ARTIFICIAL ACTIVATION OF SPIX’S YELLOW-TOOTHED CAVY (*Galea spixii* WAGLER, 1831) OOCYTES

Table 1 – Number and classification of oocytes recovered from Spix’s yellow toothed cavy ovaries..... 194

Table 2 – Evaluation of the grade of *cumulus* cell expansion and CEI of Spix’s yellow toothed cavy oocytes matured in IVM medium supplemented with two concentrations of EGF after 24 h..... 195

Table 3 – Morphometric evaluation of immature and matured Spix’s yellow toothed cavy oocytes after 24 h of IVM supplemented with EGF..... 198

Table 4 – Rate expansion of *cumulus* cells and CEI of *G. spixii* oocytes after 24 h of IVM in medium supplemented with 10 ng/mL of EGF..... 199

LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
1PB	First polar body
6-DMAP	6-dimetilaminopurina/6-dimethylaminopurine
ACP	Diluentes à base de água de coco em pó
AFP	Alexsandra Fernandes Pereira
ALRR	Ana Livia Rocha Rodrigues
ARS	Alexandre Rodrigues Silva
ARTs	Assisted reproductive technologies
ATB-ATM	Antibiotic-antimycotic
BSA	Bovine serum albumin
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CB	Cytochalasin B
CDKs	Quinases dependents de ciclina
CEI	<i>Cumulus</i> expansion index
CEMAS	Centro de Multiplicação de Animais Silvestres
CEUA	Comitê de Ética e Uso de Animais
CMXRos	MitoTracker Red®
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO ₂	Dióxido de Carbono
COC	<i>Cumulus</i> -oocyte complex
DMEM	Dulbecco's modified minimal essential medium
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
DOI	Digital object identifier
EAP	Érika Almeida Praxedes
EDTA	Ácido Etilenodiamino Tetra-acético
EG	Etilenoglicol
EGF	Epidermal growth factor
EGF10	10 ng/mL Epidermal growth factor
EGF50	50 ng/mL Epidermal growth factor
FBS	Fetal bovine serum
FSH/LH	Hormônio folículo estimulante/Hormônio luteinizante

GVBD	Oocyte in germinal vesicle breakdown
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H ₂ O ₂	Hydrogen peroxide
hCG	Gonadotrofina coriônica humana
HO•	Hydroxyl groups
IBAMA	Instituto Brasileiro do Meio Ambiente
ICMBio	Instituto Chico Mendes da Conservação da Biodiversidade
IgG	Imunoglobulina G
IOA	Internal oocyte area
IOD	Inner oocyte diameter
iPSCs	Células induzidas a pluripotência
IUCN	União Internacional para a Conservação da Natureza
IVM	<i>In vitro</i> maturation
KCl	Cloreto de potássio
KSOM	Potassium simplex optimized medium
LABMORFA	Laboratório de Morfofisiologia Animal Aplicada
LCGA	Laboratório de Conservação de Germoplasma Animal
LLVR	Luanna Lorena Vieira Rodrigues
LRMO	Lhara Ricarliany Medeiros de Oliveira
LVCA	Leonardo Vitorino Costa de Aquino
Me ₂ SO	Dimetilsulfóxido
MFO	Moacir Franco de Oliveira
MII	Metáfase II
MIV	Maturação <i>in vitro</i>
MPF	meiosis-promoting factor
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoluim bromide
NaCl	Cloreto de Sódio
OA	Ooplasm area
OD	Ooplasm diameter
OOD	Outer oocyte diameter
ORM	Oocyte recovery medium
p53	Tumor protein p53
PBS	Phosphate buffer solutions
PDT	Population doubling time

pH	Potencial hidrogeniônico
PIVE	Produção <i>in vitro</i> de embrião
POX/PRODH	Proline oxidase
PPGCA	Programa de Pós-Graduação em Ciência Animal
PSA	Perivitelline space area
PSFB	Privação de soro fetal bovino
RNAs	Ácido ribonucleico
ROS	Reactive oxygen species
RSV	Roscovitine
SCNT	Somatic cell nuclear transfer
SEM	Scanning electron microscopy
SFB	Soro fetal bovino
SLO	Samara Lima Olindo
SPD	Space perivitelline diameter
SrCl ₂	Cloreto de estrôncio
TALP	Tyrode's albumin lactate pyruvate
TCM-199	Tissue culture medium
TES	Ácido N-[Tris(hidroximetil)metil]-2-aminoetanossulfônico
TNCS	Transferência nuclear de célula somática
TRIS	Tris-hidroximetil-aminometano
UECE	Universidade Estadual do Ceará
UFC	Universidade Federal do Ceará
UFERSA	Universidade Federal Rural do Semi-Árido
UFPA	Universidade Federal do Pará
USP	Universidade de São Paulo
YLF	Yara Leticia Frutuoso e Silva
ZPT	Zona pellucida thickness

LISTA DE SÍMBOLOS

®	Marca registrada
%	Porcentagem
+	Soma
=	Igual
±	Mais ou menos
<	Menor que
>	Maior que
×	Multiplicação
≥	Igual e maior que
~	Aproximadamente
/	Divido por
$\Delta\Psi_m$	Potencial de membrana mitocondrial

SUMÁRIO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS.....	28
1 INTRODUÇÃO	28
2 FUNDAMENTAÇÃO TEÓRICA.....	30
2.1 Características anatômicas e distribuição geográfica de <i>G. spixii</i>	30
2.2 Biologia, papel ecológico e econômico de <i>G. spixii</i>	33
2.3 Aspectos fisiológicos, genéticos e reprodutivos de <i>G. spixii</i>	36
2.4 Estratégias de conservação desenvolvidas para <i>G. spixii</i>	37
2.5 Estratégias para o isolamento, cultivo <i>in vitro</i> e avaliação do ciclo celular de linhagens fibroblásticas aplicadas a roedores	42
2.6 Produção <i>in vitro</i> de embriões como estratégia reprodutiva aplicado em roedores	47
3 JUSTIFICATIVA.....	51
4 HIPÓTESES CIENTÍFICAS	52
5 OBJETIVOS	53
5.1 Objetivo Geral	53
5.2 Objetivos Específicos.....	53
6 REFERÊNCIAS BIBLIOGRÁFICAS	54
CAPÍTULO 2 – CRYOPRESERVATION AND PASSAGING OPTIMIZATION FOR Galea spixii (WAGLER, 1831) ADULT SKIN FIBROBLAST LINES: A STEP FORWARD IN SPECIES MANAGEMENT AND GENETIC STUDIES.....	65
CAPÍTULO 3 – L-PROLINE AS A CRYOPROTECTIVE AGENT FOR THE PRESERVATION OF Galea spixii SKIN FIBROBLASTS	91
CAPÍTULO 4 – EFFECT OF ROSCOVITINE AND SERUM STARVATION ON THE INDUCTION OF G₀/G₁ CELL CYCLE ARREST IN Galea spixii (WAGLER, 1831) FIBROBLASTS	119
CAPÍTULO 5 – UNDERSTANDING OOCYTE COMPETENCE TO IMPROVE IN VITRO EMBRYO PRODUCTION IN CAVIIDAE RODENTS	147
CAPÍTULO 6 – EFFECT OF EPIDERMAL GROWTH FACTOR AND 6-DIMETHYLAMINOPURINE ON IN VITRO MATURATION AND ARTIFICIAL ACTIVATION OF SPIX’S YELLOW-TOOTHED CAVY (Galea spixii WAGLER, 1831) OOCYTES	186
7 CONSIDERAÇÕES FINAIS	210
8 PERSPECTIVAS.....	212

9 ANEXOS	214
ANEXO A – CERTIFICADO DE AUTORIZAÇÃO DO PROJETO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UFERSA (CEUA/UFERSA)	214
ANEXO B – AUTORIZAÇÃO DO PROJETO PELO SISTEMA DE AUTORIZAÇÃO E INFORMAÇÃO EM BIODIVERSIDADE – SISBIO.....	215
ANEXO C – VERSÃO DO ARTIGO EXPERIMENTAL I PUBLICADO NA ACTA HISTOCHEMICA.....	218
ANEXO D – VERSÃO PUBLICADA DO ARTIGO EXPERIMENTAL II PUBLICADO NA BIOPRESERVATION AND BIOBANKING	229
ANEXO E – VERSÃO PUBLICADA DO ARTIGO EXPERIMENTAL V PUBLICADO NA ANIMALS.....	238
ANEXO F – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 2	258
ANEXO G – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 3	259
ANEXO H – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 4	260
ANEXO I – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 6.....	261
ANEXO J – RESUMO DA PUBLICAÇÃO NA REVISTA ELETRÔNICA DO SEMINÁRIO DE INICIAÇÃO CIENTÍFICA DA UFERSA – 2025	262
ANEXO K – RESUMO E CERTIFICADO DA PUBLICAÇÃO NA REVISTA ELETRÔNICA DO X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION – 2024.....	264
ANEXO L – RESUMO E CERTIFICADO DA PUBLICAÇÃO NO I SIMPÓSIO POTIGUAR EM CIÊNCIA ANIMAL – 2023.....	268
ANEXO M – PRÊMIO DA 2º MELHOR FOTO DO EVENTO NO I SIMPÓSIO POTIGUAR EM CIÊNCIA ANIMAL – 2023.....	270
ANEXO N – RESUMO EXPANDIDO PUBLICADO NO CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL – 2022	271

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

A perda de biodiversidade observada nas últimas décadas tem intensificado o desenvolvimento de estratégias para a conservação de espécies silvestres, especialmente àquelas endêmicas ou adaptadas a biomas ameaçados (SILVA et al., 2024). A fragmentação de habitats, as mudanças climáticas e a pressão antrópica comprometem a manutenção de populações naturais viáveis, tornando indispensável a adoção de ações de conservação (PEREIRA et al., 2024). Nesse contexto, a conservação *ex situ*, aliada ao avanço das biotecnologias reprodutivas e celulares, consiste em uma ferramenta essencial para a manutenção do material genético e o manejo reprodutivo de espécies silvestres.

Neste cenário, o uso de células somáticas desponta num importante papel na conservação genética, uma vez que possibilitam o cultivo *in vitro*, criopreservação e aplicações em diferentes biotecnologias reprodutivas (HILDEBRANDT et al., 2021). Entre os modelos celulares disponíveis, os fibroblastos destacam-se pela facilidade de obtenção a partir de biópsias minimamente invasivas, elevada capacidade proliferativa *in vitro* e relativa estabilidade genética ao longo das passagens celulares (MACHADO et al., 2017). Contudo, a eficiência dessas aplicações depende diretamente da padronização de protocolos específicos para cada espécie, considerando suas particularidades fisiológicas e celulares.

Para o preá-da-caatinga (*G. spixii*), um roedor silvestre neotropical adaptado ao bioma Caatinga, e que apresenta relevância ecológica, científica e conservacionista (SILVA et al., 2024), essa abordagem não é diferente. Embora seja amplamente distribuída sobre o semiárido brasileiro, a espécie ainda é pouco explorada sob a perspectiva da biotecnologia reprodutiva e celular, carecendo de protocolos padronizados que subsidiem ações de conservação *ex situ*. Considerando as particularidades fisiológicas e reprodutivas de *G. spixii* (WOODS, 1982; ROWE; HONEYCUTT, 2002), torna-se evidente a necessidade de estudos integrados que abordem, de forma sistemática, tanto a conservação celular quanto o aprimoramento das técnicas reprodutivas.

Nesse contexto, o cultivo *in vitro* e a criopreservação de fibroblastos constituem um dos pilares da conservação genética, pois permite a obtenção e o armazenamento a longo prazo de material biológico com manutenção da viabilidade e integridade funcional após a descongelação (BOLTON et al., 2022). Nesse sentido, a avaliação das condições de cultivo é fundamental para compreender a estabilidade morfofuncional das células, bem como sua

resposta aos estresses impostos pelos protocolos de criopreservação. Contudo, o processo de congelamento e descongelamento celular está associado a danos osmóticos, mecânicos e oxidativos, que podem comprometer a ultraestrutura celular, função mitocondrial e viabilidade pós-criopreservação (MOONEY et al., 2023). Assim, a busca por crioprotetores alternativos, capazes de reduzir o estresse celular e melhorar a criotolerância, representa um desafio, sobretudo em espécies silvestres pouco estudadas.

Além da conservação celular, a sincronização do ciclo em G_0/G_1 constitui um requisito fundamental para aplicações em biotecnologia reprodutiva, especialmente em técnicas como TNCS e iPSCs (CHEN et al., 2025). Protocolos baseados em inibidores farmacológicos ou na privação de fatores de crescimento têm sido explorados (VIANA; PEREIRA, 2025). Contudo, a eficiência desses métodos deve ser avaliada em cada espécie, uma vez que desequilíbrios entre sincronização e viabilidade celular podem resultar em apoptose ou alterações fisiológicas indesejáveis. Dessa forma, a definição de condições que promovam elevada sincronização celular em G_0/G_1 sem comprometer a integridade das células é essencial para o sucesso das estratégias conservacionistas.

Paralelamente às abordagens celulares, as biotecnologias reprodutivas, particularmente a maturação e a ativação oocitária *in vitro*, representam ferramentas indispensáveis para a conservação e o manejo reprodutivo de espécies não convencionais. A maturação *in vitro* de oócitos possibilita o aproveitamento de gametas imaturos obtidos de fêmeas em diferentes condições fisiológicas, ampliando o potencial reprodutivo e reduzindo a dependência de ciclos naturais (PRAXEDES et al., 2023). Contudo, a eficiência dessa técnica é altamente dependente das condições do meio de cultivo, o qual influencia diretamente a competência nuclear e citoplasmática dos oócitos (MEN, 2024).

A ativação oocitária artificial, por sua vez, constitui uma etapa crítica para o desenvolvimento embrionário inicial, especialmente em protocolos que envolvem TNCS (GONZÁLEZ et al., 2025). Diferentes agentes químicos de ativação têm sido empregados com sucesso variável entre espécies, reforçando a necessidade de ajustes específicos para cada modelo biológico (GONZÁLEZ et al., 2025; AGUIAR et al., 2025). A ausência de protocolos consolidados para espécies silvestres limita o avanço dessas biotecnologias e, consequentemente, as estratégias de conservação genética.

Portanto, essa tese sistematiza o estabelecimento de protocolos que subsidiem ações voltadas a conservação *ex situ* do preá-da-caatinga através do uso de linhagens somáticas e do uso de gametas visando o desenvolvimento de técnicas reprodutivas para a espécie.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Características anatômicas e distribuição geográfica de *G. spixii*

O *G. spixii* (**Figura 1**), conhecido popularmente como preá-de-dente-amarelo ou preá da caatinga, é um pequeno roedor silvestre histicomorfo pertencente à família Caviidae e subfamília Caviinae, a qual também abrange os gêneros *Cavia*, *Microcavia* e *Kerodon* (ADRIAN; SACHSER, 2011). Esses animais representam um grupo amplamente distribuído na América do Sul, adaptado a ambientes de clima árido ou semiárido (SILVA et al., 2024). Entre os cavídeos neotropicais, o *G. spixii* destaca-se por notável plasticidade ambiental, características que têm favorecido seu uso crescente como modelo experimental em estudos biológicos e reprodutivos. Seu porte reduzido, massa corporal e taxa metabólica conferem vantagens significativas entre as outras espécies para investigações de natureza biológica, fisiológica, celular, evolutiva e genética (SILVA; DOWNING, 1995; LOVEGROVE, 2000).



Figura 1. Exemplares de espécies de *G. spixii*. (A) Animal adulto. (B) Filhotes. Fonte: Biofaces.

Morfologicamente, o *G. spixii* apresenta corpo alongado, dorso arqueado e cauda vestigial, com comprimento total variando entre 19,5 e 28,4 cm e massa corporal entre 140 e 560 g (REIS et al., 2006). O corpo é revestido por pelagem densa e hispida, cuja coloração varia do cinza-escuro ao amarelado, tornando-se gradualmente mais clara em direção ao ventre, que é branco ou amarelado. Essa distribuição de cor tem papel adaptativo, auxiliando na camuflagem em substratos rochosos e arenosos, comuns no semiárido.

A cabeça é curta e arredondada, com orelhas pequenas e pelos pós-auriculares que constituem um traço da espécie (REIS et al., 2006). Os olhos são grandes e contornados por um anel periocular claro, também característico, que diferencia o *G. spixii* de espécies congêneras, como *Cavia aperea*, que não exibe essa marcação (REIS et al., 2006). O focinho é curto e robusto, e as vibrissas são longas, desempenhando importante papel sensorial para a locomoção em áreas rochosas e para o reconhecimento de alimentos. Os membros anteriores possuem quatro dígitos e os posteriores três, todos com garras curtas e resistentes, adequadas ao deslocamento terrestre e ao escavamento superficial (WOODS, 1982). A musculatura dos membros é compacta e bem desenvolvida, o que confere agilidade em ambientes com vegetação rala e substrato irregular. Tais características indicam uma morfologia cursorial, típica de roedores que habitam áreas abertas e secas (DUNNUN; SALAZAR-BRAVO, 2010).

A dentição segue o padrão típico dos cavídeos, composta por 20 dentes: dois pares de incisivos, nenhum canino e molariformes de coroa alta, com superfície oclusal em lâminas paralelas (LACHER, 1981). Os incisivos brancos, menos pigmentados que os de *Cavia* e *Microcavia*, e o esmalte espesso dos molariformes refletem uma especialização alimentar herbívora voltada ao consumo de vegetais fibrosos e secos. O formato das arcadas e a estrutura craniana descrita por WOODS (1984) evidenciam uma conformação adaptada ao corte e à trituração de tecidos vegetais de alta lignificação, comuns nas plantas da Caatinga.

Em virtude das características anatômicas do *G. spixii*, sua distribuição geográfica torna-se predominantemente oriental em relação à Cordilheira dos Andes. Registros documentam sua presença desde o leste da Bolívia e Paraguai até o território brasileiro, abrangendo regiões do sul do Pará, Bahia, Minas Gerais, Goiás, Mato Grosso e Distrito Federal (**Figura 2A**) (NOWAK, 1999). Embora seja amplamente distribuído geograficamente, sua maior ocorrência está associada ao bioma da Caatinga (**Figura 2B**), apesar de ser localizado em áreas de transição com o Cerrado e Mata Atlântica, revelando uma importante adaptação a mosaicos de vegetação aberta e savânica (WOODS, 1982).

Ainda, o *G. spixii* ocupa habitats predominantemente terrestres e abertos, caracterizados por solos rasos e vegetação xerófita. Costuma abrigar-se em fendas rochosas, lajeiros e buracos naturais, utilizando essas estruturas como refúgio térmico e proteção contra predadores (LACHER, 1981). Sua ocorrência em áreas com escassa disponibilidade hídrica e forte sazonalidade demonstra a eficiência de suas adaptações comportamentais e fisiológicas à vida no semiárido, o que explica sua persistência em ecossistemas submetidos a longos períodos de estiagem.

A afirmativa anteriormente descrita ocorre porque segundo QUEIROZ et al. (2020), o *G. spixii* dispõe de mecanismos termorregulatórios refinados, incluindo janelas térmicas nas regiões perioculares, auriculares e vibrissais. Essas áreas possuem alta irrigação sanguínea e atuam na dissipação de calor por radiação, reduzindo a carga térmica corporal em ambientes de temperatura elevada. Quando a radiação é excessiva, o animal aumenta a perda evaporativa, ajustando o equilíbrio térmico de modo eficiente. Essa característica, somada à coloração dorsal clara e ao comportamento diurno-crepuscular, constitui um conjunto de adaptações anatômicas e fisiológicas que favorecem sua sobrevivência no clima semiárido.

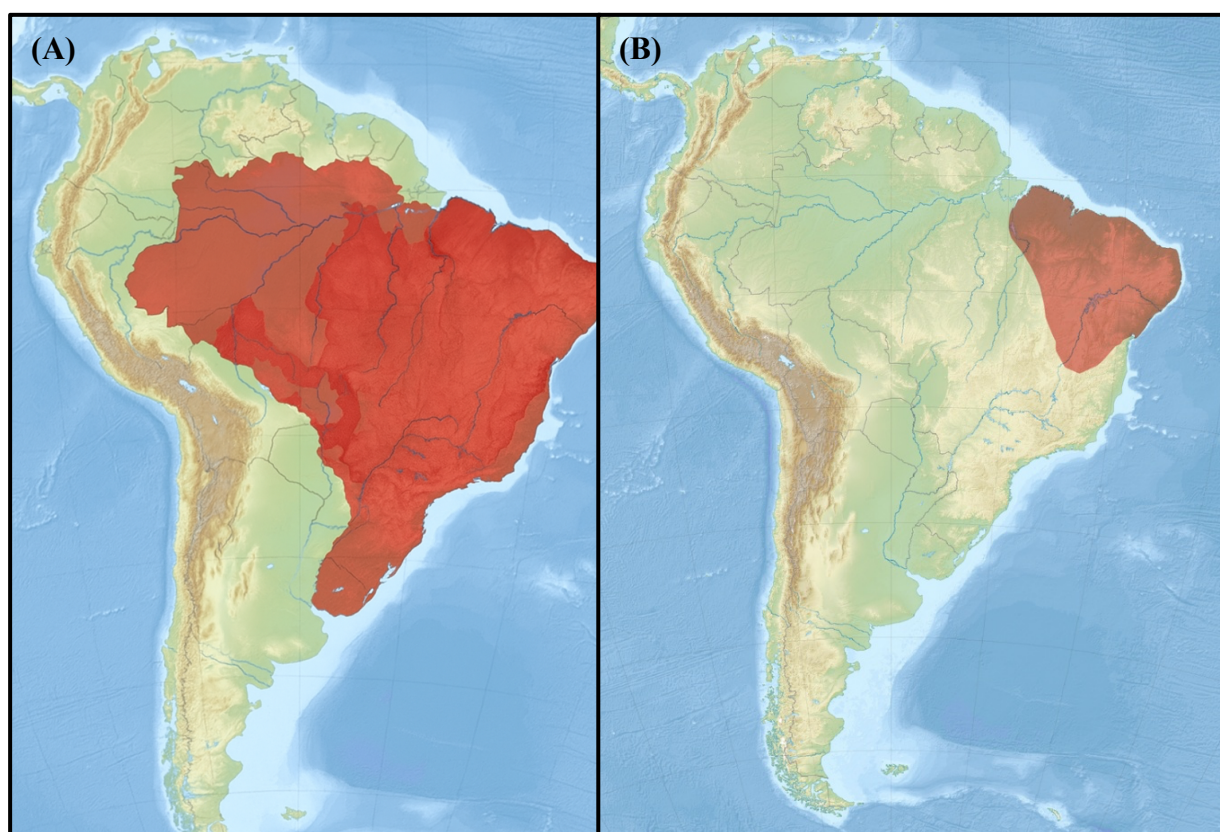


Figura 2. Registros geográficos de exemplares de *G. spixii* no território sul-americano **(A)** e sua maior ocorrência no bioma Caatinga **(B)**. Fonte: IUCN.

Dessa forma, o conjunto de características descritas para *G. spixii* reflete um notável grau de especialização às condições ambientais do semiárido, assegurando sua ampla distribuição e resiliência ecológica frente às adversidades climáticas da Caatinga. Contudo, compreender a totalidade do papel desempenhado por essa espécie exige ultrapassar a dimensão morfofuncional, integrando aspectos de sua biologia reprodutiva, hábitos alimentares, estrutura social e interações ecológicas. Tais elementos são essenciais para elucidar sua importância na

dinâmica dos ecossistemas e no equilíbrio trófico regional, além de revelar potenciais contribuições socioeconômicas associadas ao manejo sustentável da espécie.

2.2 Biologia, papel ecológico e econômico de *G. spixii*

O *G. spixii*, pertencente ao grupo de roedores histricomorfos sul-americanos, é uma espécie que teve sua biologia evolutiva debatida inicialmente nos trabalhos pioneiros de WOOD (1955). De acordo com o autor, a radiação dos caviomorfos no continente sul-americano ocorreu a partir de ancestrais norte-americanos, provavelmente oriundos da família Paramyidae, que colonizaram a América do Sul por rotas intercontinentais durante o final do Eoceno. Essa dispersão deu origem a uma diversificação adaptativa expressiva durante o Oligoceno, culminando nas linhagens atuais da superfamília Caviioidea, à qual pertencem os gêneros *Cavia*, *Kerodon*, *Microcavia* e *Galea*. Tais eventos filogenéticos explicam o conjunto de caracteres morfológicos e fisiológicos que conferem à espécie elevada capacidade de adaptação às condições ambientais extremas do semiárido brasileiro. Segundo DUNNUM; SALAZAR-BRAVO (2010), o *G. spixii* divergiu do gênero *Cavia* e *Kerodon* no Mioceno tardio ($\sim 5,9 \pm 1,6$ milhões de anos), durante a diferenciação dos biomas neotropicais (Figura 3).

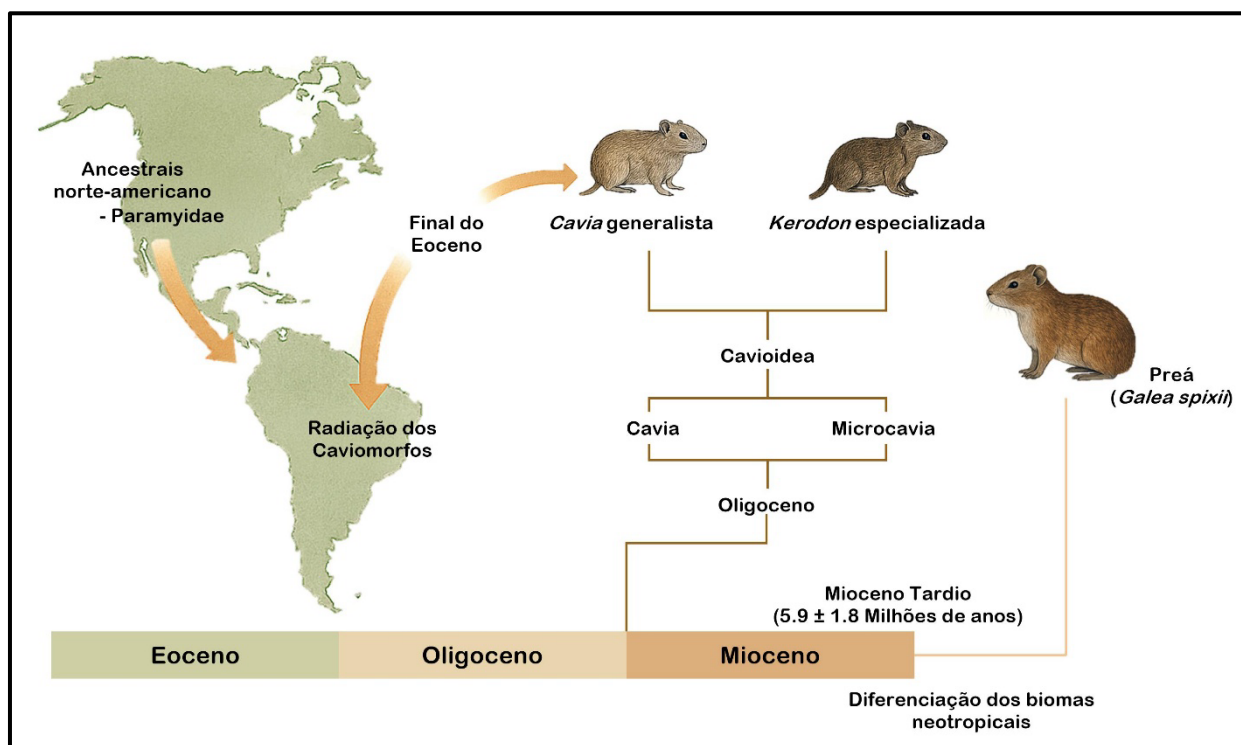


Figura 3. Distribuição evolutiva do *G. spixii* ao longo dos anos. Fonte: Própria autoria.

Dentro da família Caviidae, o gênero *Galea* ocupa posição intermediária entre as formas mais especializadas, como *Kerodon rupestris*, e as generalistas, como *Cavia aperea*, apresentando características morfofuncionais que expressam tanto ancestralidade quanto plasticidade adaptativa (ROWE; HONEYCUTT, 2002). Estudos moleculares comparativos indicam que as diferenças comportamentais e ecológicas observadas entre os caviídeos, especialmente no sistema de estrutura social, derivam mais fortemente de fatores filogenéticos do que de restrições ambientais isoladas. Por exemplo, no *G. spixii* essa herança evolutiva resultou em um comportamento social simples e em estratégias de sobrevivência baseadas na exploração eficiente de ambientes com disponibilidade hídrica limitada e alta amplitude térmica (LACHER, 1981; QUEIROZ et al., 2020).

De modo geral, o *G. spixii* é uma espécie essencialmente terrestre, de hábitos diurnos a crepusculares, que vive em pequenos grupos familiares ou isoladamente, apresentando baixa tolerância social e interações esporádicas entre indivíduos (ROWE; HONEYCUTT, 2002). Sua estrutura social é caracterizada por um sistema reprodutivo não monogâmico, sem hierarquias definidas nem vínculos duradouros entre os pares, característica comum a outras espécies de caviídeos. (NOWAK, 1999). Essa organização social mínima, associada à ausência de comportamento territorial pronunciado, reflete uma adaptação evolutiva às condições ecológicas do semiárido, onde a mobilidade e a dispersão populacional favorecem a sobrevivência em ambientes imprevisíveis (ROWE; HONEYCUTT, 2002).

Do ponto de vista trófico, *G. spixii* é um herbívoro generalista, alimentando-se de gramíneas, raízes, folhas e frutos de espécies xerófitas características da Caatinga (REIS et al., 2006). Sua dentição hipsodonte, descrita por WOOD (1955), e o esmalte espesso dos incisivos e molares estão associados a dietas ricas em sílica e fibras vegetais, típicas de plantas sujeitas à dessecação sazonal. Em períodos de estiagem, a espécie exibe comportamentos de conservação hídrica, reduzindo a ingestão de alimento e o metabolismo basal, o que assegura sua permanência em áreas onde a disponibilidade de água é limitada (QUEIROZ et al., 2020).

Ecologicamente, o *G. spixii* desempenha funções importantes nos ecossistemas onde ocorre. Seu hábito de forragear em áreas abertas e de utilizar túneis e fendas rochosas como abrigo contribui para a aeração do solo e formação de micro habitats, beneficiando invertebrados e pequenos vertebrados (LIMA et al., 2018). Além disso, atua como dispersor secundário de sementes, auxiliando na regeneração vegetal de áreas degradadas e no equilíbrio das populações de gramíneas e herbáceas. No contexto trófico, representa uma importante fonte de alimento para carnívoros de pequeno e médio porte, como *Cerdocyon thous* e *Leopardus*

tigrinus, além de aves de rapina, como *Tyto furcata*, e répteis, integrando cadeias alimentares que sustentam a diversidade faunística da Caatinga (SANTOS et al., 2021).

Essa função ecológica de espécie-chave pode indicar o potencial de *G. spixii* como bioindicador ambiental, pois sua presença e densidade populacional refletem a estabilidade dos ecossistemas semiáridos e a disponibilidade de recursos vegetais e hídricos. A manutenção de populações estáveis depende diretamente da integridade da vegetação nativa e da conectividade dos habitats, sendo a espécie sensível a distúrbios antrópicos, como desmatamento e queimadas (REIS et al., 2006). Por essas razões, o monitoramento de suas populações tem sido proposto em estudos de conservação e gestão ambiental voltados à Caatinga (QUEIROZ et al., 2020).

Sob o ponto de vista econômico, o *G. spixii* possui importância expressiva nas comunidades rurais do Nordeste brasileiro (SANTOS et al., 2021). É frequentemente utilizado como recurso alimentar em práticas tradicionais de caça de subsistência, reconhecido por seu valor nutricional elevado e baixo custo de manejo, configurando uma alternativa de proteína animal acessível (ALVES, 2009; ALVES et al., 2012). Paralelamente, o cultivo racional e controlado da espécie em cativeiro tem despertado interesse em projetos de fauno cultura e manejo sustentável, voltados tanto à geração de renda quanto à conservação *ex situ*, alinhando-se às políticas de uso sustentável da fauna silvestre (ALVES et al., 2012).

Além do papel econômico, sob uma abordagem cultural, o *G. spixii* apresenta papel simbólico e educativo relevante. Sua docilidade e aspecto morfológico peculiar têm favorecido sua inserção em programas de educação ambiental e ecoturismo, estimulando o reconhecimento da fauna regional e a valorização do bioma Caatinga (LEAL et al., 2003). Projetos de visitação e manejo didático da espécie em centros de fauna têm sido associados à sensibilização ambiental e à conscientização sobre o uso responsável dos recursos naturais. Assim, o *G. spixii* ultrapassa o valor econômico, assumindo função sociocultural que contribui para o desenvolvimento sustentável de comunidades locais.

Dessa forma, a análise integrada da biologia, ecologia e relevância econômica de *G. spixii* evidencia sua importância tanto no equilíbrio ecológico dos ecossistemas semiáridos, quanto como espécie de interesse científico e social. O conjunto de adaptações morfofisiológicas e comportamentais observadas revela uma linhagem altamente especializada e resiliente, cuja manutenção depende de ações coordenadas de conservação e manejo sustentável. Além dessas dimensões biológicas, ecológicas e econômicas, o *G. spixii* apresenta atributos fisiológicos e genéticos que favorecem seu uso em estudos voltados ao desenvolvimento de técnicas reprodutivas e à aplicação de biotecnologias de conservação.

Esses elementos fornecem base sólida para a compreensão de seus aspectos fisiológicas, genéticos e reprodutivos.

2.3 Aspectos fisiológicos, genéticos e reprodutivos de *G. spixii*

Sob os aspectos fisiológicos, o *G. spixii* é uma espécie com notável capacidade de adaptação e estabilidade, atributos essenciais para sua manutenção em condições em sistemas de criadouros científico (NOWAK, 1999). Isso ocorre porque seu peso, tempo de gestação e acelerada taxa metabólica pode fazer com que favoreça seu desempenho sob os diferentes habitats que pode ser localizado. Além disso, o comportamento reprodutivo contínuo e a ausência de sazonalidade reprodutiva marcante, observados também em fêmeas mantidas em cativeiro científico, conferem previsibilidade e repetibilidade aos estudos (VALE et al., 2023).

No que se refere às questões genéticas, o *G. spixii* ocupa posição evolutiva singular dentro dos Caviomorpha (ROWE; HONEYCUTT, 2002). Segundo MAIA (1983), o *G. spixii* apresenta cariótipo $2n = 64$ ($FN = 118$), com organização cromossômica estável e baixa variação intraespecífica. Esse padrão estrutural associado à sua estabilidade genômica e baixa taxa de mutação estrutural, o distingue como uma linhagem monofilética consolidada, geneticamente adequada e que pode ser utilizado em experimentos que envolvem a manipulação nuclear de células somáticas visando técnicas de reprogramação (DUNNUN; SALAZAR-BRAVO, 2010; ROWE; HONEYCUTT, 2002).

No âmbito reprodutivo, o *G. spixii* apresenta um sistema masculino composto por pênis cilíndrico com flexura cranial e glândulas acessórias bem desenvolvidas, incluindo próstata, glândulas vesiculares e glândulas bulbouretrais (SANTOS et al., 2012). A espermatogênese inicia-se aos 30 dias de vida, com espermatozoides maduros presentes aos 45 dias e completa maturação entre 120 e 150 dias (SANTOS et al., 2012). Essa precocidade e constância espermatogênica viabilizam experimentos com gametas em diferentes estágios de maturação, facilitando estudos de cinética espermática e criopreservação seminal (SILVA et al., 2024).

Nas fêmeas, a maturidade sexual é alcançada entre 55 e 90 dias, e o ciclo estral contínuo, de 14 a 19 dias (VALE et al., 2023). Os ovários apresentam estrutura dorsoventralmente achatada, com um número de 1100 folículos por ovário, predominando folículos primordiais e primários (PRAXEDES et al., 2017). Essa alta densidade folicular, associada à integridade morfológica das células da granulosa e oócito, faz com que a espécie possua folículos polioovulares em estágios pré-antrais, característica incomum em roedores, mas que sugere seu

potencial adaptativo relacionado à fertilidade aumentada em ambientes restritivos (PRAXEDES et al., 2017).

A fisiologia uterina e placentária de *G. spixii* também oferece particularidades de valor comparativo. A espécie apresenta útero bicornes e placenta hemocorial com subplacenta funcional persistente até o termo gestacional, que atua na invasão trofoblástica e no controle do fluxo materno-fetal (BEZERRA et al., 2018). Essa característica distingue o *G. spixii* de outros caviões, como *Cavia porcellus*, e o aproxima de modelos biomédicos utilizados em estudos de fisiopatologia placentária humana, incluindo processos de angiogênese e apoptose trofoblástica. O período gestacional varia entre 48 e 60 dias, resultando em ninhadas de dois a quatro crias (OLIVEIRA et al., 2008; VALE et al., 2023), com desenvolvimento pós-natal rápido e alta sobrevivência em cativeiro, atributos favoráveis à reprodução controlada e manutenção de colônias experimentais.

Em paralelo, Santos et al. (2021) demonstraram que o manejo tradicional de *G. spixii* em comunidades rurais do Nordeste evidencia uma domesticação incipiente da espécie. A docilidade, a adaptação alimentar e a reprodução espontânea em cativeiro são resultados de um processo empírico de manejo que reforça a resistência fisiológica e a plasticidade comportamental do *G. spixii*. Tais observações empíricas validam a viabilidade zootécnica e experimental da espécie, fortalecendo o argumento de que suas características fisiológicas e reprodutivas não apenas permitem, mas incentivam o desenvolvimento de biotecnologias voltadas à reprodução e conservação *ex situ* da espécie.

Assim, ao integrar os eixos fisiológico, genético e reprodutivo, o *G. spixii* consolida-se como uma espécie modelo promissora para o avanço das biotecnologias aplicadas à reprodução assistida e à conservação genética de roedores neotropicais. Sua combinação de estabilidade genômica, resposta hormonal previsível e competência folicular o diferencia entre os roedores histricomorfos, posicionando-o como modelo experimental em estudos envolvendo técnicas reprodutivas *in vitro*. Essas evidências constituem a base científica sobre a qual se estruturam as estratégias de conservação e manejo genético.

2.4 Estratégias de conservação desenvolvidas para *G. spixii*

As estratégias voltadas à conservação de espécies silvestres podem ser agrupadas em duas abordagens principais, sendo a *in situ* e *ex situ*, as quais embora distintas em sua aplicação, são complementares e fundamentais para a manutenção da biodiversidade. A conservação *in situ* tem como foco a preservação de populações viáveis em seus habitats naturais, assegurando

o equilíbrio ecológico, a integridade genética e o comportamento adaptativo das espécies em seus ecossistemas de origem. Por outro lado, a conservação *ex situ* envolve métodos realizados fora do ambiente natural, tais como o manejo em cativeiro, bancos genéticos e biotecnologias reprodutivas, que visam preservar o patrimônio genético e possibilitar reintroduções futuras em áreas naturais. Em conjunto, essas abordagens estruturam os pilares de conservação da fauna neotropical (**Figura 4**).

No caso de espécies endêmicas do semiárido brasileiro, como *G. spixii*, as ações *in situ* têm sido essenciais para a compreensão ecológica e comportamental necessária ao manejo sustentável (ALVES et al., 2012). Estudos conduzidos com mamíferos silvestres do Nordeste indicam que a conservação efetiva depende de uma base sólida de conhecimento sobre etnoecologia, dieta, comportamento e distribuição geográfica (PINHEIRO et al., 1989; DUNNUM; SALAZAR-BRAVO, 2010). Esses estudos têm elucidado as interações tróficas e espaciais que condicionam a sobrevivência de espécies adaptadas ao clima semiárido e orientado a implementação de medidas mitigatórias frente às pressões antrópicas (SANTOS et al., 2019; SANTOS et al., 2021).

Entre as principais ameaças à manutenção de populações naturais de pequenos roedores estão a fragmentação do habitat, a caça predatória e os atropelamentos rodoviários, fatores que comprometem a conectividade populacional e podem reduzir a variabilidade genética (REIS et al., 2006). Associadas à expansão agropecuária e às mudanças climáticas, essas ameaças reforçam a urgência de políticas voltadas à conservação *in situ* no bioma Caatinga, onde *G. spixii* desempenha papel ecológico essencial tanto como herbívoro primário quanto como presa de diversas cadeias tróficas. Dessa forma, o manejo de populações locais e o monitoramento de áreas prioritárias são medidas indispensáveis ao sucesso conservacionista (PINHEIRO et al., 1989). Nesse contexto, Alves et al. (2012) destacam que ações de conservação devem reconhecer a dimensão socioeconômica da fauna, promovendo a conscientização sobre o uso racional dos recursos naturais e incentivando práticas compatíveis com a manutenção da biodiversidade. A interação entre conhecimento científico e saber popular emerge, portanto, como elemento-chave para a eficácia das estratégias *in situ*.

Embora imprescindíveis, os esforços *in situ* não são suficientes para garantir a conservação a longo prazo de espécies silvestres, especialmente daquelas sujeitas a declínios populacionais, pressões antrópicas intensas ou perda progressiva de variabilidade genética. Neste cenário, técnicas de conservação *ex situ* tornam-se complementares e estratégicas, oferecendo alternativas para a preservação do material biológico e assegurando a disponibilidade de indivíduos e recursos genéticos para programas de reintrodução e manejo.

Tais estratégias têm ganhado relevância à medida que ameaças ambientais continuam a comprometer a estabilidade das populações naturais (MADELAIRE et al., 2025). Essa modalidade abrange a conservação de gametas, embriões, tecidos e células, bem como a manutenção de populações vivas em cativeiro sob condições controladas que reconstruam aspectos ambientais do habitat natural (HUTCHINSON et al., 2024; KHAYDUKOVA et al., 2024).

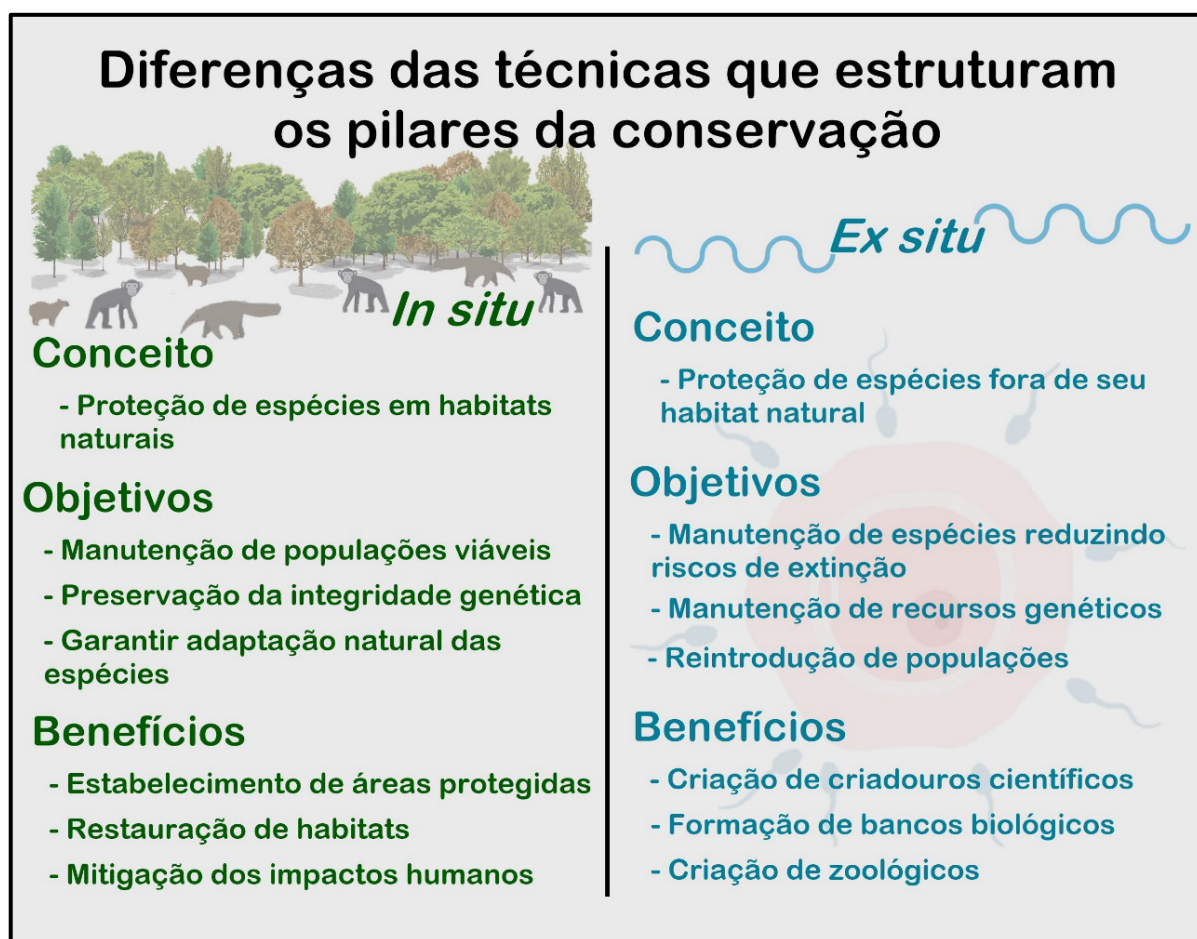


Figura 4. Estratégias de conservação desenvolvidas na conservação da biodiversidade animal.
Fonte: Própria autoria.

Em roedores silvestres, estratégias *ex situ* têm sido amplamente descritas (PRAXEDES et al., 2020). Entre elas, as abordagens *in vivo* destacam-se pela formação de criatórios com finalidades ecológicas, econômicas, culturais e científicas, constituindo importantes ferramentas para avaliação do comportamento fisiológico e reprodutivo. Em *G. spixii*, indivíduos provenientes de criatórios têm sido utilizados em estudos de caracterização anatômica e morfológica (BEZERRA et al., 2018), fornecendo informações ao planejamento

biotecnológico e ao desenvolvimento de metodologias aplicadas à reprodução (OLIVEIRA et al., 2022).

Por sua vez, as estratégias *ex situ in vitro* têm sido ainda mais amplamente empregadas em *G. spixii* (**Quadro 1**). Nessa espécie, tecidos gonadais e somáticos, gametas masculinos e femininos e células isoladas têm sido utilizados como base para a formação de bancos de recursos biológicos que subsidiam o desenvolvimento de biotecnologias reprodutivas, como a produção *in vitro* de embriões, clonagem e a obtenção de iPSCs.

No âmbito gonadal e das células gaméticas, estudos têm avaliado estruturas ovarianas e testiculares para fins de maturação *in vitro* de oócitos, criopreservação e análise morfofuncional, estabelecendo bases para o uso dessas amostras biológicas na fertilização *in vitro* e manipulações reprodutivas (PRAXEDES et al., 2017; LAGO et al., 2017). No campo dos tecidos somáticos, a criopreservação de pele, cartilagem auricular e outros tecidos de *G. spixii* tem permitido a obtenção de explantes viáveis para cultivo celular, garantindo possivelmente o acesso contínuo a linhagens somáticas para uso posterior (OLINDO et al., 2024).

Nesse contexto, insere-se a clonagem por TNCS, cuja implementação depende diretamente da qualidade dos tecidos e células destinados à manipulação nuclear (BORGES; PEREIRA, 2019). Os primeiros avanços voltados especificamente para a espécie foram apresentados por PEREIRA et al. (2021), que identificaram diferenças estruturais significativas entre tecidos de *G. spixii* e *Dasyprocta leporina*, reforçando a necessidade de protocolos individualizados e demonstrando que *G. spixii* exige refinamento próprio para alcançar uma maior eficiência no uso dos protocolos. Esses resultados iniciaram a compreensão de que o desenvolvimento de metodologias específicas para a espécie é uma condição indispensável para a evolução das abordagens biotecnológicas aplicadas à conservação.

Dessa forma, torna-se evidente que as estratégias de conservação *ex situ* aplicadas a *G. spixii* ultrapassam significativamente os limites tradicionais do manejo em cativeiro, alcançando um patamar biotecnológico avançado. A integração de protocolos de criopreservação específicos, a obtenção de linhagens somáticas viáveis e estáveis, e a capacidade de manipulação de oócitos funcionalmente competentes intensifica todas as condições técnicas e biológicas necessárias para os estudos celulares e reprodutivos na espécie.

Quadro 1. Estratégias de conservação *ex situ in vitro* desenvolvidas em *G. spixii*.

Recurso biológico	Objetivo do estudo	Principal resultado	Referência
Espermatozoide	Identificar células da linhagem espermatogênica do epitélio seminífero	A linhagem espermatogênica foi similar quando comparada a capivara e hamster	SANTOS et al. (2011)
	Avaliar as características morfológicas do processo espermatogênico	A atividade reprodutiva da espécie ocorreu aos 45 dias de idade	SANTOS et al. (2012)
	Descrever a diferenciação de células germinativas por ultraestrutura	A morfologia da cabeça dos espermatozoides é diferente de porquinho-da-índia e hamster	SANTOS et al. (2014)
	Descrever as características de espermatozoides epididimários recuperado por duas metodologias	A flutuação e a lavagem retrógrada podem ser empregadas na recuperação de espermatozoides	SILVA et al. (2016)
	Avaliar a eficiência de TES e TRIS na recuperação de espermatozoides epididimários	A solução TRIS foi mais eficiente para a recuperação espermatozoides epididimários	SILVA et al. (2017)
	Desenvolver um protocolo de criopreservação de espermatozoides epididimários	Espermatozoides foram criopreservados com sucesso em 20% gema de ovo e 6% glicerol	SILVA et al. (2019a)
	Comparar o efeito do TRIS e ACP com gema de ovo (10 e 20%) ou <i>Aloe vera</i> na criopreservação	TRIS com 10% de gema de ovo foi a solução mais eficiente na criopreservação	MOREIRA et al. (2021)
Tecido ovariano	Avaliar as características da citologia vaginal durante o ciclo estral	O ciclo estral dura em média 15 dias, e há formação/ruptura da membrana vaginal	SANTOS et al. (2016)
	Caracterizar, estimar e criopreservar a população de folículos ovarianos pré-antrais	Uma menor população folicular foi observada quando comparada a hamster e ratos	PRAXEDES et al. (2017)
Tecido testicular	Estabelecer a melhor técnica de vitrificação de tecido testicular utilizando DMSO e EG (3,0 ou 6,0 M)	3,0 M de DMSO ou EG foi a melhor concentração quando comparado a 6,0 M	LAGO et al. (2017)
	Avaliar diferentes crioprotetores (DMSO e EG) na vitrificação de tecido testicular	3,0 M de EG com 0,25 M de sacarose e 10% de SFB foi a melhor combinação de crioprotetores	SILVA et al. (2019b)
Tecido somático	Descrever a pele do pavilhão auricular apical visando a formação de criobancos	A pele diferiu quanto aos padrões morfológicos entre pacas, castor europeu e ratos	PEREIRA et al. (2021)
	Estabelecer técnicas de formação de um biobanco de amostras somáticas	Ambas as técnicas avaliadas podem ser utilizadas, mas a vitrificação em superfície sólida é mais eficiente	OLINDO et al. (2024)

**ACP: Diluente à base de água de coco em pó. TES: ácido N-[Tris(hidroximetil)metil]-2-aminoetanossulfônico. TRIS: Tris-hidroximetil-aminometano. EG: Etilenoglicol. SFB: Soro fetal bovino.

2.5 Estratégias para o isolamento, cultivo *in vitro* e avaliação do ciclo celular de linhagens fibroblásticas aplicadas a roedores

No contexto contemporâneo da conservação da biodiversidade, as biotecnologias celulares e reprodutivas emergem como ferramentas para a mitigação da perda genética, especialmente em grupos taxonômicos com elevada diversidade e vulnerabilidade ambiental, como os roedores (WU et al., 2024). Nesse cenário, o uso de estratégias celulares deixa de ser apenas um suporte experimental e passa a ocupar papel central como ferramenta de conservação *ex situ*, complementando abordagens *in vivo* e contribuindo para a redução da pressão sobre populações naturais.

A formação de biobancos de células somáticas representa um dos pilares dessas estratégias, uma vez que permite o armazenamento a longo prazo de material genético viável, passível de múltiplas aplicações futuras (HILDEBRANDT; HOLTZE, 2024). Diferentemente de abordagens baseadas exclusivamente em gametas ou embriões, os biobancos celulares somáticos oferecem maior flexibilidade operacional, possibilitando seu emprego em clonagem por TNCS, na geração de iPSCs, na produção de gametas *in vitro* e em estudos voltados à reprogramação nuclear visando a identificação de mecanismos relacionados a plasticidade celular (NAVARRO et al., 2024; MADELAIRE et al., 2025). Assim, as células somáticas configuram-se como unidades biológicas centrais para a conservação genética e para o desenvolvimento de tecnologias reprodutivas em espécies silvestres (BORGES; PEREIRA, 2019).

Em roedores de laboratório e silvestres, o uso de estratégias celulares é particularmente relevante em virtude da ampla disponibilidade de modelos experimentais, diversidade filogenética do grupo e existência de protocolos estabelecidos para isolamento, cultivo e caracterização celular. Um exemplo disso é o desenvolvimento de protocolos voltados para a caracterização de linhagens celulares já descritas tanto em tecidos fetais quanto em tecidos adultos, como em *D. leporina* (PRAXEDES et al., 2021) e em modelos amplamente utilizados em laboratórios de cultivo celular, como camundongos (XU et al., 2005), porquinho-da-índia (MEHRABANI et al., 2014) e hamsters (MEHRABANI et al., 2016). Essa abordagem aplicada a roedores de laboratório e silvestres tem consolidado essa ordem como um grupo de referência

para o desenvolvimento, padronização e refinamento de protocolos celulares aplicáveis a espécies menos estudadas do ponto de vista biotecnológico ou quando ameaçadas de extinção.

A escolha da fonte celular constitui um dos primeiros fatores para o sucesso das estratégias de conservação baseadas em células. Entre os diferentes tipos celulares avaliados em roedores, incluindo células do *cumulus*, células de Sertoli, e células epiteliais, os fibroblastos de pele destacam-se como a opção mais consistente para aplicações conservacionistas (HILDEBRANDT; HOLTZE, 2024). Essa escolha se justifica por sua obtenção minimamente invasiva, elevada capacidade proliferativa, estabilidade morfológica e genética nas passagens iniciais, além da ampla experiência acumulada nos últimos anos com esse tipo celular em programas de biobanco e quando aplicadas em estudos voltados a reprogramação celular tanto em espécies domésticas quanto em mamíferos silvestres (GAO et al., 2025). Dessa forma, o estabelecimento e a padronização de protocolos relacionados ao cultivo *in vitro* de linhagens celulares dessa natureza para *G. spixii* alinha-se tanto às evidências da literatura quanto aos princípios éticos de redução e refinamento no uso de animais quando associado aos objetivos de desenvolvimento sustentável.

Para haver sucesso na aplicação de linhagens celulares associadas ao desenvolvimento de estratégias de conservação, é necessário considerar as condições de cultivo *in vitro* que representam um segundo eixo crítico dessa abordagem, pois influencia diretamente a viabilidade, a estabilidade fisiológica e a competência funcional das células utilizadas (VIS et al., 2020). Estudos em roedores demonstram variações interespecíficas na dinâmica de crescimento celular. Por exemplo, em roedores é comum a utilização de fibroblastos entre a terceira e a oitava passagem, com relatos de redução progressiva da qualidade celular após esse intervalo (MEHRABANI et al., 2014; 2016; MA et al., 2022). Essa variabilidade reflete não apenas diferenças intra e interespecíficas, mas também a sensibilidade do fenótipo celular às condições de cultivo *in vitro* (ESLAMINEJAD et al., 2006).

A formação de biobancos de células somáticas é outro parâmetro intrinsecamente associado ao desenvolvimento de estratégias de conservação. Isso porque uma vez caracterizadas morfofuncionalmente, as células podem ser criopreservadas e posteriormente resgatadas, reduzindo o risco de senescência replicativa, minimizando o acúmulo de alterações epigenéticas indesejáveis e mantendo, a longo prazo, um estoque de linhagens utilizáveis sob diferentes abordagens biotecnológicas (HILDEBRANDT; HOLTZE, 2024; MADELAIRE et al., 2025). Essa perspectiva é particularmente relevante diante do ritmo acelerado de perda de habitats naturais, sendo que o desenvolvimento de protocolos robustos para fibroblastos para o

G. spixii insere a espécie também em uma agenda estratégica de biobancos de conservação no bioma Caatinga (BALDINI et al., 2024; GONZÁLEZ et al., 2025).

Do posto de vista metodológico, as linhagens de fibroblastos de roedores tem sido criopreservadas por congelação lenta, utilizando crioprotetores intracelulares como o dimetilsulfóxido (DMSO) na concentração de 10%, associados a 10% de soro fetal bovino (SFB). A redução gradual da temperatura visa equilibrar a saída de água celular e a entrada do crioprotetor, minimizando a formação de gelo intracelular e a injúria osmótica (PRAXEDES et al., 2023). Contudo, a associação DMSO–SFB apresenta limitações, incluindo danos à membrana plasmática, redução da viabilidade pós-descongelação e prejuízos à retomada do crescimento celular, sobretudo quando se utilizam concentrações elevadas de DMSO ou soros de composição variável (HILDEBRANDT; HOLTZE, 2024; SCIORIO et al., 2024).

Nesse contexto, crioprotetores extracelulares como a sacarose emergem como ferramentas complementares para modular o estresse osmótico durante a congelação e descongelação. Em concentrações próximas a 0,2 M, a sacarose promove desidratação controlada, eleva o ponto de transição vítrea e estabiliza estruturas de membrana, reduzindo a formação de gelo e a ruptura de bicamadas lipídicas. Em *D. leporina*, a associação de baixas concentrações de SFB com sacarose permitiu manter a viabilidade e a qualidade de fibroblastos, reduzindo a carga proteica sérica no meio de congelação e favorecendo aplicações posteriores dessas amostras (PRAXEDES et al., 2023).

Além da sacarose, aminoácidos osmoprotetores como a L-prolina despontam como candidatos promissores para a otimização da criopreservação celular. A L-prolina apresenta propriedades de estabilização de proteínas e membranas, atuando na regulação da pressão osmótica, atenuação do estresse hídrico, tamponamento de variações de pH e modulação do estado redox intracelular (SUN et al., 2012; DOU et al., 2019). Evidências indicam redução na produção de espécies reativas de oxigênio e preservação do dobramento proteico durante ciclos de congelação e descongelação (ZHANG et al., 2016). Embora concentrações de 5 mM ainda não tenham sido aplicadas especificamente a fibroblastos de roedores, estudos em células endoteliais humanas demonstram aumento significativo da integridade de membrana e da eficiência de cultivo pós-descongelação quando comparadas a protocolos baseados exclusivamente em DMSO (SUN et al., 2012; DOU et al., 2019). Em oócitos de ratos, a associação da L-prolina a crioprotetores clássicos resultou em maiores taxas de blastocistos pós-descongelação, sugerindo preservação mais eficiente da competência embrionária (ZHANG et al., 2016). Esses achados sustentam a hipótese de que a incorporação da L-prolina

possa fortalecer a base de um biobanco celular para *G. spixii*, permitindo o estabelecimento da técnica na espécie.

No âmbito das estratégias celulares aplicadas à conservação em roedores, a sincronização do ciclo celular pode ser compreendida como uma abordagem na determinação e caracterização do ciclo fisiológico das linhagens celulares obtidas. No contexto da aplicação dessas células em técnicas reprodutivas, a sincronização das linhagens em fase G₀/G₁ representa uma etapa operacional e crítica para o sucesso da TNCS. Isso porque por meio desta técnica é possível adequar o estado funcional do núcleo doador (carioplasto) às exigências impostas pelo citoplasma oocitário receptor (citoplasto), geralmente bloqueado em metáfase II. Em estudos atuais, comunicação entre núcleo–citoplasma é determinante para a manutenção da ploidia correta, para a estabilidade cromossômica após a ativação e para a eficiência global do processo de reprogramação nuclear (WU et al., 2024; LOU et al., 2025).

A sincronização em G₀/G₁ confere vantagens funcionais relevantes aos núcleos doadores, incluindo maior plasticidade epigenética, menor atividade transcricional basal e redução do risco de eventos estruturais deletérios, como condensação prematura da cromatina e ruptura do envelope nuclear (MITALIPOV et al., 2002; SANTOS et al., 2003; PANKAMMOON et al., 2025). Esses fatores contribuem diretamente para o aumento da eficiência de reprogramação e para a melhoria do desenvolvimento embrionário inicial, conforme demonstrado em diferentes modelos experimentais (CAMPBELL et al., 1996; LOI et al., 2007). Assim, a sincronização do ciclo celular deve ser compreendida como um mecanismo de padronização funcional das células somáticas, aumentando a previsibilidade e a reprodutibilidade de sua aplicação em técnicas biotecnológicas avançadas.

Ainda, populações celulares mantidas em cultivo *in vitro* apresentam progressão naturalmente assíncrona do ciclo celular, com distribuição heterogênea entre as fases G₀/G₁, S e G₂/M. Essa heterogeneidade compromete o uso técnico das células como ferramentas biotecnológicas, uma vez que núcleos em fases inadequadas estão associados a falhas de reprogramação, instabilidade genômica e redução das taxas de clivagem e de desenvolvimento embrionário subsequente (ZHUKOVA, 2024; CHEN et al., 2025). Nesse contexto, a sincronização do ciclo celular torna-se uma estratégia técnica indispensável, voltada a enriquecer a população celular em uma fase compatível com os requisitos das abordagens de reprogramação nuclear.

Entre os protocolos disponíveis, os métodos mais empregados incluem: (a) inibição por contato; (b) privação de soro fetal bovino; e (c) o uso de inibidores químicos de quinases dependentes de ciclina (CDKs), como a roscovitina (RSV) (HAYES et al., 2001; SHEN et al.,

2013; PRAXEDES et al., 2024). A eficiência dessas abordagens, contudo, é fortemente dependente da espécie, do tipo celular, do tempo de exposição e de parâmetros experimentais como confluência e composição do meio de cultivo.

Em roedores silvestres, evidências indicam limitações claras da inibição por contato como estratégia de sincronização. Em fibroblastos dérmicos de *D. leporina*, embora a inibição por contato tenha permitido alcançar aproximadamente 70% das células em G₀/G₁, essa abordagem mostrou-se menos eficiente quando comparada à privação de soro e ao uso da RSV (PRAXEDES et al., 2024). Resultados semelhantes foram descritos em ratos e camundongos, nos quais a inibição por contato é pouco utilizada e, quando aplicada, apresenta desempenho inferior ou equivalente ao observado em *D. leporina* (HAYES et al., 2001; PRAXEDES et al., 2024). Em contraste, a privação de soro por períodos entre 48 e 72 h e a exposição à RSV (10 µM por aproximadamente 12 h) resultaram em taxas de sincronização superiores a 80%, sem prejuízos à morfologia, viabilidade ou índices de apoptose celular.

Apesar de sua eficiência, a privação de soro apresenta limitações técnicas importantes. Exposições prolongadas além de 72 h não promovem ganhos adicionais de sincronização e tendem a induzir estresse metabólico, fragmentação de DNA e apoptose, comprometendo a qualidade funcional das células destinadas à reprogramação (CHO et al., 2005). De forma semelhante, a eficácia e a citotoxicidade da RSV são altamente sensíveis a variações de concentração e tempo de exposição. Revisão em felídeos silvestres demonstraram que concentrações entre 7,5 e 30 µM, com exposições de 12 a 24 h, produzem respostas variáveis entre espécies, sendo que concentrações superiores a 15 µM podem comprometer a viabilidade celular em determinadas linhagens (VIANA; PEREIRA, 2025).

Essa variabilidade interespecífica reforça a necessidade de investigar e otimizar protocolos de sincronização ajustados às características biológicas de cada espécie, especialmente no contexto de estudos de reprogramação celular e conservação genética (PEREIRA et al., 2023). Tal premissa não é diferente quando aplicado ao *G. spixii*, onde a técnica pode auxiliar para o desenvolvimento de abordagens de conservação e reprodução assistida (SILVA et al., 2024).

Dessa forma, a sincronização do ciclo em fibroblastos de *G. spixii* deve ser entendida não apenas como um procedimento técnico isolado, mas como uma etapa estruturante para o uso dessas células como ferramentas biotecnológicas. A avaliação sistemática de protocolos de sincronização, considerando viabilidade, integridade nuclear e estabilidade fisiológica, constitui um passo essencial para sustentar aplicações em reprogramação celular, TNCS e outras estratégias de conservação genética baseadas no uso de células somáticas.

Finalmente, as diferentes técnicas aplicadas a linhagens celulares estabelecidas, configura-se como uma ferramenta central para a conservação genética em roedores silvestres. A resposta a essas abordagens é marcadamente espécie-específica, o que torna indispensável a avaliação sistemática desses protocolos em fibroblastos de *G. spixii*. Portanto, o estabelecimento dessas etapas, pode possibilitar a espécie de forma estratégica no contexto da conservação de roedores neotropicais.

2.6 Produção *in vitro* de embriões como estratégia reprodutiva aplicado em roedores

As estratégias reprodutivas baseadas na produção *in vitro* de embriões (PIVE) têm se consolidado como ferramentas centrais para a conservação genética *ex situ* de mamíferos, especialmente em contextos nos quais a reprodução natural é limitada por baixa densidade populacional, fragmentação de habitats, sazonalidade reprodutiva ou inviabilidade de manejo contínuo de colônias (ARIF et al., 2025; BLACKBURN et al., 2024). Em roedores, grupo que abrange tanto modelos clássicos de laboratório quanto espécies silvestres neotropicais, a PIVE permite dissociar a geração de embriões do acasalamento natural, ampliando a flexibilidade dos programas de conservação e criando interfaces diretas com biobancos genéticos e outras biotecnologias reprodutivas aplicadas à preservação da biodiversidade.

No contexto biológico que fundamenta essas abordagens, a competência do núcleo e o citoplasma oocitário emerge como o principal determinante da eficiência do desenvolvimento embrionário *in vitro*. Evidências recentes demonstram que o oócito atua como um verdadeiro *hub* de reprogramação, por concentrar os sistemas metabólicos, epigenéticos e estruturais responsáveis por sustentar a remodelação nuclear, a reorganização do citoesqueleto e a ativação do programa embrionário inicial (SAADELDIN et al., 2025). Essa perspectiva é reforçada por análises que destacam a integração entre redes epigenéticas, metabolismo celular e dinâmicas citoplasmáticas como fatores críticos para o sucesso da reprogramação e do desenvolvimento embrionário (ZHU; NIE, 2025; PANKAMMOON et al., 2025). Assim, independentemente da aplicação subsequente, a qualidade funcional do oócito constitui o eixo central sobre o qual se constrói o sucesso das estratégias reprodutivas baseadas na PIVE, sustentando a hipótese biológica de que a padronização dessas etapas iniciais é uma condição necessária para estabelecer uma base reprodutiva robusta em espécies não-modelo, como o *G. spixii*.

Em roedores silvestres, a obtenção de oócitos imaturos representa o primeiro desafio técnico dessas estratégias. Diferentemente dos modelos laboratoriais, nos quais protocolos de superestimulação são amplamente empregados, espécies neotropicais têm demonstrado

viabilidade na recuperação de oócitos a partir de fêmeas não estimuladas, especialmente por meio da coleta ovariana pós-eutanásia associada à aspiração folicular ou ao fatiamento ovariano. Em *D. leporina*, essa abordagem resultou em proporções expressivas de oócitos viáveis (PRAXEDES et al., 2023), padrão semelhante ao observado em *Cuniculus paca* (BARROS et al., 2020). Protocolos de superestimulação, por sua vez, permanecem mais frequentes em roedores de laboratório (MA et al., 2005; CAÑÓN-BELTRÁN et al., 2024). Do ponto de vista onde a conservação da espécie é o foco, a adoção de métodos que maximizem o aproveitamento do material ovariano disponível, respeitando limites éticos e logísticos, é particularmente relevante para espécies silvestres com acesso restrito a indivíduos reprodutivos.

Após a recuperação, a maturação *in vitro* (MIV) constitui uma etapa crítica para a aquisição da competência nuclear e citoplasmática. Atualmente, é amplamente reconhecido que a MIV não se limita à progressão meiótica, mas envolve a consolidação de estoques de RNAs maternos, proteínas regulatórias, fatores epigenéticos e ajustes metabólicos e mitocondriais indispensáveis para sustentar o desenvolvimento embrionário inicial (LODDE et al., 2025; HIRAO, 2025). Em roedores de laboratório, o uso do meio TCM-199 suplementado é amplamente aceito durante a MIV, enquanto meios como o KSOM são recorrentes no cultivo embrionário pré-implantacional (CAÑÓN-BELTRÁN et al., 2024). Contudo, revisões recentes indicam que pequenas variações na composição iônica e energética do meio de cultivo podem alterar significativamente a dinâmica de maturação nuclear e citoplasmática, especialmente no que se refere ao equilíbrio entre lactato, piruvato e glicose, modulador central da respiração mitocondrial oocitária (MEN, 2024).

A modulação do ambiente redox durante a MIV constitui outro eixo fundamental para a preservação da integridade citoplasmática. A presença excessiva de espécies reativas de oxigênio compromete a formação do fuso meiótico, o alinhamento cromossômico e aumenta a incidência de aneuploidias, fatores críticos quando se objetiva gerar citoplastos funcionalmente competentes para aplicações conservacionistas (BALDINI et al., 2024). Nesse contexto, antioxidantes como a cisteamina demonstraram elevar a disponibilidade intracelular de glutatona e reduzir o estresse oxidativo, resultando em aumento das taxas de maturação em porquinhos-da-índia (WANG et al., 2019).

Além disso, fatores tróficos envolvidos na aquisição da competência oocitária também é reportado. Dentre esses, o fator de crescimento epidérmico (EGF) destaca-se por seu papel na expansão das células do *cumulus*, na retomada da meiose e na reorganização do citoesqueleto, mediado pela ativação de vias como MAPK e pela regulação de genes associados à maturação nuclear e citoplasmática (MEN, 2024; SAADELDIN et al., 2025). Em roedores, respostas

interespecies às concentrações de EGF são marcantes: em porquinhos-da-índia, concentrações acima de 50 ng/mL foram associadas a maiores taxas de MIV, enquanto em *D. leporina* concentrações de até 10 ng/mL resultaram em desempenho num melhor desempenho (PRAXEDES et al., 2023; CAÑÓN-BELTRÁN et al., 2024). Esse comportamento reforça a premissa de que espécies silvestres apresentam um próprio perfil fisiológico, exigindo a otimização dessa etapa na espécie alvo de estudo.

Superada a fase das condições de MIV, a ativação oocitária artificial representa o passo final para a construção da competência embrionária *in vitro* no contexto em que ainda não se foi realizado o desenvolvimento de técnicas reprodutivas numa espécie não modelo. De modo geral, os protocolos de ativação oocitária visam induzir oscilações controladas de cálcio intracelular capazes de desencadear a exocitose de grânulos corticais, a conclusão da meiose e a ativação do genoma embrionário (MA et al., 2005; HEYTENS, 2008). Em roedores, estudos indicam uma diversidade de protocolos para a indução desses pulsos intracelulares de cálcio. Entre os químicos utilizados, a ionomicina, etanol, ionóforos de cálcio e, especialmente, SrCl_2 representam os agentes principais usados. Além disso, agentes secundários, como o 6-DMAP, atuam bloqueando síntese proteica e impedindo o retorno prematuro ao ciclo celular, favorecendo maior estabilidade no embrião reconstruído. HEYTENS (2008) destacaram que a combinação desses estímulos determina a amplitude, a duração e o número de picos de Ca^{2+} , elementos essenciais ao sucesso da ativação.

Em roedores silvestres, a variabilidade de respostas é marcante. Em *D. prymnolopha*, FERRAZ et al. (2020) obtiveram aproximadamente 63% de clivagem quando empregado um protocolo de ativação usualmente utilizado em mamíferos maiores composto por 5 μM de ionomicina e 2 mM de 6-DMAP, enquanto em *D. leporina* 10 mM de SrCl_2 associado à 5 $\mu\text{g/mL}$ de citocalasina B, abordagem mais utilizada em roedores de laboratório, resultou em aproximadamente 20% de mórulas (PRAXEDES et al., 2023). Em murinos (MA et al., 2005), por outro lado, esse mesmo protocolo pode resultar na produção de mais de 56% de blastocistos. Um protocolo ainda mais eficiente, combinando 10 mM de SrCl_2 por 2,5 h seguido de 6-DMAP e 5 $\mu\text{g/mL}$ de citocalasina B por 3,5 h, pode elevar a taxa para cerca de 60%. Embora sejam úteis como referência conceitual, tais protocolos precisam ser ajustados para *G. spixii*, considerando sua fisiologia reprodutiva.

Essa dependência de ajustes específicos reforça um ponto central: o sucesso no desenvolvimento de técnicas reprodutivas visando a PIVE em novas espécies depende da integração funcional entre diferentes etapas do processo, particularmente no contexto da MIV de oócitos direcionada à produção embrionária por ativação artificial. Nesse sentido, os

contrastes observados entre espécies reforçam que protocolos eficientes em espécies-modelo não podem ser diretamente extrapolados para roedores silvestres sem validação sistemática, uma vez que diferenças fisiológicas, metabólicas e citoplasmáticas impactam diretamente a aquisição de competência embrionária.

Em síntese, a PIVE aplicada aos estudos de roedores deve ser compreendida como uma sequência integrada de decisões técnicas que constroem a competência citoplasmática e o núcleo e determinam o potencial de desenvolvimento embrionário. A otimização da recuperação de oócitos, o estabelecimento de sistemas de MIV ajustados à fisiologia da espécie e a otimização no sistema de produção embrionária constituem os pilares biológicos que sustentam a hipótese central deste estudo. Sob essa perspectiva, a eficiência de abordagens reprodutivas avançadas em *G. spixii* dependerá fundamentalmente do estado funcional do citoplasma oocitário, reforçando a PIVE como ferramenta estratégica para a conservação genética de roedores neotropicais.

3 JUSTIFICATIVA

O *G. spixii* é um roedor silvestre neotropical que desempenha papel ecológico relevante nos ambientes semiáridos em que ocorre, contribuindo para a dinâmica trófica, a dispersão de sementes e a manutenção da funcionalidade desses ecossistemas. Embora não esteja atualmente classificada como espécie ameaçada, o avanço de pressões antrópicas, como fragmentação de habitats, expansão agrícola e mudanças climáticas, impõe um cenário de vulnerabilidade progressiva. Nesse contexto, tornam-se fundamentais as estratégias preventivas de conservação, capazes de preservar a variabilidade genética e o potencial adaptativo da espécie antes que processos de declínio populacional se intensifiquem.

Entre essas estratégias, o uso de abordagens celulares associado às técnicas reprodutivas assistidas emerge como uma alternativa promissora para a conservação *ex situ* de espécies silvestres. O cultivo, a caracterização e a criopreservação de células somáticas, bem como a manipulação *in vitro* de oócitos e embriões, permitem dissociar a conservação genética da dependência exclusiva da reprodução natural e da manutenção contínua de populações reprodutivas. Essas ferramentas ampliam o espectro de intervenções possíveis, viabilizando o armazenamento de material biológico viável, a construção de biobancos e a aplicação de tecnologias reprodutivas avançadas.

Adicionalmente, o *G. spixii* apresenta características fisiológicas, reprodutivas e filogenéticas que a qualificam como um modelo biológico promissor para estudos em biotecnologias reprodutivas, fisiologia comparada e pesquisa translacional. A consolidação de protocolos celulares e reprodutivos espécie-específicos amplia o conhecimento sobre sua biologia e permite a transferência metodológica para outras espécies de roedores neotropicais filogeneticamente próximas, potencializando o impacto científico e conservacionista dessas abordagens.

Dessa forma, a tese justifica-se por seu caráter estratégico, preventivo e aplicado, ao integrar o uso de células somáticas e técnicas reprodutivas *in vitro* como ferramentas para a conservação genética de *G. spixii*. Ao estabelecer bases técnicas sólidas para a manipulação celular e embrionária, o estudo cria condições para aplicações em conservação *ex situ*, manejo genético e reprodução assistida, contribuindo para a consolidação de *G. spixii* como espécie-modelo relevante para o desenvolvimento de ações inovadoras de conservação da biodiversidade.

4 HIPÓTESES CIENTÍFICAS

I – Linhagens fibroblásticas de *G. spixii* podem ser estabelecidas e cultivadas por mais de oito passagens, mantendo suas características fisiológicas e genotípicas funcionais;

II – A utilização da combinação de crioprotetores extracelulares contendo 5 mM de L-prolina associada a 0,2 M de sacarose garantem uma maior viabilidade de células somáticas de *G. spixii* após a criopreservação, mantendo eficientemente sua atividade metabólica e proliferativa;

III – Células somáticas de *G. spixii* são eficientemente sincronizadas em fase G₀/G₁, onde a roscovitina é mais competente sobre a sincronização quando comparada a privação com SFB;

IV – O EGF na concentração de 50 ng/mL possui é mais eficiente na maturação nuclear de oócitos de *G. spixii*, quando comparado à concentração de 10 ng/mL de EGF

V – A utilização do 6-DMAP na ativação artificial de oócitos de *G. spixii* é mais eficiente do que protocolos sem sua inclusão, favorecendo o desenvolvimento embrionário *in vitro*.

5 OBJETIVOS

5.1 Objetivo Geral

Estabelecer etapas envolvidas no isolamento, cultivo *in vitro*, criopreservação e sincronização do ciclo celular de linhagens fibroblásticas de *G. spixii*, visando à construção de bases metodológicas para a conservação da espécie.

Estabelecer bases técnicas para a produção *in vitro* de embriões de *G. spixii*, visando à construção de bases metodológicas para a conservação e manejo reprodutivo da espécie.

5.2 Objetivos Específicos

I – Estabelecer, caracterizar e manter linhagens fibroblásticas de *G. spixii*, assegurando parâmetros morfofuncionais, viabilidade e estabilidade adequados para sua aplicação como ferramentas biotecnológicas em estratégias de conservação e reprogramação celular;

II – Criopreservar linhagens fibroblásticas de *G. spixii* utilizando diferentes soluções crioprotetoras e avaliar seus efeitos sobre viabilidade, integridade celular e potencial de uso posterior em abordagens reprodutivas e de reprogramação;

III – Avaliar e comparar métodos de sincronização do ciclo celular em G₀/G₁ (privação de soro fetal bovino vs. roscovitina) em linhagens fibroblásticas de *G. spixii*, considerando eficiência de sincronização, viabilidade e adequação para aplicações biotecnológicas;

IV – Determinar o efeito da concentração de EGF durante a MIV de oócitos de *G. spixii*, com ênfase na aquisição da competência nuclear e citoplasmática e no potencial de desenvolvimento embrionário *in vitro*;

V – Avaliar o efeito do 6-DMAP durante a ativação artificial de oócitos de *G. spixii* sobre a eficiência da ativação e o desenvolvimento embrionário inicial pré-implantacional.

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**CAPÍTULO 2 – CRYOPRESERVATION AND PASSAGING OPTIMIZATION FOR
Galea spixii (WAGLER, 1831) ADULT SKIN FIBROBLAST LINES: A STEP FORWARD
IN SPECIES MANAGEMENT AND GENETIC STUDIES**

ARTIGO EXPERIMENTAL I

**ARTIGO PUBLICADO NO PERIÓDICO INTERNACIONAL ACTA
HISTOCHEMICA**

Qualis 2017-2020: A4 (Medicina Veterinária)

Fator de Impacto: 2.4

Data da Publicação: 26 de Julho de 2024

DOI: <https://doi.org/10.1016/j.acthis.2024.152185>

Cryopreservation and passaging optimization for *Galea spixii* (Wagler, 1831) adult skin fibroblast lines: a step forward in species management and genetic studies

Leonardo Vitorino Costa de Aquino^a, Samara Lima Olindo^a, Yara Letícia Frutuoso e Silva^a, Lhara Ricarliany Medeiros de Oliveira^a, Yasmin Beatriz França Moura^a, Ana Lívia Rocha Rodrigues^a, Érika Almeida Praxedes^a, Moacir Franco de Oliveira^b, Alexandre Rodrigues Silva^c, Aleksandra Fernandes Pereira^{a*}

^aLaboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil.

^bLaboratory of Applied Animal Morphophysiology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil.

^cLaboratory of Animal Germplasma Conservation, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

*Corresponding author: Aleksandra Fernandes Pereira

Laboratory of Animal Biotechnology

Federal Rural University of Semi-Arid

Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil

Phone: +55 84 3317 8361

Email address: alexsandra.pereira@ufersa.edu.br

Institutional email of the authors: L.V.C. Aquino: leonardo.aquino03297@alunos.ufersa.edu.br; S.L. Olindo: samara.lima@alunos.ufersa.edu.br; Y.L.F. Silva: yara.silva@alunos.ufersa.edu.br; L.R.M. Oliveira: lhara.oliveira@alunos.ufersa.edu.br; Y.B.F. Moura: yasmin.moura@ufersa.edu.br; A.L.R. Rodrigues: ana.rodrigues61941@alunos.ufersa.edu.br; E.A. Praxedes: erikaalmeida@hotmail.com; M.F. Oliveira: moacir@ufersa.edu.br; A.R. Silva: alexrs@ufersa.edu.br

Abstract

The *in vitro* culture of fibroblasts is a technique based on cell isolation, physiology characterization, and cryopreservation. This technique has not been described in *Galea spixii*, offering an opportunity to learn about its cell biology and genetic diversity. Therefore, we established fibroblast lines of six *G. spixii* individuals from several passages (second, fifth, eighth, and tenth) and cryopreservation. Fibroblasts recovered from skin biopsies were identified by morphology, immunocytochemistry, and karyotyping assay. Cells before and after cryopreservation were analyzed for morphology, ultrastructure, viability, proliferation, metabolism, oxidative stress, bioenergetic potential, and apoptotic levels. After the eighth passage, fibroblasts showed morphological and karyotypic changes, although viability, metabolism and proliferation did not change. An increase in oxidative stress and bioenergetic potential from the fifth and eighth passages was observed for a longer time in the proliferative rate. Cell damage after analysis of ultrastructure, viability, proliferative rate, apoptotic levels, oxidative stress, and bioenergetic potential were verified after cryopreservation. In conclusion, fibroblasts up to the tenth passage can be cultured *in vitro*. However, cells in the fifth passage present a better quality for reproductive techniques. Additionally, optimizing the cryopreservation protocol is an essential step aiming to improve the physiological parameters of these cells.

Keywords: Rodentia, *in vitro* culture, slow freezing, physiology and cell characterization.

1. Introduction

The *in vitro* culture of fibroblasts has been an important and fundamental technique in scientific research aimed at the conservation and biological understanding of a species. This is because it is possible to use these cells in studies involving molecular and genetic knowledge by managing this somatic resource (Brugger and Basler, 2023), modeling drugs and diseases (Plikus et al., 2021; Ramklowan et al., 2023), and structural diversity of different populations, allowing to obtain crucial information in the actions related to conservation (Borges et al., 2019). Furthermore, fibroblasts offer an opportunity to develop reproductive techniques that can preserve and disseminate the genetics of species at risk (Mastromonaco et al., 2020), excluding the need for gametes, as observed in nuclear reprogramming (Ogura et al., 2000).

Considering this scenario, studies that establish fibroblasts are conducted in laboratory and wild rodents (Praxedes et al., 2021; Veraldi et al., 2022) as a conservation strategy by developing cryobanks that help maintain populations' genetic diversity (Bolton et al., 2022). Thus, identifying the cell type, viability, and senescence as a function of the number of passages and evaluating the effects and damage caused by cryopreservation is essential for establishing this cell type in rodents (Praxedes et al., 2021). Investigating the physiological behavior of these cells and their characteristics contributes to the fact that fibroblastic lines remain viable during the culture, optimizing their use.

In this sense, the Spix's yellow-toothed cavy is a wild rodent belonging to the hystricomorph suborder and may have an established fibroblastic line. In general, *G. spixii* is endemic to Brazil and is included in the order Rodentia, which comprises the guinea pig (*Cavia porcellus*), Patagonian mara (*Dolichotis patagonum*), capybara (*Hydrochoerus hydrochaeris*), hamster (*Mesocricetus auratus*), red-rumped agouti (*Dasyprocta leporina*), rats, and mice (Lovegrove, 2000). In these rodents, some studies involving the stages for establishing fibroblastic lines have been reported (Mehrabani et al., 2014; Praxedes et al., 2021). Although phylogenetically close to *G. spixii*, this rodent presents some characteristics that encourage the isolation of fibroblasts aiming to form cell biobanks.

First, *G. spixii* is a rodent that easily adapts to its habitats, presenting valuable genetics that contributes significantly to seed dispersal, soil aeration, and reforestation (Nowak, 1999). Furthermore, *G. spixii* acts in the trophic chain and is considered food for carnivores (Sales et al., 2020). Additionally, due to its reproductive characteristics and distinction from other laboratory and wild rodents in terms of size, weight, and superior metabolic rate (Rowe and Honeycutt, 2002), *G. spixii* offers an opportunity as a scientific model in breeding sites aimed at biological, physiological, cellular, evolutionary, and genetic studies. Therefore, prominence for the isolation and characterization of fibroblastic lines, which can be used aiming at basic studies and biotechnologically applied to *G. spixii* after being obtained in a minimally invasive, cheap, and fast manner (Iqbal et al., 2021), as observed in cloning by nuclear transfer and the generation of induced pluripotency stem cells (Takahashi and Yamanaka, 2006; Wakayama et al., 2000).

In rodents, such as *Cavia porcellus* (Mehrabani et al., 2014), *Mesocricetus auratus* (Mehrabani et al., 2016), and *Dasyprocta leporina* (Praxedes et al., 2021), the establishment and cryopreservation of fibroblastic lines were efficiently performed between two and eight passages. However, variations were observed in the parameters evaluated between the studied species (Xu, 2005). On the other hand, isolation and *in vitro* culture of fibroblasts can occur even in upper passages in rats and mice, as observed up to the tenth passage (Durkin et al.,

2013; Madelaire et al., 2022), and is therefore considered a metric important to be observed and validated when it is conducted in a new species (Walmsley et al., 2016). Cryopreservation is another crucial parameter that must be evaluated since this technique is described as specific for each study species (Elliott and Wang, 2017; Mooney et al., 2023).

Therefore, considering the importance of different applications from establishing a fibroblastic line and the ecological and scientific role of *G. spixii*, we established by culture time (second, fifth, eighth, and tenth passages) and cryopreservation of somatic cell lines.

2. Material and methods

2.1. Chemicals, media, and ethics statement

Reagents and media were obtained from Sigma-Aldrich (St. Louis, MO, USA), Gibco-BRL (Carlsbad, CA, USA), and Inlab (São Paulo, SP, Brazil). Anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, MA, USA). For *in vitro* culture, Dulbecco's modified minimal essential medium (DMEM) was previously prepared and filtered using a pore filter system (0.22 µm; Corning, New York, USA), with the pH adjusted between 7.2–7.4. All the protocols were approved by the Animal Use Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, nº 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1).

2.2. Animals and somatic tissues obtained from skin

We used six *G. spixii* adult males kept at the breeding site, aged one year. The animals were obtained from the Wild Animal Multiplication Center (CEMAS/UFERSA, Mossoró, RN, Brazil 5°10'S, 37° 10' W, nº 1478912). The apical ear region was identified and sterilized for skin collection using 70% alcohol, and fragments measuring 1.0–2.0 cm² were retrieved using surgical pliers (Pereira et al., 2021). Subsequently, the samples were transported to the laboratory in DMEM added with 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic (ATB-ATM) solution at 4 °C for up to 1 h.

In the laboratory, fragments were trichotomized and sectioned into 9.0 mm³ (3.0 × 3.0 × 1.0). Subsequently, the fragmented tissues were washed in media and solutions under the following sequence: i) DMEM associated with 10% FBS and 10% ATB-ATM solution; ii) 70% alcohol; and iii) DMEM supplemented with 10% FBS and 2% ATB-ATM solution. After washing, fragments were added to a dish containing eight fragments per animal, using two cell culture dishes (four fragments per dish) containing DMEM associated with 10% FBS and 2% ATB-ATM solution. Then, the dish was cultured *in vitro* under controlled temperature and atmosphere.

2.3. Experimental design

The study was divided into two experiments. *In vitro* culture was characterized from the primary system for the first experiment, and the fibroblast lines in the second, fifth, eighth, and tenth passages were established. Fibroblasts were identified and evaluated by morphology, immunocytochemistry, and karyotyping to establish the cell line. Additionally, cell physiology was assessed for viability, metabolism, proliferation, reactive oxygen species (ROS) levels, mitochondrial membrane potential ($\Delta\Psi_m$), and apoptotic levels.

In the second experiment, fibroblasts in the fifth passage were cryopreserved for the cryotolerance assay through slow freezing. A cryoprotective solution composed of DMEM supplemented with 1.3 M Me₂SO, 10% FBS, and 0.2 M sucrose was used as a cryopreservation solution (Fuentes-Mateos et al., 2023; Praxedes et al., 2023). After cryopreservation, cells were thawed and submitted to the evaluations previously described in the first experiment. Also, somatic cells were submitted to the ultrastructure assay before and after cryopreservation during this stage to assess the damage and effects caused by the slow freezing.

2.4. Establishment of the fibroblastic lines and cellular evaluations

2.4.1. Primary culture, cell isolation, and trypsinization

Cells were placed in DMEM with 10% FBS and 2% ATB-ATM for primary and permanent culture under temperature and modified atmosphere (38.5 °C; 6.5% CO₂). The dishes were monitored every 24 h, with a complete medium change. After the beginning of cell growth around the fragments, the medium was changed every 48 h, after which the fragments were removed, and cell culture was conducted until the cells reached 70–80% of their subconfluence (Klan and Gasser, 2016).

Cell culture was evaluated under the following parameters: i) number of adhered explants, ii) number of explants with subconfluence, iii) days of adhered explants, and iv) number, days, and total time for explants to reach subconfluence. After this period, evaluations under an inverted microscope (Nikon TS100, Tokyo, Japan) were performed on the cells (Praxedes et al., 2021), which were trypsinized (0.25% trypsin; 0.05% EDTA) and seeded according to the number of passages established in the experimental design.

2.4.2. Evaluation of morphological characteristics and cell type identification

Morphological characteristics were performed under an inverted microscope during the entire *in vitro* cell culture. For this, the cells' shape and nuclear and cytoplasmic extension were observed (Mehrabani et al., 2014).

The immunocytochemical assay and karyotyping were used to identify the cell type. Initially, the vimentin assay identified cells at the fifth passage using the immunocytochemical assay (Praxedes et al., 2021). Cells at a subconfluence between 60 and 70% were fixed in 4% paraformaldehyde for 10 min at 25–27 °C. Subsequently, the cells were washed three times in phosphate buffer saline solution (PBS, pH 7.4; 4 °C) for 5 min in each wash, and then antigenic retrieval was performed (100 mM Tris; 5% urea; pH 9.5) for 24 h. Later, the cells were rewashed in PBS (pH 7.4; 4 °C; 5 min) and permeabilized with 0.5% Triton X-100 for 1 h. After permeabilization, three washes were performed in PBS (pH 7.4; 4 °C; 5 min) and a solution composed of 5% bovine serum albumin (BSA), PBS, and 0.1% Tween 20, blocking non-specific binding of antibodies for 1 h in a humid chamber. Then, cells were incubated for 24 h with the primary anti-vimentin antibody (ab8979; 1:250; 1% BSA diluted in PBS and 0.1% Tween 20) for 24 h at 4 °C. The solution containing the anti-vimentin antibody was then removed, and after three washes in PBS, the secondary antibody (AlexaFluor® 488-coupled goat anti-mouse IgG, ab150113, 1:400) was added for 1 h in a humid chamber (4 °C) and protected from light. Finally, the cell nucleus was countermarked with 1 µg/mL Hoescht 33342 for 1 min, and the result obtained was visualized under fluorescence microscopy (Olympus BX51TF, Tokyo, Japan) at 20× magnification.

The Giemsa assay was used for cell karyotype analysis. It was considered that in *G. spixii* $2n = 64$ (Maia, 1984). The protocol employed was based on the one performed in *C. porcellus* with some modifications (Ford, 1956; Mehrabani et al., 2014). First, cells were cultured and fixed in colchicine (0.1%) for up to 2 h at 38.5 °C and 6.5% CO₂ after reaching 70–80% of their subconfluence. After the period, the fixative was removed, and the cells were washed in PBS, followed by trypsinization and resuspension in DMEM with 10% FBS and 2% ATB-ATM. Subsequently, the cells were centrifuged (600×g/10 min), and a KCl solution (0.075 M) was slowly added to the tube, where the cells were kept for 10 min at 35–37 °C. Then, the tube was centrifuged again (600×g/10 min), and the pellet formed was gently resuspended in 5 mL of the fixative solution (3:1 methanol: acetic acid solution), which was again submitted to centrifugation (600×g/10 min). The fixative solution was repeated three times, and at the end of the process, the pellet was resuspended in 1.5 mL fixative solution. Finally, two to four drops of cell suspension were dripped onto glass slides, previously treated with hydrolysis solution (0.1 N; HCl diluted in distilled water; 60 °C; eight minutes). The slides were air-dried, stained with Giemsa for 5 min, washed in distilled water, and kept in the air for drying and subsequent chromosome analysis. Eight to ten metaphases were identified and analyzed under conventional light microscopy (Olympus BX51TF, Tokyo, Japan) at 100× magnification.

2.4.3. Cell viability analysis

Cells (20 µL) suspended in the different passages were subjected to the staining assay using trypan blue dye (20 µL; 0.4%), diluted in PBS. The cell suspensions were placed in the Neubauer chamber to quantify non-viable cells (dead; stained in blue) due to membrane rupture and viable cells (alive; colorless) that did not present membrane rupture. Finally, the percentage of cell viability rate was obtained according to the calculation: $n^{\circ} \text{ viable cells} / n^{\circ} \text{ total number of quantified cells}$ (Mehrabani et al., 2016).

2.4.4. Functional metabolism activity and proliferative rate

Cells were subjected to a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The cells were seeded in 12-well dishes (5.0×10^4 cells/mL) for five days at 38.5 °C and 6.5% CO₂ (Praxedes et al., 2023). After culture, cells were incubated with 5 mg/mL MTT (diluted in DMEM) for 3 h at 38.5 °C and 6.5% CO₂. Me₂SO was added for MTT solubilization, and the samples were read by spectrophotometer at 595 nm (Shimadzu® UV-mini-1240, Kyoto, Japan).

The proliferative assay was based on the seeding of cells in 24-well dishes at the concentration of 1.0×10^4 cells/mL per well for 7 days (168 h), where the general average of the counts was recorded every 24 h after cell trypsinization to elaborate the cell growth curve, followed by the population doubling time (PDT) (Mehrabani et al., 2016). The PDT was estimated using the following mathematical calculation:

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

Where PDT is the time for doubling the culture (in hours), T is the incubation time, X_e is the number of cells at the end of the incubation period, $\ln$ is the Napierian logarithm, and X_b is the number of cells at the beginning of the incubation period.

2.4.4. Assessment of ROS and $\Delta\Psi_m$ levels

The levels of oxidative stress mediated by the ROS molecules of the cells were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 μ M; Invitrogen, Carls-bad, CA, USA). 2.5 mL PBS was added to an aliquot of 0.5 μ L (0.1 M) H₂DCFDA, and the cells were labeled using 50 μ L cell suspension and fluorescent probe (50 μ L; 1:1) for up to 30 min at 38.5 °C and 6.5% CO₂.

The MitoTracker Red[®] probe (CMXRos; 1 mM; Invitrogen, Carls-Bad, CA, USA) was used to evaluate the $\Delta\Psi_m$, where 750 μ L PBS was used to dilute an aliquot of 1.5 μ L (500 nM) CMXRos. Then, 50 μ L cell suspension and the fluorescent probe (50 μ L; 1:1) were obtained. Subsequently, the sample was kept for 30 min at 38.5 °C and 6.5% CO₂.

After the labeling time, aliquots containing the H₂DCFDA and CMXRos probe were centrifuged (300×g/5 min). Finally, the supernatant was removed, the pellet formed was resuspended in 50 μ L PBS, and the cells were evaluated under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). An excitation of 495 nm and emission of 520 nm were used for ROS, while 579 nm was used for excitation and 599 nm for emission of the CMXRos.

Finally, 100 cells were registered for each fluorescent probe, and the fluorescence intensity (pixels) was estimated using Image J software (National Institutes of Health, MA, USA). The background intensity was subtracted from the values obtained in the somatic cells, and the relative expression levels (arbitrary fluorescence units) were quantified. The cell was considered the experimental unit.

2.4.5. Quantification of apoptotic levels

An aliquot of cell suspension (35 μ L) was marked using a mixture of ethidium bromide dye (10 μ g/mL) and acridine orange (2 μ g/mL), diluted in 8 μ L PBS (1:1) [37]. This combined method allows the differential uptake of fluorescent DNA-binding dyes, allowing the observation of chromatin condensation in the stained nucleus (Takahashi et al., 2004), making it possible to distinguish and classify (i) viable cells with a uniform light green nucleus, (ii) cells in early apoptosis with non-uniform green nucleus, (iii) cells in late apoptosis with non-uniform orange nucleus, and (iv) necrotic cells with uniform orange/red nucleus. Finally, cells were evaluated in a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) under an absorbance of 480 nm, where images were recorded, and 300 cells were quantified in the Image J software at a magnification of 40×.

2.5. Cryopreservation and fibroblast warming

The slow freezing method was used for cell cryopreservation, as described by Praxedes et al. (2023). Cells in the fifth passage were used due the culture was already established regarding the cell type. Then, 1.0×10^5 cells/mL was recovered. Posteriorly, the cells were added to cryotubes containing the cryopreservation solution and kept for 15 min at 4 °C (Praxedes et al., 2023). Subsequently, 0.2 M sucrose was added, and the samples were kept at 4 °C for 15 min. The cells were then stored in cryotubes and transferred to the Mr. Frosty system[®] (Thermo Scientific Nalgene, Rochester, NY, USA) as a freezing vessel for 12 h in a programmable freezer at -80 °C at a cooling rate of -1 °C/min. The cryotubes were stored in liquid nitrogen (-196 °C) for two weeks, where they were subsequently thawed and evaluated for the effect of cryotolerance.

The cells were initially thawed at 25 °C for 1 min, followed by 4 min at 35–37 °C. Then, DMEM with 0.2 M sucrose was added for 15 min. Subsequently, the cells were centrifuged for 10 min at 600×g. The supernatant was removed, and the pellet was resuspended in DMEM for 15 min at 25 °C. Finally, the cells were centrifuged and cultured in DMEM with 10% FBS and 2% ATB-ATM for the evaluations described in the experimental design.

2.5.1. Ultrastructural evaluation by scanning electron microscopy

The cells were fixed in glutaraldehyde (0.4%) diluted in PBS for 2 h. Then, the fixative solution was removed, and cells were washed three times in PBS and post-fixed in osmium tetroxide (0.1%) for up to 1 h. Later, the cells were washed twice in increasing alcohol solutions (7.5%, 15%, 30%, 50%, 70%, 90%, and 100%) for 10 min for each concentration used. Finally, the cells were metalized with gold and visualized under a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

2.6. Statistical analysis

Data from six individuals were expressed as mean $\pm$ standard error (one animal/replicate) and analyzed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality using the Shapiro-Wilk test and homoscedasticity using the Levene test. The data were arcsine transformed since they did not show a normal distribution. All *in vitro* culture data were analyzed by ANOVA followed by Tukey or unpaired t-test. Significance was defined at $p < 0.05$.

3. Results

3.1. *In vitro* culture and establishment of fibroblastic lines

Eight initial explants/animal were used for *in vitro* culture to establish the fibroblastic lines, totaling forty-eight skin biopsies analyzed daily for cell growth (Table 1, Fig. 1). During *in vitro* culture, forty-five (94%) were fixed on the dish with an average period varying 4.2 days. Furthermore, it took six days (85% of the samples) for cell detachment to occur (Fig. 1A). Cell growths were observed on the dish after the eighth day of culture (Fig. 1B). It took 15 days for the cells to reach subconfluence between 70–80% (Fig. 1D) after removing the fragments (Fig. 1C), with a total culture time from the second to the tenth passages occurring in 70 days (Fig. 1E-H).

Table 1. Establishment of the physiological characteristics of the primary culture and subculture of somatic cells of six *G. spixii*.

Cellular parameters	Values (%)
No. initial explants	48 $\pm$ 0.0
Attached samples	45 $\pm$ 0.3 (94%)
Day all attached explants	4.2 $\pm$ 0.3
Day samples reached cell growth	15.0 $\pm$ 0.2
<i>In vitro</i> culture time (days)	70.0 $\pm$ 0.0

Results are expressed as mean $\pm$ standard error.

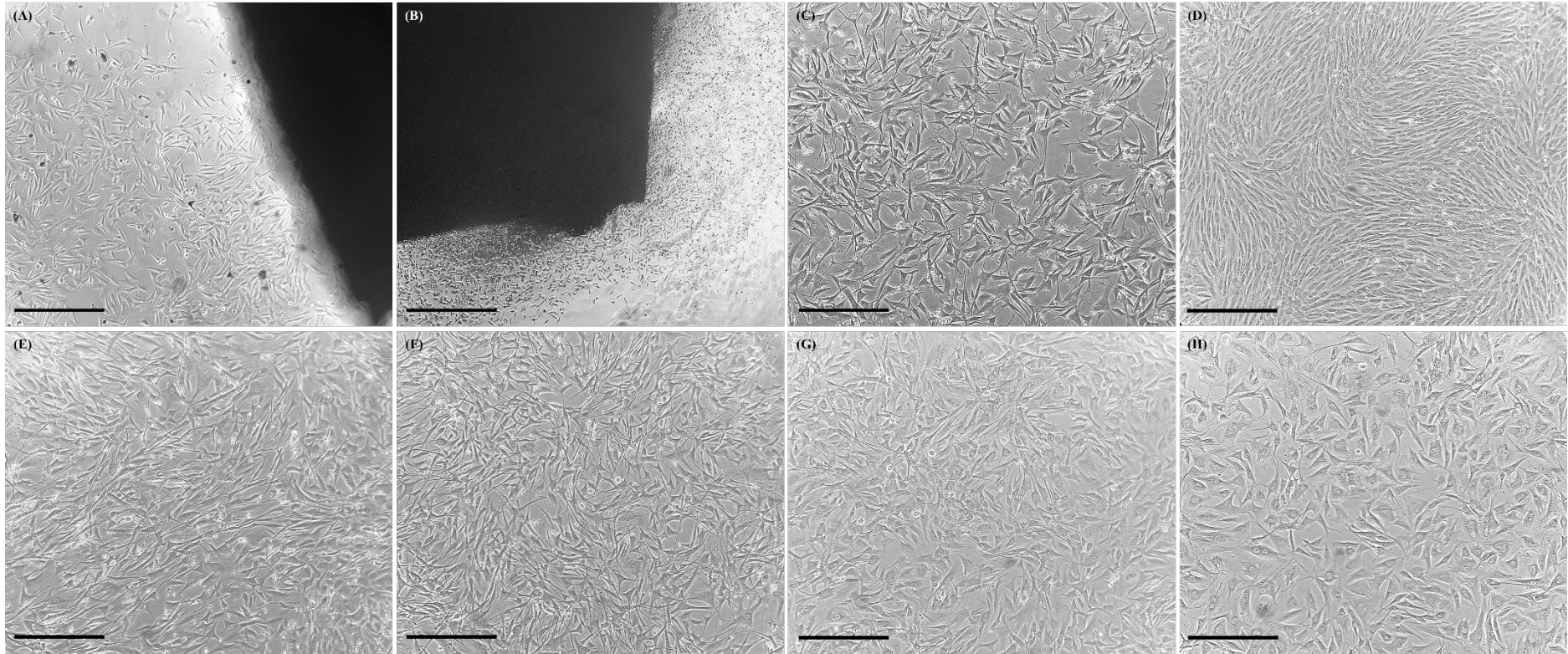


Fig. 1. Establishment of *in vitro* culture of *G. spixii* fibroblasts and their morphological characteristics in different passages. (A) Primary culture of *G. spixii* skin explant with cell shedding after 6 days of *in vitro* culture. (B) Primary culture of skin explants in cell growth after 8 days of *in vitro* culture. (C) Culture of *G. spixii* skin fibroblasts in cell growth after the removal of somatic skin explants. (D) *G. spixii* skin fibroblasts after reaching 100% subconfluence after 14 days of cell culture. (E) *In vitro* culture of *G. spixii* skin fibroblasts in the second, (F) fifth, (G) eighth, and (H) tenth passages. Bar scale = 50 μ m, mag. 4 $\times$ (B); 50 μ m, mag. 10 $\times$ (A, C, D, E, F, G, H).

3.1.1. Identification of cell type by morphology, immunocytochemistry, and chromosomes

Based on the morphological analyses, the cells presented typical fibroblast morphology from the second to the tenth passages (Fig. 1E-H). However, the cytoplasm of the fibroblasts became slender from the eighth passage onwards (Fig. 1G-H) compared to the second and fifth passages (Fig. 1E-F). The cells in the fifth passage (Fig. 2A) were identified for cell type characterization. Thus, the anti-vimentin antibody assay was used to mark the intermediate filaments of fibroblasts, thus confirming this cell type (Fig. 2B-D).

Finally, the assay determined the karyotype in the fibroblasts' second, fifth, eighth, and tenth passages (Fig. 2E-H). Therefore, the chromosomes of cells in the tenth passage showed shortening. However, the corresponding number in this species was $2n = 62$ autosomal chromosomes, with two more for the sex chromosomes.

3.1.2. Evaluation of cell viability and physiology

Fibroblasts were analyzed for their viability, metabolic rate, and proliferation capacity during the second, fifth, eighth, and tenth passages to assess cellular physiology (Fig. 3). First, concerning cell viability, a percentage of viable cells greater than 80% was observed throughout the passages (Fig. 3A). Furthermore, a metabolic rate greater than 85% between the second and tenth passages of *in vitro* culture of fibroblasts was observed (Fig. 3B).

However, despite the fibroblasts being metabolically active, the cell growth curve showed a reduction in the cell quantity (cells/mL) after the eighth passage (Fig. 3C), although the PDT was negatively affected by the number of passages performed in these cells only in the tenth passage (32.5 h) (Fig. 3D).

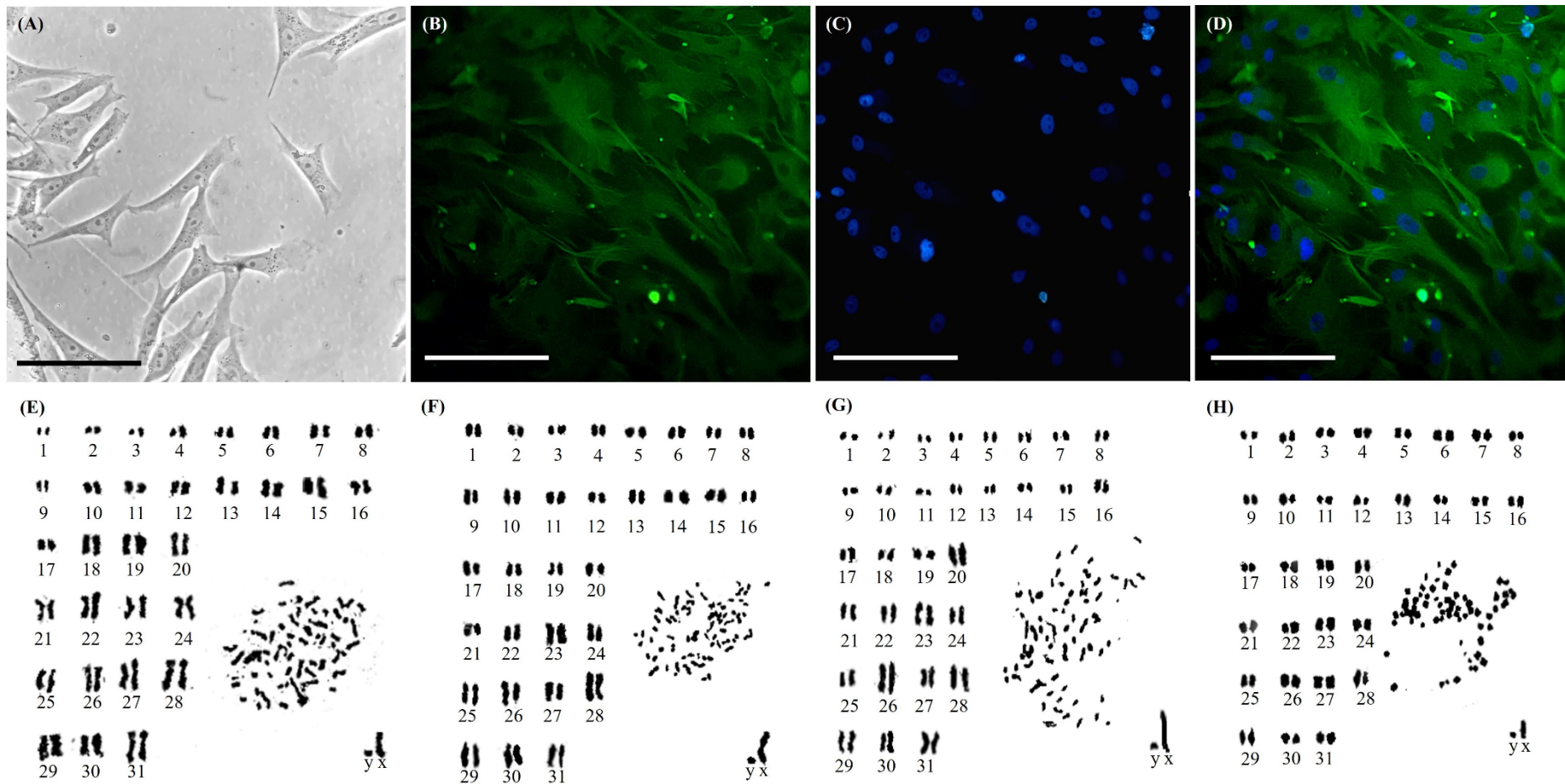


Fig. 2. Representation of the fibroblastic lineage of *G. spixii* after morphological analysis, immunostaining, and karyotyping. (A) Morphological evaluation by inverted microscope. Bar scale = 50 μ m, mag. 40 $\times$. (B) Immunostaining of cell cytoplasm by vimentin antibody. Bar scale = 50 μ m, mag. 40 $\times$. (C) Nucleus of cells stained by Hoescht 33342. Bar scale = 50 μ m, mag. 40 $\times$. (D) Composition of the cytoplasm and nucleus of *G. spixii* fibroblasts after cell type identification. Bar scale = 50 μ m, mag. 40 $\times$. (E-H) Karyotype of fibroblasts stained by Giemsa in the second (E), fifth (F), eighth (G), and tenth passages (G). Mag. 100 $\times$.

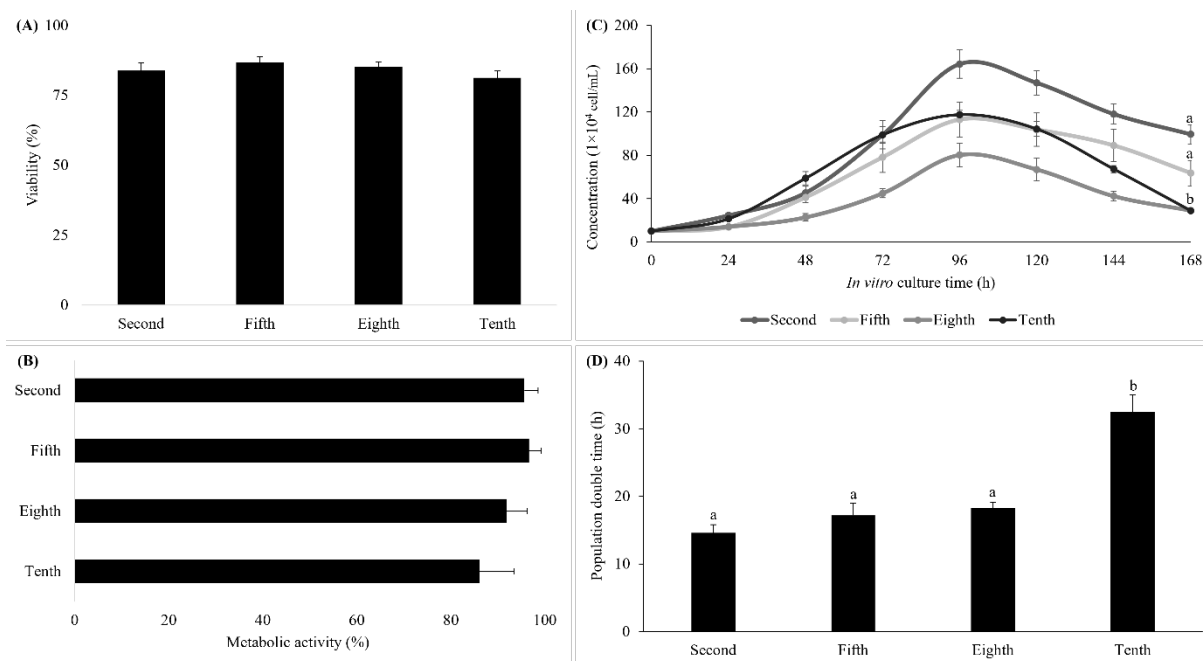


Fig. 3. Evaluation of viability and physiological rates of fibroblastic strains of *G. spixii* after the second, fifth, eighth, and tenth passages. (A) Percentage of cell viability. (B) Quantification of metabolic activity. (C) Analysis of the proliferative curve. (D) Population doubling time of fibroblasts in the second, fifth, eighth, and tenth passages. $P < 0.05$.

3.1.3. Intracellular quantification of ROS, $\Delta\Psi_m$, and cellular apoptosis

Fibroblasts were evaluated for oxidative stress, $\Delta\Psi_m$, and apoptotic levels to determine the quality of cell culture (Fig. 4; Fig. 5). Regarding the intracellular levels of ROS, cells in the fifth (1.60 ± 0.9), eighth (1.86 ± 0.8), and tenth (2.24 ± 1.0) passages presented a greater amount of ROS compared to the second passage (Fig. 4I). However, the eighth (1.30 ± 0.7) and tenth (2.37 ± 0.9) passages showed higher activity after evaluating the mitochondrial activity compared to the second (1.0 ± 0.4) and fifth (1.14 ± 0.4) passages (Fig. 4J). No difference was observed across passages and after the assessment of apoptotic levels, where the number of viable cells was superior to 80% (Fig. 5E).

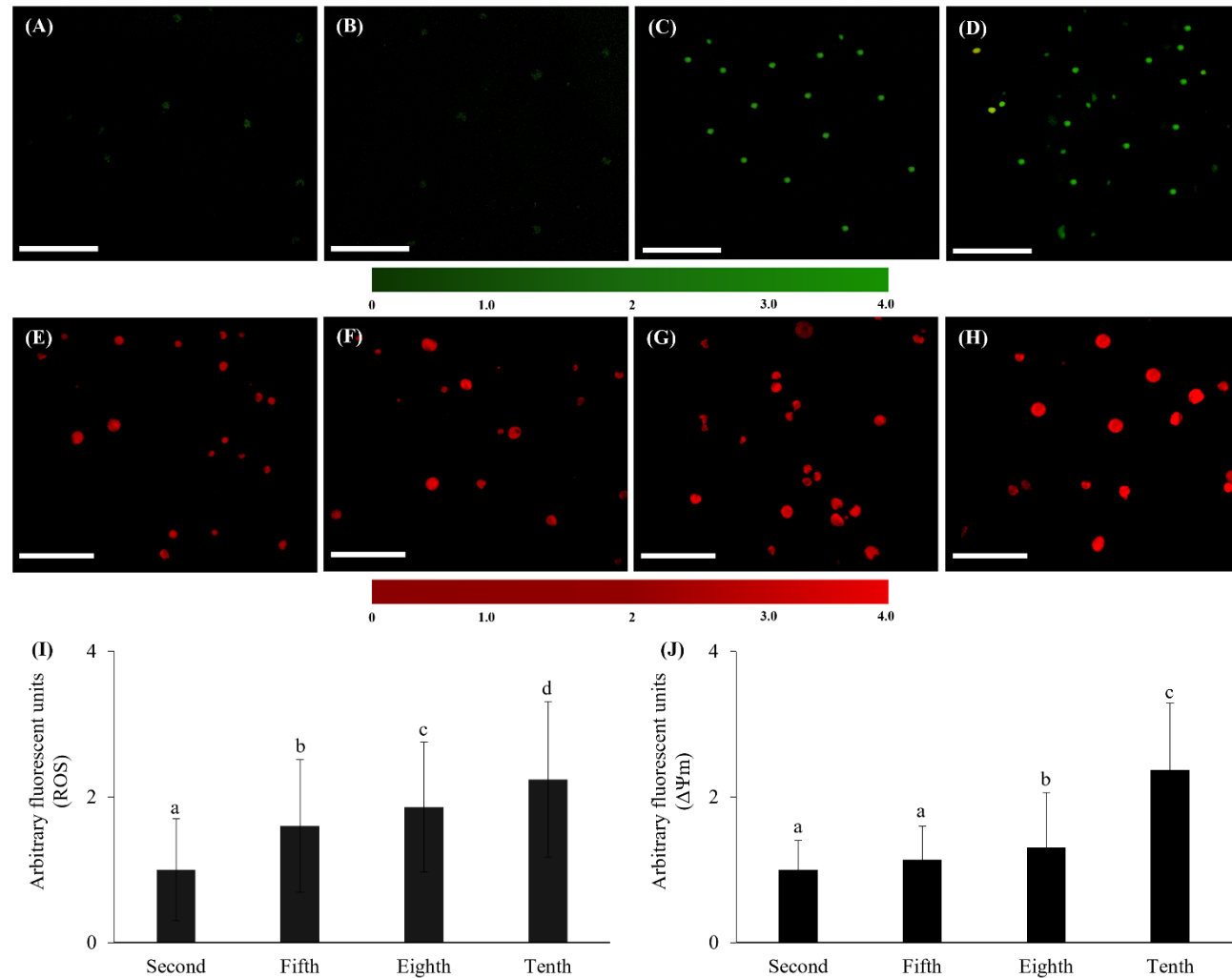


Fig. 4. Evaluation of reactive oxygen species (ROS) and mitochondrial activity of *G. spixii* adult skin fibroblasts after the second, fifth, eighth, and tenth passages of *in vitro* culture. (A-D) Representation of cells stained by the H₂DCFDA probe. Bar scale = 50 μ m, mag. 20 $\times$. (E-H) Representation of cells stained by the MitoTracker Red[®] probe (CMXRos). (I) Quantification of oxidative stress after assessment of intracellular levels of ROS. (J) Evaluation of mitochondrial activity ($\Delta\Psi_m$) of *G. spixii* adult skin fibroblasts. $P < 0.05$.

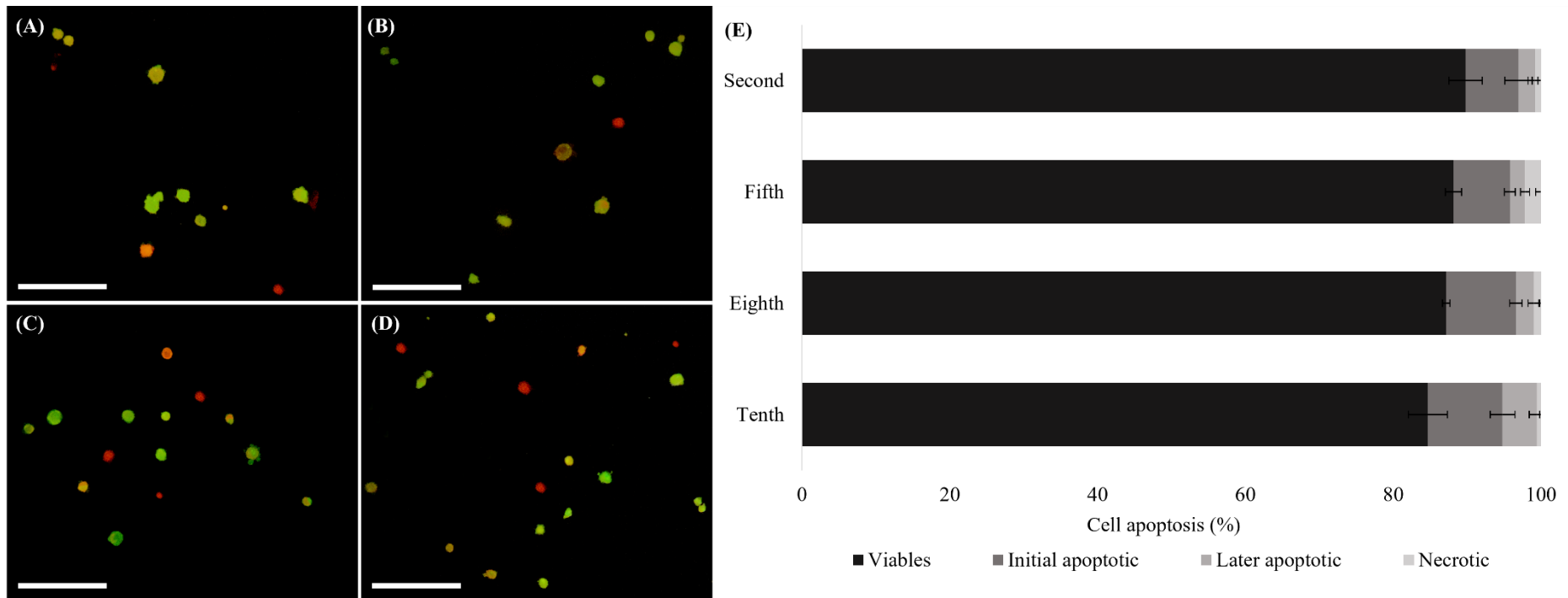


Fig. 5. Evaluation of the apoptotic levels of the *G. spixii* fibroblastic lineage after the second (A), fifth (B), eighth (C), and tenth (D) passages of cell culture. Bar scale = 50 μ m, mag. 20 $\times$. (E) Percentage of the number of viable cells in early and late apoptosis and necrotic during the ten passages of *in vitro* culture. $P > 0.05$.

3.2. Cryotolerance assay on fibroblastic lines

3.2.1. Effect of cryopreservation on cell morphology, viability, and metabolism

Despite the typical morphology of fibroblasts being maintained after the analysis by inverted microscope (Fig. 6A-A'), the viability immediately after slow freezing was reduced (70%) compared to non-cryopreserved cells (85%) (Fig. 6B). However, cell viability was recovered after *in vitro* culture (Fig. 6B). No difference was observed after the metabolic analysis of fibroblasts ($P > 0.05$; Fig. 6C), superior to 90% in both groups.

3.2.2. Effect of cryopreservation on ultrastructure, cell growth curve, and cell population doubling

In the ultrastructural analysis, fibroblasts after cryopreservation had less translucent characteristics, and the cytoplasmic membrane presented more significant irregularities and structural damage under its surface, probably caused by the effect of cryopreservation (Fig. 7A-A'). Furthermore, no difference ($P > 0.05$) was observed after analyzing the cell growth curve (Fig. 7B). However, cryopreserved cells (24.4 h) were negatively affected compared to non-cryopreserved cells (17.2 h) after slow freezing and PDT analysis (Fig. 7C).

3.2.3. Effect of cryopreservation on oxidative stress, $\Delta\Psi_m$, and apoptotic levels

A significant increase ($P < 0.05$) was observed after evaluating oxidative stress (1.49 ± 0.7) and $\Delta\Psi_m$ (1.34 ± 0.7) in cryopreserved fibroblasts compared to non-cryopreserved cells (1.00 ± 0.5 ; 1.00 ± 0.4) (Fig. 8A-A"; Fig. 8B-B"), respectively.

After apoptotic analysis, a reduction in the number of viable cells was verified after cryopreservation ($75.0\% \pm 1.1$) compared to the non-cryopreserved group ($88.2\% \pm 1.1$) (Fig. 8C"). Furthermore, an increase in the number of cells in initial ($16.4\% \pm 0.7$) and late ($5.7\% \pm 0.5$) apoptosis was observed after cryopreservation and when comparing non-cryopreserved cells ($7.6\% \pm 0.7$; $2.1\% \pm 0.5$) (Fig. 8C-C").

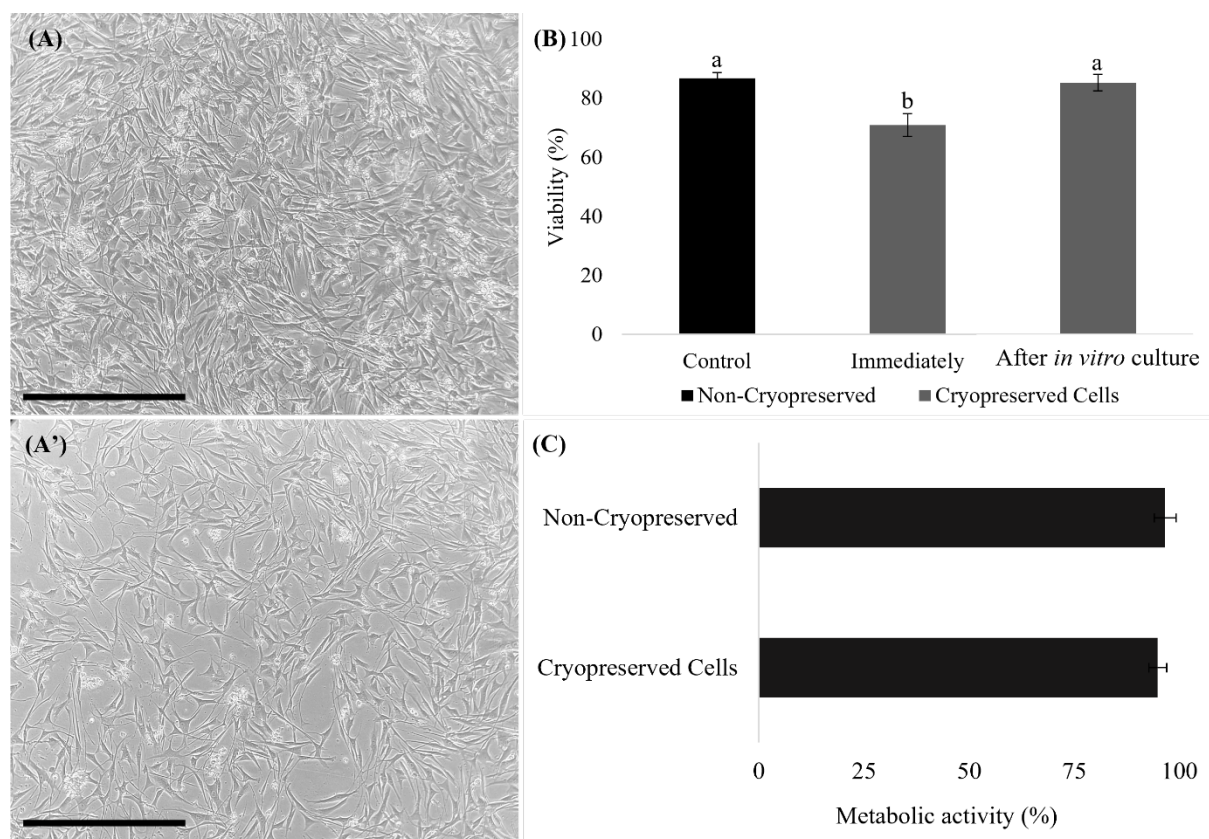


Fig. 6. Morphological, viability, and metabolism evaluation of *G. spixii* fibroblastic strains after slow freezing. (A-A') Morphological analysis of cells before and after cryopreservation. Bar scale = 50 μ m, mag. 10 $\times$. (B) Percentage of fibroblast viability before, immediately after cryopreservation and *in vitro* culture. (C) Metabolic activity of cells after cell cryopreservation. $P < 0.05$.

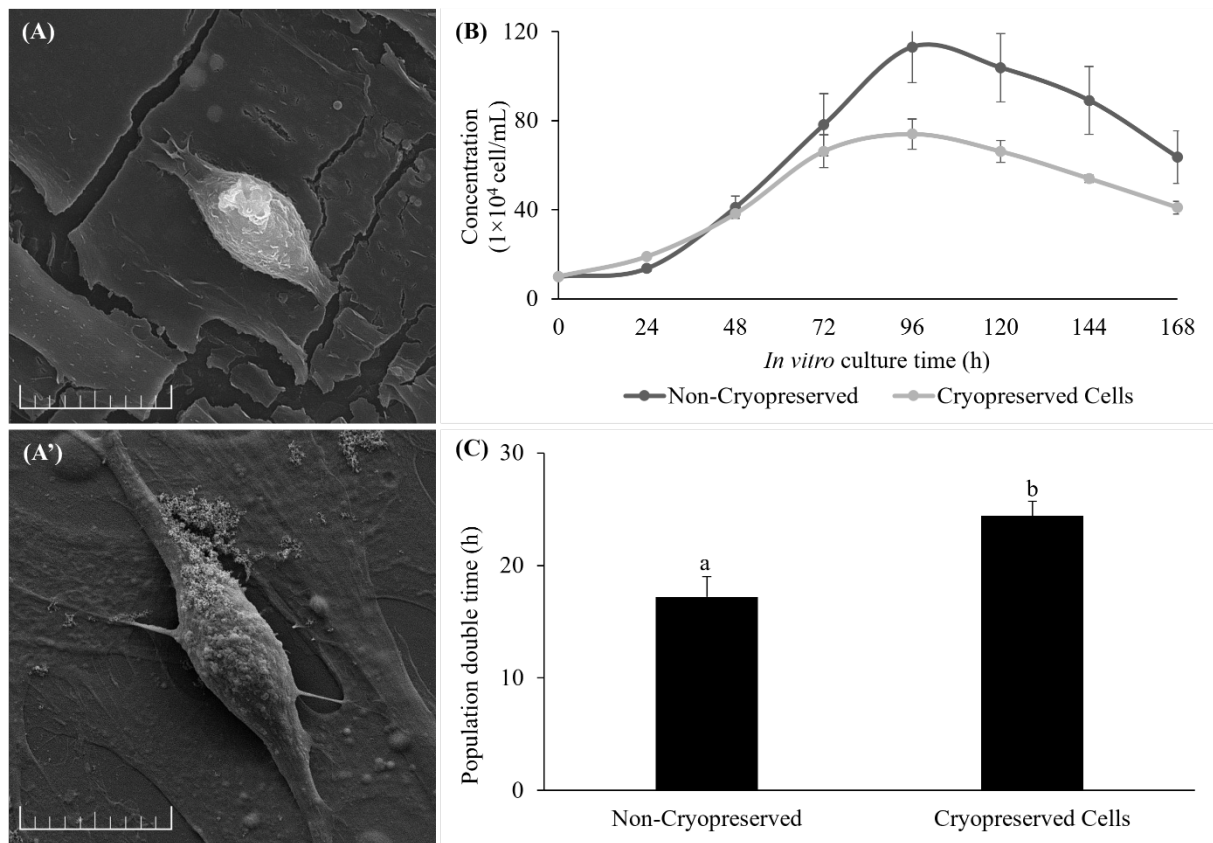


Fig. 7. Ultrastructural evaluation and proliferative rate of *G. spixii* adult skin fibroblasts after cell cryopreservation. (A-A') Ultrastructural analysis of cells before and after cryopreservation. Bar scale = 10 μm . (B) Cell concentration after evaluating the growth curve. (C) Population doubling time of fibroblasts before and after slow freezing. $P < 0.05$.

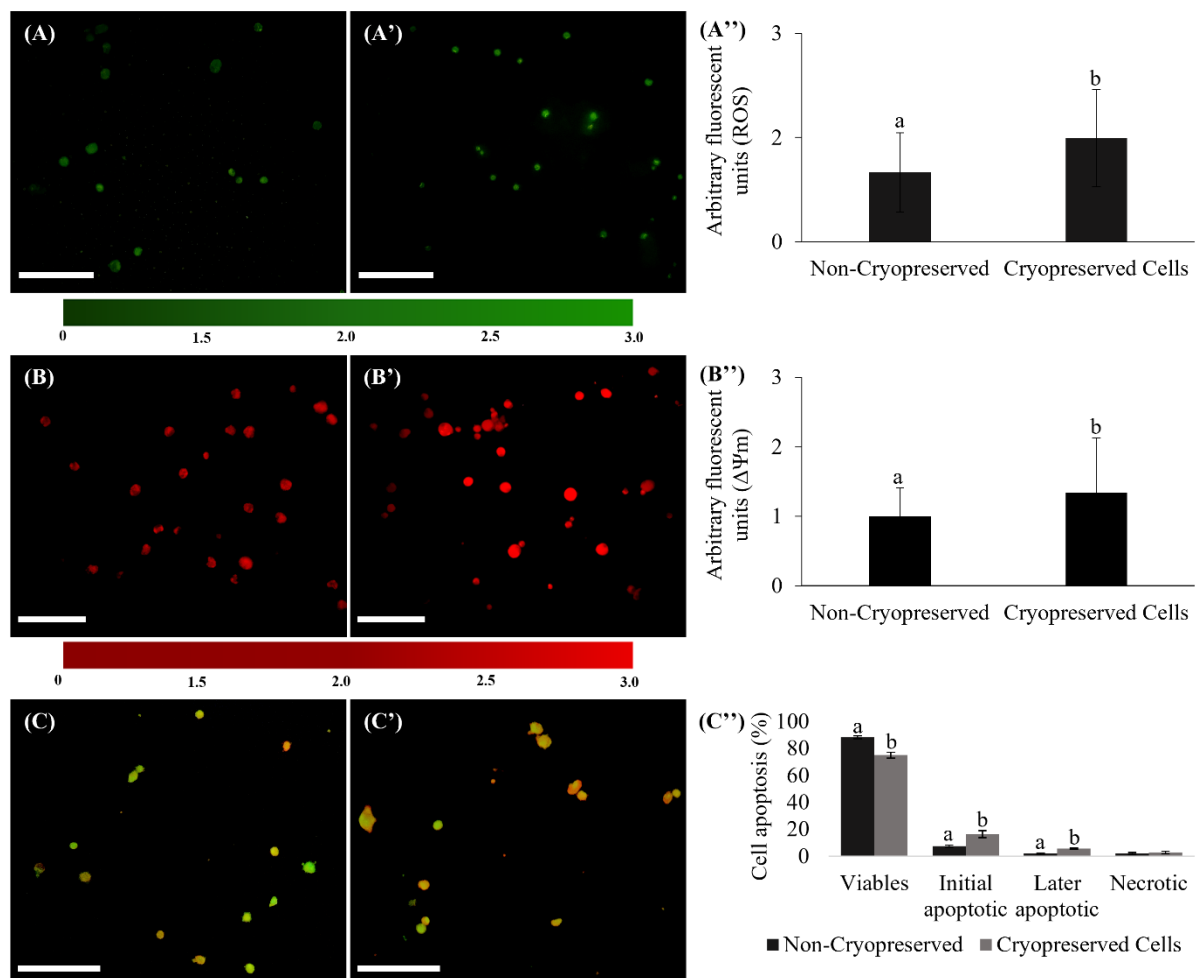


Fig. 8. Quantification of oxidative stress, mitochondrial membrane potential ($\Delta\Psi_m$), and apoptotic levels of *G. spixii* adult skin fibroblasts before and after slow freezing. (A-A'') Representative image and arbitrary units of fluorescence obtained in each cell evaluated after oxidative stress analysis. Bar scale = 50 μm , mag. 20 $\times$ (A-A'). (B-B'') Representative image and arbitrary unit of fluorescence obtained in each cell evaluated after the $\Delta\Psi_m$ evaluation. Bar scale = 50 μm , mag. 20 $\times$ (B-B'). (C-C'') Representative image and percentage of the number of fibroblasts in cellular apoptosis. Bar scale = 50 μm , mag. 20 $\times$ (C-C'). $P < 0.05$.

4. Discussion

The establishment of fibroblastic lines from *in vitro* culture represents an expandable genetic resource that can be continuously used. It is necessary to develop cell biobanks for these application purposes. Unlike gametes, somatic samples are recovered from an animal *in vivo* or *post-mortem* and used for culture to establish, identify, and physiologically characterize cell lines and cryopreservation. In this sense, fibroblast lines have been cultured from the skin of wild rodents. They are established to understand the physiological and genetic characteristics of individual cells, offering the opportunity for application in reproductive biotechnologies aiming at knowledge, reintroducing endangered populations, and in the optimization and *in vitro* development of protocols.

In our study, we established and physiologically characterized, over ten passages, fibroblastic lines from the ear skin of Spix's yellow-toothed cavy, a small wild rodent considered less worrying according to the classification of the International Union for Conservation of Nature (IUCN) (Catzefflis et al., 2016), therefore being an ideal study model for developing *in vitro* strategies since its quantitative population guarantees the highest probability of successful use of samples obtained for the conservation of this species and others phylogenetically close (Ryder and Onuma, 2018). Additionally, we used a cryopreservation protocol for *G. spixii* fibroblastic lines. Although cellular damage was observed, the tool can introduce the cryopreserved material in reproductive techniques.

Initially, we chose cell recovery through the direct culture of somatic explants instead of enzymatic methodologies for the primary culture of fibroblasts originating from *G. spixii* from the ear skin. This occurred because a good sample fixation (94%) after the beginning of the *in vitro* culture was observed, and the technique had already been used efficiently in other species (Seluanov et al., 2010; Durkin et al., 2013). Unlike mice, a low rate of cell recovery was observed after using biopsies with irregular edges, contributing to poor fixation due to tissue thickness in culture and causing damage to cellular structures after treatment by enzymatic methodologies (Klan and Gasser, 2016).

In addition, biopsies recovered from the ear apical region caused minimal damage to the animal, requiring a short time for adhesion to the dishes (4 days) and favoring a good initial cell growth rate (85%) in up to the eighth day, and still guaranteed a maximum confluence in up to 15 days. Although these results differed when compared to the culture of *C. porcellus* skin fibroblasts (Mehrabani et al., 2014), which required up to 30 days for an adequate rate of cell subconfluence, it was shown to be similar to *D. leporina*, where cells reached their maximum subconfluency within 15 days (Praxedes et al., 2021).

Regarding the morphological aspects, the somatic cells exhibited typical characteristics of fibroblasts from the second to the fifth passages; that is, spindle-shaped cytoplasm, elongated and with very adherent growth to the cell culture dish, corroborating the findings observed in *D. leporina* (Praxedes et al., 2021) and *M. auratus* (Mehrabani et al., 2016) when isolating fibroblasts from the skin. Our results were similar when comparing the morphology with fibroblasts from the skin, tail, and lung of adult and neonatal murines (Walmsley et al., 2016; Fuentes-Mateos et al., 2023). Furthermore, our cells showed a similar pattern to other reports on dermal fibroblasts from other species, such as Southern white rhinoceros (*Ceratotherium simum simum*), dromedary camel (*Camelus dromedarius*), and African savanna elephants (*Loxodonta africana*) (Madelaire et al., 2022; Van Vuuren et al., 2023).

However, we observed that fibroblast morphology changed after the morphological analysis from the eighth passage, with their cytoplasm losing its fusiform aspect and becoming slenderer. This discovery is associated with the onset of replicative senescence or spontaneous mutations that would immortalize these cells in this passage. This is justified by the cells deriving from primary and secondary cultures and may present functional limitations from the number of passages during culture. This is also observed in the spiny mouse (*Acomys cahirinus*), where cultures of primary and secondary cells present morphological and functional alterations that limit their cells in early passages, which is necessary for their immortalization (Dill et al., 2023).

In addition to morphological evaluations, fifth passage *G. spixii* fibroblasts were identified using the vimentin antibody immunostaining assay. This technique is routinely used when establishing new fibroblastic lines (Praxedes et al., 2021; Van Vuuren et al., 2023) since, unlike

epithelial cells, these cells express type III intermediate filament proteins, allowing the distinction between the two cell types.

Through karyotyping, our study evaluated fibroblast chromosomal stability in the second, fifth, eighth, and tenth passages. In this sense, the karyotype observed in this study was similar to that described in the report by Maia (1984), where the cell chromosome of the *G. spixii* fibroblast line corresponded to $2n = 64$ from 8–10 metaphases observed on the slides in each passage. However, it resulted in more significant cell genomic instability in the eighth and tenth passages, as a shortening of the chromosomal length was observed, possibly due to the wear of replicative senescence (Harley et al., 1990; Bodnar et al., 1998), which presents a shortening of telomeres caused by the number of passages in *G. spixii* cultures, unlike mouse cells (Itahana et al., 2004; Parrinello et al., 2003).

The body mass and metabolic rate of *G. spixii* are other reasons for this shortening of chromosomes observed in this study, with smaller and larger values, respectively, compared to other wild rodents (Rowe and Honeycutt, 2002). According to Gorbunova et al. (2009), rodents body mass co-evolves with transcriptional telomerase activity. Although 90% of captive mice die of cancer due to failure to utilize the telomerase mechanism (Lipman et al., 2004; Seluanov et al., 2007), *G. spixii* may have evolved its genetic system to set rigid patterns and boundaries in cell proliferation [54], as observed in primates, deer, and humans (Steinert et al., 2002; Zou et al., 2002). This justifies the chromosomal shortening of the cells in the eighth and tenth passages of cell culture and the *G. spixii* fibroblastic lines remaining superior to 80% after evaluating their viability and metabolic rate by trypan blue and MTT assay, respectively, throughout the passages. These results emphasize the importance and role of the species of the order Rodentia as an ideal and scientific model for establishing cellular and molecular studies (Gorbunova and Seluanov, 2009).

Regarding the proliferative rate, it was verified that although the cells presented a sigmoid curve, the cell density from the eighth passage was lower than the second and fifth passages. Despite this, the cells only required a longer time for their PDT on the tenth passage (32.5 h). This pattern of variation has been observed in other species of laboratory and wild rodents, such as *C. porcellus* (Mehrabani et al., 2014), *Acomys cahirinus* (Dill et al., 2023) and *D. leporina* (Praxedes et al., 2021), and may be associated with culture time and lifetime (Seluanov et al., 2010).

Regarding the *in vitro* culture system, the production of ROS and mitochondrial activity along the passages was evaluated. In this sense, there was a significant increase from the fifth passage concerning oxidative stress. However, no correlation was observed with the mitochondrial activity, which was similar in the second passage, differing only in the eighth and tenth passages. Many factors may have been responsible for the increase in the synthesis of intracellular free radicals during the passages evaluated in cells. Among them, we can observe the cellular environment, medium composition, light, temperature, and metabolic activity in how the cell passages were performed (López-Díazguerrero et al., 2005).

This statement is acceptable because fibroblasts in the eighth and tenth passages presented the highest ROS index due to greater mitochondrial activity, one of the organelles responsible for greater ROS synthesis (Turrens, 2003). Consequently, accumulations of DNA damage were generated. Although cells have repair systems for oxidative damage from p53 and glutathione (Liu et al., 2008; Vousden et al., 2009), excess ROS could cause a pro-oxidant effect, eventually leading to irreversible dysfunctions, such as cell death (López-Díazguerrero et al., 2005).

Despite what happened, no significant difference was observed after evaluating the apoptotic levels, where the cells-maintained viability superior to 80%, as observed in our study.

After establishing the fibroblastic lines, *G. spixii* cells in the fifth passage were cryopreserved to evaluate their cryotolerance under the cryopreservation medium most used in laboratory and wild rodents (Mehrabani et al., 2016; Praxedes et al., 2023). Regarding the cell morphology, no change was visually observed after cryopreservation. However, *G. spixii* fibroblasts presented irregularities on the surface of their plasmatic membrane after the ultrastructural analysis. This may have occurred due to cell dehydration during slow freezing, where cryoprotective solutions removed water from the cell interior through osmosis to prevent the formation of ice crystals (Meryman, 2007). Therefore, this mechanism is essential and necessary when using the technique since the cells require an adequate cooling rate for the ideal use of slow freezing so that their molecules and ultrastructural components remain protected by the cryoprotectants used in this study (Mazur, 2004). This may be justified by this characteristic being recovered during *in vitro* culture after cell thawing. However, there was a reduction in cell viability immediately after cryopreservation, where cells maintained metabolic rates above 90%, with no difference in the cellular quantity after analyzing the growth curve.

We also observed that fibroblasts required a longer time (24.4 ± 1.3 h) to duplicate after slow freezing compared to the non-cryopreserved group (17.2 ± 1.8 h) after the assay of the PDT. A variation in PDT before and after the slow freezing has been reported in other rodent species, such as *D. leporina* ($16.5 \pm 1.3 - 23.4 \pm 3.5$ h) (Praxedes et al., 2021). This may have occurred due to a need to optimize the concentration of cryoprotectants used, as reported in the cryopreservation of *D. leporina* skin fibroblasts (Praxedes et al., 2023). Furthermore, the biochemical and physiological conditions of the cells may be compromised during the cryopreservation with the cooling rate and exposure to hypertonic solutions, as observed in our study, where the cells required a longer time for duplication.

Another reason that could be associated with the proliferative physiology observed in *G. spixii* skin fibroblasts after cryopreservation was the oxidative stress and mitochondrial activity, which were significantly higher than in the non-cryopreserved group. This may have occurred because the intracellular water stress caused by cryopreservation and after culture can continuously increase the number of ROS molecules (Martindale and Holbrook, 2002) because of the byproduct of the aerobic metabolism of the fibroblast mitochondria from the skin of this small rodent, corroborating our findings (Malpique et al., 2009). Because of the accumulation of free radicals, such as $O_2^{\bullet-}$, H_2O_2 , and $\bullet OH$, there could be damage to the lipids of biological membranes, proteins, core acids, and other biological macromolecules (Lee et al., 2014), causing or inducing cellular apoptosis (Lee et al., 2014; Sastre et al., 2000). This justifies the reduction in the number of viable cells and increases in the number of cells in early and late apoptosis, as observed after cryopreservation of the fibroblastic lines of *G. spixii*.

5. Conclusions

This was the first study describing the establishment, physiological characterization, and cryopreservation of new cell lines from *G. spixii*. This wild rodent plays a significant role in ecology and science. Therefore, cells up to the fifth passage are the most suitable for cellular and molecular studies, as greater genetic, morphological, and physiological stability was observed. Although we have efficiently used the cryopreservation of the fibroblastic line of *G. spixii*, optimizing the development of protocols and cryopreservation solutions for the species is still necessary. Finally, our results offer an opportunity for managing this somatic resource,

allowing the cellular, molecular, and genetic knowledge of the species. This information can help in actions related to the conservation of this species and others phylogenetically close to *G. spixii* since this small wild rodent stands out as an ideal model for scientific study, enabling the increase of *in vitro* techniques, such as nuclear reprogramming.

CRedit authorship contribution statement

Leonardo VC Aquino: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft. **Samara L Olindo:** Methodology. **Yara Letícia F Silva:** Methodology. **Lhara RM Oliveira:** Methodology. **Yasmin BF Moura:** Methodology. **Ana Livia R Rodrigues:** Methodology. **Erika A Praxedes:** Methodology. **Moacir F Oliveira:** Methodology. **Alexandre R Silva:** Formal analysis, Investigation. **Alexsandra F Pereira:** Conceptualization, Formal Analysis, Investigation, Resources, Data Curation, Writing – Review & Editing, Supervision, Funding acquisition.

Acknowledgments

The authors thank the Center for Wild Animals Multiplication (CEMAS/UFERSA) for providing the biological samples used in this study.

Conflict of Competing Interest

The authors declare having no conflict of interest.

Data availability

The data that has been used is confidential.

Statement of Funding

This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil (CNPq, nº 309078/2021-0).

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**CAPÍTULO 3 – L-PROLINE AS A CRYOPROTECTIVE AGENT FOR THE
PRESERVATION OF *Galea spixii* SKIN FIBROBLASTS**

ARTIGO EXPERIMENTAL II

**ARTIGO PUBLICADO NO PERIÓDICO INTERNACIONAL BIOPRESERVATION
AND BIOBANKING**

Qualis 2017-2020: A4 (Medicina Veterinária)

Fator de Impacto: 1.4

Data da Publicação: 14 de Abril de 2025

DOI: <https://doi.org/10.1089/bio.2024.0006>

L-proline as a Cryoprotectant Agent for Preservation of *Galea spixii* Skin Fibroblasts

Leonardo V.C. Aquino, Luanna L.V. Rodrigues, Samara L. Olindo, Yara L.F. Silva, Lhara R.M. Oliveira, Yasmin B.F. Moura, Alessandra F. Pereira*

Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil.

***Address correspondence to:** Alessandra F. Pereira, Ph.D., Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Costa e Silva, Mossoró, RN, Brazil, 59625900, Email: alexsandra.pereira@ufersa.edu.br

Running title: L-Proline Guarantees the Cell Quality.

Abstract

Somatic cell biobanking is a promising strategy for developing reproductive techniques. Although cryopreservation, a technique used for creating the biobanks, has been performed on *Galea spixii*, structural and physiological damage to its cells highlights the need to optimize the cryoprotective solution being used. Therefore, the osmoprotective activity of 5 mM L-proline as an alternative cryoprotectant for the conservation of *G. spixii* fibroblasts was evaluated. The concentration was defined based on previous studies conducted on mammalian cells. Cells derived from the skin of six individuals cultured until the fifth passage were cryopreserved under the following treatments: (i) control (non-cryopreserved); (ii) solution with 10% dimethyl sulfoxide (Me₂SO), 10% fetal bovine serum (FBS) and 0.2 M sucrose; (iii) solution with 10% Me₂SO, 10% FBS, and 5 mM L-proline; (iv) solution with 10% Me₂SO, 10% FBS, 0.2 M

sucrose, and 5 mM L-proline. Tests were conducted to analyze the morphology, viability, metabolism, proliferation, apoptosis, reactive oxygen species (ROS) levels, and mitochondrial membrane activity ($\Delta\Psi_m$). With respect to viability, a reduction in the number of viable cells ($72.3\% \pm 1.2$) was observed in the group containing sucrose compared to the control ($86.7\% \pm 2.0$) and L-proline ($88.4\% \pm 1.8$; $87.8\% \pm 2.1$). After apoptotic analysis, a reduction in the number of viable cells was observed in the group with sucrose alone ($74.6\% \pm 4.1$) compared to the control ($88.2\% \pm 1.1$). The ROS (1.03 ± 0.5 ; 1.07 ± 0.5) and $\Delta\Psi_m$ (0.99 ± 0.42 ; 1.22 ± 0.73) values observed in the groups with L-proline were similar to that observed in the control (1.00 ± 0.5 ; 1.00 ± 0.4). Moreover, no difference was observed between groups for morphology, metabolism, and proliferation. Thus, L-proline is a cryoprotectant agent that can be used during the cryopreservation of *G. spixii* fibroblasts alone or in association with sucrose. Additionally, we developed an adequate biobank for this species in which stored cells could be used for reproductive techniques.

Keywords: Rodentia, Spix's yellow-toothed cavy, cryoinjury, osmolyte cryoprotectant, somatic cells.

Introduction

Cellular biobanks are a collection of isolated and *in vitro* cultured somatic cells. This enables the formation of an organized and searchable collection of biological samples¹, and it provides relevant information for the management, optimization, and development of protocols for wild species. With respect to the laboratory and wild rodents, biobanks offer applications in the treatment of genetic diseases and development of regenerative therapies². Furthermore, somatic cell banks offer an opportunity for the conservation of species³, and genetic reintroduction of populations threatened with extinction through assisted reproduction techniques⁴, as observed

in cloning by somatic cell nuclear transfer⁵, and the induction of cells to pluripotency⁶. Additionally, with the formation of cellular banks for wild rodents, it is possible to implement other techniques irrespective of limiting factors, such as reproductive dysfunction, unexpected deaths, geographic isolation, age and sex of the animal⁷.

Spix's yellow-toothed cavy (*Galea spixii*) is a wild rodent that was initially used to develop a biobank from fibroblastic lines⁸. *G. spixii* is a herbivorous rodent native to the Caatinga biome, which can be geographically located in other biomes, such as in Brazil, Paraguay, Bolivia, and east of the Andes Mountains⁹. This species has a length varying between 22.5 cm and 23.5 cm¹⁰, with uniform colored fur, gray back, white belly, and no tail¹¹. *G. spixii* contributes to the maintenance of local fauna and flora¹². Despite environmental disturbances associated with hunting this rodent¹³, its population is considered stable¹⁴. This correlates with its adaptive, physiological, reproductive, and genetic characteristics¹⁵. Therefore, it stands out as an ideal study model for developing protocols involving the formation of biobanks at scientific breeding sites, and among other wild rodents.

In *G. spixii*, the development of a biobank of somatic tissues has already been elucidated, as observed in the structural and morphological description of the skin, which is an organ from which somatic cells can be isolated in a minimally invasive, inexpensive, and rapid manner¹⁶. The establishment and cryopreservation of fibroblastic lines is another technique implemented for the wild rodent⁸. Post cell cryopreservation, changes were observed in the viability and proliferation rate of the cells, owing to an imbalance in their cellular redox system and an increased number of apoptotic cells, probably caused by osmotic stress and ice crystals formation during cryopreservation. This may have caused lipid peroxidation, which occurs an

important event in cell membrane damage during cryopreservation¹⁷. Therefore, it is necessary to optimize this process.

The first parameter evaluated was the selection of the cryoprotective solution. In *G. spixii*, the first protocol established was based on other phylogenetically close species^{3,18}, where the cryoprotectant solution was composed of 10% dimethyl sulfoxide (Me₂SO), 10% fetal bovine serum (FBS) and 0.2 M sucrose⁸. However, post *in vitro* culturing of the cryopreserved fibroblastic lines⁸, their viability, metabolism, and proliferative rate were affected, probably due to the osmotic stress generated by the formation of intracellular and extracellular ice crystals¹⁷. In this study, only the cryopreservation mechanisms of the cryoprotectants used were studied, such as Me₂SO, which promotes the osmotic dehydration of the cells through its penetration into the cytosol, preventing the formation of intracellular ice crystals¹⁹. Moreover, sucrose, a disaccharide, promotes the stabilization of the lipid membrane as a result of direct interactions with the polar components of the cell bilayer, preventing the aggregation of intracellular molecules and osmotic stress¹⁷.

However, it is also necessary to consider species-specific characteristics, as cryopreservation protocols are dependent on them; for *G. spixii* this is not different, where physiological characteristics, such as size, weight, and metabolic rate, differ from other laboratory and wild rodents¹⁵. Therefore, considering the damage observed after cryopreservation of the *G. spixii* fibroblasts, as well as the need to optimize the cellular cryopreservation protocol for this species, selecting new cryoprotective agents can help promote greater membrane stabilization and control osmotic stress. Thus, L-proline may alternatively be used because in red blood cells¹⁹, lung carcinoma cells²⁰, endothelial cells²¹, and mouse oocytes²², as it was found to have multifunctionality properties post cryopreservation.

L-proline, a natural amino acid is structurally formed by a non-polar cyclic pyrrolidine side chain with an amino group¹⁹. In endothelial cells, L-proline has been implicated as a cell survival factor at a concentration of 5 mM²¹, as it protects cells against osmotic stress and maintains cell cycle progression through the binding and transmission of signal transporters that can participate in cell control and death^{20,23}. Furthermore, studies have shown that L-proline can balance cell turgor pressure, reduce water stress, and thus control the acidity of the medium, as it stabilizes proteins in the cell cytosol, preventing denaturation, aggregation, or dissociation caused by cryopreservation stress²³.

Considering the positive characteristics of 5 mM L-proline, we hypothesized that such agent could improve the viability and physiological quality of *G. spixii* fibroblasts. However, the isolated or synergistic effect with sucrose needs to be investigated. Therefore, the aim of this study was to evaluate the synergistic interaction with sucrose or the isolated presence of L-proline on the viability and physiology of fibroblasts of *G. spixii*.

Materials and Methods

Reagents and solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco BRL (Carlsbad, CA, USA). The study protocol was approved by the Animal Use Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, No. 23091.010566/2017-20) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, No. 60428-1).

Animals, collection, and processing of skin biopsies

Biopsies from the peripheral ear region were recovered from six adult males of *G. spixii* (1 year of age) that were kept in scientific breeding facilities located at the Wild Animal Multiplication

Center (CEMAS/UFERSA, Mossoró, RN, Brazil 5° 10'S, 37°10' W, No. 1478912). To simultaneously collect all skin biopsies, samples sterilized with 70% alcohol and sizes ranging from 1.0 to 2.0 cm² were obtained with surgical pliers. The samples were then transported to the laboratory in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic (ATB-ATM) solution at 4 °C for up to 1 h. In the laboratory, skin biopsies were processed by first removing the hair and then cutting to obtain sections of 9.0 mm³ (3.0 × 3.0 × 1.0 mm) for *in vitro* culture.

Primary culture of skin biopsies and cell subculture

After processing the skin biopsy samples, a primary culture was set up and it was incubated at 38.5 °C and 6.5% CO₂⁸. When the cells reached a cellular confluency ranging between 70–80%, they were subcultured and cell lines were established⁸. Trypsinization was then performed using 0.25% trypsin with 0.05% EDTA. This was considered as the first cell passage. After the fifth passage, cells were cryopreserved as described in the experimental design.

Experimental design

To evaluate the effect of L-proline during cryopreservation, fibroblasts were stored at low temperatures (-196 °C) using the slow freezing technique^{26,27}. Fibroblasts were cryopreserved under the following experimental conditions: (i) control (non-cryopreserved); (ii) solution with 10% Me₂SO, 10% FBS, and 0.2 M sucrose; (iii) solution with 10% Me₂SO, 10% FBS, and 5 mM L-proline; (iv) solution with 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. The L-proline concentration used here was based on the cryopreservation of the human endothelial cell lines²². In this study, 5 mM L-proline did not cause negative effects after the cells were recultured and stored at low temperatures, compared to higher concentrations that generated high osmotic stress.

We evaluated the presence, absence, and synergism of L-proline in a standard cryopreservation medium^{3,18}. This allowed us to visualize its effects in reducing or controlling osmotic stress during the cryopreservation of fibroblastic lines of *G. spixii*. Finally, the non-cryopreserved and cryopreserved cells were evaluated for their morphology, as well as their physiological characteristics, such as viability, metabolism, proliferation, apoptosis, levels of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi_m$).

Slow freezing and thawing of fibroblasts

Fibroblasts were cryopreserved at low temperatures. A concentration of 1.0×10^5 cells/mL was recovered from *in vitro* cultures, and the cells were counted using a Neubauer chamber⁸. The cells were then added to cryotubes containing DMEM, 10% Me₂SO, and 10% FBS, and maintained for 15 min at 4 °C. Post incubation, 0.2 M sucrose and 5 mM L-proline were added and it was further incubated for 15 min at 4 °C^{3,22}. The Mr. Frosty system[®] (Thermo Scientific Nalgene, Rochester, NY, USA), a programmable freezer, was then used for freezing the cells held in a container for 12 h at -80 °C (1 °C/min). The sample was stored in cryotubes in liquid nitrogen (-196 °C) for up to two weeks.

The cryovials were thawed by initially incubating at 25 °C for 1 min, followed by 4 min in a temperature controlled double boiler (35–37 °C). To the thawed cells, DMEM and 0.2 sucrose or 5 mM L-proline was added, according to the experimental groups^{3,22} and it was maintained at 4 °C for 15 min. Post incubation, the cell suspension was centrifuged for 10 min at 600×g. The supernatant was removed, the cells were resuspended in DMEM, 10% FBS, and 2% ATB-ATM and incubated for 15 min at 25 °C, followed by centrifugation (10 min; 600×g). Post centrifugation, the cells were recovered and subjected to *in vitro* culture using a previously

described medium (DMEM, 10% FBS, and 2% ATB-ATM) and evaluated according to the experimental design.

Morphological evaluation and cell viability assay using trypan blue

The morphology of the fibroblasts, before and after cryopreservation, the nuclear shape, and cytoplasmic extension of the cells, was evaluated using an inverted microscope (Nikon TS100, Tokyo, Japan)²⁸.

A cell suspension (20 μ L) of fibroblasts was recovered before and after cryopreservation (immediately after thawing and *in vitro* culturing) to determine the percentage of viable cells. For this, the cells were stained using trypan blue (20 μ L; 0.4%), where non-viable cells (dead; stained in blue) and were those with ruptured membranes, and viable cells (alive; colorless) were those with intact cell membrane²⁸. The Neubauer chamber was used for cell counting and quantification was carried out under an optical light microscope (Olympus CX23, Boston, USA; magnification 10 $\times$) using the following formula: number of viable cells/number of total quantified cells.

Metabolic and proliferative activity of cells

The metabolic activity of the cells was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay²⁷. A concentration of 5.0×10^4 cells/mL was recovered and seeded in 12-well dishes. The cells were incubated for 5 days in an incubator (38.5 °C with 6.5% CO₂). Post incubation, the MTT solution (5 mg/mL) was added, and after 3 h under modified temperature and atmosphere, the cells were analyzed at 595 nm using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan).

To evaluate the proliferation rate, cells were seeded in 24-well dishes at an initial concentration of 1.0×10^4 cells/mL for 7 days (168 h). At intervals of 24 h, post trypsinization the average cell count was obtained which was used to generate a growth curve and population doubling time (PDT)²⁶, which was estimated using the following mathematical calculation:

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

For PDT, time is expressed in hours (h), where T is the incubation time, ln is Napier's logarithm, X_e is the number of cells at the end of the incubation period, and X_b is the number of cells at the start of the incubation period.

Assessment of apoptotic levels

Apoptotic levels of the fibroblasts were evaluated using a cell suspension (35 μ L) in which they were labeled with ethidium bromide (10 μ g/mL) and acridine orange (2 μ g/mL) diluted in 8 μ L of phosphate buffer saline solution (PBS)²⁹. Analysis was carried out using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at an absorbance of 480 nm, (i) viable cells were visible as a uniform green nucleus; (ii) cells at an initial stage of apoptosis were visible as non-uniform green nucleus; (iii) cells at a late stage of apoptosis were visible as a uniform orange cell nucleus; (iv) cells undergoing necrosis were visible as a uniform red cell nucleus²⁶. Images were captured and 300 cells were quantified using the ImageJ software (National Institutes of Health, Bethesda, MA, USA) at a magnification of 20 $\times$.

Quantification of ROS and $\Delta\Psi_m$ levels

Oxidative stress mediated by ROS molecules as well as $\Delta\Psi_m$ was quantified using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 μ M; Invitrogen,

Carlsbad, CA, USA) and MitoTracker Red[®] (CMXRos; 500 nM; Invitrogen, Carlsbad, CA, USA). Approximately, 50 μ L of cell suspension was labeled with fluorescent probes. After 30 min of incubation (38.5 °C and 6.5% CO₂), the cells were processed, as 100 cells per group and the probes were analyzed using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan)⁸.

Statistical analysis

Data obtained from the six adult males of *G. spixii* were expressed as mean $\pm$ standard error (one animal/one replicate) and analyzed using the GraphPad Prism software (Version 10.2.3, Graph-Pad Prism Software Incorporation, La Jolla, USA). Normality was assessed using the Shapiro-Wilk test, whereas homoscedasticity was assessed using the Levene test. Data that were not normally distributed were transformed using arcsine. Data were compared using analysis of variance, followed by Tukey's test or an unpaired t-test. Statistical significance was set at $p < 0.05$.

Results

No phenotypic changes were observed in cellular morphology between the experimental groups (Fig. 1A-D). This indicates that the cells maintained their fusiform shape, were elongated, and had a centralized nucleus. Moreover, we observed that after cryopreservation and *in vitro* culture, fibroblasts exposed to the cryoprotectant solution containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline were more adherent to the surface than those in the group containing only (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose. This can be due to the lower viability observed after the trypan blue assay (Fig. 2A), which demonstrated that immediately after cryopreservation, the cell viability rate in the solution with (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose ($72.3\% \pm 1.2$) was lower than that in the (i) non-cryopreserved group ($86.7\% \pm 2.0$). Moreover, the groups

containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline ($88.4\% \pm 1.8$) and (iv) 10% Me₂SO, 10% FBS, 0.2 sucrose, and 5 mM L-proline ($87.8\% \pm 2.1$) were similar only to the (i) non-cryopreserved (Fig. 2A) group. However, no differences were observed among the experimental groups after *in vitro* culture (Fig. 2A).

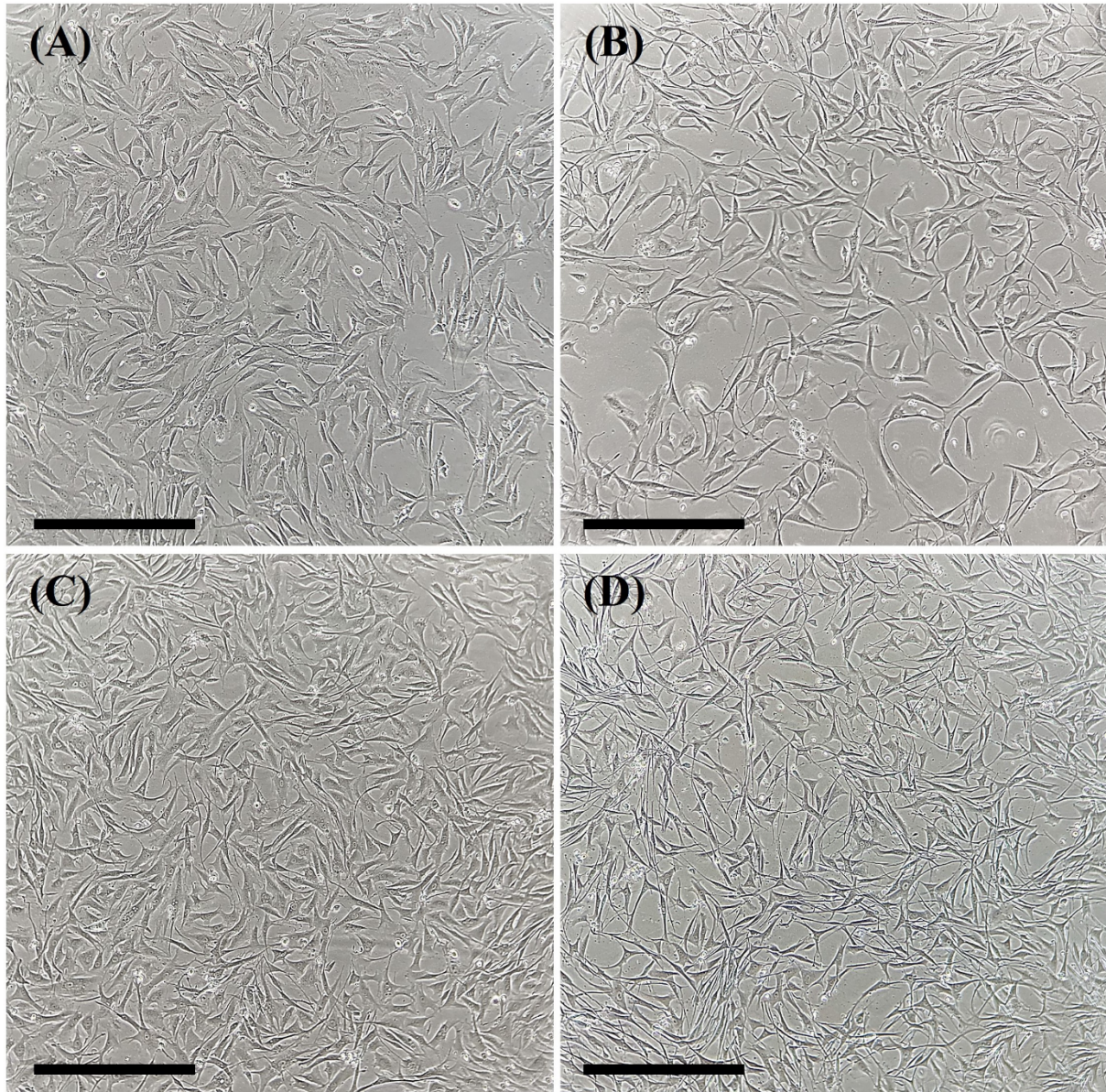


Fig. 1. Representative images of groups of fibroblasts isolated from *G. spixii* ear skin before and after slow freezing. (A) Control (non-cryopreserved). (B) 10% Me₂SO, 10% FBS, and 0.2 M sucrose. (C) 10% Me₂SO, 10% FBS, and 5 mM L-proline. (D) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. Bar scale = 50 μ m, magnitude 40 $\times$.

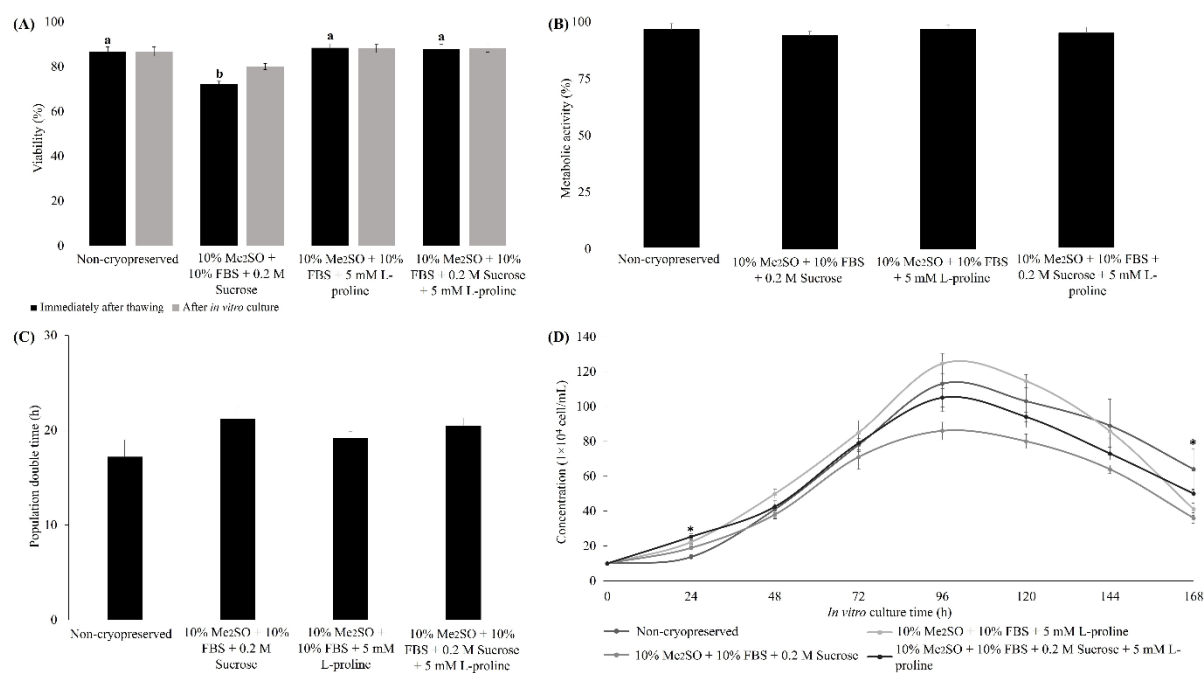


Fig. 2. Viability analysis (immediately after cryopreservation and after *in vitro* culture) and physiological characteristics of *G. spixii* skin fibroblasts before and after slow freezing. (A) Quantification of cell viability by trypan blue assay. (B) Percentage of metabolic activity mediated by MTT assay. (C) Analysis of population doubling time. (D) Evaluation of the cell growth curve. ^{a,b,*}Subscript indicates difference between the experimental groups ($p < 0.05$).

With respect to cellular metabolism and the proliferation rate after cryopreservation, it was initially evident that cell metabolism was greater than 90% in all experimental groups (Fig. 2B, $p > 0.05$), demonstrating physiological functionality of each evaluated group. Similar results were obtained when comparing the experimental groups after PDT analysis, in which the proliferation rate varied between 17 and 20 h (Fig. 2C, $p > 0.05$). Although the metabolism and proliferation rate between experimental groups did not differ, it was observed that after 24 h of cell growth curve analysis (Fig. 2D), only the groups containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline (22.3 ± 1.3 cell/mL) and (iv) 10% Me₂SO, 10% FBS, 0.2 sucrose, and 5 mM L-proline (25.3 ± 1.6 cell/mL) had a higher cell concentration compared to the (i) non-cryopreserved (13.7 ± 1.4 cell/mL) group. Furthermore, after 168 h of evaluation, only the

group containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline (41.1 ± 3.5 cell/mL) and (iv) 10% Me₂SO, 10% FBS, 0.2 sucrose, and 5 mM L-proline (50.0 ± 2.7 cell/mL) were similar to the (i) non-cryopreserved (63.6 ± 11.8 cell/mL) group, while the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose (36.0 ± 3.0 cell/mL) had a lower cell concentration (Fig. 2D).

After apoptotic analysis of the cryopreserved cells (Fig. 3A-D), only the group with (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline ($78.4\% \pm 3.4$) and (iv) 10% Me₂SO, 10% FBS, 0.2 sucrose, and 5 mM L-proline: $78.8\% \pm 3.1$ was similar to the (i) non-cryopreserved group ($88.2\% \pm 1.1$, Fig. 3E). Furthermore, group (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose ($74.6\% \pm 4.1$) showed a lower number of viable cells compared to the (i) non-cryopreserved group ($88.2\% \pm 1.1$, Fig. 3E). This result was no different after evaluating the levels of ROS and $\Delta\Psi_m$ (Fig. 4), where only the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose showed an increase in ROS (2.31 ± 0.9) and $\Delta\Psi_m$ (1.27 ± 0.5) compared to the (i) non-cryopreserved group (1.00 ± 0.5 ; 1.00 ± 0.4), while the groups containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline (1.03 ± 0.5 and 0.99 ± 0.42) and (iv) Me₂SO, 10% FBS, 0.2 sucrose, and 5 mM L-proline (1.07 ± 0.5 and 1.22 ± 0.73) were similar.

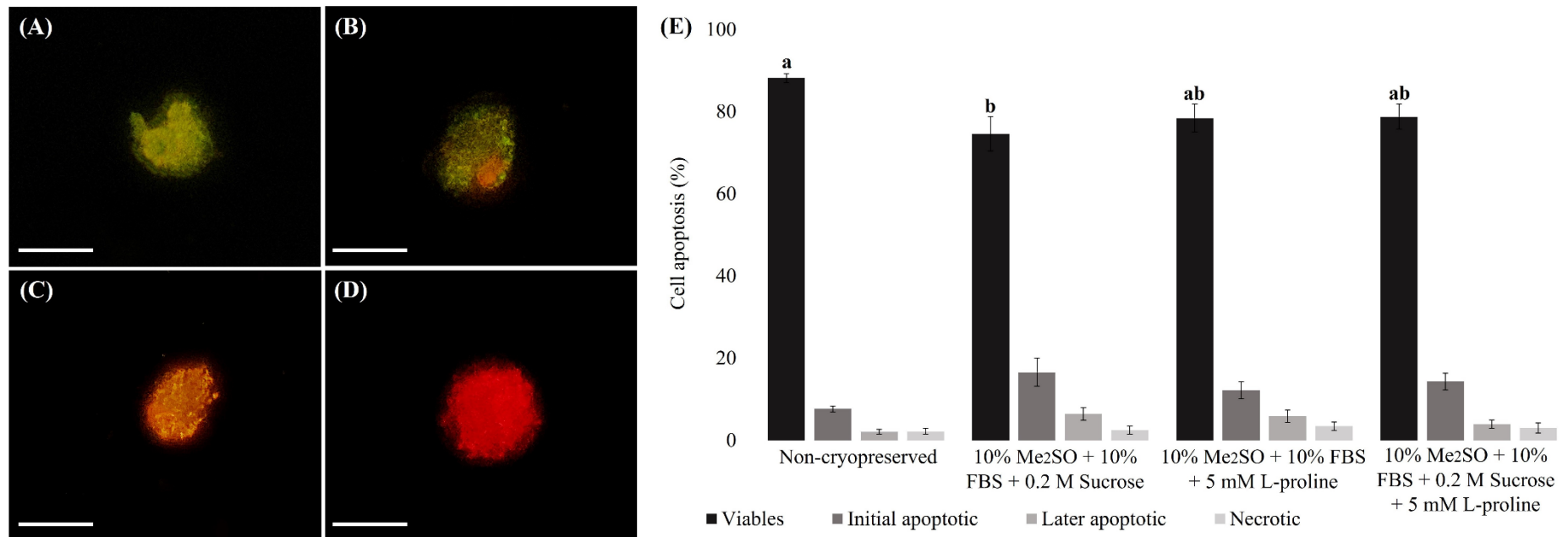


Fig. 3. Assessment of the percentage of apoptotic levels after *in vitro* culture of *G. spixii* skin fibroblast lines. (A) Viable cell. (B) Initial apoptosis. (C) Late apoptosis. (D) Necrotic cell. (E) Percentage after assessment of apoptotic levels. Bar scale = 100 μ m, magnitude 100 $\times$. ^{a,b}Subscript indicates difference between the experimental groups ($p < 0.05$).

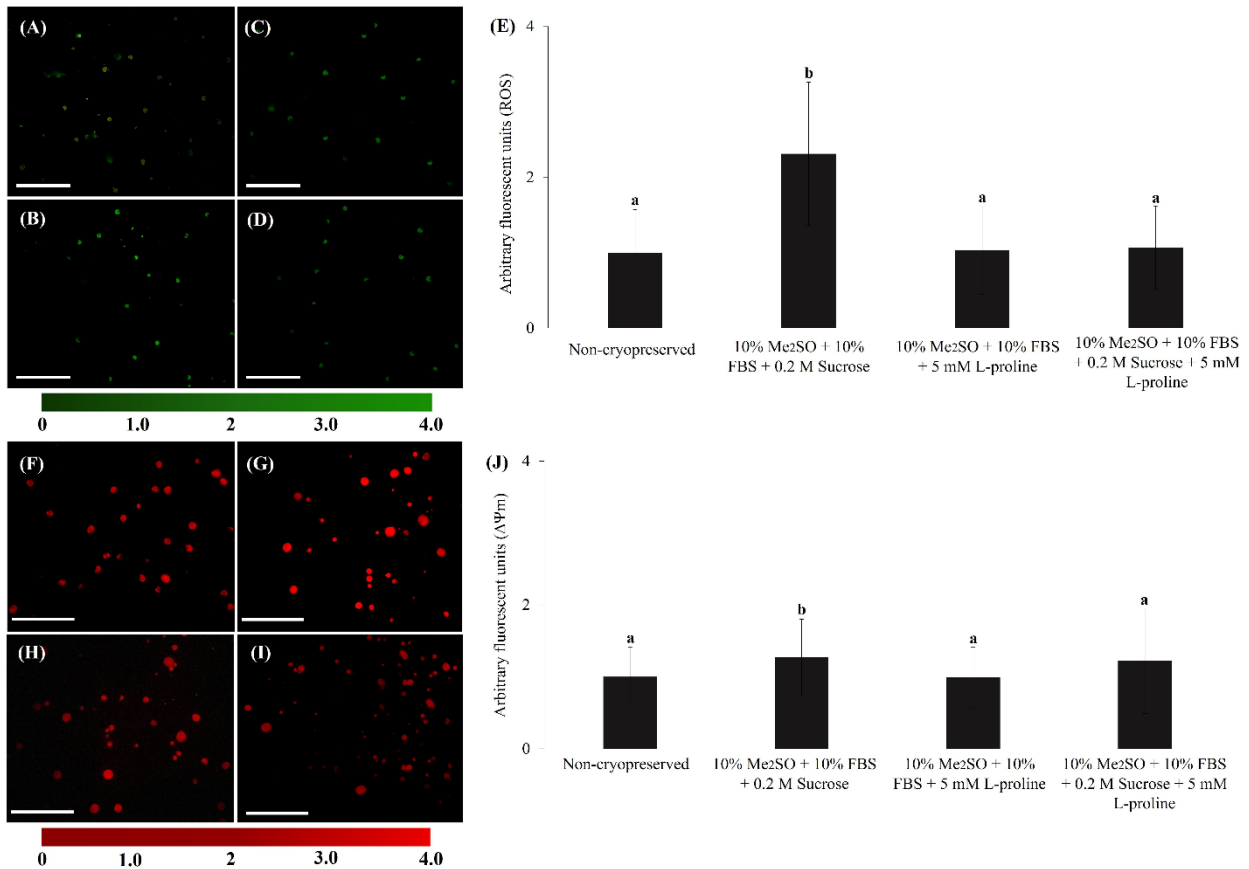


Fig. 4. Assessment of intracellular levels of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) of *G. spixii* fibroblasts before and after slow freezing. (A-D) Representative image of the ROS assessment of the groups (Control [Non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 μ m, magnitude 20 $\times$. (E) Arbitrary fluorescence units obtained after evaluating cellular oxidative stress. (F-I) Representative image of the $\Delta\Psi_m$ assessment of the groups (Control [non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 μ m, magnitude 20 $\times$. (J) Arbitrary fluorescence units obtained after evaluating the mitochondrial activity of fibroblasts. ^{a,b}Subscript indicates difference between the experimental groups ($p < 0.05$).

Discussion

The formation of cell biobanks of wild rodents through cryopreservation is a technique that can significantly contribute to studies aimed at conservation³, as they can help maintain genetic resources. The samples can be used for basic and applied studies, such as to gain information about the physiological, reproductive, biological, and molecular nature of the species³⁰. In *G. spixii*, cryopreservation of the somatic cells occurred for the first time in fibroblastic lines isolated from the skin by slow freezing⁸. Although this was a promising technique, cryopreservation by slow freezing resulted in morphological, structural, and physiological damage to the fibroblasts stored at low temperatures. Additionally, cryopreservation caused an imbalance in the cellular redox system, altering the ROS levels and $\Delta\Psi_m$. In other laboratory and wild rodents, such as *Cavia porcellus*¹⁸, *Mesocricetus auratus*²⁶, and *Dasyprocta leporina*²⁷, characteristics similar to those of *G. spixii* were also verified, which led to a reduction in the quality, viability, and stability of cells stored in cellular biobanks.

Due to the damage generated in the fibroblastic lines of *G. spixii*, we sought, in this study, alternatives that could improve the quality of this cell type after cryopreservation. As the first parameter evaluated, the selection of components of the cryoprotectant solution was an essential factor to be improved during slow freezing³¹ as this could guarantee greater viability and stability of cells during *in vitro* culture and cryopreservation. In our study, we used a protocol usually applied to the cryopreservation of wild mammalian cells³, which was composed of 10% Me₂SO, an intracellular cryoprotectant with multimodal action that promotes multiple effects and actions on specific and nonspecific targets²¹. Furthermore, Me₂SO acts through the osmotic dehydration of cells, reducing the formation of ice crystals during nucleation and thawing events, which could lead to cell death³².

In this same cryopreservation protocol, sucrose was also used at a concentration of 0.2 M, whereby stabilizing the lipid membrane, this disaccharide could interact directly with the cellular components present in the lipid bilayer, avoiding the aggregation of intracellular ice formed, and providing cytoprotection²¹. Although cryoprotectants are widely used in the cryopreservation of cells in wild rodents³³, many studies have demonstrated that these agents are not always efficient, such as Me₂SO, which, when reaching different cellular compartments, can cause negative effects that are required for the physiological activity of cells, causing changes in the enzymatic reactions of mitochondria, modifications in transport mechanisms, and ionic exchange³³. Therefore, an increase in the release of electrons can increase ROS synthesis, leading to oxidative stress, as observed in some experimental groups in our study.

To optimize slow freezing during the cryopreservation of *G. spixii* fibroblasts, with the aim of minimizing the deleterious effects caused by these cryoprotective solutions, L-proline was used either alone or synergistically with sucrose as an alternative to synthetic congeners and their limitations in terms of toxicity and cytoprotective function on cryopreserved *G. spixii* fibroblastic lines were studied. This is because L-proline is a natural amino acid synthesized by microorganisms in response to exposure to cold, whose active ingredient eliminates oxidizing molecules³⁴ and performs some biological functions, such as protein synthesis³⁵, metabolism³⁶, maintenance of cellular structure²⁵, and regulation of the intracellular redox state³⁷.

Using this approach, we initially observed no phenotypic differences between the fibroblasts of the experimental groups after morphological evaluation. This result was not different from those of other studies involving somatic cells from laboratory and wild rodents^{3,18}. However, after analyzing the viability of these cells using the membrane exclusion test, we observed that immediately after cryopreservation, the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2

M sucrose had a lower viability than the (i) non-cryopreserved group and the group containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. A similar example was observed in the study carried out by Jesus et al.³⁸, where superior viability of HaCaT cells compared to the control group composed only of Me₂SO was observed. According to Bojic et al.³¹, this may have occurred because of the close interactions between the cells and the cryopreservation medium, where the cells were much more susceptible to damage caused by changes during the freezing and thawing phases.

In *M. auratus*²⁶, a reduction in cell viability was observed immediately after cryopreservation. This was due to the association with the osmotic limits of the cells and the cryoprotective solutions used in the study (10% Me₂SO and 90% FBS). In our study, L-proline guaranteed a greater interaction between the molecules of the cell cytosol, as well as the extracellular environment to which this natural amino acid was subjected. This response was corroborated by Pi et al.³⁹, by combining and observing the synergistic effect of L-proline during T-cell cryopreservation.

The results previously obtained are important because, in our study, we also observed that after cryopreservation and *in vitro* culture of *G. spixii* fibroblastic lines, the groups containing only (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline in the first 24 h of evaluation of the cell growth curve (cells/mL) were much superior to the (i) non-cryopreserved. However, after 168 h, the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose showed a lower number of cells (cells/mL) than the (i) non-cryopreserved group. This result can be justified because after reculturing, the structural integrity of the *G. spixii* fibroblasts was not positively supported when they were only cryopreserved in a cryoprotectant solution containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M

sucrose, however the cells that were cryopreserved with (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline were less affected by slow freezing.

Therefore, cryopreserved cells containing L-proline can recover more quickly from injury caused by the formation of ice crystals during freezing and thawing. In contrast to the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose, *G. spixii* fibroblasts lost their biochemical activity although they maintained intact cell membranes. These results corroborated those of a study on endothelial cells²². When compared with the control group (Me₂SO) and the other osmolytes evaluated, cells cryopreserved in medium containing L-proline were much more adherent to the *in vitro* culture dishes.

Although (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline promoted an increase in cell viability as well as in the proliferation rate, after analyzing the cell growth curve in comparison with the (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose, we did not observe differences after analyzing the population doubling time and metabolism. These results suggest that the mentioned cryoprotective agents, at adequate concentrations, are not toxic to fibroblastic lines isolated from the skin of *G. spixii*. Furthermore, cryoprotective solutions can act positively in reducing and controlling the formation of ice crystals, as well as in processes related to recrystallization after thawing cells, especially those groups composed of L-proline that allow greater cell viability and proliferation after cryopreservation²¹.

In addition, the apoptotic index of *G. spixii* fibroblasts was analyzed. In our study, this assay made it possible to predict cell death, which has been associated with an osmotic stress event

during the slow freezing of cells⁴⁰. This assay also allowed the quality and stability of the cells to be observed during *in vitro* culture²⁸. Therefore, we observed that in the group containing (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose, the number of viable cells was lower than that in the (i) non-cryopreserved, and the number of viable cryopreserved fibroblasts containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline remained same. This may be because the group containing only sucrose may have initiated biochemical pathways related to programmed cell death, such as caspase-3 and caspase-7, which are highly active during cell death process^{41,42}.

Moreover, unlike L-proline, sucrose is a disaccharide with an action similar to that of trehalose, allowing only the stabilization of the lipid membrane as a result of direct interactions of the polar components of the cell bilayer¹⁷. L-proline can exert different biological functions, such as inducing the control of p53 genes and synthesis of enzymes, such as proline oxidase (POX/PRODH), located in the inner membrane of the mitochondria, and is responsible for catalyzing the transfer of electrons, and regulating intracellular redox³⁷, thereby regulating the *in vitro* culture of cells.

The previously described justification can be confirmed because in our study, the groups containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline maintained ROS and $\Delta\Psi_m$ levels similar to those in the (i) non-cryopreserved group, while it was higher in the group composed of (ii) 10% Me₂SO, 10% FBS, and 0.2 M sucrose. This may have occurred because studies showed that free and polypeptide-bound proline can react with hydrogen peroxide (H₂O₂) and hydroxyl groups (HO•) to form stable free radicals²⁵. For example, a study that observed the antioxidant action

of 5 mM L-proline⁴³, found that after 30 min the H₂O₂ levels reduced by more than 90% when compared to the control.

In addition, L-proline was added to the culture of human keratinocytes, and it was demonstrated that L-proline protected cells against induced apoptosis⁴⁴. Therefore, it is suggested that this molecule reduces oxidative stress, and ROS and $\Delta\Psi_m$ levels in the experimental groups containing (iii) 10% Me₂SO, 10% FBS, and 5 mM L-proline and (iv) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline, ensuring, greater osmotic control and balance of deplasmolysis pressure and cell turgidity^{20,44}.

Conclusions

This is the first study to optimize a technique for the cryopreservation of fibroblasts of *G. spixii* using L-proline. In our study, *G. spixii* cells stored at low temperatures, after thawing, or with sucrose remained viable and functional. Moreover, the experimental groups with L-proline were better at maintaining cell viability, proliferative rate, and control of ROS and $\Delta\Psi_m$ levels. Therefore, L-proline either alone or synergistically with sucrose can be an alternative for cryopreservation of *G. spixii* fibroblasts. Thus, it was possible to develop an adequate cellular biobank for *G. spixii*, where the stored cells could later be used to obtain biological, genetic, and reproductive knowledge of the species, as observed in cloning by somatic cell nuclear transfer and inducing pluripotency in cells.

Acknowledgments

The authors thank the Center for Wild Animals Multiplication (UFERSA) for providing biological material.

Author contributions

LVC Aquino: Conceptualization, methodology, writing, original draft, and investigation. LLV Rodrigues: Methodology and investigation. SL Olindo: Methodology and investigation. YLF Silva: Methodology and investigation. LRM Oliveira: Methodology and investigation. YBF Moura: Methodology and investigation. AF Pereira: Conceptualization, methodology, writing, original draft, supervision, project administration, and funding acquisition.

Authors' Disclosures

The authors reported no potential conflict of interest.

Funding Statement

This study was partially funded by the Coordination of Improvement of Higher Education Personnel, Brazil (CAPES, Financial Code 001), and the National Council for Scientific and Technological Development (CNPq, No. 309078/2021-0).

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**CAPÍTULO 4 – EFFECT OF ROSCOVITINE AND SERUM STARVATION ON THE
INDUCTION OF G₀/G₁ CELL CYCLE ARREST IN *Galea spixii* (WAGLER, 1831)
FIBROBLASTS**

ARTIGO EXPERIMENTAL III

**ARTIGO SUBMETIDO NO PERIÓDICO INTERNACIONAL ANIMAL
REPRODUCTION**

Qualis 2017-2020: A2 (Medicina Veterinária)

Fator de Impacto: 2.1

Data da Submissão: 22 de Dezembro de 2025

**Effect of roscovitine and serum starvation on the induction of G₀/G₁ cycle arrest in
Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) fibroblasts**

G₀/G₁ arrest in Galea spixii fibroblasts

Leonardo Vitorino Costa de Aquino¹ (<https://orcid.org/0000-0001-7192-7581>), Luanna Lorena Vieira Rodrigues¹ (<https://orcid.org/0000-0002-1764-1205>), Ana Beatriz Morais de Oliveira¹ (<https://orcid.org/0009-0004-3993-8407>), Sueli de Oliveira Lima¹ (<https://orcid.org/0000-0002-7085-5624>), Samara Lima Olindo¹ (<https://orcid.org/0009-0000-0435-7881>), Sarah Sant'Anna Maranhão² (<https://orcid.org/0000-0002-5379-2780>), Maria Claudia dos Santos Luciano² (<https://orcid.org/0000-0002-7871-2513>), Cristiana Libardi Miranda Furtado³ (<https://orcid.org/0000-0002-8711-8225>), Claudia Pessoa² (<https://orcid.org/0000-0002-4344-4336>), Alexsandra Fernandes Pereira^{1*} (<https://orcid.org/0000-0003-2137-854X>)

¹Laboratory of Animal Biotechnology, Federal University of Semi-Arid, Mossoro, RN, Brazil.

²Experimental Oncology Laboratory, Federal University of Ceara, Fortaleza, CE, Brazil.

³Genetics, Ecology and Evolution Department, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

***Corresponding author:** alexandra.pereira@ufersa.edu.br

Conflicts of interest: The authors have no conflict of interest to declare.

Financial support: LVCA, LLVR, SOL receive grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). CP, AFP and CLMF receive grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Author contributions: LVCA: Conceptualization, Data curation, Methodology, Writing – original draft & review; LLVR: Methodology; ABMO: Methodology; SLO: Methodology; SOL: Methodology; SSAM: Methodology; MCSL: Methodology; CLMF: Methodology; CP: Writing – Review & Editing; AFP: Conceptualization, Methodology, Supervision, Writing – Original draft & Review, Writing – Review & Editing.

ABSTRACT

G₀/G₁ synchronization is a nuclear reprogramming strategy that enables the selection of quiescent cells with greater genomic stability for use as donor nuclei. Methods such as chemical inhibitors, including roscovitine (RSV), and serum starvation (SS) have shown effectiveness in various models, including wild rodents. However, validating these protocols requires considering cell line and species-specific features. In Spix's yellow-toothed cavy, a neotropical rodent of ecological and reproductive interest with an established cell biobank, G₀/G₁ synchronization offers an opportunity to study cell cycle regulation and develop protocols for assisted reproduction. This study evaluated the effects of RSV (7.5, 10, and 15 μ M for 6–24 h) and SS (24–96 h) on morphology, viability, apoptosis, reactive oxygen species (ROS), and cell cycle distribution in Spix's yellow-toothed cavy fibroblasts. Morphology was preserved, and viability remained above 85% in all RSV-treated groups. Although 7.5 μ M (90.7% $\pm$ 1.5) and 15 μ M (91.6% $\pm$ 1.2) induced high G₀/G₁ rates, 10 μ M (92.2% $\pm$ 1.9; 90.0% $\pm$ 0.8) provided the best balance between synchronization and apoptosis compared to control (86.3% $\pm$ 0.7; 97.9% $\pm$ 0.3). In the SS group, viability was maintained up to 24 h (95.3% $\pm$ 0.7), with colony

formation, loss of adherence, and increased apoptosis after 48 h. Synchronization with SS was effective at 24 h ($90.3\% \pm 1.1$), higher than control ($82.3\% \pm 1.0$). Both treatments significantly increased ROS. RSV (10 μ M/12 h) and SS (24 h) were the most efficient and safe synchronization protocols for Spix's yellow-toothed cavy fibroblasts.

Keywords:

Caviidae; conservation strategies; cell cycle synchronization; chemical inhibitors.

Introduction

The cell cycle is a fundamental process for maintaining homeostasis, proliferation, and differentiation in eukaryotic cells (Viana and Pereira, 2025). Progression through the G₁, S, G₂, and M phases depends on the precise activity of cyclin-dependent kinases (CDKs) and their regulatory proteins (Anglada et al., 2025). These mechanisms ensure that the cell performs the growth, DNA replication, and chromosomal segregation stages in the correct order and timing through checkpoint controls that safeguard genomic integrity and prevent the propagation of molecular damage (Pankammoon et al., 2025).

Despite being extensively characterized in established cell lines, studies of cell cycle dynamics still face significant technical limitations. Cell populations cultured under standard conditions exhibit asynchronous progression, with cells found in different stages of the cycle. This heterogeneity complicates the analysis of specific molecular events and compromises the reproducibility and accuracy of methods that require synchronized cell populations (Chen et al., 2025). Cell cycle synchronization is a valuable experimental strategy for enriching *in vitro* cell populations in the G₀/G₁ phase (Praxedes et al., 2020), a requirement for both somatic cell nuclear transfer (SCNT) and the generation of induced pluripotent stem cells (iPSCs).

Among the synchronization protocols, the most employed methods include contact inhibition, serum starvation (SS), and chemical CDK inhibitors, such as roscovitine (RSV)

(Hayes et al., 2001; Praxedes et al., 2024). However, the efficiency of each method varies according to species, cell type, and duration of exposure. In wild rodents, when evaluating fibroblasts from *Dasyprocta leporina*, Praxedes et al. (2024) demonstrated that contact inhibition, although yielding 70% cells in the G₀/G₁, exhibited lower efficiency compared to other methods. Similar results have been observed in rats and mice, where contact inhibition is not widely adopted. When applied, it produces outcomes comparable to those found in *D. leporina* (Hayes et al., 2001; Praxedes et al., 2024). In contrast, both SS for 48 to 72 h and RSV (10 µM for 12 h) achieved high synchronization rates (>80%) without compromising morphology, viability, or apoptosis.

Nonetheless, prolonged SS beyond 72 h does not yield further improvements and may induce an increased cellular stress. Similarly, RSV efficacy and cytotoxicity are highly sensitive to variations in concentration and exposure time. Reviewing synchronization protocols in wild felines, Viana and Pereira (2025) found that RSV between 7.5 and 30 µM, with exposure times ranging from 12 to 24 h, displayed variable effectiveness depending on the species, with concentrations above 15 µM potentially compromising cell viability in specific lines.

Given interspecies variability, the development of synchronization protocols tailored to species-specific biological traits is essential for advancing reprogramming studies. This is particularly relevant for Spix's yellow-toothed cavy, a neotropical rodent of the family Caviidae, primarily distributed in Brazil's semiarid region but also occurring in other Brazilian biomes, Paraguay, and the Eastern Andes (Silva et al., 2024). With diurnal and crepuscular habits and an herbivorous diet, this species plays an important ecological role in seed dispersal, soil aeration, and trophic network stability. Although its population is considered stable (Catzefflis et al., 2016), ongoing threats such as habitat degradation, and climate change highlight its relevance as a strategic model for conservation and assisted reproduction efforts (Silva et al., 2024).

Beyond its ecological relevance, Spix's yellow-toothed cavy has been proposed as a promising experimental model for the development of reproductive biotechnologies aimed at conservation within the Hystricomorpha suborder. The availability of an established cell biobank (Aquino et al., 2024) and validated cryopreservation protocols (Aquino et al., 2025) support its potential application in SCNT and iPSC technologies. However, limited knowledge regarding fibroblast cell cycle kinetics and the effects of synchronization strategies remains a key constraint (Viana and Pereira, 2025). To address this gap, we compared RSV exposure (7.5–15 μ M for 6–24 h) and SS (24–96 h) in *G. spixii* fibroblast lines to establish efficient protocols for G₀/G₁ phase synchronization.

Methods

Reagents and solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco BRL (Carlsbad, CA, USA), except as otherwise indicated.

Bioethics, animals, and processing of skin biopsies

All experimental procedures were approved by the Ethics Committee on Animal (CEUA/UFERSA, no. 23091.010566/2017-20) and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1). The study was conducted using six adult males (1 year old), each weighing between 250 and 350 g. The animals were housed at the Center for the Multiplication of Wild Animals (CEMAS/UFERSA, no. 1478912, Mossoró, Brazil; 5°10'S, 37°10'W), where they had free access to fruits, feed, and water.

The animals were manually restrained for the collection of skin biopsies. The apical region of the ear was chosen as the donor site due to its accessibility and minimal invasiveness. After asepsis with 70% ethanol, tissue fragments ranging from 1.0 to 2.0 cm² were excised using sterile surgical forceps (Aquino et al., 2024). Immediately following excision, the samples

were placed in sterile tubes containing Dulbecco's modified minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution, and kept at 4 °C (Pereira et al., 2021). Transport to the laboratory occurred within one hour of collection. Once in the laboratory, the biopsies were cleaned to remove residual hair and trimmed into explants of 9.0 mm³ (3.0 × 3.0 × 1.0 mm) to initiate *in vitro* primary culture procedures.

Establishment, subculture, cryopreservation, and post-thaw recovery of fibroblasts

Following the processing of skin biopsies, primary cultures were established to isolate fibroblast cell lines. The cultures were incubated at 38.5 °C and 6.5% CO₂. Once cells began to detach and reached 70–80% confluence, they were dissociated using 0.25% trypsin with 0.05% EDTA (Praxedes et al., 2021). Each enzymatic dissociation was considered one cell passage. Fibroblast lines were established and cryopreserved after five passages (Aquino et al., 2024). Fibroblast cell lines were cryopreserved according to the protocol described by Aquino et al. (2025). Briefly, cells were harvested from cultures at a concentration of 1.0×10^5 cells/mL, and transferred to cryotubes containing the cryoprotective solution, frozen at –80 °C for 12 h using the Mr. Frosty[®] system and stored in liquid nitrogen (–196 °C) for two weeks. Post-cryopreservation thawing and processing were performed according to Aquino et al. (2025).

Experimental design

To evaluate cell cycle synchronization efficiency, this study adopted an experimental design based on protocols established for rodents and wild mammals, including *D. leporina* (Praxedes et al., 2024), domestic and wild felids, and large carnivores (Wittayarat et al., 2013; Rodrigues et al., 2023). The central hypothesis was that RSV and SS could effectively induce G₀/G₁ synchronization when concentrations and exposure times were optimized for the target

species. Fibroblasts at the fifth passage post-cryopreservation were cultured to ~70% confluency and characterized by growth curve, metabolic parameters, and population doubling time to ensure cell line stability.

Cells were allocated into experimental conditions: (a) control, cells maintained at 50–60% confluency without synchronization; (b) RSV-treated cells exposed to 7.5, 10, or 15 μM for 6, 12, or 24 h; and (c) SS-treated cells cultured in DMEM containing 0.5% FBS for 24, 48, 72, or 96 h, totaling fourteen groups. Post-treatment analyses included cell viability (trypan blue), morphology, apoptosis (acridine orange/ethidium bromide), intracellular ROS levels, and cell cycle distribution by flow cytometry.

Cell proliferation dynamics and metabolic activity

The cell growth curve and the metabolic assay were used to determine the proliferative rate and metabolic activity of fibroblasts (Praxedes et al., 2021). For the cell growth curve, cells were seeded in 24-well plates at an initial density of 1.0×10^4 cells/mL and incubated for seven days (168 h) at 38.5 °C and 6.5% CO₂. Cell counts were performed every 24 h, allowing the construction of the growth curve and the calculation of population doubling time (PDT), estimated using the equation: $T \times \ln_2 / \ln(X_e/X_b)$, where T is the total incubation time (in hours), X_e is the number of cells at the end of the incubation period, X_b is the number of cells at the beginning, and ln is the natural logarithm. For the metabolic analysis, cells were seeded in 12-well plates at a concentration of 5.0×10^4 cells/mL and incubated for five days (38.5 °C; 6.5% CO₂). After, an MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5 mg/mL diluted in DMEM was added and incubated for an additional 3 h (Aquino et al., 2025). Then, DMSO was added to solubilize the resulting formazan crystals, and absorbance was measured at 595 nm using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan).

Evaluation of cell viability and morphology under *in vitro* culture

A 20 μL aliquot of cell suspension was collected after each cycle synchronization treatment to assess cell viability (Aquino et al., 2025). The cells were stained with 0.4% trypan blue (20 μL), distinguishing between viable and non-viable cells based on plasma membrane integrity. Non-viable cells, which stained blue, indicated disrupted membranes, whereas viable cells remained colorless, maintaining intact membranes. Cell counting and viability were performed using a Neubauer chamber under a light microscope (Olympus CX23, Boston, USA; 10 $\times$ magnification). Morphological evaluation of fibroblasts was conducted throughout the entire culture period using an inverted microscope (Leipzig IMx, Phoenix Optics, Germany). Assessments focused on cell shape, nuclear morphology, and cytoplasmic extension.

Nuclear integrity by cellular apoptosis

Apoptotic levels were assessed using differential fluorescent staining (Aquino et al., 2025). A 35 μL aliquot of the cell suspension was incubated with a dye mixture composed of 10 $\mu\text{g/mL}$ ethidium bromide and 2 $\mu\text{g/mL}$ acridine orange, diluted in 8 μL of phosphate-buffered saline solution (PBS 1 $\times$). This combination of fluorescent DNA-binding dyes enables discrimination of different stages of cell death based on plasma membrane integrity and chromatin condensation, as both selectively bind to DNA.

Analyses were performed using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) with an excitation wavelength of 480 nm. Cells were classified into four categories based on the fluorescence patterns: (i) viable cells with uniform light green nuclei; (ii) early apoptotic cells with irregular green fluorescence and chromatin condensation; (iii) late apoptotic cells with orange nuclei and signs of fragmentation; and (iv) necrotic cells with red or intense orange nuclei, indicating loss of membrane integrity. Representative images of adhered cells were recorded, and for each experimental condition, a total of 300 cells from suspension were

quantified using ImageJ software (National Institutes of Health, Bethesda, USA) under 20× magnification.

Measurement of intracellular ROS levels using H₂DCFDA fluorescent probe

The quantification of ROS levels was performed using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 μM; Invitrogen, Carlsbad, CA, USA) (Aquino et al., 2024). The staining solution was prepared using 2.5 mL PBS added to a 0.5 μL H₂DCFDA (0.1 M). The cells were labeled by incubating a mixture containing 50 μL of the cell suspension and 50 μL of the fluorescent probe (1:1 ratio) for 30 min at 38.5 °C and 6.5% CO₂. After, samples were centrifuged at 300×g for 5 min. The supernatant was discarded, and the cell pellet resuspended in 50 μL PBS. The cells were analyzed using a fluorescence microscope (excitation at 495 nm and emission at 520 nm).

Images of 100 cells were recorded for each experimental condition, and fluorescence intensity was quantified in pixels using the ImageJ software. Background fluorescence values were subtracted from the total signal, and the relative ROS levels were expressed in arbitrary fluorescence units (AFU). Each cell was considered an experimental unit.

Quantification of G₀/G₁, S and G₂/M phases by flow cytometry

The samples were centrifuged at 600×g for 10 min, and the supernatant was discarded. The cells were fixed by the slow addition of 500 μL of cold 70% ethanol (4 °C), as described by Praxedes et al. (2024), and stored at -20 °C for up to five days until flow cytometric analysis. For sample preparation, ethanol was removed by two successive washes in PBS (500 μL), followed by centrifugation at 400×g for 10 min each. Subsequently, the cells were stained with a solution containing propidium iodide (5 μg/mL; Thermo Scientific, Waltham, Massachusetts,

USA) and RNase A (50 µg/mL; Thermo Scientific, Waltham, Massachusetts, USA) (Praxedes et al., 2024). The samples were incubated for 40 min before data acquisition.

The analysis was performed using a Guava EasyCyte flow cytometer (Guava Technologies, Stamford, Lincolnshire, UK), employing a gating strategy based on forward scatter (FSC) and DNA content, with unstained controls used for calibration. For each sample, 10.000 events were analyzed, and each experimental condition was biologically replicated six times (Rodrigues et al., 2023).

Statistical analysis

Data obtained from the six biological replicates (one animal per replicate) were expressed as mean $\pm$ standard error of the mean and analyzed using the GraphPad Prism software (version 10.4.2; GraphPad Software Inc., La Jolla, CA, USA). The Shapiro–Wilk test was applied to assess data normality, and the Levene test was used to verify the homogeneity of variances. As the data did not meet the assumption of normal distribution, an arcsine transformation was applied. Subsequently, all data were subjected to analysis of variance (ANOVA), followed by Tukey’s post hoc test for multiple comparisons. Differences were considered significant when $p < 0.05$.

Results

Proliferative and metabolic dynamics of fibroblasts before cycle synchronization

Proliferative evaluation of six fibroblast lines revealed that cells exhibited a lag phase within the first 24 h, followed by exponential growth between 48 and 96 h, indicating efficient recovery of proliferative capacity (Fig. 1A). After 96 h, cultures reached a plateau, with a reduction in cell number observed at 168 h. MTT assays performed on day five confirmed functional viability, with metabolic activity exceeding 95% across all lines (Fig. 1B). These

results were consistent with the growth curves and PDT, which averaged approximately 19 h (Fig. 1B).

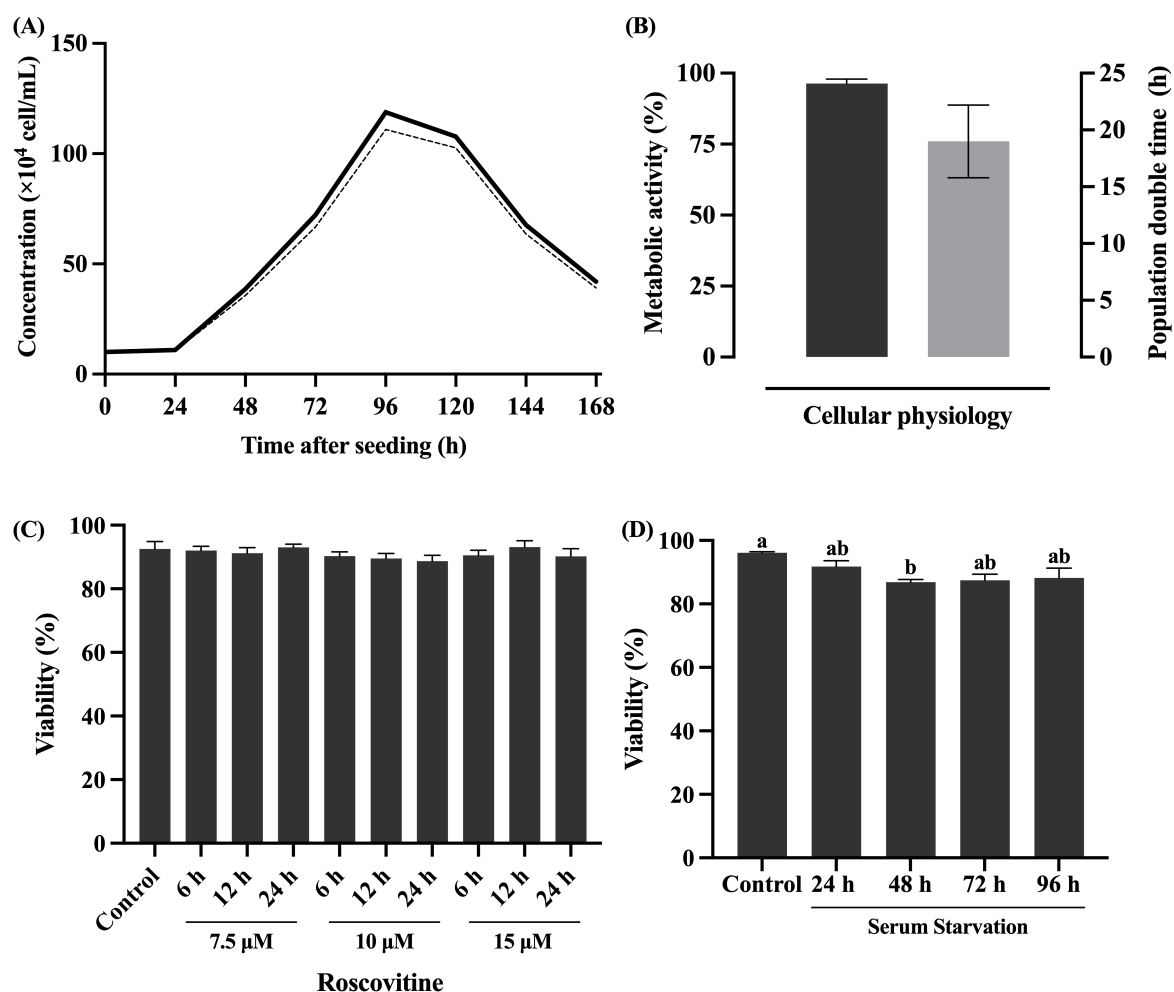


Fig. 1. Physiological assessment of Spix's yellow-toothed cavy fibroblasts before synchronization treatments by growth curve (A), metabolic activity (B) PDT assay, and viability after cell cycle synchronization treatments using different concentrations and exposure times of RSV (C) and SS (D). Mean $\pm$ standard error. ^{a,b}: $P < 0.05$.

Evaluation of synchronization methods on cell viability and morphology

Fig. 1C-D presents the results regarding cell viability following cell cycle synchronization methods. In RSV exposure, regardless of the concentration (7.5, 10, and 15 μ M) and incubation time (6, 12, and 24 h), cell viability remained above 85%, indicating

that the inhibitor did not compromise cell integrity (Fig. 1C). In contrast, the SS elicited a different response: during the first 48 h, an initial reduction in cell viability was observed, possibly due to adaptation to the low-FBS environment. However, this condition was partially reversed, with a gradual recovery in viability observed up to 96 h of culture, ultimately reaching levels above 85% (Fig. 1D).

The results regarding cell viability may be directly related to the morphological patterns observed following synchronization treatments. This association is plausible, as RSV did not compromise cell viability regardless of concentration (7.5, 10, and 15 μ M) or exposure time (6, 12, and 24 h), and cellular morphology was also preserved. The images presented in Fig. 2A–D'' show that fibroblasts exposed to RSV exhibited a typical morphology, with well-defined cytoplasmic extensions, central nuclei, and no evident signs of cellular retraction under the evaluated conditions.

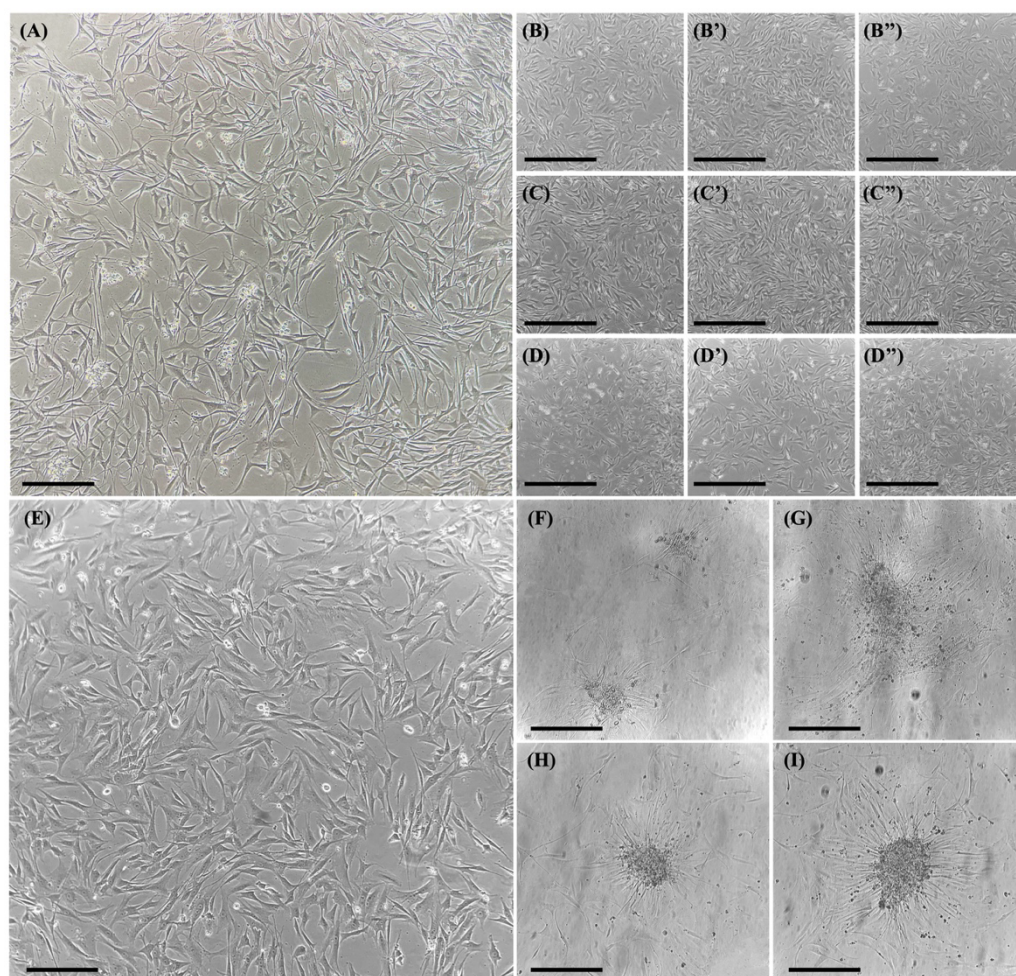


Fig. 2. Morphological analysis of Spix's yellow-toothed cavy fibroblasts before and after cell cycle synchronization using RSV (**A–D''**) and SS (**E–I**). (**A**) Control group (no treatment). (**B–B''**) Cells treated with 7.5 μM RSV for 6 h, 12 h, and 24 h. (**C–C''**) Cells treated with 10 μM RSV for 6 h, 12 h, and 24 h. (**D–D''**) Cells treated with 15 μM RSV for 6 h, 12 h, and 24 h. (**E**) Control group (no treatment). (**F–I**) Cells subjected to SS for 24 h (**F**), 48 h (**G**), 72 h (**H**), and 96 h (**I**). Scale bar = 100 μm ; magnification = 20 $\times$.

In contrast, the SS method induced progressive morphological alterations over the course of the culture. As evidenced in Fig. 2E–I, corresponding to 24, 48, 72, and 96 h of treatment, a gradual reduction in the extent of cytoplasmic projections was observed, along with the loss of monolayer organization, increased nuclear density in certain regions, and clear signs of cellular retraction. By 72 h (Fig. 2H), cells displayed a more compact morphology, with irregular contours and a reduced cytoplasmic area. At 96 h (Fig. 4I), although cell viability had been partially restored, many cells exhibited features compatible with morphological stress, including reduced substrate adhesion.

Effect of synchronization methods on the apoptotic levels

The analysis of apoptotic levels in cells subjected to different cycle synchronization protocols revealed distinct responses among the groups (Fig. 3A–D). In RSV, the 7.5 μM concentration induced a slight reduction in the proportion of viable cells after 24 h of exposure ($90.9\% \pm 1.6$) compared to the control group ($97.9\% \pm 0.3$) (Fig. 3E). However, the percentages of cells in early and late apoptosis, as well as necrosis, did not differ in this group. When evaluating the 10 μM concentration, RSV did not induce changes in cell viability over time, maintaining stable viability rates at 6 h ($91.6\% \pm 1.5$), 12 h ($92.2\% \pm 1.9$), and 24 h ($92.9\% \pm 1.9$), with no differences compared to the control or the other concentrations (Fig. 3E). Nevertheless, after 6

h of exposure at this concentration, an increase in the percentage of cells undergoing early apoptosis was observed ($6.6\% \pm 0.9$), which was higher than in the control ($1.3\% \pm 0.3$). However, this effect did not persist at later time points.

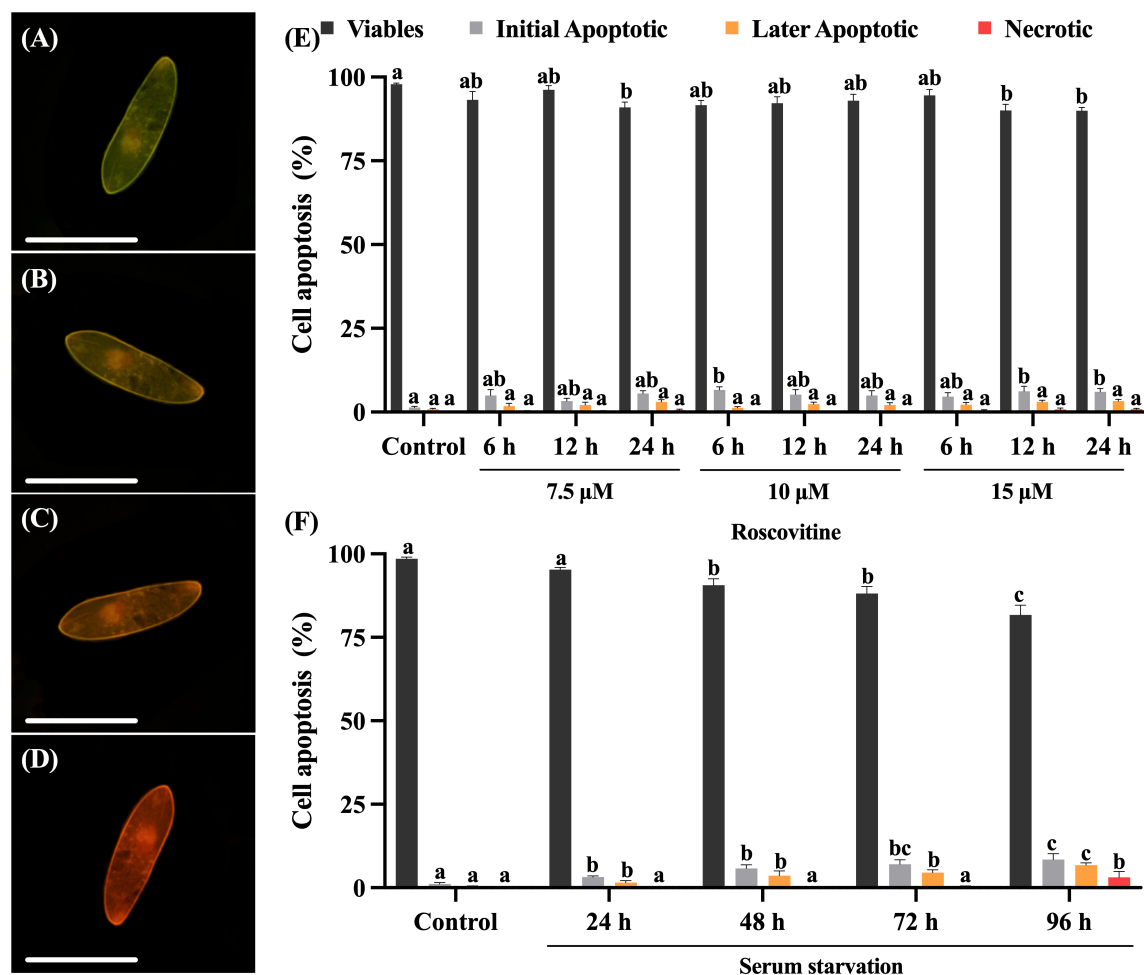


Fig. 3. Apoptotic profile of Spix's yellow-toothed cavy fibroblast lines before and after cell cycle synchronization (A–D). Representative images of viable cells (A), early apoptotic (B), late apoptotic (C), and necrotic cells (D). (E–F) Graphical representations of apoptotic cell percentages after different synchronization treatments using RSV (E) and SS (F). Scale bar = 100 μ m, 40 $\times$ mag. Mean $\pm$ standard error. ^{a,b}: Values with different superscript letters within columns significantly differ ($P < 0.05$).

Conversely, the 15 μ M RSV induced more pronounced adverse effects beginning at 12 h of exposure, with a reduction in cell viability ($90.0\% \pm 1.9$) compared to the control. Additionally, increased levels of early apoptosis were detected at both 12 h ($6.2\% \pm 1.5$) and 24 h ($6.0\% \pm 1.0$), indicating that higher concentrations of the inhibitor may progressively compromise cell integrity (Fig. 3E). These findings suggest that, although RSV exerts minimal impact on cell viability at moderate concentrations, higher concentrations and prolonged exposure can trigger relevant apoptotic effects. The application of the SS led to progressive alterations in the apoptotic profile, as shown in Fig. 3F. After 24 h SS, there was a modest yet increase in the proportion of cells in early ($3.2\% \pm 0.3$) and late apoptosis ($1.4\% \pm 0.6$) compared to the control group ($1.1\% \pm 0.5$ and $0.3\% \pm 0.1$, respectively). Despite these changes, overall cell viability remained high ($95.3\% \pm 0.6$), with no significant difference from the control ($98.6\% \pm 0.5$).

As exposure time progressed to 48 and 72 h, cell viability decreased, reaching $90.6\% \pm 1.9$ and $88.1\% \pm 2.1$, respectively. Simultaneously, there was a marked increase in early apoptotic cells ($5.8\% \pm 1.0$ and $7.1\% \pm 1.3$) and late apoptotic cells ($3.6\% \pm 1.4$ and $4.4\% \pm 0.9$), indicating a time-dependent progression of cellular impairment. The highest level of cellular damage was observed after 96 h SS, with a sharp decline in viability ($81.7\% \pm 2.9$), accompanied by significant increases in early apoptosis ($8.5\% \pm 1.8$), late apoptosis ($6.7\% \pm 0.6$), and necrosis ($3.1\% \pm 1.7$). These findings suggest that although SS can effectively induce cycle synchronization, prolonged application may severely compromise cellular integrity, culminating in cell death.

Effect of cell cycle synchronization methods on intracellular ROS levels

The results of ROS quantification following the different synchronization methods are presented in Fig. 4. In RSV (Fig. 4A-D''), regardless of concentration (7.5, 10, or 15 μ M) and exposure time (6, 12, or 24 h), the relative fluorescence levels were consistently higher compared to the control group, with average intensities ranging from 1.26 ± 0.40 to 1.57 ± 0.71 AFU (Fig. 4E).

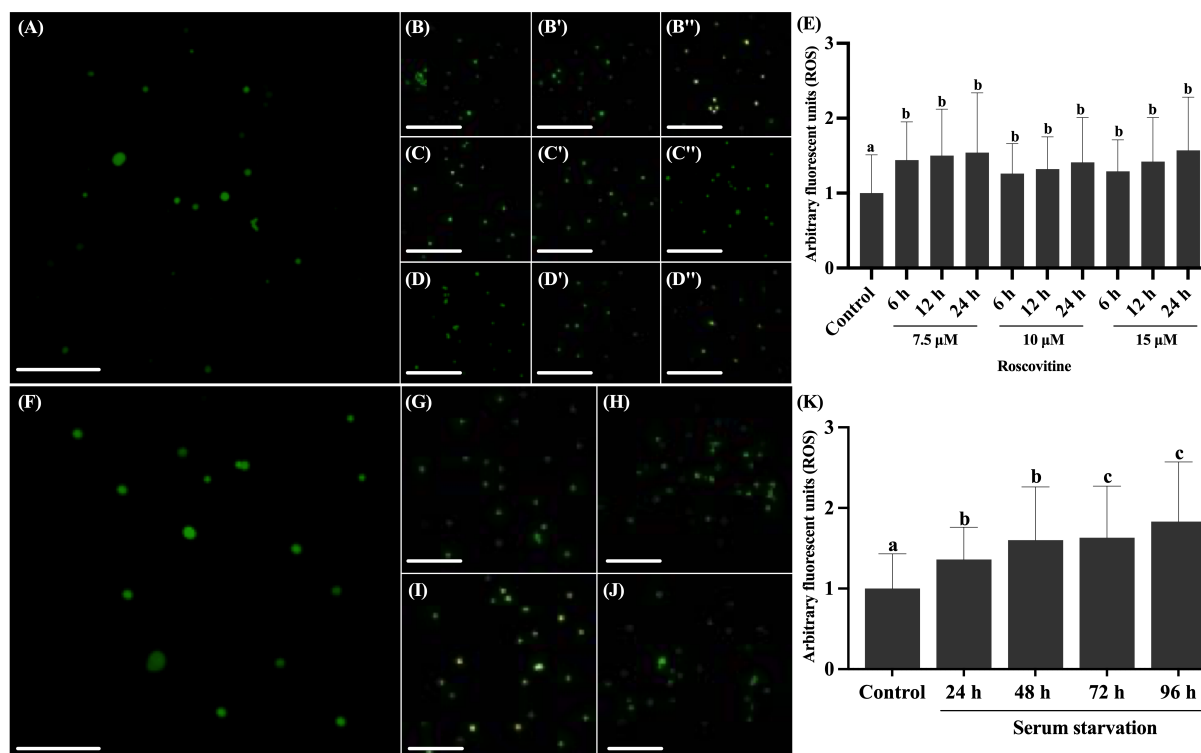


Fig. 4. Quantification of intracellular ROS levels in Spix's yellow-toothed cavy fibroblasts after cell cycle synchronization with RSV and SS. (A) RSV control (no treatment). (B–B'') Cells treated with 7.5 μ M RSV for 6 h (B), 12 h (B'), and 24 h (B''). (C–C'') Cells treated with 10 μ M RSV for 6 h (C), 12 h (C'), and 24 h (C''). (D–D'') Cells treated with 15 μ M RSV for 6 h (D), 12 h (D'), and 24 h (D''). (E) ROS levels after RSV treatments. (F) SS control (no treatment). (G–J) Cells subjected to SS for 24 h (G), 48 h (H), 72 h (I), and 96 h (J). (K) ROS levels after SS. Scale bar = 100 μ m; magnification = 20 $\times$. Mean $\pm$ standard error. ^{a,b}: $P < 0.05$.

In contrast, the results obtained from the SS revealed a progressive increase in ROS levels as the duration of SS was extended (Fig. 4F-J). A significant rise in relative fluorescence was observed during the first 48 h of culture, reaching 1.60 ± 0.66 AFU compared to the control group (1.00 ± 0.43 AFU) (Fig. 4K). This increase became even more pronounced after 72 h SS, with ROS levels peaking at 1.83 ± 0.74 AFU (Fig. 4K). These findings indicate that nutrient restriction, particularly the reduction of FBS, compromises cellular redox homeostasis and promotes the accumulation of ROS.

Effect of synchronization methods on the G₀/G₁, S, and G₂/M phases

The effects of different cell cycle synchronization strategies on the distribution of cell cycle phases were observed in Fig. 5A and Fig. 6A. The analysis revealed that both RSV and SS were effective in inducing a significant accumulation of cells in the G₀/G₁, indicating successful cell cycle arrest. Under RSV, regardless of the concentration used (7.5, 10, or 15 μ M), there was an increase in the percentage of cells in the G₀/G₁ compared to the control group ($86.3\% \pm 0.7$). The best synchronization results were observed after 12 h, with values exceeding 89%, reaching a peak of $91.6\% \pm 1.2$ at 15 μ M (Fig. 5B). Exposure to 7.5 μ M and 10 μ M for 12 h resulted in $89.9\% \pm 1.7$ and $90.0\% \pm 0.8$ of cells in G₀/G₁, respectively.

However, after 24 h of treatment, a reduction in the proportion of cells in the G₀/G₁ phase was observed at all concentrations tested, suggesting a partial release from the arrest or the onset of transition to subsequent cycle phases (Fig. 5B). At 7.5 μ M, this proportion dropped to $83.0\% \pm 2.1$; at 10 μ M, to $83.7\% \pm 2.4$; and at 15 μ M, to $82.2\% \pm 1.0$, all statistically comparable to the control. Regarding the S and G₂/M phases, no differences were detected across most experimental conditions (Fig. 5B). Nevertheless, after 24 h of exposure to 7.5 μ M, a modest increase in cells in the G₂/M phase ($6.5\% \pm 1.2$) was observed compared to 12 h ($2.3\% \pm 0.2$), suggesting potential cell cycle progression. A similar trend was noted at 15 μ M, with an

increase in S phase cells after 24 h ($6.1\% \pm 0.7$) compared to 12 h ($3.1\% \pm 0.5$), although without significant compromise to synchronization efficiency (Fig. 5B).

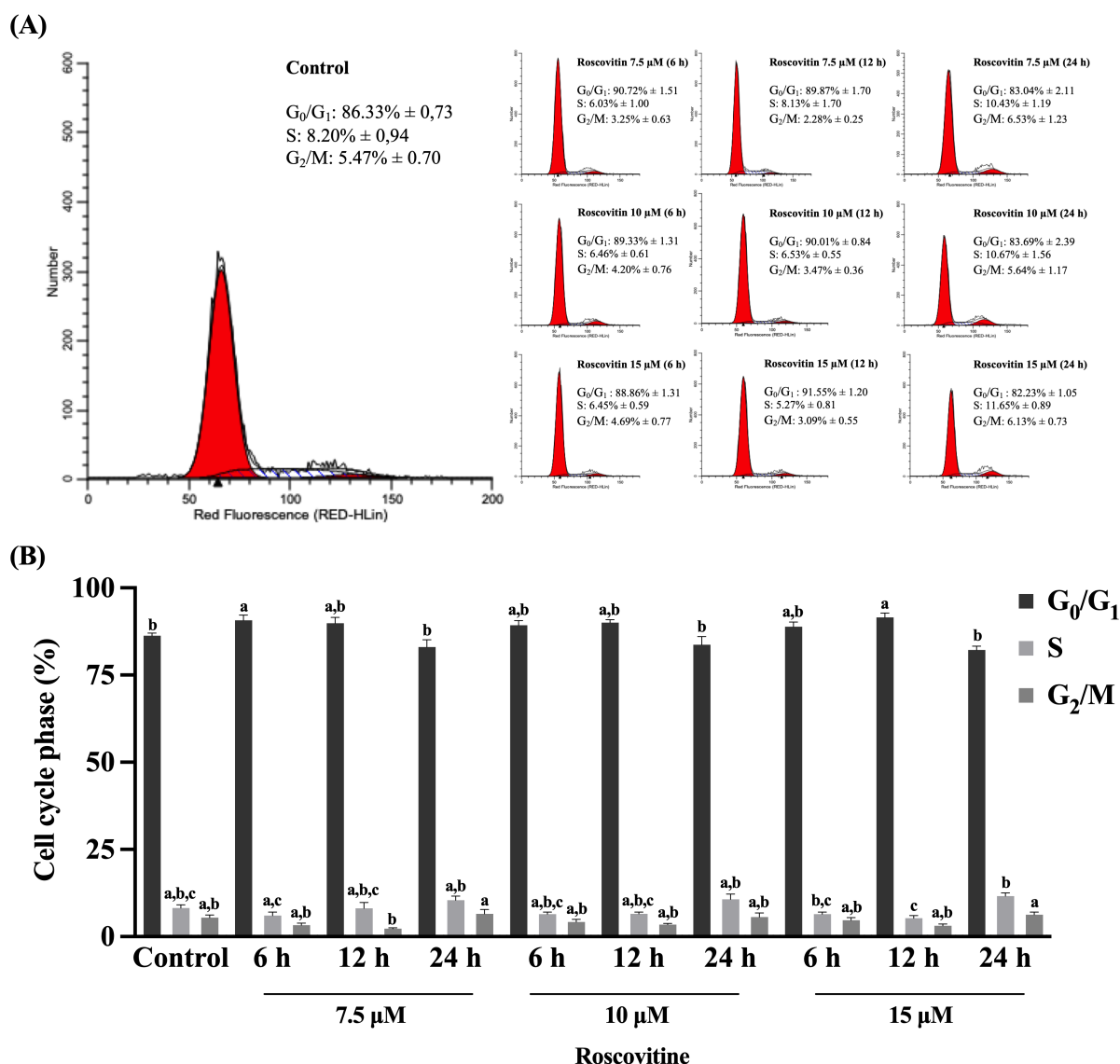


Fig. 5. Cell cycle analysis of Spix's yellow-toothed cavy fibroblast lines before and after treatment with different concentrations and exposure times of RSV. **(A)** Representative histograms of cell cycle stages assessed by flow cytometry. **(B)** Quantitative graph showing the percentage of cells in G_0/G_1 , S, and G_2/M phases. mean $\pm$ standard error. ^{a,b}: Values with different superscript letters within columns significantly differ ($P < 0.05$).

The SS protocol also promoted effective arrest in G_0/G_1 , with cell percentages above 88% at all evaluated time points (24, 48, 72, and 96 h), reaching a maximum of $90.3\% \pm 1.1$ (Fig. 6B). A progressive reduction in the S phase population falling below 6% further supports the efficiency of G_1 -phase arrest. No differences were found in G_2/M phase proportions, indicating that this protocol predominantly induced a cell cycle block at G_0/G_1 without compensatory accumulation in other phases. Collectively, these data indicate that both RSV and SS were effective in synchronizing fibroblast lines in the G_0/G_1 phase. However, RSV showed superior performance over shorter durations (especially at 12 h), while SS produced stable and sustained results up to 96 h. Therefore, the choice between methods may depend on the desired synchronization duration and the sensitivity of the cells to culture conditions.

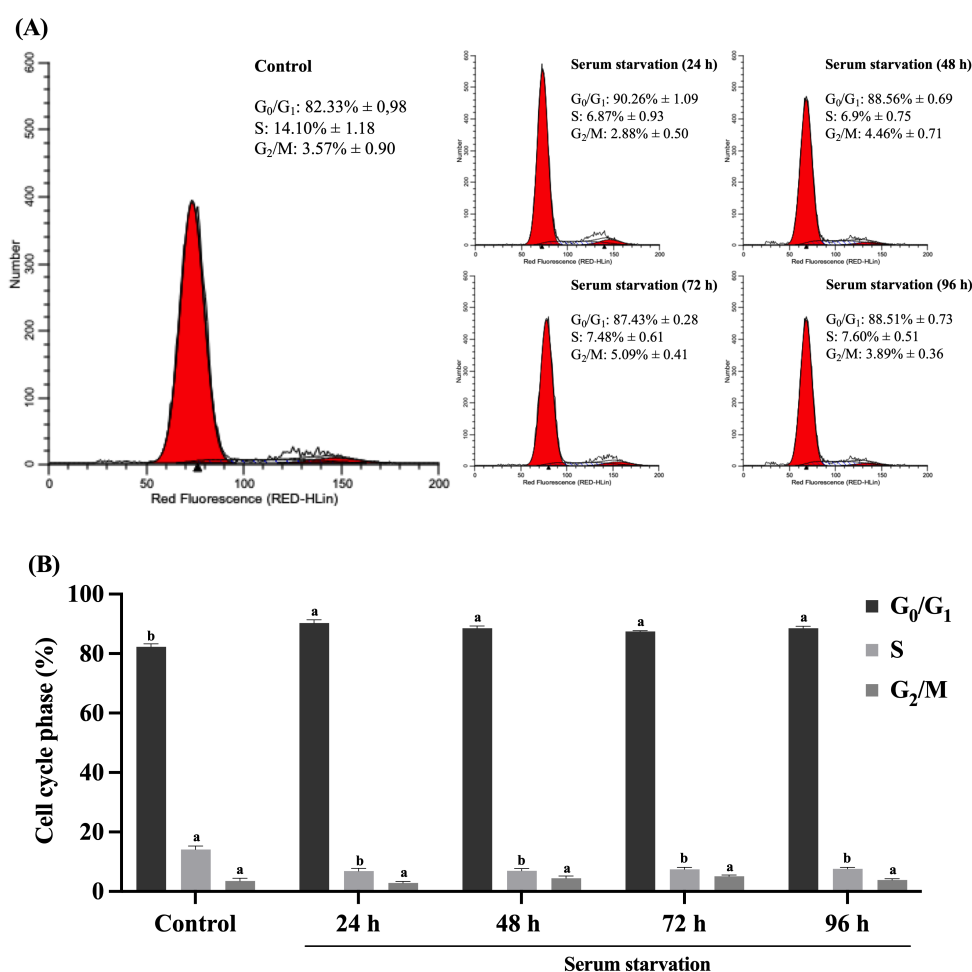


Fig. 6. Cell cycle analysis of Spix's yellow-toothed cavy fibroblast lines before and after treatment with different SS exposure times. **(A)** Representative histograms of cell cycle stages assessed by flow cytometry. **(B)** Quantitative graph showing the percentage of cells in G₀/G₁, S, and G₂/M phases. Mean $\pm$ standard error. ^{a,b}: Values with different superscript letters within columns significantly differ ($P < 0.05$).

Discussion

In this study, we evaluated different cell cycle synchronization strategies to identify the most effective approach for maintaining *G. spixii* fibroblasts in the G₀/G₁ phase. RSV efficiently promoted G₀/G₁ accumulation across all tested concentrations, with a 12 h exposure yielding optimal results, consistent with its selective inhibition of CDKs 1, 2, and 5 and its transient, reversible arrest of the cell cycle (Chen et al., 2025). Under these conditions, fibroblasts preserved normal morphology, including well-defined cytoplasmic extensions, monolayer organization, and centrally positioned nuclei, comparable to control, while cell viability remained above 85%. In contrast, prolonged exposure and higher RSV concentrations (15 μ M) increased apoptosis and ROS production, indicating oxidative stress associated with extended inhibitor exposure, as reported in human fibroblasts and tumor cell lines (Wesierska-Gadek et al., 2008).

SS treatment was also effective in inducing G₀/G₁ cell cycle arrest, with more than 88% of cells in this phase across all analyzed time points. However, SS durations beyond 48 h induced pronounced adverse cellular effects, including reduced viability, increased apoptosis, elevated ROS levels, and progressive morphological alterations detectable as early as 24 h. These changes comprised cytoplasmic retraction, loss of cell adhesion, monolayer disorganization, and increased nuclear density. The cellular damage observed after 48 h of SS was associated with sustained ROS overproduction, leading to mitochondrial and cytoplasmic

alterations that precede cell death. This condition promotes functional uncoupling between the nucleus, mitochondria, and cytoplasm, compromising morphological integrity prior to measurable viability loss and reinforcing morphology as an early and sensitive indicator of stress (Zeidler et al., 2017). Accordingly, our findings highlight the relevance of morphological assessment as a complementary criterion for evaluating the safety and applicability of synchronization protocols, particularly in species for which these approaches are still being established.

Analysis of apoptotic responses revealed that RSV induced concentration and time-dependent effects on fibroblast cell lines. Reductions in cell viability were evident after 24 h of exposure to 7.5 μ M and 10 μ M, whereas at 15 μ M, viability declined as early as 12 h, suggesting a cytotoxic response. This profile is consistent with findings in fibroblasts of *Panthera temminckii* and *P. pardus*, in which higher RSV concentrations (>15 μ M) resulted in reduced viability and increased apoptosis (Wittayarat et al., 2012). These effects are likely related to RSV's mechanism of action, which includes CDK inhibition, p53 stabilization, and suppression of the NF- κ B pathway, culminating in activation of mitochondrial apoptotic pathways mediated by proteins such as p53AIP3 (Liu et al., 2012). In our model, this process was accompanied by increased ROS, indicating that oxidative stress acted as a cofactor in the induction of cell death.

In contrast, SS elicited a biphasic apoptotic response. During the first 24 h, increases in early and late apoptosis were observed, while overall cell viability remained comparable to control, suggesting a transient adaptive response to trophic factor deprivation. However, this adaptation was not sustained, as prolonged SS (48–72 h) led to marked reductions in viability and progressive increases in apoptotic and necrotic cells. By 96 h, these effects were exacerbated, with a further rise in early and late apoptosis and necrosis. These findings corroborate with Zeidler et al. (2017), who reported that nutrient depletion leads to disorganization of the mitochondrial respiratory chain, forcing cells to activate compensatory

metabolic pathways. Although initially protective, these mechanisms become progressively ineffective in cell lines with limited metabolic flexibility. This pattern appears to apply to *G. spixii* fibroblasts, rodent characterized by a high basal metabolic rate and reduced tolerance to stress (Rowe and Honeycutt, 2002).

This hypothesis is supported by Seluanov et al. (2010), who indicate that cells derived from small rodents tend to exhibit greater susceptibility in culture, with increased sensitivity to redox instability and reduced regenerative capacity under unfavorable conditions, as observed in our study through the analysis of ROS levels. Rowe and Honeycutt (2002) emphasize that rodents with lower body mass possess intensified metabolism and higher tissue turnover rates, implying cells with elevated trophic demands and heightened susceptibility to bioenergetic instability. Under SS, cells rapidly exhaust their internal resources, leading to mitochondrial overload and apoptosis, necrosis, or premature senescence.

Regarding the cell cycle analyses, our study showed that both methodologies evaluated were effective in inducing G₀/G₁ phase arrest in *G. spixii* fibroblasts. However, the stability of this synchronization and its physiological impacts on the cells varied depending on the method and parameters used. RSV demonstrated particularly efficient performance at concentrations of 7.5 µM and 10 µM, promoting the accumulation of 80% to 90% of cells in the G₀/G₁ phase after 12 h of exposure. This dynamic response profile was similar to the data reported by Praxedes et al. (2024), who used synchronization methods in fibroblasts of *D. leporina*. In that model, a similarly high proportion (86% of cells in G₀/G₁) was observed after 12 h of exposure to RSV, followed by a progressive decline in efficiency after 24 h. This finding reinforces the hypothesis that small-bodied wild rodents may share physiological and metabolic traits that limit the tolerance window to RSV, even at moderate concentrations, requiring synchronization protocols with optimized exposure times to prevent adverse responses.

This study also evaluated the effects of the highest concentration of RSV (15 μ M) on fibroblasts. This dose promoted the highest synchronization rate observed, with 91% of cells in the G₀/G₁ phase after 12 h of exposure. However, this peak in efficiency was accompanied by a significant physiological cost: cell viability assays indicated a marked reduction in the number of viable cells from this same period onward, along with an increase in early apoptosis levels. These findings suggest that, although higher concentrations of RSV enhance the effectiveness of cell cycle arrest induction, they exceed the physiological tolerance threshold of *G. spixii* fibroblasts, compromising mitochondrial integrity and activating apoptotic pathways. This dissociation between technical efficiency and biological safety is consistent with the findings of Wittayarat et al. (2013), who reported viability loss in fibroblasts of wild felids exposed to high doses of RSV (>15 μ M). According to the authors, the compound's action ultimately leads to the activation of apoptotic mechanisms.

In contrast to RSV, SS efficiently induced G₀/G₁ cycle arrest within the first 24 h, indicating effective blockade in response to SS. However, this response became structurally and physiologically unstable after 48 h of exposure, with progressive cellular alterations observed as treatment duration increased. These findings suggest that, although initially effective, SS exerts its effects indirectly by modifying the culture microenvironment. The deprivation of trophic components such as IGF, EGF, lipids, and calcium-binding proteins disrupts signaling pathways required for mitochondrial integrity and oxidative metabolism, leading to increased oxidative stress (Chen et al., 2025).

From this perspective, fibroblasts derived from small rodents, such as Spix's yellow-toothed cavy, whose basal metabolic rate is naturally elevated (Rowe and Honeycutt, 2002; Seluanov et al., 2010), may require a continuous supply of nutrients and growth factors to maintain functional integrity, making them more susceptible to morphological and physiological alterations under prolonged SS. Therefore, the morphophysiological

heterogeneity of species should be considered when employing synchronization protocols. Although the method demonstrated an initial ability to induce synchronization, its application beyond 48 h proved detrimental to the maintenance of cell viability and mitotic stability.

Conclusion

Both RSV and SS effectively induce G₀/G₁ cell cycle arrest in *G. spixii* fibroblasts, but with markedly different impacts on cellular integrity. RSV, particularly at 7.5 μ M and 10 μ M for 12 h, emerged as the safest and most efficient short-term protocol, achieving high synchronization rates with minimal effects on cell viability, whereas exposure to 15 μ M caused increased deleterious effects. In contrast, SS was effective only up to 24 h, as prolonged deprivation severely compromised cell morphology, increased oxidative stress, and activated programmed cell death pathways. These findings support the development of species-specific synchronization protocols for small wild mammals and underscore the importance of accounting for physiological, bioenergetic, and adaptive traits when applying biotechnological approaches.

Acknowledgments

The authors thank the Center for Wild Animals Multiplication (CEMAS/UFERSA) for providing biological samples. This study was partially financed by the CAPES (no. 001) and CNPq (no. 309078/2021-0).

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CAPÍTULO 5 – UNDERSTANDING OOCYTE COMPETENCE TO IMPROVE *IN VITRO* EMBRYO PRODUCTION IN CAVIIDAE RODENTS

ARTIGO DE REVISÃO

ARTIGO SUBMETIDO NO PERIÓDICO INTERNACIONAL ZYGOTE

Qualis 2017-2020: B3 (Medicina Veterinária)

Fator de Impacto: 1.4

Data da Submissão: 11 de Janeiro de 2026

Understanding oocyte competence to improve *in vitro* embryo production in Caviidae rodents

Running headline: Oocyte competence in Caviidae rodents

Leonardo Vitorino Costa de Aquino¹ and Alexsandra Fernandes Pereira^{1*}

¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil.

* All correspondence to: Alexsandra Fernandes Pereira

Laboratory of Animal Biotechnology

Federal Rural University of Semi-Arid

Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil

Phone: +55 84 3317 8361

E-mail address: alexsandra.pereira@ufersa.edu.br

Abstract

In vitro embryo production has emerged as a valuable tool for both reproductive research and conservation programs in non-traditional animal models, including Caviidae rodents. However, in this group, the efficiency of these protocols remains limited, largely due to low oocyte maturation and developmental competence. A comprehensive understanding of oocyte competence is therefore essential for the optimization of *in vitro* embryo production systems in these species. Oocyte competence is a multifactorial trait established during folliculogenesis and regulated by complex interactions between intrinsic oocyte factors and the surrounding somatic cells, as well as endocrine, paracrine, and environmental signals. In this review, we summarize the biological mechanisms governing follicular development and oocyte maturation in Caviidae rodents, highlighting their implications for oocyte quality and developmental potential. We further discuss current and emerging technologies used to assess oocyte competence, including morphological, metabolic, and cellular approaches. In addition, we examine strategies and biotechnological interventions aimed at enhancing oocyte competence and improving maturation outcomes, such as culture system optimization, supplementation with bioactive factors, and modulation of follicular and oocyte signaling pathways. By integrating biological regulation with technological advances, this review provides a comprehensive study for understanding oocyte competence in Caviidae rodents and underscores its relevance for improving *in vitro* embryo production efficiency in these species.

Keywords: Cavids, Wild and non-traditional rodent models, Oocyte developmental potential, Oocyte maturation, Oocyte quality assessment.

Introduction

The Caviidae family comprises a diverse group of South American rodents, encompassing three subfamilies subdivided into six genera (Table 1). Owing to their distinctive genetic characteristics, remarkable ecological adaptability, and evolutionary success across a wide range of habitats, caviids have become valuable biological models for scientific research (Lall *et al.*, 2022; Silva *et al.*, 2024). In addition, these species play an important ecological role in maintaining the balance of fauna and flora within their native environments (Silva *et al.*, 2024). Collectively, these attributes, combined with reproductive particularities, confer upon rodents of the family Caviidae a particular suitability for investigations in physiology, cell biology, and the development of assisted reproductive techniques (ARTs, Silva *et al.*, 2024; Oliveira and Pereira, 2025).

Within this context, *in vitro* embryo production (IVEP) has emerged as an increasingly relevant tool for reproductive research and conservation-oriented programs in cavid species (Santos *et al.*, 2025). IVEP offers powerful opportunities for reproductive management, particularly for non-traditional animal models and species of conservation concern (Oliveira *et al.*, 2025a). Furthermore, it provides a robust experimental platform for the development, application, and optimization of advanced reproductive technologies involving female gamete manipulation, thereby expanding the translational potential of ARTs in these rodents (Praxedes *et al.*, 2023; Aquino *et al.*, 2025). A summary of the main reproductive techniques employed in different caviid species is synthesized in Fig. 1.

Despite its promise, the application of IVEP in Caviidae rodents remains limited by considerable interspecific variation in body weight, size, nutritional requirements, metabolic rate, gestation length, and litter size (Rowe and Honeycutt, 2002). Such biological diversity poses significant challenges to the standardization and optimization of IVEP protocols across species. Although caviids share close phylogenetic relationships, species-specific differences in reproductive biology, particularly those related to the nutritional, metabolic, and endocrine demands required for the oocyte to intrinsically acquire meiotic competence, play a decisive role in determining IVEP outcomes (Oliveira and Pereira, 2025). These processes are governed by complex and tightly regulated interactions among multiple intrinsic and extrinsic factors (Gilchrist and Thompson, 2007), many of which remain incompletely understood, ultimately contributing to the reduced efficiency of IVEP in these species.

Given these limitations, increasing attention has been directed toward studies of both *in vivo* and *in vitro* folliculogenesis as key strategies for improving IVEP efficiency in Caviidae

rodents (Orozco-Galindo *et al.*, 2025). A comprehensive understanding of follicular development offers several advantages, most notably the ability to identify and characterize the biochemical and molecular status of the oocyte, an essential determinant of oocyte quality and developmental competence from maturation through the blastocyst stage (Chandra and Sharma, 2020). During the acquisition of oocyte competence, highly regulated processes, such as the accumulation and storage of maternal RNAs, ribosomes, proteins, and cytoplasmic organelles, are required to support nuclear and cytoplasmic maturation (Mermillod and Marchal, 1999), thereby ensuring the oocyte's capacity to sustain early embryonic development.

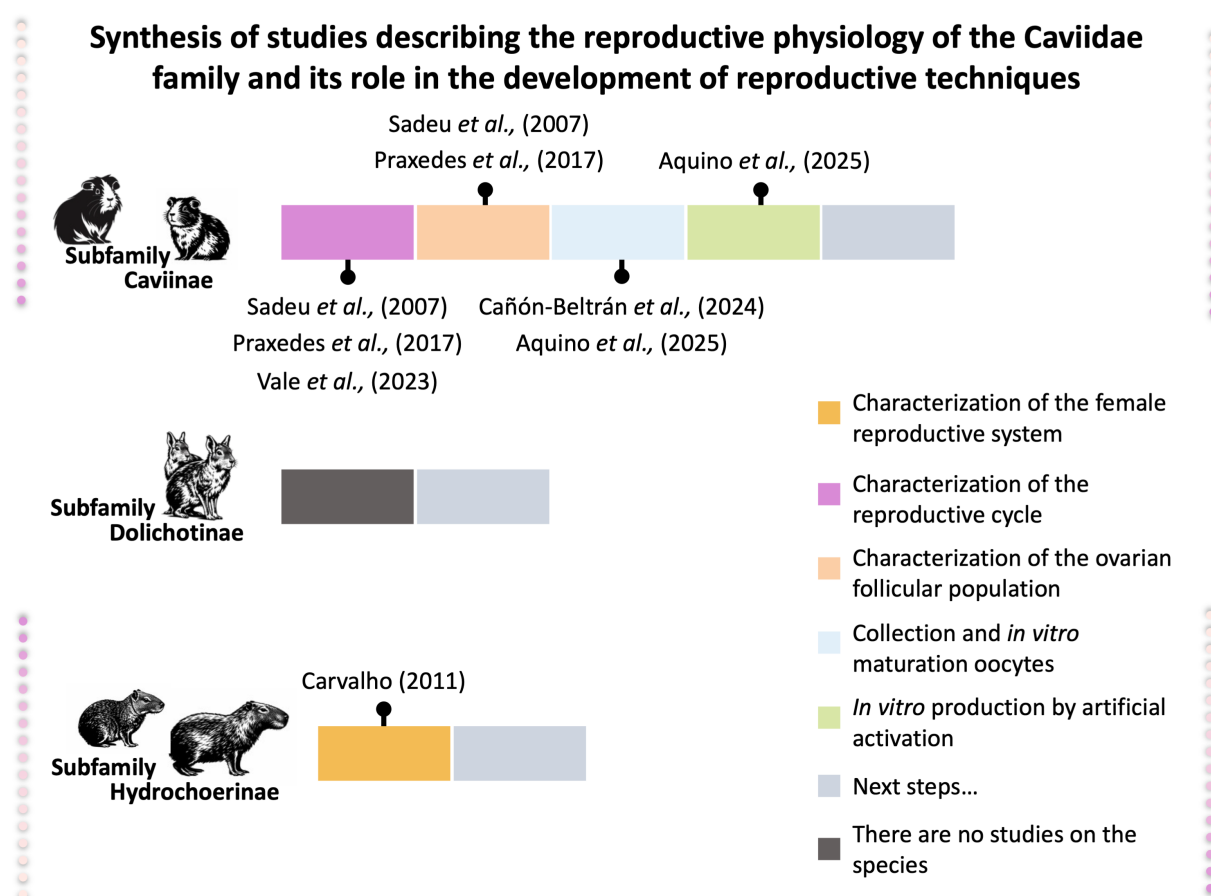


Fig. 1. A representative overview of the main studies developed on the reproductive physiology of rodents belonging to the Caviidae family, focusing on understanding reproductive processes and developing reproductive techniques.

In caviids, *in vivo* folliculogenesis was first described in the guinea pig (*C. porcellus*; Sadeu *et al.*, 2007), providing foundational insights into ovarian dynamics in this family. Subsequent studies extended this knowledge to other cavid species, including the Spix's yellow-toothed cavy (*G. spixii*; Praxedes *et al.*, 2017), through the characterization of ovarian follicle

populations and developmental dynamics. Although these investigations have contributed essential baseline information and supported the initial establishment of *in vitro* culture systems for cavid oocytes and embryos, oocyte maturation and embryo production rates under *in vitro* conditions generally do not exceed 50%. These outcomes remain substantially lower than those reported for rats and mice, species within the same phylogenetic order, which display higher efficiencies in embryo production following artificial activation (Ma *et al.*, 2005), *in vitro* fertilization (González *et al.*, 2025), somatic cell nuclear transfer (Mizutani *et al.*, 2016), and the generation of transgenic models (Kasatskin *et al.*, 2022).

In light of the persistent limitations in IVEP efficiency observed in cavid species, alongside the evident gaps in knowledge regarding the mechanisms underlying their reproductive physiology, this review aimed to systematize and critically integrate existing studies and recent scientific advances related to the regulatory factors and biological processes governing the acquisition of oocyte competence. Particular emphasis is placed on mechanisms operating during folliculogenesis and oocyte maturation, as well as on emerging methodologies for assessing and enhancing oocyte quality and developmental potential. By consolidating biological insights with technological advances, this review underscores the central role of oocyte competence in improving IVEP protocols and advancing their application across rodent species of the Caviidae family.

Regulation and factors involved in *in vivo* folliculogenesis in cavids

In rats and mice, meiotic competence is progressively acquired during *in vivo* oocyte growth and depends on the coordinated accumulation of molecular regulators that sustain early embryogenesis (Zeide *et al.*, 2022a). In cavid species, the comparatively lower efficiency of *in vitro* oocyte development likely reflects, at least in part, an incomplete acquisition of key translational and post-translational regulatory layers that normally mature within the follicular niche (Mohammed and Alwaid, 2022). Thus, elucidating the *in vivo* determinants that regulate oocyte-somatic cell communication, and the follicular microenvironment is central to improving assisted reproductive outcomes, including IVEP, in cavids. This notion is supported by evidence linking oocyte and follicular gene-expression signatures to fertilization success, embryonic development, and offspring production following embryo transfer (Zeide *et al.*, 2022a).

Folliculogenesis in cavids is shaped by species-specific reproductive traits and by general biological drivers that directly influence the probability that an oocyte collected from

antral follicles will express full meiotic and developmental competence under *in vitro* conditions (Ozawa *et al.*, 2001; Samaniego *et al.*, 2025).

Table 1. List of species belonging to the Caviidae family.

Species ^{a,b,d}	Conservation status ^e	Population local ^{d,e}	Fur color ^d	Number of offspring/pregnancies ^{b,c}
Subfamily Caviinae (Genus <i>Cavia</i> , <i>Galea</i> and <i>Microcavia</i>)				
<i>C. aperea</i>	Least Concern	Argentina, Brazil, Bolivia, Colombia, and Venezuela	Grayish	1-5
<i>C. fulgida</i>	Least Concern	Brazil	Grayish and dark brown	1-4
<i>C. intermedia</i>	Largely Depleted	Brazil	Grayish and yellowish	1-3
<i>C. magna</i>	Least Concern	Brazil and Uruguay	Light yellow and black	1-3
<i>C. porcellus</i>	Least Concern	Argentina, Brazil, Colombia, and Venezuela	Varied tonality	1-4
<i>C. tschudii</i>	Least Concern	Bolivia, Chile, and Peru	-	-
<i>G. flavidens</i>	Least Concern	Brazil	-	-
<i>G. musteloides</i>	Data Deficient	Bolivia	-	-
<i>G. spixii</i>	Least Concern	Brazil	Yellowish	1-2
<i>G. monasteriensis</i>	Least Concern	Bolivia	-	-
<i>M. australis</i>	Least Concern	Argentina, Chile, and Paguay	Grayish yellow	1-3

<i>M. niata</i>	Least Concern	Bolivia	Olive-gray and smooth	-
<i>M. shiptoni</i>	Least Concern	Argentina	-	-
Subfamily Dolichotinae (Genus <i>Dolichotis</i>)				
<i>D. patagonum</i>	Near Threatened	Argentina and Chile	Rizzly gray and patch	1-3
<i>D. salinicola</i>	Least Concern	Argentina, Bolivia and Paaguay	Dark gray to white	1-3
Subfamily Hydrochoerinae (Genus <i>Hydrochoerus</i> and <i>Kerodon</i>)				
<i>H. hydrochaeris</i>	Least Concern	South American		
<i>H. isthmus</i>	Data Deficient	Colombia, Panama and Venezuela	Brown	1-3
<i>K. acrobata</i>	Data Deficient	Brazil	Reddish	1-3
<i>K. rupestris</i>	Least Concern	Brazil	Gayish and yellow	1-3

^{a,b,c}Subscripts indicate references: ^aRowe and Honeycutt, (2002); ^bSadeu *et al.*, (2007); ^cVale *et al.*, (2023). ^dThe IUCN Red List of Threatened Species (2023); ^eLacher (1981)

These drivers include estrous-cycle length, follicular dynamics (including wave patterns), follicle and oocyte size, and the timing/quality of *cumulus*-oocyte interactions (Gatica *et al.*, 2019). Additional determinants encompass the capacity for energy-reserve accumulation, and the synthesis and storage of maturation regulators, such mRNAs, ribosomes, proteins, and organelles, required for nuclear and cytoplasmic maturation (Saadeldin *et al.*, 2025; Hirao, 2025). Nutritional status and metabolic supply (amino acids, lipids, carbohydrates), endocrine signaling, and locally acting growth factors further modulate the oocyte's trajectory toward meiotic competence, particularly in species with distinct metabolic rates and reproductive pacing, as emphasized in the broader framing of your manuscript regarding interspecific biological diversity and its consequences for protocol standardization in Caviidae (Rowe and Honeycutt, 2002; Gilchrist and Thompson, 2007).

Across the estrous cycle, oocytes of Caviidae rodents progressively acquire developmental competence during folliculogenesis. In these species, follicular growth extends for approximately 15–20 days and largely overlaps with the estrogenic phase (Adrian and Sachser, 2011). Within Caviinae (e.g., *Cavia*, *Microcavia*, *Galea*), folliculogenesis commonly occurs in two successive waves, favoring the gradual intracellular accumulation of essential substrates and enabling oocytes to reach maximal developmental size near ~17 days after estrus (Sadeu *et al.*, 2007; Praxedes *et al.*, 2017; Vale *et al.*, 2023). This “oocyte capacitation” (*sensu in vivo*) reflects the progressive assembly of the molecular and cytoplasmic machinery needed for meiotic maturation, fertilization, and early embryonic development (Chandra and Sharma, 2020). However, when oocytes are abruptly removed from the follicle, their native metabolic homeostasis and intercellular signaling context are disrupted with an effect that can be exacerbated when collections originate from heterogeneous follicular populations that include oocytes still completing capacitation. In this way, incomplete establishment of the molecular framework required to sustain embryogenesis becomes a key constraint on developmental competence in caviids (Gilchrist and Thompson, 2007).

Follicle and oocyte diameter are robust phenotypic proxies of competence because they integrate the history of somatic support, nutrient availability, and temporal exposure to endocrine/paracrine cues (Johnston *et al.*, 2025). In caviids, follicular and oocyte dimensions increase progressively during follicular growth, reflecting coordinated acquisition of meiotic and developmental competence. Classical comparisons indicate larger follicular and oocyte diameters in guinea pigs (*C. porcellus*) relative to *G. spixii*, underscoring interspecific variability within Caviidae (Sadeu *et al.*, 2007; Praxedes *et al.*, 2017). More recently, species-specific analyses in *G. spixii* have reinforced that oocyte morphometrics align with nuclear

maturation, cytoplasmic organization, and developmental potential, supporting follicle size and oocyte diameter as functional indicators of competence in this model (Aquino *et al.*, 2025). Interspecific differences likely reflect distinct oogenetic programs, including timing of nucleolar compaction and chromatin remodeling (Weir, 1974). Importantly, such size-competence relationships are conserved across mammals, as similar positive associations have been reported in other models (*D. leporina*, *Pecari tajacu*), supporting follicular expansion as a prerequisite for competence acquisition (Borges *et al.*, 2020; Praxedes *et al.*, 2023).

Mechanistically, competence acquisition depends on the intensity and integrity of oocyte-granulosa/*cumulus* communication. *In vivo*, this communication is sustained by intimate soma-germline contacts that enable bidirectional exchange of ions, metabolites, and regulatory signals (Richani *et al.*, 2024). The centrality of oocyte-derived paracrine control over the somatic compartment is well established: mammalian oocytes produce multiple growth factors during folliculogenesis, and GDF-9 is described as obligatory for follicular growth beyond the primary stage, with a model in which secreted GDF-9 orchestrates key physiological responses in granulosa and theca compartments (Erickson and Shimasaki, 2000; Richani *et al.*, 2024). This oocyte-centric signaling framework provides a conceptual basis for interpreting why competence can be compromised when oocytes are removed prematurely or when somatic support is disrupted during collection and handling (Erickson and Shimasaki, 2000).

A critical, and often underappreciated, layer of competence relates to the spatial organization of the oocyte cortex, an emergent structural/functional domain that integrates signals from *cumulus* contact and coordinates maturation events. Components of the oocyte cortical domain (e.g., transzonal projections, cortical actin networks, ERM proteins, Fyn kinases, acto-myosin mechanics) are functionally linked to organelle distribution, spindle alignment/positioning, cortical granule maintenance, and polar body extrusion. Disruptions in these cortical components are associated with defective spindle alignment and meiotic arrest, chromosome segregation errors, and broader developmental failure (Coticchio *et al.*, 2025). The emerging role of the oocyte cortical domain in maturation. Although these mechanisms are not yet comprehensively mapped in caviids, they are directly relevant for IVEP because *in vitro* collection necessarily alters *cumulus* contact patterns and may destabilize cortex-dependent maturation dynamics (Coticchio *et al.*, 2025).

Consistent with this, oocyte competence is strongly influenced by *cumulus*-oocyte interactions within the follicular environment (Sutton and Gilchrist, 2003). In contrast, *in vitro* systems require removal of the *cumulus*-oocyte complex (COC) from antral follicles, interrupting soma-germline coupling and potentially precipitating premature meiotic

resumption (Mohammed *et al.*, 2024). In mice, premature nuclear maturation can compromise subsequent embryo development (Eppig and Downs, 1987), and analogous constraints are plausible in cavid species given the dependence on extended *in vivo* capacitation windows.

At the endocrine level, folliculogenesis and meiotic resumption are regulated by gonadotropin-driven cascades acting primarily through granulosa cells (Zeide *et al.*, 2022b). In guinea pigs, inhibin emerges as a major regulator of follicular development via control of FSH secretion: passive immunization against inhibin increases plasma FSH and increases the number of small follicles (100–200 μm), supporting a mechanistic link between inhibin tone, gonadotropin dynamics, and follicle recruitment/growth in this cavid model (Ozawa *et al.*, 2001). These findings are particularly informative for cavid species because they (i) connect a defined endocrine regulator to measurable follicular outcomes and (ii) help explain why species-specific endocrine demands can shape competence acquisition trajectories.

When meiotic resumption occurs prematurely *in vitro* (“spontaneous maturation”), competence can be impaired through loss of essential metabolites and regulatory support normally delivered by *cumulus* cells (Chandra and Sharma, 2020). Accordingly, prematuration strategies, such as transient meiotic inhibition after COC recovery have been proposed to prolong oocyte-*cumulus* communication, support continued accumulation/maintenance of key intracellular components (mRNAs, proteins), and improve competence (Gilchrist and Thompson, 2007; Saadeldin *et al.*, 2025). Although such strategies remain underexplored in cavid species, their rationale is strengthened by the broader mechanistic literature linking intact soma-germline coupling and cortical organization to successful maturation and developmental competence (Coticchio *et al.*, 2025).

Finally, advancing reproductive tools to identify and evaluate meiotic competence is an essential next step for cavid species. This includes (i) deeper mapping of oocyte-derived growth-factor pathways that coordinate follicle development (e.g., GDF-9–centered networks) (Erickson and Shimasaki, 2000). The role of oocyte in (ii) endocrine profiling and manipulation frameworks grounded in cavid biology (e.g., inhibin–FSH coupling in *C. porcellus*) (Ozawa *et al.*, 2001) and (iii) integration of structural competence markers that reflect cytoplasmic/cortical readiness for fertilization and embryo development (Coticchio *et al.*, 2025). By anchoring IVEP optimization in the *in vivo* biology of folliculogenesis rather than in generalized rodent protocols this section strengthens the logic that oocyte competence is the primary bottleneck and the most actionable target for improving IVEP performance in Caviidae (Gilchrist and Thompson, 2007; Orozco-Galindo *et al.*, 2025).

Technologies for assessing oocyte developmental competence in Cavid

The acquisition of oocyte developmental competence in cavid results from a progressive and highly integrated process established during *in vivo* folliculogenesis through dynamic interactions among the oocyte, *cumulus* and granulosa cells, endocrine signals, and local factors within the follicular microenvironment. As discussed in the previous section, subtle alterations in these regulatory mechanisms including oocyte-somatic cell communication within the ovarian follicle, metabolic support, and hormonal control exert a direct impact on oocyte biological quality and its ability to complete meiotic maturation and sustain early embryonic development (Erickson and Shimasaki, 2000; Orozco-Galindo *et al.*, 2025).

Within this context, technologies aimed at assessing oocyte developmental competence play a central role in reproductive research involving cavid, particularly in light of the persistent limitations observed in IVEP efficiency. Unlike well-established domestic animal models, cavid exhibit species-specific patterns of follicular growth, endocrine dynamics, and oocyte maturation, making the careful application of tools capable of accurately identifying the functional status of recovered oocytes indispensable (Lall *et al.*, 2022; Latham, 2025).

Among the most widely employed approaches for this purpose are morphological, functional/metabolic, and molecular assessments, which provide complementary information regarding oocyte developmental stage and competence potential. Morphological characterization of the follicular population and COCs remain a fundamental initial step, as parameters such as follicular and oocyte diameter, *cumulus* integrity and organization, and cytoplasmic homogeneity reflect the history of somatic support and the progression of *in vivo* oocyte capacitation (Samaniego *et al.*, 2025; Mohammed *et al.*, 2024). In cavid, these markers have proven particularly informative considering the previously described interspecific variability in follicular dynamics (Sadeu *et al.*, 2007; Praxedes *et al.*, 2017).

However, the assessment of oocyte competence cannot be limited to structural morphology alone. Growing evidence indicates that functional parameters including nuclear maturation, mitochondrial activity, redox balance, and the maintenance of communication through gap junctions between the oocyte and *cumulus* cells are critical determinants of developmental competence (Richani *et al.*, 2024; Ebrahimi *et al.*, 2025). These functional attributes directly reflect the efficiency of soma-germline metabolic coupling, the integrity of which is established during *in vivo* folliculogenesis and is frequently compromised following oocyte removal from the follicular environment.

In addition, molecular and transcriptomic approaches have gained prominence as refined tools for assessing oocyte competence, enabling the identification of gene-expression

signatures associated with maturation, metabolism, and developmental potential. Although still limited in caviids, these strategies offer promising perspectives for elucidating the mechanisms that constrain the efficiency of *in vitro* reproductive technologies in these species (Mohammed *et al.*, 2024; Latham, 2025).

Finally, the characterization of preantral follicles and the contributions of antral follicle manipulation have emerged as complementary approaches for both assessing and enhancing oocyte competence. Studies across mammalian species demonstrate that the analysis of follicular activity at early developmental stages and the recovery of oocytes from viable antral follicles including under suboptimal or *post-mortem* conditions can substantially expand the understanding of reproductive potential and support conservation strategies and *in vitro* embryo production (Ozawa *et al.*, 2001; Johnston *et al.*, 2025).

Thus, the integration of morphological, functional, and molecular approaches constitutes the conceptual foundation for robust assessment of oocyte developmental competence in caviids, establishing a direct link between the regulatory mechanisms of *in vivo* folliculogenesis and the development of more efficient and biologically appropriate reproductive technologies for this taxonomic group.

Morphological characterization of the follicular population

The development of effective *in vitro* culture and manipulation systems in caviids requires a species-oriented understanding of the morphological and morphometric features of follicular and oocyte growth established *in vivo*. Morphological characterization of the follicular population constitutes a fundamental step in assessing oocyte developmental competence, as it integrates information on follicular dynamics, somatic cell support, and endocrine regulation accumulated throughout folliculogenesis.

In caviids, ovarian follicles at distinct developmental stages primordial, primary, secondary, and antral are consistently observed; however, their relative abundance, spatial distribution, and growth dynamics vary markedly among species (Sadeu *et al.*, 2007; Praxedes *et al.*, 2017). Quantitative analyses have revealed pronounced interspecific differences in follicle number and ovarian organization across species within the order Rodentia, highlighting substantial biological heterogeneity. A comparative summary of these patterns is presented in Table 2. For instance, two genetic groups of *C. porcellus* exhibit approximately 26–58 follicles per ovary throughout the estrous cycle (Fernández *et al.*, 2022), whereas the *G. spixii* displays a considerably larger follicular reserve, estimated at ~416 follicles per ovarian pair (Praxedes *et al.*, 2017). In rats, follicle numbers are even more disparate, ranging from approximately

2.000 to 15.000 follicles per ovarian pair (Dean, 2002; McClellan *et al.*, 2003). Collectively, these contrasts reflect species-specific folliculogenic trajectories, encompassing differences in follicle recruitment, growth duration, and ovulatory strategies.

Accordingly, morphometric parameters such as follicular diameter, oocyte diameter, and the organization of granulosa cell layers have emerged as robust morphological indicators of oocyte competence in cavid. The progressive increase in follicle and oocyte size during follicular growth mirrors the gradual acquisition of meiotic and developmental competence, as it reflects sustained somatic support, nutrient availability, and prolonged exposure to endocrine and paracrine cues (Praxedes *et al.*, 2017; Samaniego *et al.*, 2025). Recent comparative analyses in *C. porcellus* demonstrated strong associations between follicle size, oocyte morphometry, cytoplasmic organization, and functional competence markers, reinforcing the predictive value of these morphological traits (Samaniego *et al.*, 2025). Comparable relationships have also been described in *G. spixii*, supporting the use of follicular and oocyte dimensions as practical selection criteria in cavid models.

Beyond size-based metrics, the structural organization of the follicle and the COC provides additional insight into oocyte developmental potential. In cavid, well-organized granulosa and *cumulus* cell layers reflect efficient oocyte-somatic cell communication established during *in vivo* folliculogenesis. In contrast, alterations in follicular architecture including the occurrence of polyovular follicles reported in several cavid species may negatively affect oocyte quality and subsequent *in vitro* manipulation (Sadeu *et al.*, 2007; Praxedes *et al.*, 2017). Polyovularity, likely resulting from incomplete germ cell disaggregation during early follicle formation, has been associated with impaired oocyte maturation and reduced efficiency of assisted reproductive technologies in rodents (Iguchi *et al.*, 1990). Comparative evidence further indicates that, despite phylogenetic proximity to other rodent models, cavid exhibit distinct follicular morphological patterns, highlighting that folliculogenesis in this family is governed by a complex interplay among follicle size, proliferative activity, estrous cycle stage, and endocrine environment (Hirshfield and Midgley, 1978; Lall *et al.*, 2022). This biological diversity helps explain why *in IVEP* outcomes in cavid remain more variable and generally lower than those reported for well-established laboratory rodents such as rats and mice (Ma *et al.*, 2005; Mochida *et al.*, 2019).

Table 2. Morphological characteristics of the follicular population of species belonging to the order Rodentia.

Species (Family)	Number of follicles			Follicle diameter (µm)			Oocyte diameter (µm)			Nuclear diameter (µm)		
	Primordial	Primary	Secondary	Primordial	Primary	Secondary	Primordial	Primary	Secondary	Primordial	Primary	Secondary
<i>C. porcellus</i> (Caviidae) ^a	606	364	410	-	67.9	188.9	-	44.8	72.8	-	-	-
<i>Cryptomys</i> <i>hottentotus</i> (Bathyergidae) ^b	72	72	54	-	37.7	81.7	22.0	25.3	44.8	-	-	-
<i>Dasyprocta</i> <i>leporina</i> (Dasyproctidae) ^c	3692	708	18	18.62	23.7	88.5	12.2	14.2	52.8	6.1	6.7	22.3
<i>Lagostomus</i> <i>maximus</i> (Chinchillidae) ^d	374	13	7	24.5	68.7	127.6	-	34.7	53.7	-	-	-
<i>Mesocricetus</i> <i>auratus</i> (Cricetidae) ^e	-	-	273	26.0	56.0	125.0	23.4	32.2	63.6	-	-	-
<i>Mus musculus</i> (Cricetidae) ^e	7565	-	-	17	52.1	104.0	12.6	28.8	53.8	-	-	-

Neotomodon

<i>alstoni</i>	100	-	110	-	-	83.3	-	-	-	-	-	-
(Cricetidae) ^f												
<i>Galea spixii</i>	71	140	9	16.6	28.3	123.7	12.4	18.9	50.1	5.8	7.0	14.3
(Caviidae) ^g												

^{a,b,c}Subscripts indicate references: ^aSadeu *et al.* (2007); ^bWillingstorfer *et al.* 1998; ^cSantos *et al.* (2018); ^dEspinosa *et al.* (2011); ^eGriffin *et al.* (2006); ^fPraxedes *et al.* (2017); ^gLuis-Díaz *et al.* (2008).

Overall, morphological characterization of the follicular population provides a critical framework for evaluating oocyte developmental competence in cavid. When interpreted in the context of species-specific follicular dynamics, morphological parameters not only support the selection of higher-quality oocytes but also guide the design and refinement of *in vitro* culture conditions. Consequently, understanding how follicular morphology and development are maintained or altered under *in vitro* conditions, particularly in preantral and antral follicles, represents a key step toward improving oocyte competence and the overall efficiency of assisted reproductive technologies in this taxonomic group.

Characterization of nuclear, metabolic, and functional maturation

Morphological characterization of the follicular population, as discussed in the previous subsection, represents a relevant initial step for identifying potentially competent oocytes in cavid; however, accumulated experience with *in vitro* maturation (IVM) systems indicates that morphology alone is not sufficient to consistently predict the true developmental potential of the oocyte (Mohammed *et al.*, 2024; Samaniego *et al.*, 2025). In this context, the assessment of nuclear, metabolic, and functional maturation emerges as a necessary extension of morphological analysis, allowing progression from structural description toward an understanding of the cellular processes that underpin developmental competence (Zeide *et al.*, 2022a; Richani *et al.*, 2024).

Nuclear maturation, typically assessed by meiotic progression from the germinal vesicle stage to metaphase II, remains a widely used criterion in studies involving cavid, particularly in the absence of well-established molecular markers for these species (Yao *et al.*, 2023; Cañón-Beltrán *et al.*, 2024). However, evidence derived from mammalian models demonstrates that attainment of the MII stage alone does not ensure proper chromosomal organization or meiotic spindle stability, both of which are essential for embryonic competence (Zeide *et al.*, 2022a; Coticchio *et al.*, 2025). Recent reviews emphasize that correct cytoplasmic polarization and integrity of the oocyte cortical domain are closely associated with spindle migration and positioning, as well as with asymmetric polar body extrusion, and that these events are particularly sensitive to perturbations imposed by *in vitro* culture conditions (Coticchio *et al.*, 2025). Although such mechanisms have been predominantly described in classical experimental models, they provide a useful conceptual framework for interpreting observations in cavid, in which gradual improvements in IVM systems have been associated with more consistent rates of meiotic progression and embryonic activation (Aquino *et al.*, 2025; Cañón-Beltrán *et al.*, 2024).

In parallel, metabolic maturation represents a central component of oocyte competence and is directly related to the oocyte's capacity to meet the high energetic demands of meiosis, fertilization, and early embryonic cleavages (Horta *et al.*, 2025; Mohammed *et al.*, 2024). Recent studies highlight that metabolic parameter, such as mitochondrial activity, redox balance, and availability of energetic substrates provide more refined functional information than morphology alone (Horta *et al.*, 2025; Mohammed *et al.*, 2024). In caviids, the metabolic dependence of the oocyte on *cumulus* cells is particularly relevant, as oocytes display limited capacity for glucose metabolism and rely on soma-germline coupling for the provision of pyruvate, lactate, and amino acids (Richani *et al.*, 2024; Ebrahimi *et al.*, 2025). Experimental evidence indicates that culture interventions capable of modulating the metabolic environment are associated with improved maturation and activation outcomes, reinforcing the role of metabolism as a functional determinant of competence (Yao *et al.*, 2023; Aquino *et al.*, 2025).

Functional maturation, in turn, reflects the integration of nuclear and metabolic maturation and is closely linked to the maintenance of communication between the oocyte and cumulus cells (Eppig *et al.*, 2002; Richani *et al.*, 2024). The integrity of gap junctions and appropriate remodeling of the extracellular matrix within the COC are fundamental for synchronizing maturation events (Zeide *et al.*, 2022b; Chow *et al.*, 2025). Qualitative alterations in this matrix, including imbalances in glycosaminoglycan composition, have been associated with incomplete *cumulus* expansion and reduced functional efficiency in *in vitro* systems (Chow *et al.*, 2025). Although much of this evidence derives from classical experimental models, studies in caviids evaluating the response of the COC to different culture conditions indicate that preservation of these interactions is a critical factor for successful maturation (Yao *et al.*, 2023; Cañón-Beltrán *et al.*, 2024).

Importantly, available studies in caviids have largely focused on the establishment and optimization of culture media and maturation conditions (Yao *et al.*, 2023), assessing outcomes such as meiotic progression (Cañón-Beltrán *et al.*, 2024), embryonic activation, and early development (Aquino *et al.*, 2025). These studies provide indirect functional evidence that oocyte competence can be modulated *in vitro*, while also highlighting the limitations of current systems, particularly when oocytes are recovered from follicles at different developmental stages (Mohammed *et al.*, 2024). Accordingly, the variability observed in IVM outcomes in caviids suggests that part of the constraints on developmental competence may be related not only to culture conditions but also to the intrinsic maturation status of the oocyte at the time of recovery (Samaniego *et al.*, 2025).

Within this framework, integrated assessment of nuclear, metabolic, and functional maturation not only complements morphological characterization of the follicular population but also reveals the need for strategies that preserve, or partially reconstruct, the *in vivo* follicular microenvironment (Zeide *et al.*, 2022b; Richani *et al.*, 2024). This realization opens the door to approaches that extend beyond the maturation of isolated oocytes, directing attention toward culture systems capable of maintaining follicular organization and soma-germline interactions at earlier stages of development. In this regard, *in vitro* culture of preantral follicles emerges as a promising strategy to deepen our understanding of the determinants of oocyte competence in mammals, as addressed in the following subsection (Yao *et al.*, 2023; Latham, 2025).

Contributions of in vitro culture of preantral follicles

Since the first successful live birth obtained from the *in vitro* culture of preantral follicles in laboratory rodents (O'Brien *et al.*, 2003), this approach has become established as a highly relevant experimental tool for the study of reproductive physiology, the acquisition of oocyte developmental competence, and the development of assisted reproductive technologies. Two-dimensional (2D) and three-dimensional (3D) culture systems have been widely explored, leading to significant advances in the understanding of the mechanisms regulating follicular growth (Xu *et al.*, 2006), cell-cell communication (Jee *et al.*, 2012), and oocyte maturation under controlled conditions (Luciano *et al.*, 2025).

Historically, *in vitro* preantral follicle culture has been applied in several mammalian species; however, its development and optimization have been predominantly focused on murine models, where the technique has demonstrated potential for the production of developmentally competent oocytes and for applications in reproductive toxicology (Liu *et al.*, 2025), ovarian failure studies (Wang *et al.*, 2020), and fertility preservation (Zhang *et al.*, 2017). In these models, 2D culture systems based on maintaining follicles on rigid substrates have been widely employed due to their technical simplicity; however, they are frequently associated with loss of three-dimensional architecture and disruption of cellular interactions essential for proper follicular development (Adam *et al.*, 2004; Koohestani *et al.*, 2019). In response to these limitations, 3D systems based on bioengineered matrices have been developed, allowing improved preservation of follicular organization, communication between granulosa cells and the oocyte (Smitz and Cortvrindt, 2004), and the mechanical dynamics of the ovarian microenvironment (Higuchi *et al.*, 2015; Candelaria *et al.*, 2025).

More recently, relevant advances have also been reported in wild rodent species, expanding the scope of this technique within the order Rodentia. Pioneering studies in *Dasyprocta leporina* demonstrated, for the first time, the feasibility of *in vitro* culture of preantral follicles enclosed within fresh and vitrified ovarian tissue using media such as TCM199 supplemented with FSH, resulting in significant preservation of follicular morphology, viability, and the proportion of primordial and primary follicles after culture (Praxedes *et al.*, 2023). These findings represent an important milestone, as they demonstrate that even in wild rodents with reproductive physiology distinct from classical laboratory models, it is possible to establish initial culture systems capable of preserving follicular structural integrity and sustaining follicle survival *in vitro*.

Although *D. leporina* belongs to the family Dasyproctidae rather than Caviidae, these findings have direct conceptual implications for the advancement of reproductive biotechnologies in hystricomorph rodents as a whole. Similarities in general ovarian organization patterns, the predominance of preantral follicles in the ovarian reserve, and shared challenges related to follicular activation and the acquisition of oocyte competence suggest that progress achieved in agoutis may serve as a methodological and experimental reference for the future development of protocols adapted to cavid species.

In addition, studies in *D. leporina* reinforce the importance of species-specific optimization of culture systems, as factors such as FSH concentration and source, basal medium composition (Praxedes *et al.*, 2023), culture duration, and the physiological status of ovarian tissue exert decisive effects on follicular responses (Figueiredo *et al.*, 2018; Luciano *et al.*, 2025). These results corroborate previous observations in other mammals, indicating that preantral follicle activation and growth depend on complex interactions among endocrine, paracrine, and mechanical signals that are difficult to reproduce in simplified systems (Figueiredo *et al.*, 2011; 2018).

Within the family Caviidae, although consolidated studies on *in vitro* culture of preantral follicles leading to the production of mature oocytes are still lacking, available evidence suggests that this approach represents a promising avenue for overcoming limitations observed in the IVM of oocytes recovered from antral follicles. The maintenance of the oocyte within its native follicular microenvironment for extended periods offers a unique opportunity to preserve oocyte-follicle communication, thereby promoting a progressive and coordinated acquisition of nuclear, metabolic, and functional competence, while reducing the heterogeneity observed in maturation outcomes. Thus, *in vitro* culture of preantral follicles emerges not only as an experimental tool for studying folliculogenesis in wild rodents but also as an integrative strategy

capable of linking the morphological characterization of the follicular population, discussed previously, to future efforts aimed at improving oocyte competence and IVEP efficiency in caviids.

Taken together, advances in *in vitro* preantral follicle culture highlight the potential of this approach to preserve the follicular microenvironment, sustain cellular interactions, and promote a more gradual and coordinated acquisition of oocyte competence. Nevertheless, the application of this strategy still faces significant technical challenges, particularly with respect to progression of follicular growth to advanced stages and the consistent generation of fully competent oocytes for IVEP. In this context, approaches based on the manipulation and *in vitro* culture of antral follicles emerge as a complementary and strategic alternative, allowing intervention at a more advanced stage of follicular development, when the oocyte already exhibits greater structural and functional maturity. The analysis of these contributions is therefore essential to understand how different levels of follicular intervention can be integrated to optimize oocyte competence in caviids, as discussed in the following subsection.

Contributions of in vitro manipulation and culture of antral follicles

During follicular growth, the progressive accumulation of maternal factors, such as proteins, messenger RNAs, and cytoplasmic organelles, is essential to support meiotic progression, fertilization, and early embryonic development (Takashima *et al.*, 2021). For this process to be completed successfully, the oocyte must maintain a fully functional molecular and metabolic machinery, sustained by coordinated interactions with granulosa and theca cells within the antral follicle. Consequently, the *in vitro* manipulation and culture of antral follicles have emerged as strategic approaches for investigating the regulatory mechanisms underlying oocyte functional activity and developmental competence.

It is well established that oocyte competence is not acquired abruptly, but rather gradually throughout folliculogenesis, as a result of tightly regulated interactions between the oocyte and its surrounding somatic cells (Orisaka *et al.*, 2021). In this context, antral follicles represent a particularly informative developmental window, as the oocyte has already achieved substantial growth, accumulated critical cytoplasmic reserves, and established complex endocrine and paracrine signaling networks. Thus, experimental manipulation of antral follicles allows direct intervention at a stage in which the oocyte exhibits greater structural and functional maturity, while still retaining dependence on the follicular microenvironment.

Follicular development at the antral stage is tightly controlled by pituitary gonadotropins, especially FSH, in concert with intraovarian regulators that collectively shape

the biochemical and mechanical properties of the follicular niche. Within this regulatory framework, the analysis of gene expression patterns has become a valuable tool for assessing follicular developmental status and oocyte competence (Takashima *et al.*, 2021). Although oocytes display limited transcriptional activity during early follicular stages, entry into the growth phase is marked by a pronounced increase in transcriptional output and protein synthesis, reflecting the preparation of the oocyte for subsequent meiotic and embryonic events (Mtango *et al.*, 2008).

In caviids, direct molecular data derived from antral follicles remain scarce; however, initial insights have been provided by immunohistochemical studies in *G. spixii*. These investigations demonstrated the presence of leptin receptors in follicular cells and suggested that leptin signaling contributes to oocyte maturation through mitogen-activated protein kinase (MAPK) pathways (Macêdo *et al.*, 2018). Although limited in scope, such findings highlight the relevance of metabolic and endocrine cues in modulating follicular function and oocyte competence in cavid species.

Evidence from other rodent models further reinforces the importance of intraovarian signaling during antral follicle development. In mice, disruption of FSH receptor signaling results in arrested follicular development at early stages, underscoring the indispensable role of gonadotropin-mediated pathways in follicle growth and survival (Kumar *et al.*, 1997; Dierich *et al.*, 1998). In addition to FSH, locally acting factors including androgens, insulin-like growth factor (IGF) signaling, activins, and oocyte-derived growth factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are critical for maintaining granulosa cell viability, follicular expansion, and oocyte developmental competence (Takashima *et al.*, 2021). For example, ablation of androgen receptor signaling in murine follicles leads to granulosa and theca cell apoptosis, antral follicle arrest, and premature ovarian failure (Shiina *et al.*, 2006), illustrating how local endocrine disruptions can profoundly compromise follicular integrity and oocyte quality.

At the molecular level, the expression of genes associated with follicle growth and oocyte function represents an additional indicator of developmental competence. In rodents, genes such as NOBOX, KITL, and NLRP5 play essential roles in oocyte development and in regulating bidirectional communication between the oocyte and somatic cells (Mtango *et al.*, 2008; Monk *et al.*, 2017). Quantification of these transcripts has therefore been widely used as a molecular proxy for meiotic competence during *in vitro* oocyte maturation in established models. Although comparable datasets are not yet available for caviids, these molecular

frameworks provide a valuable reference for future studies aimed at characterizing competence-associated gene networks in this family.

Collectively, the available evidence underscores that the manipulation and culture of antral follicles offer a powerful experimental platform for probing the endocrine, paracrine, and molecular determinants of oocyte competence. In caviids, the limited understanding of reproductive physiology and follicular signaling likely contributes to the scarcity of molecular data and to the variability observed in IVM outcomes. Nevertheless, incremental advances in the manipulation of antral follicles have begun to reveal how targeted interventions at this developmental stage can modulate meiotic progression and early developmental potential.

Importantly, these observations indicate that limitations in oocyte competence are not solely attributable to intrinsic follicular heterogeneity, but also reflect constraints imposed by current *in vitro* systems. This recognition supports the development and application of targeted technologies to enhance oocyte developmental competence. Accordingly, the following section focuses on emerging and established technological strategies aimed at actively improving oocyte quality and developmental potential in caviids, integrating lessons from other rodent models while considering the unique biological features of this family.

Technologies to enhance oocyte developmental competence

Despite the intrinsic limitations imposed by the recovery of oocytes from heterogeneous follicular populations, substantial technological advances have been developed to enhance oocyte developmental competence following the collection of COCs, with the ultimate goal of improving IVEP outcomes. This progress is particularly relevant in non-model and wild rodents, in which IVEP represents a strategic tool for species conservation, genetic resource banking, and the advancement of fundamental studies in reproductive, cellular, and molecular biology (Oliveira and Pereira, 2025).

In general, IVEP involves a sequence of procedures designed to mimic key aspects of female reproductive physiology. The process typically begins with the recovery and selection of immature oocytes obtained either *in vivo* or *post-mortem*, followed by IVM, during which oocytes acquire the nuclear and cytoplasmic competence required to support fertilization and early embryonic development. Although IVEP protocols are well established in mice and rats, in wild rodents the process remains inherently artificial, as oocytes are frequently retrieved from follicles that have not completed *in vivo* capacitation and therefore lack the full complement of molecular and metabolic mechanisms required for optimal developmental competence (Gilchrist and Thompson, 2007).

To mitigate these constraints, significant efforts have been directed toward the optimization of IVM media, with particular emphasis on mimicking essential biochemical and paracrine signals present within the follicular environment. Supplementation strategies involving gonadotropins, antioxidants, growth factors, and signaling pathway modulators have been extensively explored across different rodent models. In this context, epidermal growth factor (EGF) has emerged as a central component of IVM systems due to its role in *cumulus* cell expansion, meiotic resumption, cytoplasmic maturation, and maintenance of oocyte ultrastructural integrity. Recent studies in *G. spixii* have demonstrated that species-specific adjustment of EGF concentration is essential, as lower doses improved *cumulus* cell viability and oocyte morphometric quality, whereas higher concentrations were associated with reduced developmental outcomes (Aquino *et al.*, 2025).

Parallel advances have also been reported in other South American rodents, including species of the genus *Dasyprocta*, in which IVM protocols and subsequent fertilization or artificial activation steps have been progressively refined. These studies indicate that, although these species belong to different families within the order Rodentia, shared physiological constraints allow methodological insights to be transferred across taxa (Ferraz *et al.*, 2020; Praxedes *et al.*, 2023). Importantly, these investigations have expanded the focus beyond IVM alone, incorporating improvements in sperm selection methods, fertilization strategies, artificial activation, and embryo culture systems, thereby reinforcing the need to view oocyte competence as a dynamic property shaped across multiple stages of IVEP (Oliveira *et al.*, 2025c; 2025d).

In parallel with culture media optimization, new methods for evaluating immature oocytes have gained prominence as essential tools for refining pre-IVM selection criteria. Traditional morphological assessment, while useful, has proven insufficient to accurately predict developmental competence. Consequently, functional and quantitative approaches have been increasingly adopted. In caviids, these methods have provided valuable insights into oocyte quality, revealing subtle alterations in the oocyte microenvironment that are not detectable by morphology alone (Yao *et al.*, 2023; Cañón-Beltrán *et al.*, 2024).

Collectively, these technological advances highlight a transition from purely descriptive or empirical IVM protocols toward more integrative and physiology-driven strategies. By combining optimized culture media with refined oocyte evaluation tools, recent studies in caviids and other rodents of the order Rodentia have laid the foundation for a more consistent acquisition of oocyte developmental competence. These efforts not only enhance IVEP efficiency but also create a conceptual bridge between follicular biology, oocyte maturation,

and downstream reproductive technologies, setting the stage for further innovations aimed at improving fertilization efficiency and embryonic development, as discussed in the following subsections.

Optimization of IVM media

The optimization of IVM media represents a central challenge for improving oocyte developmental competence in cavid, given the marked divergence between the *in vivo* follicular microenvironment and artificial culture systems. Oocyte maturation requires the coordinated regulation of ionic balance, energy metabolism, redox homeostasis, cytoskeletal dynamics, and paracrine signaling mediated by *cumulus* cells. As emphasized in recent reviews, the historical evolution of mammalian culture media reflects a gradual shift from empirically derived formulations toward more physiologically informed systems; however, no single medium can universally meet the dynamic and species-specific requirements of oocyte maturation (Men, 2024).

In cavid, particularly *C. porcellus*, IVM protocols have traditionally relied on TCM-199 supplemented with inorganic salts, sodium bicarbonate, buffering agents (HEPES), L-glutamine, sodium pyruvate, and fetal bovine serum (FBS), under controlled temperature and CO₂ conditions. Although this formulation supports meiotic resumption, reported nuclear maturation rates remain modest, typically ranging from 20% to 40%, reflecting incomplete acquisition of cytoplasmic competence and suboptimal coordination between nuclear and cytoplasmic maturation (Wang *et al.*, 2019). These outcomes highlight the limitations of applying generic mammalian media to species with distinct folliculogenesis dynamics and strong soma-germinal dependence.

Efforts to improve IVM efficiency in cavid have therefore focused on targeted supplementation strategies aimed at recreating key biochemical and paracrine signals of the follicular niche. Antioxidant supplementation has emerged as a particularly effective approach. In *C. porcellus*, combined treatment with cysteamine and leukemia inhibitory factor (LIF) significantly increased the proportion of oocytes reaching metaphase II (61.8%) compared with control groups (17.5%), whereas either compound alone produced only marginal improvements (Wang *et al.*, 2019). These findings underscore the importance of redox regulation during IVM and demonstrate that synergistic supplementation is required to effectively support cytoplasmic maturation. Importantly, the same study showed that inhibition of ROCK signaling impaired microfilament organization and reduced maturation rates, reinforcing the sensitivity of the oocyte cytoskeleton to culture conditions.

Growth factor supplementation has also proven to be a critical determinant of IVM success in cavid. Cañón-Beltrán *et al.* (2024) demonstrated that supplementation with EGF and insulin-like growth factor I (IGF-I) exerts dose-dependent effects on oocyte quality in *C. porcellus*. In addition to increased nuclear maturation rates, optimized concentrations of these growth factors improved *cumulus* cell viability, reduced apoptotic indices, and preserved cytoplasmic features associated with competence, including mitochondrial distribution and cortical granule organization. These results extend previous observations by linking growth factor supplementation not only to meiotic progression but also to structural and functional markers of cytoplasmic maturation.

Specificity in growth factor requirements is further illustrated by studies in *G. spixii*. Aquino *et al.* (2025) showed that lower concentrations of EGF improved *cumulus* cell viability, oocyte morphometric parameters, and maturation outcomes, whereas higher concentrations were associated with reduced developmental indicators. This dose-dependent response highlights the risk of directly extrapolating protocols from laboratory rodents or even closely related species and reinforces the need for fine-tuned optimization tailored to the reproductive physiology of each cavid model.

Hormonal supplementation and temporal control of meiotic progression represent additional layers of IVM optimization in cavid. Yao *et al.* (2023) demonstrated that the combination of FSH, luteinizing hormone (LH), sulfur-containing amino acids (L-cysteine and cystine), and transient cell-cycle modulators such as roscovitine and IBMX significantly improved nuclear maturation rates in *C. porcellus*, reaching values close to 70%. Complementary work by Yao *et al.* (2021) further showed that chromatin configuration at the germinal vesicle stage is closely associated with oocyte growth and IVM outcome, indicating that medium optimization must be considered in parallel with the intrinsic maturation status of the oocyte at recovery.

Taking together, current evidence in cavid indicates that optimization of IVM media cannot rely on single-factor supplementation or static formulations. Instead, effective strategies integrate redox modulation, growth factor signaling, endocrine support, and controlled regulation of meiotic timing, all adjusted to species-specific physiological constraints. As highlighted by Men (2024), continued refinement of culture media requires moving beyond universal recipes toward systems that accommodate the dynamic needs of the oocyte–*cumulus* unit. In cavid, such integrative approaches have already yielded substantial gains in maturation efficiency and oocyte quality, while also revealing the remaining limitations of current IVM systems. These advances provide a critical foundation for the development of complementary

technologies aimed at enhancing oocyte developmental competence, including improved selection and evaluation of immature oocytes, discussed in the following subsection.

New methods for evaluating immature oocytes

Considering the complex and gradual nature of oocyte competence acquisition discussed in the previous sections, the ability to reliably evaluate immature oocytes remains a central challenge for improving IVEP, particularly in non-model species, such as Caviidae rodents. Oocyte competence emerges from the coordinated progression of follicular growth, metabolic maturation, cytoplasmic remodeling, and bidirectional communication with surrounding somatic cells, rather than from a single isolated event. Consequently, several methodological approaches have been developed to assess immature oocytes, aiming to identify gametes with higher developmental potential prior to IVM. A summary of the different approaches taken can be seen in Fig. 2.

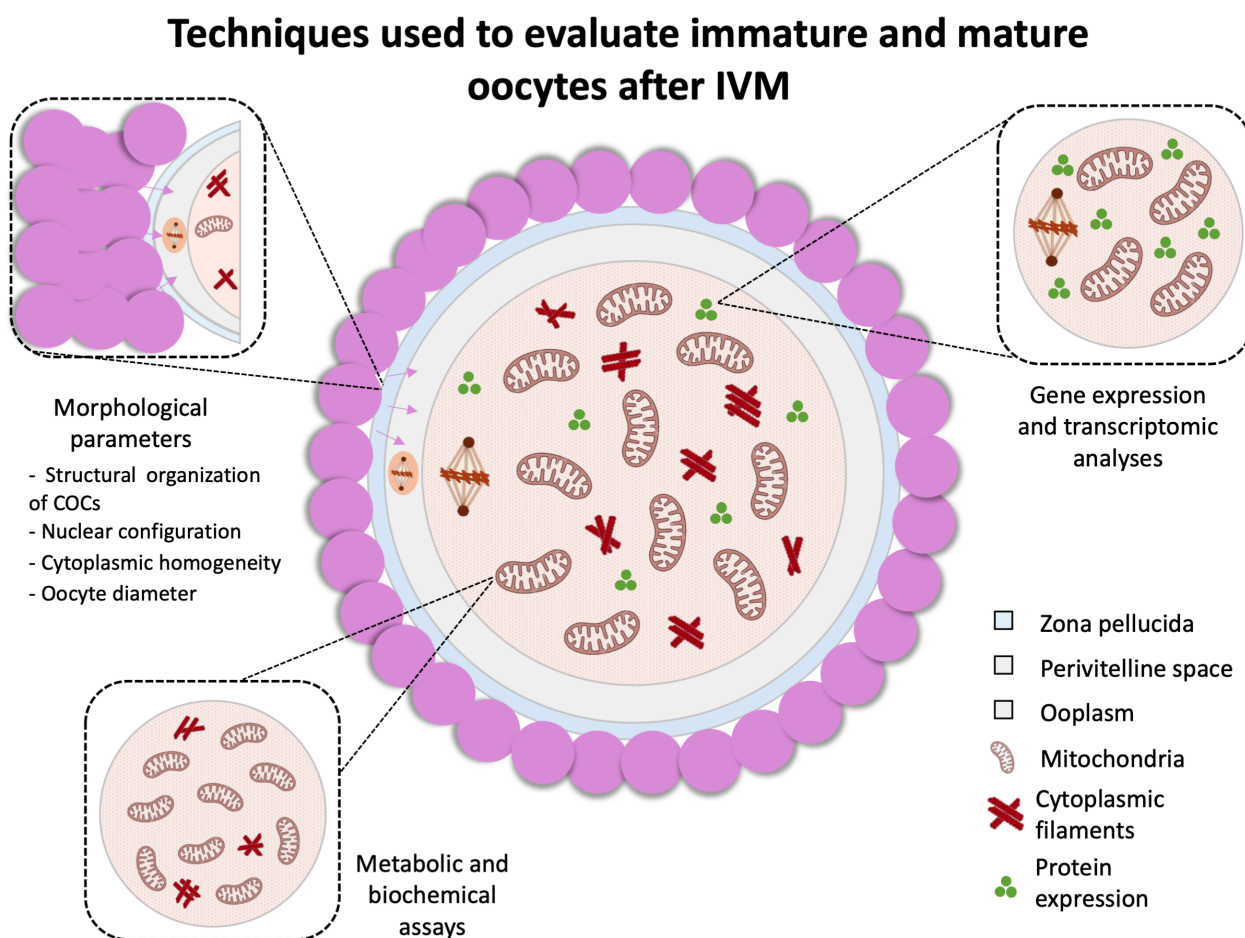


Fig. 2. Schematic representation of the evaluation of immature and mature oocytes, focusing on the selection of higher quality gametes and their application in the development of ARTs.

Traditionally, morphological assessment has represented the primary strategy for evaluating immature oocytes due to its non-invasive nature and operational simplicity. Parameters such as oocyte diameter, cytoplasmic homogeneity, nuclear configuration, and, notably, the structural organization of the COCs are consistently associated with developmental competence across mammalian species (Lemseffer *et al.*, 2022). Recent studies reinforce the central role of *cumulus* cells as metabolic and regulatory mediators, highlighting that their density, multilayer organization, and preservation of transzonal projections reflect functional oocyte–somatic cell coupling and support competence acquisition (Sudewo *et al.*, 2025; Hirao, 2025). Although partial morphological descriptions exist for some caviids, systematic application of these criteria remains limited, indicating their potential value as an accessible first-line evaluation tool in these species.

Beyond general morphology, the extrusion of the first polar body has long been employed as an indicator of nuclear maturation and meiotic progression. Polar body extrusion reflects critical meiotic events, including germinal vesicle breakdown, chromosome segregation, and cytoskeletal reorganization. However, growing evidence indicates that nuclear maturation alone does not guarantee full cytoplasmic competence, underscoring the need to interpret this parameter cautiously and in conjunction with additional markers (Liu *et al.*, 2025; Chen *et al.*, 2025).

In parallel with morphological criteria, metabolic and biochemical assays have gained prominence as non-invasive approaches to evaluate immature oocytes. The brilliant cresyl blue (BCB) test remains one of the most widely used methods to infer oocyte growth status based on glucose-6-phosphate dehydrogenase (G6PDH) activity. Oocytes retaining BCB staining generally exhibit reduced enzymatic activity and higher meiotic competence, making this assay particularly useful for selecting oocytes from heterogeneous populations (Lemseffer *et al.*, 2022; Chen *et al.*, 2025). Additionally, recent studies emphasize the importance of mitochondrial function, redox balance, and energy metabolism as determinants of oocyte quality, demonstrating that modulation of metabolic pathways during IVM directly influences maturation efficiency and developmental outcomes (Yarmohammadi *et al.*, 2026; Liu *et al.*, 2025). Although such approaches have not yet been validated in caviids, their non-invasive nature and functional relevance make them highly attractive for future application in wild and non-model species.

Molecular and transcriptomic analyses provide a deeper understanding of the regulatory processes underlying the acquisition of oocyte competence. In well-established mammalian models, the expression of genes involved in folliculogenesis, oocyte–somatic cell

communication, and early embryonic development, such as NOBOX, GDF9, BMP15, KITL, and NLRP5 has been widely correlated with oocyte quality (Chen *et al.*, 2025). However, in caviids, molecular markers of competence remain largely unexplored, reflecting both technical limitations and gaps in fundamental knowledge of the reproductive physiology of these species. At the present stage, molecular approaches should therefore be regarded as comparative and hypothesis-generating tools, offering a framework for future species-specific validation in the model species under investigation.

Advances in biophysical and structural analyses have further expanded the scope of oocyte evaluation. Techniques such as zona pellucida birefringence analysis and polarized light microscopy of the meiotic spindle provide indirect information on cytoplasmic organization and maturation status (Lemseffer *et al.*, 2022). More recently, attention has shifted toward the oocyte cortical domain as a dynamic regulator of spindle positioning, polar body extrusion, and cytoskeletal remodeling. Alterations in cortical architecture and mechanical properties have been associated with impaired maturation and reduced developmental competence, suggesting that cortical organization may serve as an emerging non-invasive indicator of oocyte quality (Coticchio *et al.*, 2025). Collectively, these approaches represent promising alternatives, particularly for species in which gamete availability is limited, as they may enhance oocyte selection strategies and support the efficient application of reproductive technologies.

Recent approaches have increasingly favored integrated evaluation strategies, in which morphological, metabolic, molecular, and biophysical parameters are considered in a complementary manner rather than relying on isolated indicators. Within this context, fluid-based methods, such as the analysis of follicular and cumulus-conditioned media, have gained attention as innovative and minimally invasive alternatives for inferring oocyte quality and follicular functionality (Ozturk *et al.*, 2025). These approaches are particularly relevant for non-model species, as they allow the gradual refinement of selection criteria while accounting for species-specific biological variability.

Overall, the methods discussed in this section indicate that, although mechanistic markers of oocyte competence remain poorly characterized in Caviidae rodents, a consistent set of approaches validated in other mammalian models is already available and can serve as a comparative foundation. These strategies provide a solid conceptual and technical basis for future adaptation and validation in caviids. Thus, current limitations in IVEP efficiency in these species appear to be less related to the lack of evaluative tools and more associated with the absence of species-specific integration among morphological, functional, and molecular criteria. Addressing this gap will be essential for advancing reproductive biotechnologies in

Caviidae rodents, with direct implications for conservation efforts, reproductive biobanking, and the establishment of these species as emerging experimental models.

Final considerations

Cavids comprise a diverse group of South American rodents within the order Rodentia that play key ecological roles in native ecosystems and have increasingly attracted attention as non-traditional models for reproductive biology and assisted reproductive technologies. In this review, we synthesized current knowledge on cavid reproductive physiology, with particular emphasis on *in vivo* folliculogenesis, oocyte-somatic cell interactions, and the progressive acquisition of oocyte developmental competence. By integrating biological regulation with advances in *in vitro* manipulation strategies, we provide a comprehensive framework for understanding how intrinsic oocyte factors and follicular microenvironmental cues collectively determine developmental potential under *in vitro* conditions.

The evidence compiled herein reinforces that oocyte competence in cavids is a multifactorial and dynamic trait, acquired gradually throughout follicular development and modulated by endocrine, paracrine, metabolic, and cellular interactions. Morphological, metabolic, and molecular indicators of competence, together with improvements in *in vitro* maturation systems, culture media optimization, and functional assessment tools, emerge as complementary approaches for enhancing oocyte quality and embryonic development. Importantly, recent technological advances highlight a shift from empirical protocols toward more integrative, physiology-driven strategies that better reflect the complexity of the follicular niche.

Despite these advances, the application of *in vitro* reproductive technologies in cavids remains limited and uneven, largely due to the concentration of studies in a small number of species, particularly *C. porcellus* and *G. spixii*. Expanding research to other members of the Caviidae family is essential to capture the biological diversity of the group and to refine species-appropriate reproductive protocols. A broader comparative approach will be critical for improving IVEP efficiency and consistency, supporting the application of complementary technologies, such as *in vitro* fertilization and somatic cell nuclear transfer, and strengthening the role of cavids as experimental models for reproductive biotechnology, conservation, and genetic resource preservation.

Acknowledgements

Leonardo Vitorino Costa de Aquino is a recipient of a grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Financial Code 001). Alexsandra Fernandes Pereira is recipient of a grant from CNPq (no. 309078/2021-0). None of the authors has any conflict of interest to declare.

Authors's Disclosures

The authors reported no potential conflict of interest.

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CAPÍTULO 6 – EFFECT OF EPIDERMAL GROWTH FACTOR AND 6-DIMETHYLAMINOPURINE ON *IN VITRO* MATURATION AND ARTIFICIAL ACTIVATION OF SPIX’S YELLOW-TOOTHED CAVY (*Galea spixii* WAGLER, 1831) OOCYTES

ARTIGO EXPERIMENTAL IV

ARTIGO PUBLICADO NO PERIÓDICO INTERNACIONAL ANIMALS

Qualis 2017-2020: A1 (Medicina Veterinária)

Fator de Impacto: 2.7

Data da Publicação: 15 de Agosto de 2025

DOI: <https://doi.org/10.3390/ani15162403>

Effect of Epidermal Growth Factor and 6-Dimethylaminopurine on *In Vitro* Maturation and Artificial Activation of Spix's Yellow-Toothed Cavy (*Galea spixii* Wagler, 1831) Oocytes

Leonardo Aquino¹, Samara Olindo¹, Yara Letícia Silva¹, Vinícius Silva¹, Lhara Oliveira¹, Moacir Oliveira², Alessandra Pereira^{1,*}

¹ Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil; leonardovt@live.com (L.A.); samara.lima@alunos.ufersa.edu.br (S.O.); yara.silva@alunos.ufersa.edu.br (Y.L.S.); viniciusd647@gmail.com (V.S.); lharagirs@hotmail.com (L.O.); alessandra.pereira@ufersa.edu.br (A.P).

² Center of Multiplication of Wild Animals, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil; moacir@ufersa.edu.br (M.O.)

* Correspondence: alessandra.pereira@ufersa.edu.br.

Simple Summary: *In vitro* maturation (IVM) and artificial oocyte activation are essential steps in the development of assisted reproductive technologies (ARTs), such as somatic cell nuclear transfer. While these techniques are well established in laboratory rodents like mice and rats, they remain underdeveloped in wild species such as Spix's yellow-toothed cavy. Advancing ARTs in this species could support its conservation and help preserve biodiversity. This study evaluated the effects of two concentrations of epidermal growth factor (EGF; 10 or 50 ng/mL) in IVM medium and the use of 6-dimethylaminopurine (6-DMAP) during artificial oocyte activation. Results showed that 10 ng/mL EGF enhanced *cumulus* cell viability and improved oocyte morphometric quality compared to 50 ng/mL. Although 6-DMAP positively influenced cleavage rates, it did not significantly affect morula formation. In conclusion, 10 ng/mL EGF is recommended for IVM in Spix's yellow-toothed cavy oocytes, and 6-DMAP shows potential to support early embryonic development, contributing to the establishment of ART protocols for this species.

Received: 7 July 2025

Revised: 10 August 2025

Accepted: 14 August 2025

Published: 15 August 2025

Citation: Aquino, L.V.C.; Olindo, S.L.; Silva, Y.L.F.; Silva, V.D.; Oliveira, L.R.M.; Oliveira, M.F.; Pereira, A.F. Effect of Epidermal Growth Factor and 6-Dimethylaminopurine on *In Vitro* Maturation and Artificial Activation of Spix's Yellow-Toothed Cavy (*Galea spixii* Wagler, 1831) Oocytes. *Animals* 2025, 15, 2403. <https://doi.org/10.3390/ani15162403>

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Abstract: The successful application of assisted reproductive technologies (ARTs), such as *in vitro* maturation (IVM) and artificial oocyte activation, require species-specific adaptations. Although these methods are routinely used in laboratory rodents, their use in wild or non-model species remains limited, such as the Spix's yellow-toothed cavy, a Neotropical species of ecological and reproductive interest. This study evaluated the effects of different concentrations of epidermal growth factor (EGF; 10 or 50 ng/mL) on IVM (Experiment 1) and of 6-dimethylaminopurine (6-DMAP) on artificial oocyte activation (Experiment 2). EGF at 10 ng/mL ($93.8\% \pm 1.6$; $84.9\% \pm 0.7$) promoted greater viability and less apoptosis in *cumulus* cells, compared to 50 ng/mL ($83.0\% \pm 1.6$; $78.9\% \pm 2.7$), maintaining *cumulus* expansion, ultrastructural integrity and better morphometric quality of oocytes.

Thus, this concentration was used in the next step, where oocytes were activated with or without 6-DMAP. After five days, the presence of 6-DMAP increased cleavage rates ($69.3\% \pm 5.0$) compared to activation without the compound ($53.5\% \pm 3.5$), without significantly affecting morula formation ($13.2\% \pm 3.1$ to $17.3\% \pm 2.9$). It is concluded that EGF improves the oocyte microenvironment, while 6-DMAP enhances cleavage, being initial steps in the development of ARTs for Spix's yellow-toothed cavy.

Keywords: Wildlife; Neotropical rodents; *Ex-situ* conservation; *In vitro* embryo production; oocyte viability; embryonic development

1. Introduction

The Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) is a Neotropical rodent with diurnal and crepuscular habits and a varied plant-based diet [1]. Native to the Caatinga, it also occurs in other Brazilian biomes, Paraguay, and the eastern Andes [2]. Its ecological adaptability supports genetic diversity and contributes to seed dispersal, soil aeration, and food web dynamics [3,4]. Despite the Spix's yellow-toothed cavy population being regarded as stable [5], individuals face significant threats due to habitat disturbance and heavy hunting pressure. Moreover, cavyes serve as valuable models for developing conservation strategies aimed at protecting other critically endangered species within the suborder Hystricomorpha.

Among wildlife conservation strategies, assisted reproductive technologies (ARTs) have gained significant attention [6]. One of these ARTs is *in vitro* maturation (IVM) combined with artificial oocyte activation, both of which are essential steps in the development of techniques, such as somatic cell nuclear transfer [7]. With the advent of nuclear reprogramming—where a somatic cell's epigenetic landscape is altered by the remarkable reprogramming capacity of an enucleated oocyte to initiate embryonic development [8]—somatic cell nuclear transfer (SCNT) has emerged as a powerful biotechnological tool for the multiplication, conservation, and scientific study of reproductive biology in both laboratory and wild rodents [9].

However, the successful application of SCNT, including IVM and artificial oocyte activation, depends on species-specific adaptations [10]. While these techniques are well established in laboratory rodents, their application in wild or non-model species remains scarce and underexplored. Regarding IVM, it is clear that optimized protocols are essential, as oocyte development depends on precise conditions such as temperature [11], duration [12], and culture medium composition and supplementation [7] to acquire meiotic competence. Specifically, culture systems for both laboratory and wild rodents often include epidermal growth factor (EGF) due to its role as one of the 13 families of secondary proteins that regulate *cumulus* cell expansion [13,14]. These *cumulus* cells are crucial for signaling the resumption of meiosis I, facilitating the

formation of the first polar body, metaphase plate alignment, and cytoplasmic maturation [15,16]. Studies on rodents have shown that EGF concentrations between 10 and 50 ng/mL enhance IVM efficiency [7,17], whereas higher doses may negatively affect oocyte quality and maturation success. Therefore, it is especially important to evaluate the effects of EGF concentration during the IVM of Spix's yellow-toothed cavy oocytes.

It is important to emphasize that, besides oocyte in IVM, the sensitivity of embryonic development during artificial activation is a critical factor [8]. In both laboratory and wild rodents, a protocol combining 10 mM strontium chloride (SrCl₂) with 5 µg/mL cytochalasin B (CB) for 6 h has been used for artificial activation of red-rumped agouti oocytes, yielding a morula formation rate of 25% [7]. Although blastocysts were not observed, these results suggest potential for optimizing embryonic production protocols for this genus. In contrast, artificial activation protocols in laboratory rodents have demonstrated higher efficiency, with blastocyst rates varying according to the methods applied. For instance, Ma et al. [18] reported two commonly used murine protocols: the first, using 10 mM SrCl₂ and 5 µg/mL CB for 6 h, achieved a 56.3% blastocyst rate; the second, more effective protocol applied 10 mM SrCl₂ for 2.5 h, followed by 2 mM 6-dimethylaminopurine (6-DMAP) and 5 µg/mL CB for 3.5 h, reaching a 60.1% blastocyst rate. These findings illustrate that artificial activation efficiency varies between species, underscoring the importance of tailoring and evaluating such protocols specifically for Spix's yellow-toothed cavy during the development of its reproductive technologies.

Therefore, this study aimed to evaluate the effects of two concentrations of EGF (10 or 50 ng/mL) in IVM medium and the use of 6-DMAP during artificial activation of Spix's yellow-toothed cavy oocytes. To achieve this, we evaluated the microenvironment quality and matured oocytes by analyzing *cumulus* cell expansion, ultrastructure, viability, and apoptosis levels, alongside morphometric parameters, extrusion of the first polar body, nuclear stage, and embryonic development kinetics.

2. Materials and Methods

2.1. Chemicals and media

Unless otherwise stated, all chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco-BRL (São Paulo, SP, Brazil).

2.2. Bioethics and animals

All experimental procedures were approved by the Animal Use Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.010566/2017-20) and the Chico Mendes Institute for Biodiversity Conservation (no. 60428-1). Nineteen healthy adult females, aged one year and weighing 250–350 g, were used in the study. They were housed in a controlled environment with access to fruit and water at the Center of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W), which is

registered in Brazil in the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912).

2.3. Collection, transport of ovaries and recovery of the cumulus-oocyte complexes

Females were subjected to a 12-hour fasting period prior to ovary collection. Anesthesia was induced using a combination of xylazine hydrochloride (Xilazin® 2%, 1 mg/kg, Manufacturer Syntec, São Paulo, SP, Brazil) and ketamine hydrochloride (Ketamine® 10%, 15 mg/kg, Vetnil, São Paulo, SP, Brazil). Euthanasia was then carried out via intravenous administration of potassium chloride (Chlorure de potassium® 19.1%, 2.56 mEq/kg, Halexistar, Goiânia, GO, Brazil) following established protocols [19].

After ovary collection using surgical scissors, the ovaries were transported to the laboratory in a pre-warmed saline solution (0.9% NaCl, w/v) at 30 °C, supplemented with 0.05 mg/mL penicillin, and maintained for up to 30 min [11]. For cumulus-oocyte complex (COC) retrieval, the ovaries were transferred to *in vitro* culture plates (60 × 15 mm) containing oocyte recovery medium (ORM). The ORM consisted of tissue culture medium (TCM-199) supplemented with 0.2 mM sodium pyruvate (w/v), 1% antibiotic-antimycotic (v/v), and 10% fetal bovine serum (FBS, v/v).

Following ovarian slicing, oocytes were recovered and morphologically evaluated under a stereomicroscope based on cumulus cell compaction, the number of surrounding cell layers, and ooplasm homogeneity. COCs were classified into three categories [12]: Type A – oocytes surrounded by three or more layers of cumulus cells; Type B – oocytes with one to three layers of cumulus cells; and Type C – oocytes with few or no cumulus cells, including denuded oocytes. Only COCs classified as Type A and Type B were selected for use in the experiments.

2.4. Experimental design

Two experiments were conducted to evaluate the effects of different concentrations of EGF (10 or 50 ng/mL) in the IVM medium (Experiment 1) and the influence of 6-DMAP on artificial oocyte activation (Experiment 2). In the first experiment, seven females were used across three replicates (with two to three animals per replicate) to evaluate the effect of EGF on the oocyte microenvironment and nuclear maturation. Recovered oocytes were randomly allocated to IVM media supplemented with one of two EGF concentrations: 10 ng/mL (EGF10 group) and 50 ng/mL (EGF50 group). The selected concentrations were based on previous studies in other rodent models, such as guinea pigs and red-rumped agouti, in which EGF concentrations between 10 and 50 ng/mL enhanced oocyte maturation and embryonic development outcomes, while higher doses negatively impacted efficiency [7,17]. Analyses focused on cumulus cell expansion and ultrastructural characteristics using scanning electron microscopy (SEM). In addition, cumulus cell viability and apoptosis levels were evaluated to assess the quality of the *in vitro* culture environment. Oocytes were also examined for morphometric parameters and nuclear maturation, the latter determined by the extrusion rate of the first polar body (1PB) and classification of the nuclear stage in matured structures.

Following the selection of the optimal EGF concentration, a second experiment was conducted to evaluate the developmental competence of oocytes based on their ability to progress to early embryonic stages after artificial activation. Twelve females were used across four replicates (three animals per replicate). Recovered oocytes were matured *in vitro* using the previously determined optimal EGF concentration, and their quality was assessed according to the parameters previously described. Oocytes exhibiting 1PB extrusion were selected, subdivided into groups, and subjected to one of two artificial activation protocols: 10 mM SrCl₂ + 5 µg/mL CB for 6 h (SrCl₂+CB group), or 10 mM SrCl₂ for 2.5 h followed by 5 µg/mL CB + 2 mM 6-DMAP for 3.5 h (SrCl₂+CB+6-DMAP group). In this study, 6-DMAP was evaluated during artificial activation using a standard protocol employed in the artificial activation of murine oocytes (Ma et al., 2005). Finally, embryonic culture was conducted in a simple optimized and potassium-enriched medium (KSOM) for five days [17,20]. During this period, we monitored the kinetics of embryonic development and performed ultrastructural analyses to assess cleavage and embryo production rates.

2.5. *In vitro* maturation of oocytes

For the oocyte IVM, recovered COCs were initially washed three times in ORM, followed by two additional washes in the IVM medium. Groups of approximately 20–25 oocytes were then cultured in 100 µL droplets of maturation medium, covered with mineral oil, and incubated at 38.5 °C in an atmosphere of 6.5% CO₂ [21]. The maturation medium consisted of ORM supplemented with 0.1 mM cysteamine and 10 mIU/mL FSH/LH (Pluset®, Hertape-Calier, Barcelona, Spain) [7]. EGF was added to the IVM medium at concentrations of 10 or 50 ng/mL. After 24 h, matured structures were evaluated according to the parameters described in the experimental design.

2.6. Assessments of the microenvironment and oocyte maturation

2.6.1. Expansion of *cumulus* cells

Matured oocytes were evaluated for *cumulus* cell expansion under a stereomicroscope, following the classification method described by Zhao et al. [22]. COCs were scored on a scale from 0 to 4, as follows: Score 0 – no observable expansion; Score 1 – minimal expansion, with rounded *cumulus* cells predominantly adhered to the substrate; Score 2 – expansion observed in the outer layers of *cumulus* cells, forming two distinct layers; Score 3 – expansion of all layers except the corona radiata, forming more than three layers; Score 4 – full expansion of both the *cumulus oophorus* and *corona radiata* cell complexes.

Moreover, the *cumulus* expansion index (CEI) was calculated for each EGF concentration tested. This index represents the mean expansion score within each group and was determined using the formula proposed by Fagbohun and Downs [23]. Groups with a CEI value approaching 0 were indicative of minimal *cumulus* expansion and suboptimal *in vitro* culture

conditions, whereas values approaching 4 reflected complete expansion and were associated with higher oocyte quality [23].

2.6.2. Ultrastructure assessment by SEM

Matured oocytes exposed to different EGF concentrations were analyzed by scanning electron microscopy (SEM) to assess cellular quality. Sample preparation followed the protocol described by Li et al. [24], with minor modifications. Briefly, structures were fixed in 2.5% glutaraldehyde in saline phosphate buffer solutions (PBS; pH 7.2) at 4 °C for 2 h. After time, cells were washed twice for 5 min in 2% cacodylate buffer and postfixed in 1% osmium tetroxide for 2 h at 25 °C. Subsequently, structures were washed in PBS and dehydrated for 5 min in increasing ethanol solutions (7.5%, 15%, 30%, 50%, 70%, 90% and 100%). After washing in ethanol, structures were placed on glass slides and metallized in gold for visualization using a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bou-ches-du-Rhône, France).

2.6.3. Viability and apoptotic levels of *cumulus* cells

Matured oocytes were denuded by mechanical pipetting in ORM medium containing 0.1% hyaluronidase (v/v). Next, 50 µL of the cell suspension was collected and stained with Trypan blue (0.2%) diluted in PBS [7]. The *cumulus* cell suspension was then analyzed for viability using a Neubauer chamber. Non-viable cells (dead; stained blue) were distinguished from viable cells (alive; colorless), based on the rupture of the cell membrane. The percentage of viable cells was calculated using the formula: no. of viable cells/no. total cells quantified × 100.

The apoptotic levels of *cumulus* cells were determined using fluorescent dyes, following the method described by Rodrigues et al. [25] with modifications. For this, 50 µL of cell suspension from denuded oocytes containing *cumulus* cells was collected and labeled with ethidium bromide (5 µg/mL) and acridine orange (1 µg/mL), diluted in 8 µL of PBS. Through differential dye uptake, chromatin condensation in the nucleus of each cell was identified [26]. Cells were classified based on their fluorescence as follows: i) Viable cells that are uniformly stained green; ii) Initial apoptosis when the nucleus is non-uniform green; iii) Late apoptosis when the nucleus is uniform orange; and (iv) Necrosis when the cells present their nuclei in red. For this, a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) was used with an absorbance of 480 nm. Finally, images were saved, and 300 cells were counted for each replicate using Image J (National Institutes of Health, MA, USA) software at 20× magnification.

2.6.4. Morphometric and 1PB evaluation of oocytes

Morphometric analyses involved the morphometric evaluation of oocytes with and without the presence of 1PB after 24 h of IVM. Images at 20× magnification were captured using an inverted microscope (Leipzig IMx, Phoenix Optics, Germany). Pixel distances were converted using

ImageJ software to determine the outer oocyte diameter (OOD), inner oocyte diameter (IOD), zona pellucida thickness (ZPT), and ooplasm diameter (OD) [27]. Based on these parameters, the following measurements were calculated using mathematical formulas: space perivitelline diameter (SPD; $OID - OD$), internal oocyte area (IOA; $\pi \times (IOD/2)^2$), ooplasm area (OA; $\pi \times (OD/2)^2$), and perivitelline space area (PSA; $IOA - OA$) [27].

2.6.5. Nuclear stage assessment

The nuclear stage was identified after visualizing the extrusion of 1PB from the oocytes. Cells were fixed in 4% paraformaldehyde for 15 min. Then, oocytes were washed in PBS supplemented with 0.4% bovine serum albumin (BSA). Subsequently, cells were labeled with 10 $\mu\text{g/mL}$ Hoescht 33342 for 15 min [28]. After time, oocytes were transferred to glass slides and viewed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) to observe structures in metaphase II, the nuclear stage in which they were considered mature (Yao et al., 2021). For fluorescence microscopy, the excitation wavelength was 510 nm, while the emission wavelength was 590 nm.

2.7. Artificial activation, ultrastructure analysis and embryonic kinetic patterns

For artificial activation, denuded oocytes that had shown 1PB extrusion were used. The oocytes were first washed three times in ORM medium. Then, they were artificially activated in calcium-free Tyrode's albumin lactate pyruvate (TALP) medium, supplemented with 10 mM SrCl_2 and 5 $\mu\text{g/mL}$ CB for 6 h at 38.5 °C and 6.5% CO_2 . For the 6-DMAP group, oocytes were initially incubated at 38.5 °C and 6.5% CO_2 in TALP medium containing 10 mM SrCl_2 for 2.5 h. After this period, the oocytes were washed three times in ORM medium and transferred to drops containing TALP medium with 5 $\mu\text{g/mL}$ CB and 2 mM 6-DMAP for 3.5 h at 38.5 °C and 6.5% CO_2 , following the protocol described by Ma et al. (2005). The TALP medium was composed of salts, 10 mM sodium lactate (w/v), 3 mg/mL bovine serum albumin fraction V (w/v) and 1% antibiotic-antimycotic (v/v).

After artificial activation, presumed zygotes were washed again in ORM medium and placed in 60 μL drops containing KSOM, which was supplemented with 1% essential amino acids, 0.5% non-essential amino acids, and 15 mM BSA. The structures were cultured for five days, with D0 defined as the day of artificial activation. On D2, cleavages were identified, and the rate for each group was recorded. Additionally, on D4 and D5, the production rates of morulae and blastocysts were observed.

Furthermore, during embryonic culture, one structure at each stage of embryonic development was recovered, fixed, and processed as previously described for ultrastructural evaluation by SEM of oocytes.

2.8. Statistical analysis

All data are expressed as the mean $\pm$ standard error and were analyzed using StatView 5.0 (SAS Institute Inc, Cary, NC, USA). The normality of the data was verified using the Shapiro–Wilk test, while homoscedasticity was verified using Levene's test. The *cumulus* expansion, *cumulus* viability, 1 PB presence, embryonic development rates were examined by Fisher's exact test, and the CEI and morphometric oocytes were examined by analysis of variance and Tukey's test. Differences were considered statistically significant when the $p < 0.05$

3. Results

After slicing 38 ovaries (Figure 1A), the number of oocytes recovered, and their morphological classification are detailed in Table 1. This table shows the proportion of each oocyte type obtained from the ovaries used in this study.

Table 1. Number and classification of oocytes recovered from Spix's yellow toothed cavy ovaries.

Experiment	No. ovaries	Classification of immature oocytes			Oocytes type A/No. Ovaries	Oocytes type B/No. Ovaries	Oocytes type C/No. Ovaries	Total Oocytes/O varies
		Type A (%)	Type B (%)	Type C (%)				
I	14	52.7 $\pm$ 5.5 (78/148)	18.9 $\pm$ 3.7 (28/148)	28.4 $\pm$ 2.1 (42/148)	5.6 $\pm$ 1.6	2.0 $\pm$ 0.0	3.0 $\pm$ 0.5	10.67 $\pm$ 2.05
II	24	44.6 $\pm$ 5.0 (107/240)	50.0 $\pm$ 2.3 (120/240)	5.4 $\pm$ 3.2 (13/240)	4.5 $\pm$ 0.2	5.0 $\pm$ 0.8	0.5 $\pm$ 0.3	10.00 $\pm$ 0.43

Note: Type A) Oocytes that had three or more layers of *cumulus* cells; Type B) These were oocytes that had between 1 – 3 layers of *cumulus* cells; Type C) Oocytes that had few or no *cumulus* cells or were naked. Experiment I: Three repetitions; Experiment II: Four repetitions.

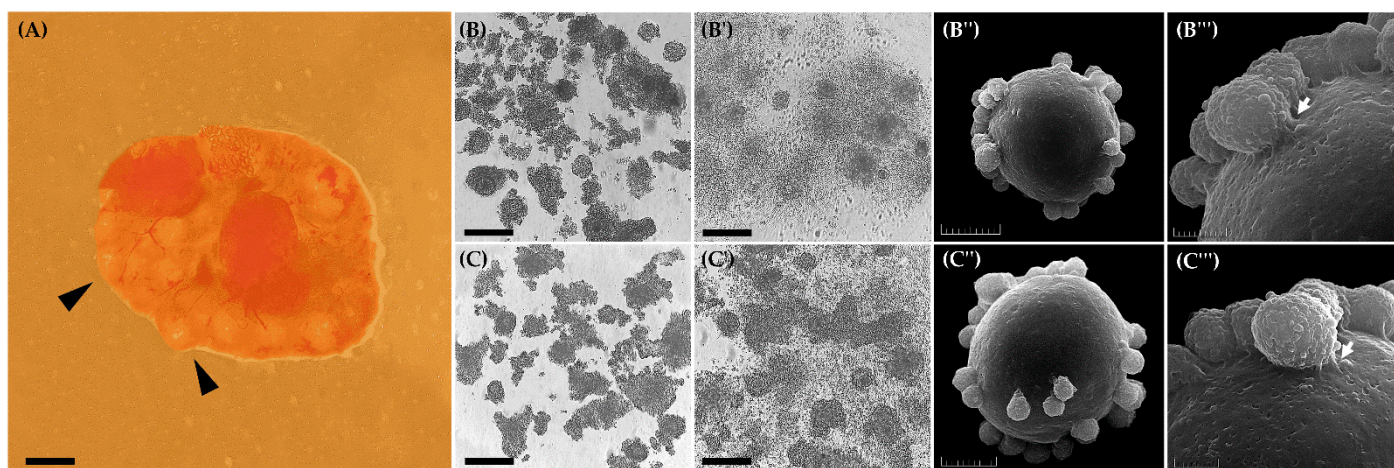


Figure 1. Representation of Spix's yellow toothed cavy ovaries and oocytes before and after IVM with EGF as a supplement to evaluate oocyte competence acquisition. (A) Ovary containing follicles (arrow-triangle) before processing by slicing. (B-B') Immature oocytes and *cumulus* cell expansion after 24 h of IVM with 10 ng/mL EGF. (B''-B''') Ultrastructural analysis showing transzonal projections (arrow) of *cumulus* cells

surrounding the oocyte under the zona pellucida in IVM medium with 10 ng/mL EGF. (C-C') Immature oocytes and *cumulus* cell expansion after 24 h of IVM with 50 ng/mL EGF. (C''-C''') Ultrastructural analysis showing transzonal projections (arrow) of *cumulus* cells surrounding the oocyte under the zona pellucida in IVM medium with 50 ng/mL EGF. Scale bar = 100 μ m (A); 50 μ m, mag. 20 $\times$ (B, B', C, C'); 20 μ m (B'', C''); 5 μ m (B''', C''').

3.1. Experiment I: Effect of EGF on *cumulus* cell expansion and ultrastructural visualization of oocytes

Figure 1 shows immature oocytes before (Figure 1B and C) and after (Figure 1B' and C') IVM with 10 and 50 ng/mL of EGF. It was observed that after the IVM process, neither concentration of EGF significantly influenced the different degrees of *cumulus* cell expansion (Table 2; $P > 0.05$). However, it is important to note that 10 ng/mL and 50 ng/mL EGF resulted in more than 40% of the evaluated oocytes achieving a score of 4. Additionally, this result was considered positive as the CEI analysis showed oocytes with indices up to 3.4, where a score of 4 represents the maximum value on the scale used to determine oocyte quality in this study (Table 2).

Table 2. Evaluation of the grade of *cumulus* cell expansion and CEI of Spix's yellow toothed cavy oocytes matured in IVM medium supplemented with two concentrations of EGF after 24 h.

Groups	No. Oocytes	Grade of <i>cumulus</i> cell expansion (%)					CEI
		Score 0	Score 1	Score 2	Score 3	Score 4	
EGF10	54	-	1.8 ± 1.5 (1/54)	13.0 ± 1.2 (7/54)	29.6 ± 4.2 (16/54)	55.6 ± 2.6 (30/54)	3.4 ± 0.1
EGF50	52	-	9.6 ± 0.3 (5/52)	11.5 ± 3.5 (6/52)	34.6 ± 9.0 (18/52)	44.2 ± 7.5 (23/52)	3.1 ± 0.1
<i>P</i>	-	-	0.1093	1.000	0.6784	0.3314	0.120

Note: CEI, *cumulus* expansion index was assessed according to a subjective scoring system, ranging from 0 to 4, considering 0 the absence of expansion and 4 the expansion of all layers. The calculation of the average expansion was performed as described by Fagbohun and Downs [23]. According to example: 55 COCs presented 20 COCs with a score of 0; 20 COCs with a score of 1; 10 COCs with a score of 2; 5 COCs with a score of 3 and 0 COCs with a score of 4, this group would present the CEI calculated: $[(0 \times 20) + (1 \times 20) + (2 \times 10) + (3 \times 5) + (4 \times 0)]/55 = 1.0$. Three repetitions.

Ultrastructural analysis of oocytes after IVM with 10 ng/mL or 50 ng/mL EGF (Figure 1B'' and C'') revealed *cumulus* cells responsible for the nutrition, growth, and gradual acquisition of machinery essential for proper oocyte development and subsequent embryonic stages. Additionally, the analysis visualized highly specialized transzonal cytoplasmic projections that penetrate the zona pellucida (Figure 1B''' and C'''). These projections form gap junctions with the oocytes, facilitating the formation of COCs, which are crucial for the acquisition of

competence and the intricate relationship between oocytes and *cumulus* cells.

3.2. Experiment I: EGF effect on viability and apoptotic levels of *cumulus* cells

Cell viability was assessed using the Trypan blue assay (Figure 2A). It was observed that the IVM medium supplemented with 10 ng/mL EGF ($93.8\% \pm 1.6$; 982/1047) ensured greater *cumulus* cell viability compared to the medium with 50 ng/mL EGF ($83.0\% \pm 1.6$; 643/775). In addition to cell viability, the apoptotic levels of *cumulus* cells were analyzed (Figure 2B and C). It was observed that in the EGF10 group ($84.9\% \pm 0.7$; 764/900), the cells were more viable compared to the EGF50 group ($78.9\% \pm 2.7$; 710/900) (Figure 2D). This difference may be attributed to the higher number of cells in early apoptosis observed in the EGF50 group ($15.1\% \pm 2.4$; 136/900) compared to the EGF10 group ($7.9\% \pm 1.1$; 71/900) after 24 h of IVM (Figure 2D). No significant differences were observed later between the groups regarding the number of cells in later apoptotic and necrosis (Figure 2D).

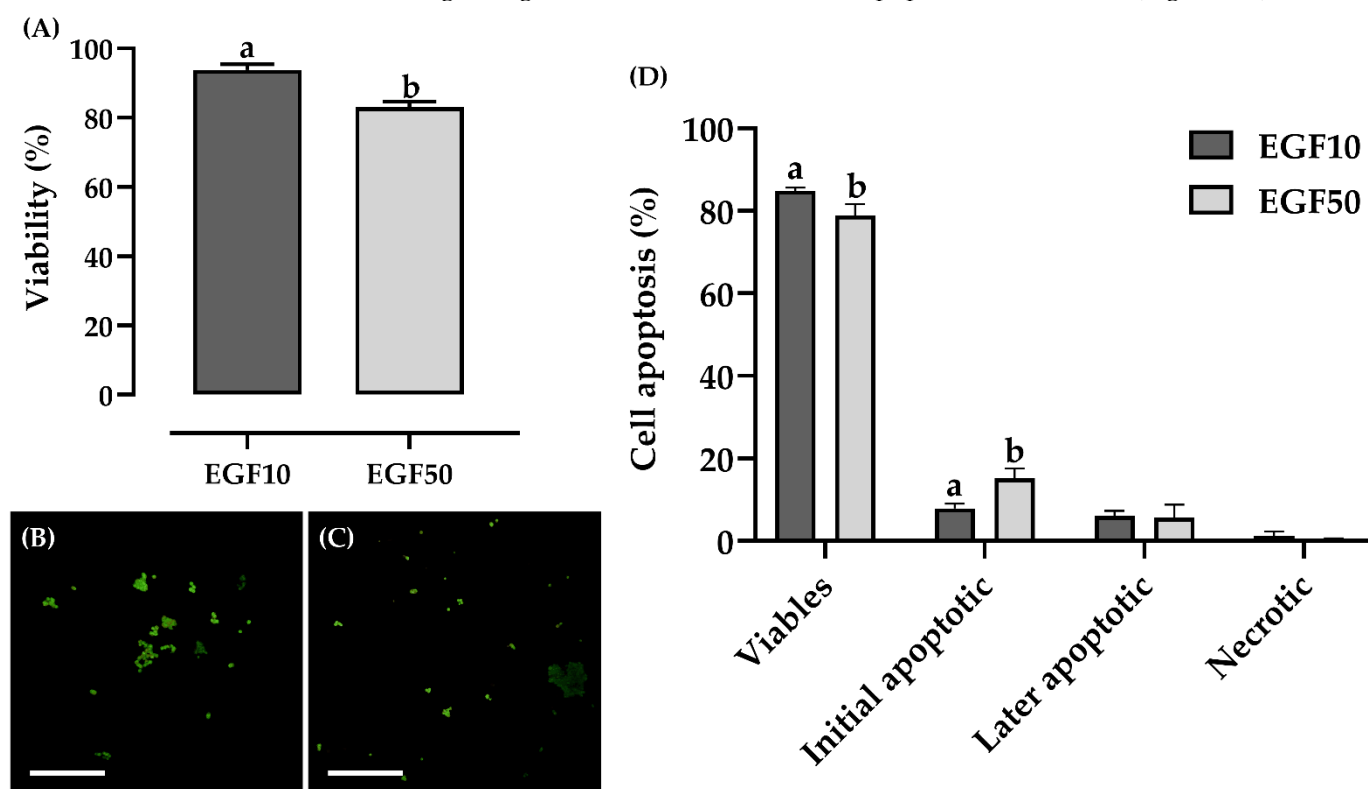


Figure 2. Assessment of viability and apoptotic levels of *cumulus* cells after 24 h of IVM of Spix's yellow toothed cavy oocytes. (A) Percentage of cell viability by Trypan blue assay. (B) Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 10 ng/mL EGF. (C) Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 50 ng/mL EGF. (D) Rate of apoptotic levels of *cumulus* cells under 10 and 50 ng/mL EGF. ^{a,b}Values with different superscript letters within columns significantly differ ($P < 0.05$). Scale bar = 50 μ m (B, C), mag. 20 $\times$. Three repetitions.

3.3. Experiment I: Effect of EGF on morphometric parameters and nuclear maturation

After 24 h of IVM, a morphometric evaluation was performed on immature oocytes and those showing extrusion of the 1PB (Figure 3A and B). The results are presented in Table 3. It was observed that oocytes matured in IVM medium supplemented with 10 ng/mL EGF had a larger diameter compared to those supplemented with 50 ng/mL EGF, regarding IOD ($78.8 \pm 5.2 \mu\text{m}$ vs. $74.9 \pm 2.6 \mu\text{m}$), ZPT ($9.4 \pm 0.8 \mu\text{m}$ vs. $8.5 \pm 0.9 \mu\text{m}$), and OD ($69.6 \pm 4.2 \mu\text{m}$ vs. $64.8 \pm 2.8 \mu\text{m}$). Consequently, oocytes matured with 10 ng/mL EGF exhibited a larger IOA ($15502 \pm 2466 \mu\text{m}^2$ vs. $13877 \pm 957 \mu\text{m}^2$) and OA ($12058 \pm 1630 \mu\text{m}^2$ vs. $10412 \pm 901 \mu\text{m}^2$) compared to those with 50 ng/mL EGF.

Regarding the evaluation of nuclear maturation, the extrusion rate of the 1PB was observed, as well as the analysis of the nuclear stage of oocytes matured *in vitro* (Figure 3B, C, and D). In this context, it was observed that there was no difference between oocytes subjected to *in vitro* maturation with 10 ng/mL EGF ($64.8\% \pm 2.4$; 35/54) and 50 ng/mL EGF ($55.8\% \pm 1.7$; 29/52) after 1PB extrusion. Additionally, no differences were observed between the groups after evaluating the nuclear stage ($63.0\% \pm 2.9$; 34/54 vs. $51.9\% \pm 1.5$; 27/52) (Figure 3D). Although this was the case, it is important to note that the results obtained in this study were promising and represent a significant step towards implementing *in vitro* techniques in this species.

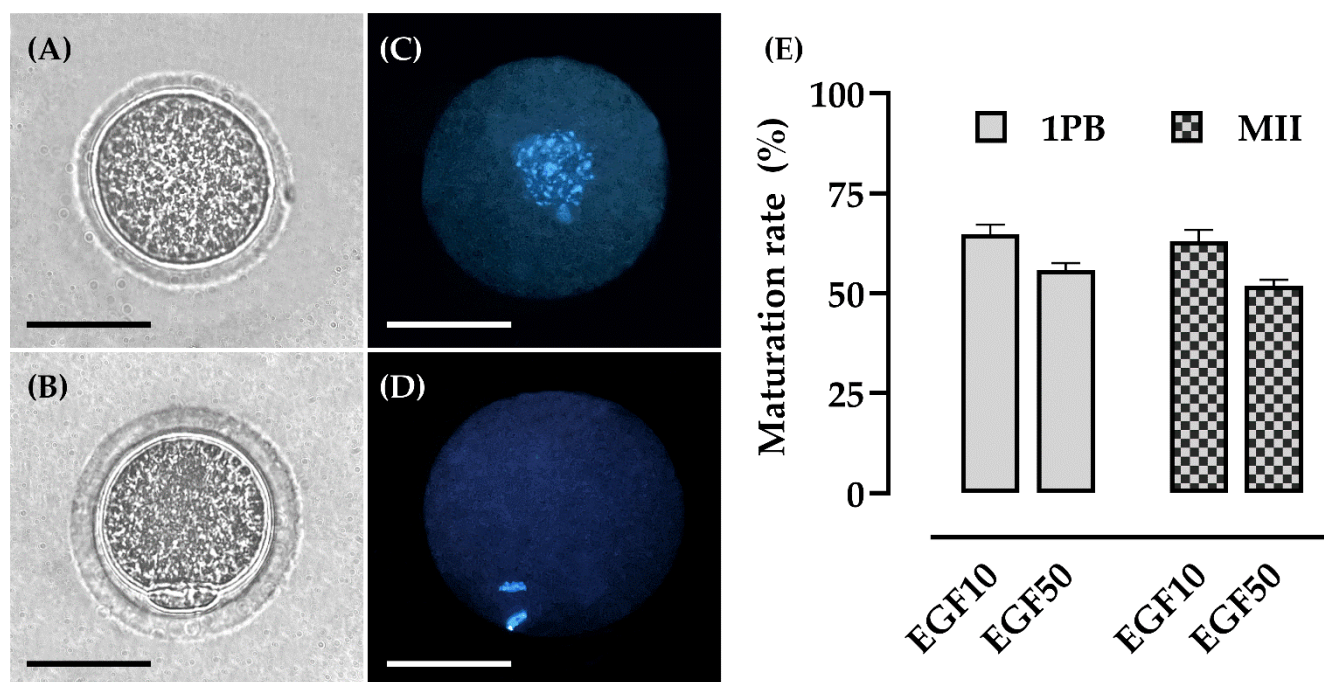


Figure 3. Representative image of Spix's yellow toothed cavy oocytes before and after 24 h of IVM containing different concentrations of EGF. (A) Immature oocyte. (B) Oocyte after 1PB extrusion. (C) Oocyte in germinal vesicle breakdown (GVBD) configuration. (D) Oocyte in metaphase II. (E) Percentage of oocyte rate after nuclear maturation assessment. Scale bar = 100 μm , mag. 20 $\times$ (A, B, C, D). Three repetitions.

Table 3. Morphometric evaluation of immature and matured Spix's yellow toothed cavy oocytes after 24 h of IVM supplemented with EGF.

Groups	1PB	No. Oocytes	OOD (μm)	IOD (μm)	ZPT (μm)	OD (μm)	SPD (μm)	IOA (μm^2)	OA (μm^2)	PSA (μm^2)
EGF10	-	19	92.8 $\pm$ 4.5 ^a	76.4 $\pm$ 2.9 ^{ab}	9.3 $\pm$ 0.5 ^{ab}	69.2 $\pm$ 2.4 ^a	7.2 $\pm$ 2.3 ^a	14431 $\pm$ 1087 ^{ab}	11833 $\pm$ 805 ^{ab}	2598 $\pm$ 840 ^a
	+	35	95.7 $\pm$ 6.9 ^a	78.8 $\pm$ 5.2 ^a	9.4 $\pm$ 0.8 ^a	69.6 $\pm$ 4.2 ^a	9.2 $\pm$ 3.4 ^a	15502 $\pm$ 2466 ^a	12058 $\pm$ 1630 ^a	3443 $\pm$ 1437 ^a
EGF50	-	23	93.9 $\pm$ 0.2 ^a	76.7 $\pm$ 2.6 ^{ab}	8.9 $\pm$ 0.8 ^{ab}	69.0 $\pm$ 3.3 ^a	7.7 $\pm$ 2.7 ^a	14564 $\pm$ 1021 ^{ab}	11813 $\pm$ 1101 ^{ab}	2751 $\pm$ 941 ^a
	+	29	94.3 $\pm$ 1.7 ^a	74.9 $\pm$ 2.6 ^b	8.5 $\pm$ 0.9 ^b	64.8 $\pm$ 2.8 ^b	10.1 $\pm$ 2.7 ^a	13877 $\pm$ 957 ^b	10412 $\pm$ 901 ^b	3464 $\pm$ 926 ^a

Note: ^{a,b}Values with different superscript letters within columns significantly differ ($P < 0.05$). OOD, outer oocyte diameter; IOD, inner oocyte diameter; ZPT, zona pellucida thickness; OD, ooplasm diameter; SPD, space perivitelline diameter; IOA, internal oocyte area; OA, ooplasm area; PSA, perivitelline space area. The morphometric calculation for each parameter evaluated on the oocytes was performed as described by Saadeldin et al. [43]. Three repetitions.

3.4. Experiment II: Assessment of the microenvironment and quality of oocytes after IVM

Considering the benefits of EGF10 observed in the previous experiment, oocytes used in the second experiment were subjected to IVM in medium supplemented with 10 ng/mL EGF. To ensure the appropriate microenvironment and nuclear maturation of the oocytes that were artificially activated, cells were analyzed across four replications for expansion grade, viability, and apoptotic levels of *cumulus* cells. Additionally, 1PB extrusion was observed, and cells exhibiting this structure were subjected to artificial activation to evaluate embryonic development rates over five days.

The results regarding the grade of expansion of *cumulus* cells and CEI can be seen in Table 4. Concerning viability, as assessed by the trypan blue assay, oocytes matured in the presence of 10 ng/mL EGF exhibited a viability rate greater than 90% (2970/3279). Additionally, 97% (878/900) of *cumulus* cells were viable following the apoptotic assay. Finally, regarding 1PB extrusion, oocytes matured with EGF10 showed a rate of 64% (146/227). These oocytes were then randomly subdivided to evaluate the effect of the presence or absence of 6-DMAP, as per the artificial activation protocol previously described in the experimental design.

Table 4. Rate expansion of *cumulus* cells and CEI of *G. spixii* oocytes after 24 h of IVM in medium supplemented with 10 ng/mL of EGF.

Group	No. Oocytes	Grade of <i>cumulus</i> cell expansion (%)					CEI
		Score 0	Score 1	Score 2	Score 3	Score 4	
EGF10	227	1.3 $\pm$ 0.9 (3/227)	6.6 $\pm$ 1.2 (15/227)	21.1 $\pm$ 3.9 (48/227)	27.1 $\pm$ 3.4 (66/227)	41.9 $\pm$ 2.9 (95/227)	3.0 $\pm$ 0.03

Note: CEI, *cumulus* expansion index was assessed according to a subjective scoring system, ranging from 0 to 4, considering 0 the absence of

expansion and 4 the expansion of all layers. The calculation of the average expansion was performed as described by Fagbohun and Downs [23]. According to example: 55 COCs presented 20 COCs with a score of 0; 20 COCs with a score of 1; 10 COCs with a score of 2; 5 COCs with a score of 3 and 0 COCs with a score of 4, this group would present the CEI calculated: $[(0 \times 20) + (1 \times 20) + (2 \times 10) + (3 \times 5) + (4 \times 0)]/55 = 1.0$. Four repetitions.

3.5. Experiment II: Effect of 6-DMAP on activation and kinetics of embryonic development

After artificial activation, it was observed through analysis of embryonic development kinetics and ultrastructure (Figure 4) that zygotes contained 2-cell and 3-7 cell cleavages (Figure 4A and B). Additionally, the digestion of the zona pellucida in the zygotes was visualized after sample processing by SEM (Figure 4A'). The ultrastructural analysis of the zygotes revealed the external surfaces of the blastomeres, which were spherical and characterized by a uniform presence of microvilli (Figure 4A' and B'). These microvilli function to increase the surface area for greater absorption of the surrounding medium. During the evaluation of embryonic development kinetics over five days, artificially activated oocytes achieved cleavages beyond 8 cells (Figure 5A). For the first time in this species, compacted morulae were also obtained (Figure 5B). Regarding embryonic development kinetics, 6-DMAP did not affect the number of cleavages in 2-cell, 3-7 cell, and >8 cell zygotes (Figure 5C). However, the presence of 6-DMAP ($69.3\% \pm 5.0$; 52/75) positively influenced the total number of cleaved zygotes compared to the group without the secondary activator ($53.5\% \pm 3.5$; 38/71) (Figure 5D), resulting in the production of compacted morulae at

rates of $13\% \pm 3.1$ (5/38) in the group without 6-DMAP and $17\% \pm 2.9$ (9/52) in the group supplemented with 6-DMAP (Figure 5D).

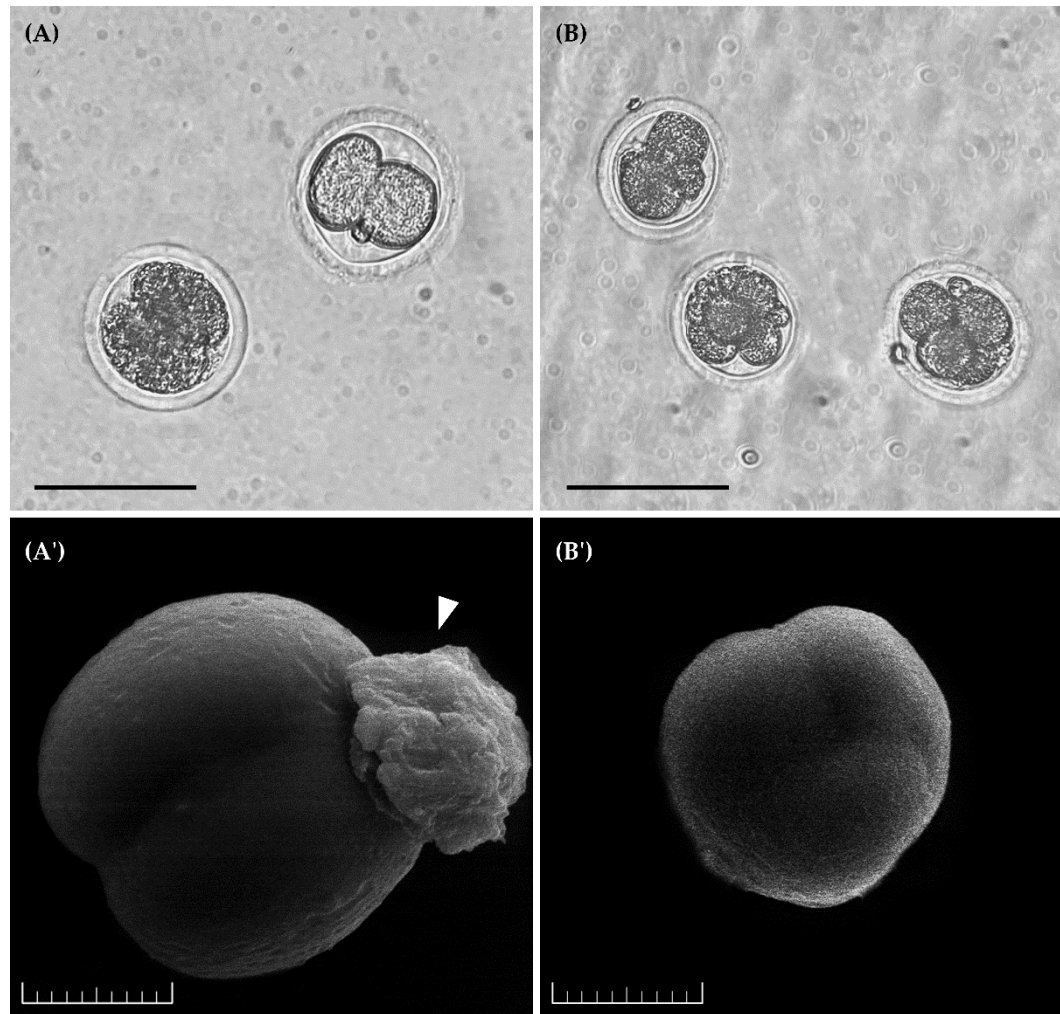


Figure 4. Representative image of cleaved zygotes after artificial activation and two days of embryonic culture. (A) Zygotes with 2 cleavages. (B) Zygotes with 3-7 cleavages. (A') Ultrastructure of zygotes with two cleavages. The arrow-triangle indicates remains of the degraded zona pellucida after processing for visualization in SEM. (B') Ultrastructure of cleavages containing three blastomeres and their microvilli. Scale bar = 100 μm , mag. 20 $\times$ (A, B); 20 μm (A', B'). Four repetitions.

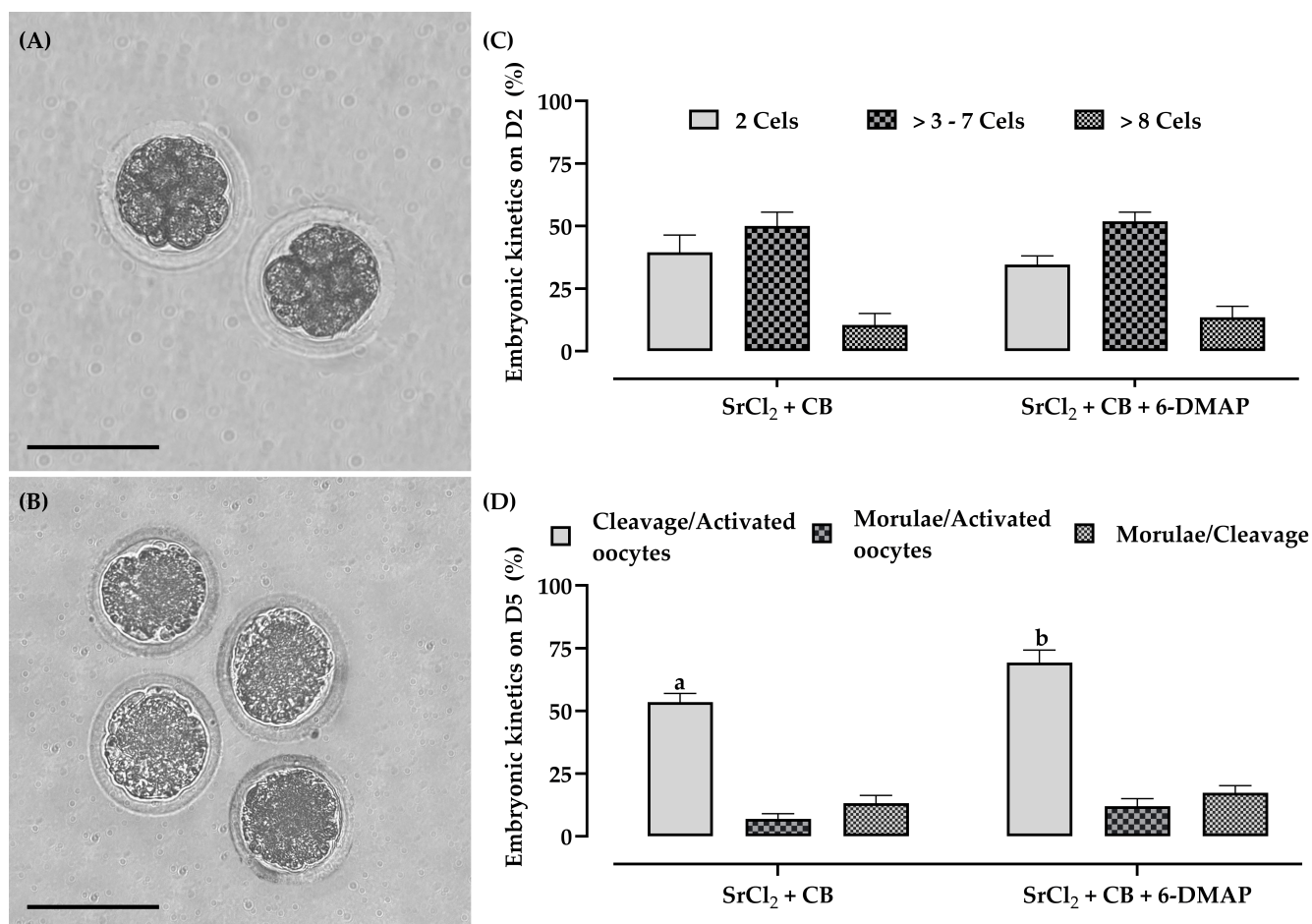


Figure 5. Embryonic development rate of artificially activated *G. spixii* oocytes. (A) Zygotes with cleavages >8 cells after two days of embryonic culture. (B) Compacted morulae after five days of embryonic culture. (C) Percentage of cleaved zygotes after two days of *in vitro* culture. (D) Percentage of cleavages with more than 8 blastomeres and production of morulae. Scale bar = 100 μ m, mag. 20 $\times$ (A, B). ^{a,b}Values with different superscript letters within columns significantly differ ($P < 0.05$).

4. Discussion

We established, over seven repetitions, *in vitro* manipulation strategies for Spix's yellow toothed cavy oocytes and embryos, evaluating the effect of EGF at two concentrations (10 or 50 ng/mL) during IVM. We analyzed the influence of EGF on the quality of the microenvironment and nuclear maturation of oocytes. EGF interacts with specific receptors that play essential roles in the endocrine, paracrine, and autocrine processes of oocytes and surrounding cells, positively influencing differentiation, communication, and nuclear and cytoplasmic maturation [15,16]. After selecting the optimal EGF concentration, we assessed the matured structures using a established artificial activation protocol for red-rumped agoutis, mice, and rats [7,18]. At this stage, we analyzed the influence of the presence or absence of 6-DMAP, a molecule that inhibits serine/threonine kinases and seems to enhance the efficiency of artificial activation for greater *in vitro* embryo production [29,30].

Regarding *cumulus* cell expansion, our study observed that both EGF concentrations of 10 ng/mL and 50 ng/mL resulted in more than 40% expansion of the *cumulus* oophorus complex and the corona radiata, achieving a Score of 4. This was subsequently justified by the CEI values, which ranged between 3.1 and 3.4. In contrast, a study by Praxedes et al. [7] with red-rumped agouti demonstrated that 10 ng/mL EGF in conjunction with 10 mIU/mL FSH resulted in a $31.8\% \pm 1.3$ rate of oocytes with Score 3 and $15.9\% \pm 0.9$ with Score 4, reaching a CEI of up to 3.6. This discrepancy may be attributed to the fact that, although phylogenetically close, there are intrinsic biological and reproductive differences between these species [31,32]. In Spix's yellow toothed caviar, EGF may be more efficiently utilized for *cumulus* cell expansion without affecting the CEI, whereas in red-rumped agouti, there may be lower efficiency in the activation of these molecules at this concentration. For example, in mice, when paracrine and juxtacrine components of the ovulatory signal were inactivated by knockout of EGF-like transmembrane peptides AREG and EREG [33,34], reduced efficiency in *cumulus* cell expansion and oocyte maturation was observed.

Another justification is that while FSH exerts an inhibitory effect on the initial resumption of meiosis, it induces the final stages of meiotic maturation [22]. This is mediated through the levels of expression of natriuretic peptide precursor type C (NPPC) in *cumulus* cells and the positive regulation by natriuretic peptide receptor 2 (NPR2), influenced by the actions of FSH and EGF. The oocytes in these species may respond differently to the treatments observed in each species [35], which could affect *cumulus* cell expansion. For example, in red-rumped agouti, a higher rate of Score 3 was observed compared to Score 4 and when compared to Spix's yellow toothed caviar. For this reason, we evaluated Spix's yellow toothed caviar oocytes in our study for their ultrastructural characteristics, viability, and apoptotic levels as quality indicators of the *in vitro* environment.

Regarding viability after Trypan blue staining, oocytes in medium supplemented with 10 ng/mL EGF had a higher rate of viable cells compared to 50 ng/mL EGF. Consequently, we performed an analysis of apoptotic levels of these cells using ethidium bromide and acridine orange assays. In this context, we observed that the medium supplemented with 50 ng/mL EGF showed a higher initial apoptosis index in *cumulus* cells compared to 10 ng/mL EGF. This result may reflect the metabolic activity of oocyte mitochondria involved in the production of reactive oxygen species (ROS), leading to a higher incidence of apoptosis in *cumulus* cells [36]. Therefore, a better antioxidant supplementation than the cysteamine used in our study may be needed.

Additionally, during the acquisition of meiotic competence of mouse oocytes *in vitro*, EGF promotes a significant and rapid increase in phosphorylation within the first 30 min of the event compared to oocytes matured *in vivo* [37]. This suggests that this metabolic activity may lead to increased ATP production, negatively affecting the quality of *cumulus* cells through subsequent ROS molecule synthesis [38]. Consequently, this results in a lower cellular viability rate, as observed after Trypan blue staining and apoptosis assays. Another associated reason is that it has

been demonstrated *in vitro* that *cumulus* cells can also be affected by the accumulation of glycosylations derived from transmembrane proteins (AREG, EREG, and BTC) in response to EGF [39,40]. Therefore, it is possible that an excess of these factors could cause significant damage to the *cumulus* cells of Spix's yellow toothed cavy oocytes.

Although supplementation with 50 ng/mL EGF reduced the viability rate of *cumulus* cells, our study did not observe any morphological changes from the SEM analysis. Our results revealed that oocytes matured under both EGF supplementation conditions maintained the necessary machinery for development and oocyte competence. This is justifiable as we could observe the highly specialized transzonal cytoplasmic projections of *cumulus* cells penetrating the zona pellucida of Spix's yellow toothed cavy oocytes [15]. This likely ensures the interface and intricate bidirectional communication between the microenvironment and the oocyte, which are crucial for providing the necessary nutrition and support for meiotic acquisition [15]. This result is corroborated by our study, where, in the initial attempt to perform IVM of Spix's yellow toothed cavy oocytes using EGF as a supplement, we observed nuclear maturation rates ranging from 55% to 64% for the extrusion of the 1PB, while 51% to 63% corresponded to the metaphase II nuclear stage. These rates are similar when compared to guinea pigs [11,17], paca (*Cuniculus paca*) [41], *D. prymnolopha* [42], and red-rumped agouti [7].

In addition to the parameters analyzed, we performed, for the first time, morphometric evaluations on Spix's yellow toothed cavy oocytes regarding structures that exhibited or did not exhibit the extrusion of the 1PB after IVM with 10 or 50 ng/mL EGF. According to Saadeldin et al. [43], assessing oocyte morphometry can also be a decisive step in evaluating the quality and meiotic competence for adequate embryonic development following artificial activation. Thus, a larger ooplasm diameter (OD) and a smaller space perivitelline diameter (SPD) are expected to indicate better oocyte quality [43]. In our study, we initially observed that Spix's yellow toothed cavy oocytes exhibited a diameter ranging between 92.8 and 94.3 μm . Additionally, after IVM, oocytes matured in media containing 10 ng/mL EGF demonstrated a larger OD compared to those matured with 50 ng/mL EGF, although no significant difference was observed in SPD. Notably, this result is interesting because, in *in vivo* systems, oocytes from small antral follicles are usually not responsive to EGF signaling cascades [44], whereas COCs from Graafian follicles can respond to progressively increasing EGF levels, enabling better embryonic development [45,46]. Therefore, the acquisition of EGF signaling capability by *cumulus* cells in relation to oocytes aligns with the mechanisms governing oocyte meiotic competence, and these processes are likely interrelated [46,47].

Based on the previous justifications, we selected 10 ng/mL EGF as the ideal concentration for use during the IVM of Spix's yellow toothed cavy oocytes and considered this concentration for evaluating artificial activation in the presence or absence of 6-DMAP. Initially, regarding the artificial activation protocol for embryonic development, we chose 10 mM SrCl_2 , which could induce repetitive intracellular calcium releases, similar to what occurs during *in vitro* fertilization in mice and rats, which are laboratory rodents phylogenetically close to Spix's yellow toothed cavies.

In these species, 10 mM SrCl_2 has mimicked natural conditions similar to the role of spermatozoa on the oocyte, providing an opportunity for the reconstruction of cloned embryos or in physiological studies [48,49]. Additionally, in our study, we also used 5 $\mu\text{g/mL}$ CB. This compound is included during cloning and prevents the formation and extrusion of the second polar body, resulting in diploid embryos. In mice, its addition has significantly enhanced blastocyst development compared to artificial activation without this compound [18]. This occurs because, in diploid mouse embryos, a lower incidence of apoptosis is observed compared to haploid embryos [50]. Therefore, we hypothesize that its addition could improve the quality of the embryos produced in our experiment.

In our study, we also evaluated the presence or absence of 6-DMAP as a secondary activator. The addition of this activator was based on previous observations that the inclusion of 6-DMAP in mouse activation could enhance the efficiency of artificial activation, achieving blastocyst rates of up to 60%. This secondary activator may reduce the activity of the meiosis-promoting factor (MPF), likely improving process efficiency. In our protocol, which combined 10 mM SrCl_2 for 2.5 hours followed by 5 + 2 mM 6-DMAP for 3.5 hours (SrCl_2 +CB+6-DMAP), we observed a higher overall cleavage rate compared to the group without 6-DMAP, although only a 13% to 17% rate of compact morulae was noted. This outcome might be due to the fact that while Spix's yellow toothed cavies is phylogenetically close to other laboratory and wild rodents, there are still gaps in our understanding of reproductive techniques in this species. Therefore, implementing optimized protocols by improving gas conditions, temperature, supplements, and IVM duration could be a potential alternative for enhancing this step in this species. The previously described observation could justify the need for further investigation, as studies with mice have shown that treatment with chorionic gonadotropin hormones (hCG) between 13 and 18 could result in 30% to 60% artificial activation rates of mature oocytes (Ma et al. 2005). Hence, further investigation into this process is warranted.

5. Conclusions

This was the first study to establish the steps involved in the *in vitro* manipulation of oocytes and embryos of Spix's yellow toothed cavies aimed at multiplication, conservation, and reproductive investigation of the species. We concluded that EGF supplemented with 10 ng/mL is the ideal concentration for use in oocyte IVM, allowing for greater viability of *cumulus* cells and a lower incidence of cellular apoptosis. Additionally, EGF10 also ensured better morphometric quality of the analyzed structures and achieved 60% nuclear maturation.

Furthermore, we observed that under a protocol of artificial activation typically employed in laboratory rodents, we produced morulae for the first time, although optimization of this step is still needed. With this information, new approaches related to the development of reproductive biotechnologies associated with the conservation and multiplication of Spix's yellow toothed cavies can be undertaken, enabling advancements in ARTs.

Author Contributions: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft, L.A.; Methodology, S.O.; Methodology, Y.L.S.; Methodology, V.S.; Methodology, L.O.; Methodology, Investigation, M.O.; Conceptualization, Formal analysis, Investigation, Data curation, Writing – Review & Editing, Supervision, Funding acquisition, A.P.

Funding: This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil (CNPq, no. 309078/2021-0).

Institutional Review Board Statement: All procedures were approved by the UFERSA Animal Ethics Committee, Opinion no. 23091.010566/2017-20, and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The authors thank the Center for Wild Animals Multiplication (CEMAS/UFERSA) for providing the biological samples used in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

1PB	First polar body
6-DMAP	6-dimethylaminopurine
ARTs	Assisted reproductive technologies
BSA	BSA bovine serum albumin
CB	Cytochalasin B
CEI	<i>Cumulus</i> expansion index
CEMAS	Centre of Multiplication of Wild Animals
CEUA	Animal Use Ethics Committee
COCs	<i>Cumulus</i> -oocyte complex
EGF	Epidermal growth factor
GVBD	Oocyte in germinal vesicle breakdown
hCG	Chorionic gonadotropin hormones
IOA	Internal oocyte area
IOD	Inner oocyte diameter
IVM	<i>In vitro</i> maturation
KSOM	Simple optimized and potassium-enriched medium
MPF	Meiosis-promoting factor
OA	Ooplasm area
OD	Ooplasm diameter
OOD	Outer oocyte diameter
ORM	Oocyte recovery medium
PBS	Phosphate buffer solutions
PSA	Perivitelline space area
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer

SEM	Scanning electron microscopy
SPD	Space perivitelline diameter
SrCl ₂	Strontium chloride
TALP	Tyrod's albumin lactate pyruvate
UFERSA	Federal Rural University of Semi-Arid

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7 CONSIDERAÇÕES FINAIS

Os resultados obtidos nesta tese representam avanços consistentes na compreensão dos fatores que determinam a qualidade, a estabilidade e a competência funcional de estruturas celulares, como os fibroblastos, oócitos e embriões de *G. spixii*. Ao abordar de forma integrada o cultivo, a criopreservação e a manipulação funcional dessas células, o estudo estabelece bases técnicas e conceituais para o uso de estratégias celulares e reprodutivas *in vitro* aplicadas ao manejo genético, à conservação *ex situ* e a abordagens de reprogramação celular na espécie. Os capítulos apresentados constituem, assim, pilares estruturantes para o desenvolvimento progressivo de biotecnologias reprodutivas em *G. spixii*, com potencial de aplicação para espécies filogeneticamente próximas. Portanto:

CAPÍTULO 2 – CARACTERIZAÇÃO MORFOLÓGICA, IMUNOCITOQUÍMICA E FISIOLÓGICA DE FIBROBLASTOS DE *G. spixii*: A padronização das condições de cultivo e caracterização dos fibroblastos adultos de *G. spixii* revelou que linhagens até a 5ª passagem apresentam maior estabilidade morfológica, genética e fisiológica. Além disso, a criopreservação (10% de DMSO e 0,2 M de sacarose) provocou alterações ultraestruturais, redução de viabilidade e aumento de estresse celular, demonstrando a necessidade do desenvolvimento de protocolos otimizados e espécie-específicos para a formação de biobancos somáticos de *G. spixii*.

CAPÍTULO 3 – EFEITO DA L-PROLINA NA CRIOPRESERVAÇÃO E FORMAÇÃO DE UM BIOBANCO CELULAR PARA *G. spixii*: A introdução da L-prolina em meios de congelamento lento mostrou-se uma inovação para roedores neotropicais, produzindo melhor equilíbrio osmótico, menor produção de espécies reativas de oxigênio, menor dano fisiológico e maior atividade mitocondrial após a descongelamento. Esses resultados consolidam a L-prolina como um aditivo promissor para criopreservação de células somáticas de *G. spixii*, contribuindo diretamente para o desenvolvimento de biobancos e para aplicações biotecnológicas avançadas.

CAPÍTULO 4 – EFEITO DAS TÉCNICAS DE SINCRONIZAÇÃO SOBRE O CICLO CELULAR DE FIBROBLASTOS DE *G. spixii*: A roscovitina, especialmente nas concentrações de 7,5 μ M e 10 μ M por 12 h, mostrou-se o protocolo de curto prazo mais seguro e eficiente, promovendo elevadas taxas de sincronização com efeitos mínimos sobre a viabilidade. Em contraste, a exposição a 15 μ M intensificou efeitos deletérios, evidenciando

que o aumento da concentração pode comprometer a estabilidade fisiológica das células. A privação de soro, por sua vez, apresentou eficácia restrita até 24 h, uma vez que períodos prolongados comprometeram severamente a morfologia celular, elevaram o estresse oxidativo e ativaram vias de morte celular programada.

CAPÍTULO 5 – COMPREENDENDO A COMPETÊNCIA DOS OÓCITOS PARA MELHORAR A PRODUÇÃO DE EMBRIÕES *IN VITRO* EM ROEDORES CAVIIDAE:

A competência oocitária em roedores da família Caviidae configura-se como um processo complexo, gradual e finamente regulado, estabelecido ao longo da foliculogênese e fortemente dependente das interações entre o oócito e as células somáticas que o circundam. As evidências reunidas indicam que as limitações observadas na eficiência da produção *in vitro* de embriões decorrem, sobretudo, da aquisição incompleta da competência nuclear, citoplasmática e metabólica. Indicadores morfológicos, funcionais e moleculares mostram-se complementares na identificação de oócitos com maior potencial de desenvolvimento. A ampliação das investigações para outros caviídeos é fundamental para o refinamento de estratégias reprodutivas. Esse progresso contribuirá para o aumento da eficiência da produção *in vitro* de embriões para ações de conservação e para a consolidação desses roedores como modelos em biotecnologia reprodutiva.

CAPÍTULO 6 – EFEITO DO EGF E 6-DMAP SOBRE A QUALIDADE DE MATURAÇÃO OOCITÁRIA E PRODUÇÃO *IN VITRO* DE EMBRIÕES DE *G. spixii*:

A avaliação da competência oocitária de *G. spixii* demonstrou que o tratamento com 10 ng/mL de EGF proporciona melhor viabilidade das células do *cumulus*, maior integridade morfológica do oócitos e taxas mais elevadas de maturação nuclear. A ativação artificial com SrCl₂, citocalasina B e 6-DMAP durante 6 h resultou na melhor taxa de clivagem registrada para a espécie (69,3% ± 5,0%), representando um marco para a aplicação de protocolos de ativação oocitária em Caviidae. Esses achados fortalecem a base necessária para obtenção de oócitos competentes para a produção *in vitro* de embriões.

8 PERSPECTIVAS

Os resultados desta tese ampliam de forma consistente as possibilidades de aplicação de estratégias celulares e reprodutivas *in vitro* no contexto da conservação genética e do manejo biotecnológico de *G. spixii*. A partir da padronização de protocolos para obtenção, cultivo, sincronização e criopreservação de células somáticas, bem como do estabelecimento de condições para a maturação e ativação *in vitro* de oócitos, torna-se possível estruturar uma base técnica sólida para a utilização dessas células como ferramentas funcionais em diferentes abordagens de reprodução assistida e conservação *ex situ*.

Nesse contexto, uma perspectiva aplicada imediata consiste na avaliação experimental da integração entre carioplastos e citoplastos obtidos sob condições otimizadas, permitindo investigar a capacidade de reconstrução embrionária e os estágios iniciais do desenvolvimento *in vitro*, visando a clonagem por TNCS. Essas análises possibilitarão compreender, de forma controlada, a eficiência das estratégias celulares empregadas, bem como os limites biológicos associados à compatibilidade núcleo-citoplasma, com ênfase nas dinâmicas epigenéticas do núcleo transferido e nos eventos iniciais de ativação do genoma embrionário. Adicionalmente, a recuperação e obtenção de oócitos competentes representam etapas também importantes para a produção *in vitro* de embriões por fertilização *in vitro*.

Paralelamente, as linhagens fibroblásticas estabelecidas e funcionalmente caracterizadas nesta tese configuram um recurso estratégico para abordagens adicionais de reprogramação celular, incluindo a indução de pluripotência e aplicações futuras em gametogênese *in vitro*. Essas perspectivas ampliam o uso de *G. spixii* como modelo experimental em biologia reprodutiva comparada, ao mesmo tempo em que fortalecem o potencial de aplicação dessas tecnologias em programas de conservação genética. Os protocolos de criopreservação desenvolvidos consolidam ainda o papel dessas linhagens celulares como componentes de biobancos somáticos, capazes de sustentar estratégias de manejo reprodutivo e conservação a médio e longo prazo, especialmente em ecossistemas vulneráveis como a Caatinga.

Adicionalmente, estudos comparativos envolvendo outras espécies da família Caviidae e do grupo Hystricomorpha poderão elucidar particularidades celulares e reprodutivas relevantes à reprogramação nuclear, contribuindo para o refinamento de abordagens espécie-específicas. Em síntese, o uso integrado de células somáticas, oócitos e embriões como ferramentas biotecnológicas, associado ao avanço de técnicas reprodutivas *in vitro*, configura um caminho promissor para ampliar o entendimento sobre a plasticidade celular de *G. spixii* e

para viabilizar a aplicação prática dessas tecnologias em estratégias de conservação genética e reprodução assistida de roedores histricomorfos.

9 ANEXOS

ANEXO A – CERTIFICADO DE AUTORIZAÇÃO DO PROJETO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UFERSA (CEUA/UFERSA)



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

Mossoró, 28 de setembro de 2017.

PARECER

O projeto intitulado “*Caracterização morfológica de tecidos somáticos derivados de preás (Galea spixii Wagler, 1831)*” protocolo n. 23091.010566/2017-20 sob a responsabilidade de *Alexsandra Fernandes Pereira*, o qual envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da lei 11.794 de 8 de outubro de 2009 e com as normas editadas pelo Conselho Nacional de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal Rural do Semi-Arido –UFERSA em reunião de 26/09/2017 com o parecer número 18/2017.

Vigência do projeto	Outubro 2017 a setembro de 2019
Espécie/linhagem	Galea spixii
N. de Animais	08
Peso/idade	6 meses, 2kg
Sexo	Machos e Fêmeas
Origem	CEMAS UFERSA

Emanuelle Fontenele Rabelo
Emanuelle Fontenele Rabelo

Presidente CEUA-UFERSA

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



Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 60428-1	Data da Emissão: 11/10/2017 18:26	Data para Revalidação*: 10/11/2018
* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Alessandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: CARACTERIZAÇÃO MORFOLÓGICA DE TECIDOS SOMÁTICOS DERIVADOS DE PREÁS (<i>Galea spixii</i> WAGLER, 1831)	
Nome da Instituição : UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Envio para o CEUA	10/2017	10/2017
2	Treinamentos	10/2017	12/2017
3	Revisão de Literatura	10/2017	09/2019
4	Seleção de animais	01/2018	02/2018
5	Obtenção de amostras teciduais (n=4)	03/2018	10/2018
6	Processamento histológico	03/2018	12/2018
7	Análise histológica	03/2018	12/2018
8	Obtenção de amostras teciduais (n=4)	01/2019	10/2019
9	Processamento histológico e análises	01/2019	10/2019
10	Envio de resumos e artigos	11/2019	12/2019
11	Análise dos resultados	11/2019	12/2019
12	Envio de relatórios	12/2019	12/2019

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NAO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
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6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen .
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	MOACIR FRANCO DE OLIVEIRA	Pesquisador	325.949.504-59	552984 SSP-RN	Brasileira
2	Alexandre Rodrigues Silva	Pesquisador	702.982.543-87	91002308901 SSP-CE-CE	Brasileira

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Página 1/3



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Dados do titular

Nome: Alexsandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: CARACTERIZAÇÃO MORFOLÓGICA DE TECIDOS SOMÁTICOS DERIVADOS DE PREÁS (<i>Galea spxii</i> WAGLER, 1831)	
Nome da Instituição : UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	MOSSORO	RN	Centro de Multiplicação de Animais Silvestres	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas ex situ	<i>Galea spxii</i>

Material e métodos

1	Amostras biológicas (Outros mamíferos)	Fragmento de tecido/órgão
2	Método de captura/coleta (Outros mamíferos)	Puça
3	Método de marcação (Outros mamíferos)	"Tags"

Destino do material biológico coletado

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

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Página 2/3



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Dados do titular

Nome: Alexandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: CARACTERIZAÇÃO MORFOLÓGICA DE TECIDOS SOMÁTICOS DERIVADOS DE PREÁS (<i>Galea spixii</i> WAGLER, 1831)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

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Página 3/3

ANEXO C – VERSÃO DO ARTIGO EXPERIMENTAL I PUBLICADO NA ACTA HISTOCHEMICA

Acta Histochemica 126 (2024) 152185



Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Cryopreservation and passaging optimization for *Galea spixii* (Wagler, 1831) adult skin fibroblast lines: A step forward in species management and genetic studies

Leonardo Vitorino Costa de Aquino^a, Samara Lima Olindo^a, Yara Letícia Frutuoso e Silva^a,
Lhara Ricarliany Medeiros de Oliveira^a, Yasmin Beatriz França Moura^a,
Ana Lívia Rocha Rodrigues^a, Érika Almeida Praxedes^a, Moacir Franco de Oliveira^b,
Alexandre Rodrigues Silva^c, Alexsandra Fernandes Pereira^{a,*}

^a Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

^b Laboratory of Applied Animal Morphophysiology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

^c Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

ARTICLE INFO

Keywords:

Rodentia
in vitro culture
Slow freezing
Cell characterization

ABSTRACT

Background: *In vitro* culture of fibroblasts is a technique based on cell isolation, physiological characterization, and cryopreservation. This technique has not been described for *Galea spixii*, therefore, it can be used to learn about its cellular biology and genetic diversity.

Objective: We established fibroblast lines of six *G. spixii* individuals from several passages (second, fifth, eighth, and tenth) and cryopreserved them.

Methods: Fibroblasts recovered from skin biopsies were identified based on morphology, immunocytochemistry, and karyotyping. The cells were analyzed for morphology, ultrastructure, viability, proliferation, metabolism, oxidative stress, bioenergetic potential, and apoptosis before and after cryopreservation.

Results: After the eighth passage, the fibroblasts showed morphological and karyotypic changes, although their viability, metabolism, and proliferation did not change. An increase in oxidative stress and bioenergetic potential from the fifth to the eighth passages were also observed. Post cryopreservation, cell damage with respect to the ultrastructure, viability, proliferative rate, apoptotic levels, oxidative stress, and bioenergetic potential were verified.

Conclusion: Fibroblasts up to the tenth passage could be cultured *in vitro*. However, cells at the fifth passage were of better quality to be used for reproductive techniques. Additionally, optimization of the cryopreservation protocol is essential to improve the physiological parameters of these cells.

1. Introduction

In vitro culture of fibroblasts is an important and fundamental technique in scientific research aimed at the conservation and biological understanding of a species (Machado et al., 2017). Moreover, it is possible to use these cells in studies involving molecular and genetic knowledge by managing this somatic resource (Brugger and Basler, 2023), modeling drugs and diseases (Plikus et al., 2021; Ramklowan

et al., 2023), and in understanding the structural diversity of different populations, allowing crucial information to be obtained to conservation (Borges and Pereira, 2019). Furthermore, fibroblasts can be used to develop reproductive techniques that can preserve and disseminate the genetics of at-risk species (Mastromonaco and Songssasen, 2020), excluding the need for gametes, as has been observed in nuclear reprogramming (Ogura et al., 2000).

Therefore, studies that establish fibroblasts have been conducted in

* Correspondence to: Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró, RN 59625-900, Brazil

E-mail addresses: leonardo.aquino03297@alunos.ufersa.edu.br (L.V.C. Aquino), samara.lima@alunos.ufersa.edu.br (S.L. Olindo), yara.silva@alunos.ufersa.edu.br (Y.L.F. Silva), lhara.oliveira@alunos.ufersa.edu.br (L.R.M. Oliveira), yasmin.moura@ufersa.edu.br (Y.B.F. Moura), ana.rodrigues61941@alunos.ufersa.edu.br (A.L.R. Rodrigues), erikaalmeida@hotmail.com (É.A. Praxedes), moacir@ufersa.edu.br (M.F. Oliveira), alexrs@ufersa.edu.br (A.R. Silva), alexandra.pereira@ufersa.edu.br (A.F. Pereira).

<https://doi.org/10.1016/j.acthis.2024.152185>

Received 22 March 2024; Received in revised form 15 July 2024; Accepted 16 July 2024

Available online 26 July 2024

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laboratories and in wild rodents (Praxedes et al., 2021; Veraldi et al., 2022) as a conservation strategy by developing cryobanks that help maintain population genetic diversity (Bolton et al., 2022). Thus, identifying cell type, viability, and senescence as a function of the number of passages and evaluating the effects and damage caused by cryopreservation are essential for establishing this cell type in rodents (Praxedes et al., 2021). Investigating the physiological behavior of these cells and their characteristics will help establish that fibroblastic lines remain viable during culture, thereby optimizing their use.

The Spix's yellow-toothed cavy is a wild rodent belonging to the hystricomorph suborder (Oliveira et al., 2023) and may have an established fibroblastic line. In general, *G. spixii* is endemic to Brazil and belongs to the order Rodentia, which includes the guinea pig (*Cavia porcellus*), Patagonian mara (*Dolichotis patagonum*), capybara (*Hydrochoerus hydrochaeris*), hamster (*Mesocricetus auratus*), red-rumped agouti (*Dasyprocta leporina*), rats, and mice (Lovegrove, 2000). Studies involving the stages of establishing fibroblastic lines have been reported (Mehrabani et al., 2014; Praxedes et al., 2021). Although phylogenetically similar to *G. spixii*, this rodent has characteristics that encourage the isolation of fibroblasts to form cell biobanks.

First, *G. spixii* is a rodent that easily adapts to its habitat, presenting valuable genetics that contribute significantly to seed dispersal, soil aeration, and reforestation (Nowak, 1999). This species feeds on the leaves, stems, fruits, vines, roots, tubers, and tree bark (Mendes, 1987). Furthermore, *G. spixii* acts in the trophic chain and is considered a food source for carnivores (Sales et al., 2020). In addition, this species is considered an important wild rodent for the maintenance and functionality of the ecosystems in which it is located, especially in the Caa-tinga biome, a region in which it is considered a native species (Silva et al., 2023).

Additionally, because of its reproductive characteristics and distinction from other laboratory and wild rodents in terms of size, weight, and metabolic rate (Rowe and Honeycutt, 2002), *G. spixii* can be a scientific model for breeding sites for biological, physiological, cellular, evolutionary, and genetic studies (Queiroz et al., 2020). Therefore, *G. spixii* is used for the isolation and characterization of fibroblastic lines, which is obtained from the skin in a minimally invasive, inexpensive, and rapid manner (Iqbal et al., 2021) and have potential to be used for cloning by somatic cell nuclear transfer (Takahashi and Yamanaka, 2006; Wakayama et al., 2000). Using this technique, it is possible to understand the reproductive mechanisms inherent to *G. spixii*, as well as other phylogenetically close species. In addition, these cells can assist in the development and optimization of techniques associated with the induction of cellular pluripotency to obtain gametes *in vitro* (Hayashi et al., 2012; Cowl et al., 2024). Both procedures can contribute significantly to the conservation and genetic maintenance of populations, as previously observed in mice (Hikabe et al., 2016; Hamazaki et al., 2020).

In rodents such as *Cavia porcellus* (Mehrabani et al., 2014), *Mesocricetus auratus* (Mehrabani et al., 2016), and *Dasyprocta leporina* (Praxedes et al., 2021), the establishment and cryopreservation of fibroblastic lines was efficiently performed between second and eighth passage. However, variations have been observed in the parameters evaluated for the studied species (Xu, 2005). Isolation and *in vitro* culture of fibroblasts can occur even in the higher passages in rats and mice, as observed up to the tenth passage (Durkin et al., 2013; Madelaire et al., 2022), and is therefore considered an important metric for observation and validation when it is conducted in a new species (Walmsley et al., 2016). Cryopreservation is another crucial parameter that must be evaluated as this technique is specific to each species studied (Elliot et al., 2017; Mooney et al., 2023).

Therefore, considering the importance of different applications, from establishing a fibroblastic line to the ecological and scientific role of *G. spixii*, we established the culture time (second, fifth, eighth, and tenth passages) and cryopreservation of somatic cell lines.

2. Materials and methods

2.1. Chemicals, media, and ethics statement

All reagents and media were obtained from Sigma-Aldrich (St. Louis, MO, USA), Gibco-BRL (Carlsbad, CA, USA), and Inlab (São Paulo, SP, Brazil). Anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, England). For *in vitro* culture, Dulbecco's modified minimal essential medium (DMEM) was previously prepared and filtered using a pore filter system (0.22 µm; Corning, New York, USA), with the pH adjusted between 7.2–7.4. All protocols were approved by the Animal Use Ethics Committee of the Federal Rural University of the Semi-Arid (CEUA/UFERSA, no. 23091.010566/2017–20) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428–1).

2.2. Animals and somatic tissues obtained from skin

We used six *G. spixii* adult males maintained at the breeding site. The animals were obtained from the Wild Animal Multiplication Center (CEMAS/UFERSA, Mossoró, RN, Brazil 5°10'S, 37° 10' W, n° 1478912). The apical ear region was identified and sterilized for skin collection using 70 % alcohol and fragments measuring 1.0–2.0 cm² were retrieved using surgical pliers (Pereira et al., 2021). Subsequently, the samples were transported to the laboratory in DMEM supplemented with 10 % fetal bovine serum (FBS) and 2 % antibiotic-antimycotic (ATB-ATM) solution at 4 °C within 1 h.

In the laboratory, fragments were trichotomized and sectioned into 9.0 mm³ (3.0 × 3.0 × 1.0). Subsequently, the fragmented tissues were washed with media and solutions in the following sequence: i) DMEM supplemented with 10 % FBS and 10 % ATB-ATM solution, ii) 70 % alcohol, and iii) DMEM supplemented with 10 % FBS and 2 % ATB-ATM solution. After washing, eight fragments per animal were placed onto two cell culture dishes (four fragments per dish) containing DMEM supplemented with 10 % FBS and 2 % ATB-ATM solution. The dish was then cultured *in vitro* under controlled temperature and atmospheric conditions.

2.3. Experimental design

This study was carried using two experiments. *In vitro* culture was characterized using the primary system for the first experiment, and fibroblast lines in the second, fifth, eighth, and tenth passages were established. Fibroblasts were identified and evaluated by morphology, immunocytochemistry, and karyotyping to establish a cell line. Additionally, cell physiology was assessed for viability, metabolism, proliferation, reactive oxygen species (ROS) levels, mitochondrial membrane potential (ΔΨm), and apoptotic levels.

In the second experiment, fibroblasts at the fifth passage were cryopreserved by slow freezing for the cryotolerance assay. A cryoprotective solution composed of DMEM supplemented with 1.3 M dimethyl sulfoxide (Me₂SO), 10 % FBS, and 0.2 M sucrose was used as a cryopreservation solution (Fuentes-Mateos et al., 2023; Praxedes et al., 2023). After cryopreservation, the cells were thawed and evaluated as described in the first experiment. In addition, cells were subjected to an ultrastructural assay before and after cryopreservation to assess the damage and effects caused by slow freezing.

2.4. Establishment of the fibroblastic lines and cellular evaluations

2.4.1. Primary culture, cell isolation, and trypsinization

Cells were placed in DMEM with 10 % FBS and 2 % ATB-ATM solution for primary and permanent culture under a modified atmosphere (38.5 °C; 6.5 % CO₂). The culture dishes were monitored every 24 h and the media was changed each time. Post cell growth around the fragments, the medium was changed every 48 h, after which the fragments

were removed, and the cells were cultured until they reached 70–80 % subconfluence (Khan and Gasser, 2016).

Cell culture was evaluated using the following parameters: i) number of adhered explants; ii) number of explants at subconfluence; iii) days of adhered explants; and iv) number, days, and total time taken for explants to reach subconfluence. Subsequently, the cells were evaluated under an inverted microscope (Nikon TS100, Tokyo, Japan) (Praxedes et al., 2021), trypsinized (0.25 % trypsin, 0.05 % EDTA), and seeded according to the number of passages established in the experimental design.

2.4.2. Evaluation of morphological characteristics and cell type identification

Morphological characteristics were examined using an inverted microscope. The cell shape and nuclear and cytoplasmic extensions were observed (Mehrabani et al., 2014).

Immunocytochemical assays and karyotyping were performed to identify cell types. Initially, a vimentin assay was used to identify cells at the fifth passage using an immunocytochemical assay (Praxedes et al., 2021). Cells at a subconfluence between 60 % and 70 % were fixed in 4 % paraformaldehyde for 10 min between 25 and 27 °C. Subsequently, the cells were washed three times in phosphate buffer saline solution (PBS, pH 7.4; 4 °C) for 5 min in each wash, and then antigenic retrieval was performed (100 mM Tris; 5 % urea; pH 9.5) for 24 h. Post which the cells were rewashed in PBS (pH 7.4; 4 °C; 5 min) and permeabilized with 0.5 % Triton X-100 for 1 h. After permeabilization, three washes were performed in PBS (pH 7.4; 4 °C; 5 min) and a solution composed of 5 % bovine serum albumin (BSA), PBS, and 0.1 % Tween 20, was used to block the non-specific binding of antibodies for 1 h in a humid chamber. The cells were then incubated for 24 h with the primary anti-vimentin antibody (ab8979; 1:250; 1 % BSA diluted in PBS and 0.1 % Tween 20) for 24 h at 4 °C. The solution containing the anti-vimentin antibody was then removed, and after three washes in PBS, the secondary antibody (AlexaFluor® 488-coupled goat anti-mouse IgG, ab150113, 1:400) was added. It was incubated for 1 h in a humid chamber (4 °C) and protected from light. Finally, the cell nucleus was countermarked with 1 µg/mL Hoechst 33342 for 1 min and the result obtained was visualized using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 20× magnification.

The Giemsa assay was used for karyotype analysis. It was considered that in *G. spixii* $2n = 64$ (Maia, 1984). The protocol used was similar to that described for *C. porcellus* with some modifications (Ford and Hamerton, 1956; Mehrabani et al., 2014). First, cells were cultured and fixed in colchicine (0.1 %) for up to 2 h at 38.5 °C and 6.5 % CO₂ after reaching 70–80 % of their subconfluence. After this period, the fixative was removed and the cells were washed in PBS, followed by trypsinization and resuspension in DMEM with 10 % FBS and 2 % ATB-ATM solution. Subsequently, the cells were centrifuged (600×g/10 min) and a KCl solution (0.075 M) was slowly added to the tube, where the cells were incubated for 10 min between 35 and 37 °C. The tube was then centrifuged again (600×g/10 min) and the pellet formed was gently resuspended in 5 mL of a fixative solution (3:1 methanol: acetic acid solution), which was then centrifuged (600×g/10 min). Resuspension in the fixative solution was repeated three times and at the end of the process, the pellet was resuspended in 1.5 mL of fixative solution. Finally, two to four drops of cell suspension were dripped onto glass slides, previously treated with hydrolysis solution (0.1 M; HCl diluted in distilled water; 60 °C; eight min). The slides were air-dried, stained with Giemsa stain for 5 min, washed in distilled water, air-dried, and stored at room temperature for subsequent chromosomal analysis. Eight to ten metaphase cells were identified and analyzed using the conventional light microscope (Olympus BX51TF, Tokyo, Japan) at 100× magnification.

2.4.3. Cell viability analysis

Cells (20 µL) suspended in the different passages were subjected to

the staining assay using trypan blue dye (20 µL; 0.4 %) diluted in PBS. The cell suspensions were placed in a Neubauer chamber to quantify non-viable cells (dead; stained blue) due to membrane rupture and viable cells (alive; colorless) that did not present membrane rupture. Finally, percentage cell viability was calculated as follows: n° viable cells/ n° total number of quantified cells (Mehrabani et al., 2016).

2.4.4. Functional metabolism activity and proliferative rate

Cells were subjected to 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) assay. The cells were seeded in 12-well dishes (5.0×10^4 cells/mL) for five days at 38.5 °C and 6.5 % CO₂ (Praxedes et al., 2023). After culture, the cells were incubated with 5 mg/mL MTT (diluted in DMEM) for 3 h at 38.5 °C and 6.5 % CO₂. Me₂SO was added for MTT solubilization and the samples were measured at 595 nm using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan).

The proliferation assay was based on the seeding of cells in 24-well dishes at a concentration of 1.0×10^4 cells/ mL per well for 7 days (168 h), where the general average of the counts was recorded every 24 h after cell trypsinization to elaborate the cell growth curve, followed by the population doubling time (PDT) (Mehrabani et al., 2016). PDT was estimated using the following mathematical calculations:

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

where, PDT is the time required to double the culture (h), T is the incubation time, X_e is the number of cells at the end of the incubation period, $\ln$ is the Napierian logarithm, and X_b is the number of cells at the beginning of the incubation period.

2.4.5. Assessment of ROS and $\Delta\Psi_m$ levels

The levels of oxidative stress mediated by the ROS molecules of the cells were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 µM; Invitrogen, Carlsbad, CA, USA). Approximately, 2.5 mL PBS was added to an aliquot of 0.5 µL (0.1 M) H₂DCFDA and the cells were labeled using 50 µL cell suspension and a fluorescent probe (50 µL; 1:1) for up to 30 min at 38.5 °C and 6.5 % CO₂.

The MitoTracker Red® probe (CMXRos; 1 mM; Invitrogen, Carlsbad, CA, USA) was used to evaluate the $\Delta\Psi_m$, where 750 µL PBS was used to dilute an aliquot of 1.5 µL (500 nM) CMXRos. Post which, 50 µL cell suspension and the fluorescent probe (50 µL; 1:1) were obtained. Subsequently, the sample was kept for 30 min at 38.5 °C and 6.5 % CO₂.

After labeling, aliquots containing the H₂DCFDA and CMXRos probes were centrifuged (300×g/5 min). Finally, the supernatant was removed, the pellet formed was resuspended in 50 µL PBS, and the cells were evaluated under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). Excitation at 495 nm and emission at 520 nm were used for ROS, whereas excitation at 579 nm and emission at 599 nm were used for CMXRos.

Finally, 100 cells were registered for each fluorescent probe and the fluorescence intensity (pixels) was estimated using the ImageJ software (National Institutes of Health, MA, USA). The background intensity was subtracted from the values obtained from the somatic cells and the relative expression levels (arbitrary fluorescence units) were quantified. The cells were considered experimental units.

2.4.6. Quantification of apoptotic levels

An aliquot of cell suspension (35 µL) was marked using a mixture of ethidium bromide dye (10 µg/mL) and acridine orange (2 µg/mL) diluted in 8 µL PBS (1:1) (Praxedes et al., 2021). This combined method allows for the differential uptake of fluorescent DNA-binding dyes, allowing the observation of chromatin condensation in the stained nucleus (Takahashi et al., 2004), distinguishing and classifying (i) viable cells with a uniform light-green nucleus, (ii) cells in early apoptosis with non-uniform green nuclei, (iii) cells in late apoptosis with non-uniform orange nuclei, and (iv) necrotic cells with uniform orange/red nuclei. Finally, the cells were evaluated under a fluorescence microscope

Table 1

Establishment of the physiological characteristics of the primary culture and subculture of somatic cells of six *G. spixii*.

Cellular parameters	Values (%)
No. initial explants	48 ± 0.0
Attached samples	45 ± 0.3 (94 %)
Day all attached explants	4.2 ± 0.3
Day samples reached cell growth	15.0 ± 0.2
<i>In vitro</i> culture time (days)	70.0 ± 0.0

Results are expressed as mean ± standard error.

(Olympus BX51TF, Tokyo, Japan) at an absorbance of 480 nm, where images were recorded, and 300 cells were quantified using ImageJ software at a magnification of 40×.

2.5. Cryopreservation and fibroblast warming

The slow-freezing method was used for cell cryopreservation, as described by [Praxedes et al. \(2023\)](#). Cells in the fifth passage were used because the culture had already been established based on the cell type. Then, 1.0×10^5 cells/mL was recovered. Cells were then added to cryotubes containing the cryopreservation solution and incubated for 15 min at 4 °C ([Praxedes et al., 2023](#)). Subsequently, 0.2 M sucrose was added, and the samples were kept at 4 °C for 15 min. The cells were then stored in cryotubes and transferred to the Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, NY, USA), a freezing vessel, for 12 h in a programmable freezer at −80 °C at a cooling rate of −1 °C/min. The cryotubes were stored in liquid nitrogen (−196 °C) for two weeks, where they were subsequently thawed and evaluated for the effect of cryotolerance.

The cells were initially thawed at 25 °C for 1 min, followed by 4 min between 35–37 °C. DMEM with 0.2 M sucrose was added and the cells were incubated in the medium for 15 min. Subsequently, the cells were centrifuged for 10 min at 600×g. The supernatant was removed, and the pellet was resuspended in DMEM for 15 min at 25 °C. Finally, the cells were centrifuged and cultured in DMEM supplemented with 10 % FBS and 2 % ATB-ATM solution for evaluation, as described in the experimental design.

2.5.1. Ultrastructural evaluation by scanning electron microscopy

The cells were fixed in glutaraldehyde (0.4 %) diluted in PBS for 2 h. The fixative solution was removed, the cells were washed three times in PBS, and post-fixed in osmium tetroxide (0.1 %) for up to 1 h. The cells were then washed twice with increasing concentrations of alcohol solutions (7.5 %, 15 %, 30 %, 50 %, 70 %, 90 %, and 100 %) for 10 min at each concentration. Finally, cells were stained with gold and visualized using a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

2.6. Statistical analysis

Data from six individuals were expressed as mean ± standard error (one animal/replicate) and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality using the Shapiro-Wilk test and homoscedasticity using Levene's test. The data were arcsine-transformed because they did not exhibit a normal distribution. All *in vitro* culture data were analyzed by ANOVA followed by Tukey or unpaired t-test. Statistical significance was defined as $P < 0.05$. Additionally, a power analysis (G*Power 3.1.9.7, Heinrich Heine University Düsseldorf, Germany; [Faul et al., 2007](#); [Faul et al., 2009](#)) was conducted for each assessment, considering reliability when the power effect exceeded 80 %.

3. Results

3.1. *In vitro* culture and establishment of fibroblastic lines

Eight initial explants/animals were used for *in vitro* culture to establish fibroblastic lines and a total of forty-eight skin biopsies were analyzed daily for cell growth ([Table 1](#), [Fig. 1](#)). During *in vitro* culture, forty-five (94 %) cells were fixed on the culture dish for an average period of 4.2 days. Furthermore, it took six days (85 % of the samples) for cell detachment to occur ([Fig. 1A](#)). Cell growth was observed after the eighth day of culture ([Fig. 1B](#)). It took 15 days for the cells to reach subconfluence between 70 % and 80 % ([Fig. 1D](#)) after removing the fragments ([Fig. 1C](#)), with a total culture time of 70 days from the second to the tenth passage ([Fig. 1E-H](#)).

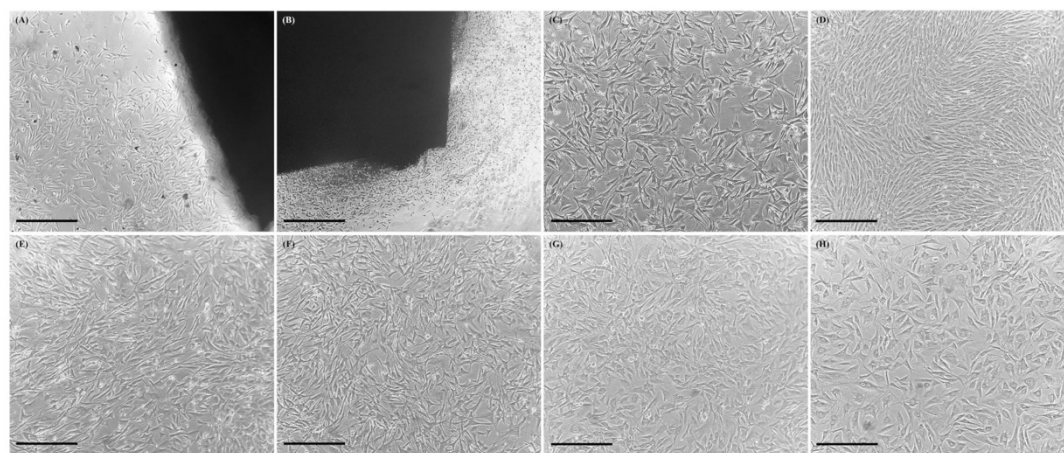


Fig. 1. Establishment of *in vitro* culture of *G. spixii* fibroblasts and their morphological characteristics in different passages. (A) Primary culture of *G. spixii* skin explant with cell shedding after 6 days of *in vitro* culture. (B) Primary culture of skin explants in cell growth after 8 days of *in vitro* culture. (C) Culture of *G. spixii* skin fibroblasts in cell growth after the removal of somatic skin explants. (D) *G. spixii* skin fibroblasts after reaching 100 % subconfluence after 14 days of cell culture. (E) *In vitro* culture of *G. spixii* skin fibroblasts in the second, (F) fifth, (G) eighth, and (H) tenth passages. Bar scale = 50 µm, mag. 4× (A, C, D, E, F, G, H).

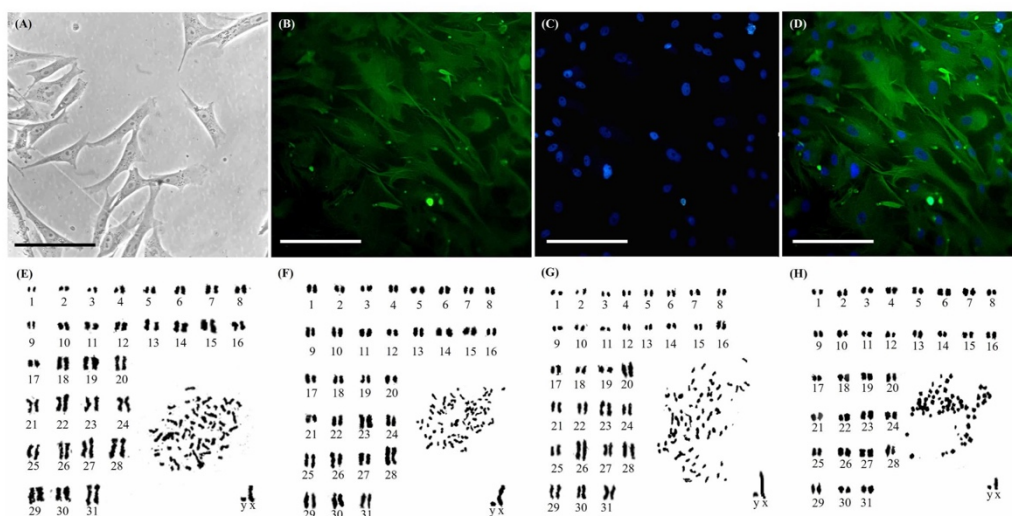


Fig. 2. Representation of the fibroblastic line of *G. spixii* after morphological analysis, immunostaining, and karyotyping. (A) Morphological evaluation by inverted microscope. Bar scale = 50 μ m, mag. 40 $\times$. (B) Immunostaining of cell cytoplasm by vimentin antibody. Bar scale = 50 μ m, mag. 40 $\times$. (C) Nucleus of cells stained by Hoescht 33342. Bar scale = 50 μ m, mag. 40 $\times$. (D) Composition of the cytoplasm and nucleus of *G. spixii* fibroblasts after cell type identification. Bar scale = 50 μ m, mag. 40 $\times$. (E-H) Karyotype of fibroblasts stained by Giemsa in the second (E), fifth (F), eighth (G), and tenth passages (G). Mag. 100 $\times$.

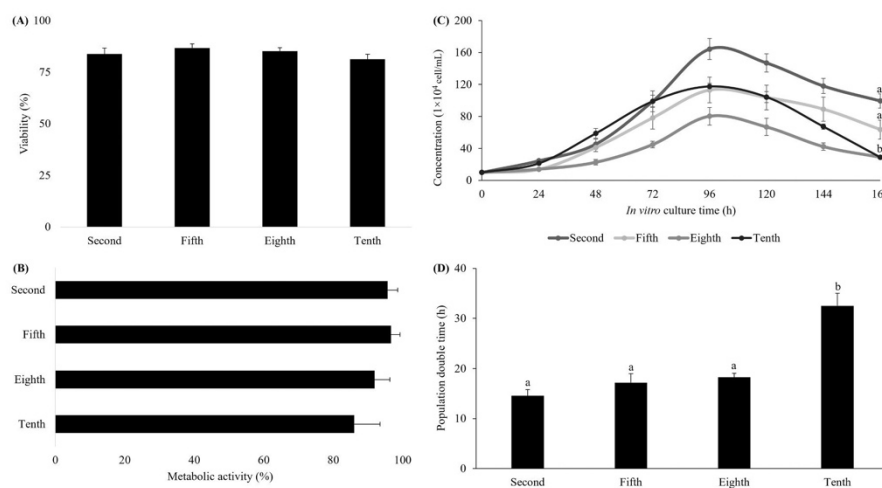


Fig. 3. Evaluation of viability and physiological rates of fibroblastic lines of *G. spixii* after the second, fifth, eighth, and tenth passages. (A) Percentage of cell viability. (B) Quantification of metabolic activity. (C) Analysis of the proliferative curve. (D) Population doubling time of fibroblasts in the second, fifth, eighth, and tenth passages. $P < 0.05$.

3.1.1. Identification of cell type by morphology, immunocytochemistry, and chromosomes

Based on morphological analyses, the cells presented typical fibroblast morphology from the second to the tenth passage (Fig. 1E-H). However, the cytoplasm of the fibroblasts became slender from the eighth passage onwards (Fig. 1G-H), compared to the second and fifth passages (Fig. 1E-F). Cells at the fifth passage (Fig. 2A) were identified for cell type characterization. Thus, an anti-vimentin antibody assay was

used to mark the intermediate filaments of fibroblasts, confirming this cell type (Fig. 2B-D).

Finally, the assay determined the karyotype of the fibroblasts during the second, fifth, eighth, and tenth passages (Fig. 2E-H). Therefore, the chromosomes of the cells during the tenth passage showed shortening. However, the corresponding number in this species was found to be $2n = 62$ autosomal chromosomes, with two more sex chromosomes.

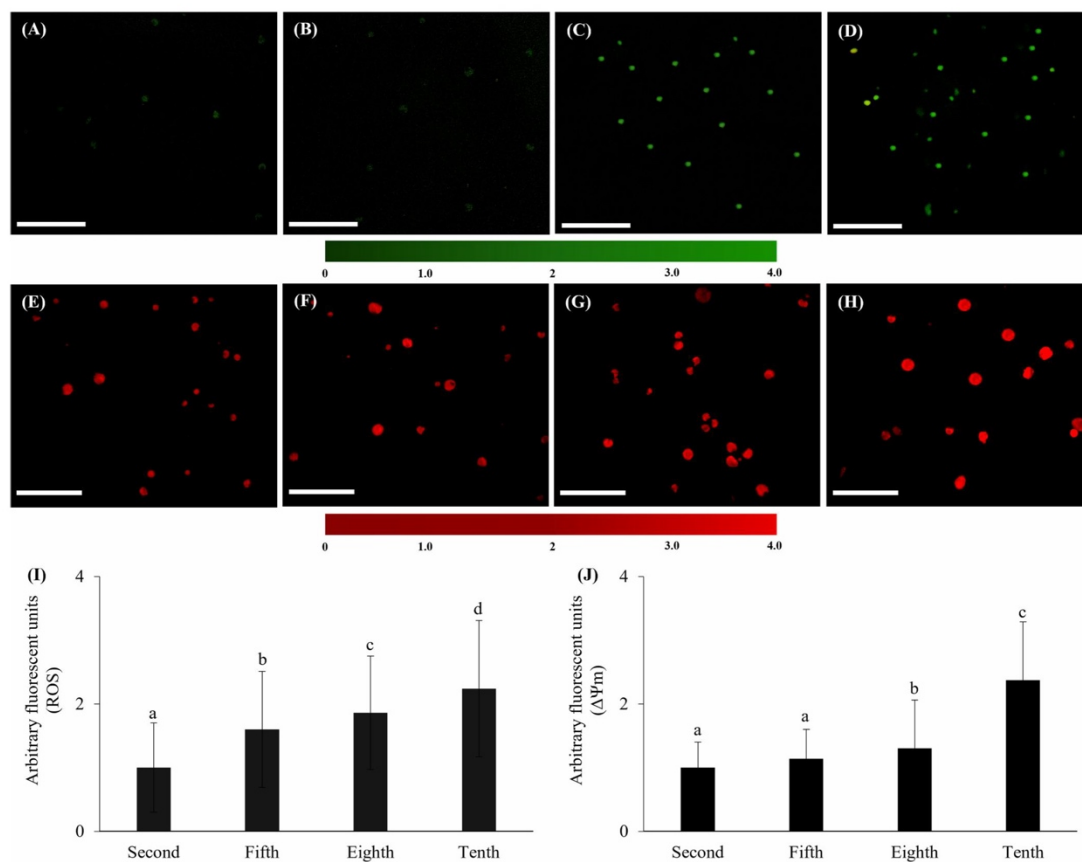


Fig. 4. Evaluation of reactive oxygen species (ROS) and mitochondrial activity of *G. spixii* adult skin fibroblasts after the second, fifth, eighth, and tenth passages of *in vitro* culture. (A-D) Representation of cells stained by the H₂DCFDA probe. Bar scale = 50 μ m, mag. 20 $\times$. (E-H) Representation of cells stained by the MitoTracker Red[®] probe (CMXRos). (I) Quantification of oxidative stress after assessment of intracellular levels of ROS. (J) Evaluation of mitochondrial activity ($\Delta\Psi_m$) of *G. spixii* adult skin fibroblasts. $P < 0.05$.

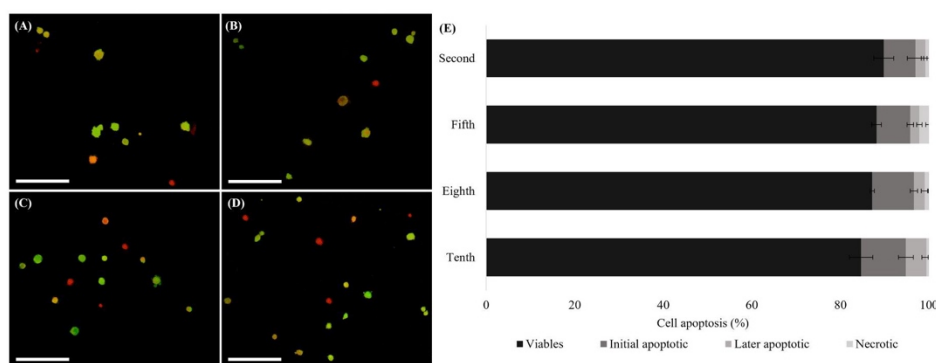


Fig. 5. Evaluation of the apoptotic levels of the *G. spixii* fibroblastic lines after the second (A), fifth (B), eighth (C), and tenth (D) passages of cell culture. Bar scale = 50 μ m, mag. 20 $\times$. (E) Percentage of the number of viable cells in early and late apoptosis and necrotic during the ten passages of *in vitro* culture. $P > 0.05$.

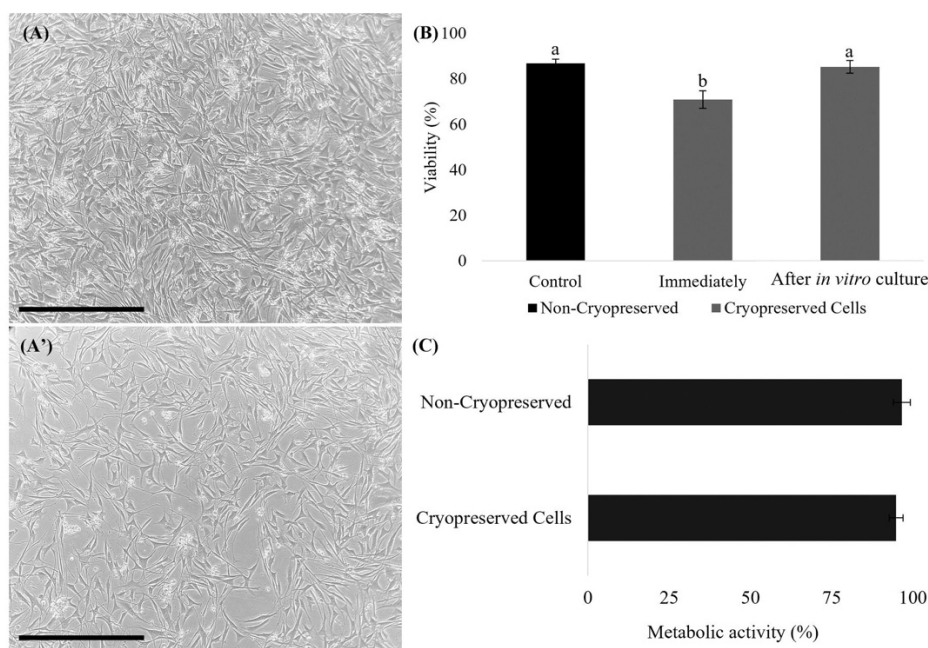


Fig. 6. Morphological, viability, and metabolism evaluation of *G. spixii* fibroblastic lines after slow freezing. (A-A') Morphological analysis of cells before and after cryopreservation. Bar scale = 50 μm, mag. 10×. (B) Percentage of fibroblast viability before, immediately after cryopreservation and *in vitro* culture. (C) Metabolic activity of cells after cell cryopreservation. $P < 0.05$.

3.1.2. Evaluation of cell viability and physiology

The fibroblasts were analyzed for viability, metabolic rate, and proliferation capacity during the second, fifth, eighth, and tenth passages to assess their cellular physiology (Fig. 3). First, a percentage of viable cells greater than 80 % was observed throughout the passages (Fig. 3A). Furthermore, a metabolic rate greater than 85 % was observed between the second and tenth passages of *in vitro* fibroblast culture (Fig. 3B).

However, despite the fibroblasts being metabolically active, the cell growth curve showed a reduction in cell quantity (cells/mL) after the eighth passage (Fig. 3C), and PDT was negatively affected by the number of passages performed in these cells only in the tenth passage (32.5 h) (Fig. 3D).

3.1.3. Intracellular quantification of ROS, $\Delta\Psi_m$, and cellular apoptosis

Fibroblasts were evaluated for oxidative stress, $\Delta\Psi_m$, and apoptotic levels to determine the quality of cell culture (Fig. 4; Fig. 5). With respect to the intracellular levels of ROS, cells in the fifth (1.60 ± 0.9), eighth (1.86 ± 0.8), and tenth (2.24 ± 1.0) passages presented a greater amount of ROS compared to the second passage (Fig. 4I). However, cells in the eighth (1.30 ± 0.7) and tenth (2.37 ± 0.9) passage showed higher activity after evaluating the mitochondrial activity compared to the second (1.0 ± 0.4) and fifth (1.14 ± 0.4) passage (Fig. 4J). No differences were observed across passages or after the assessment of apoptotic levels, where the number of viable cells was greater than 80 % (Fig. 5E).

3.2. Cryotolerance assay on fibroblastic lines

3.2.1. Effect of cryopreservation on cell morphology, viability, and metabolism

Despite the typical morphology of fibroblasts being maintained after analysis using an inverted microscope (Fig. 6A-A'), the viability

immediately after slow freezing reduced (70 %) compared to that of non-cryopreserved cells (85 %) (Fig. 6B). However, cell viability was recovered after *in vitro* culture (Fig. 6B). No difference was observed in the metabolic analysis of fibroblasts ($P > 0.05$; Fig. 6C), which was greater than 90 % in both groups.

3.2.2. Effect of cryopreservation on ultrastructure, cell growth curve, and cell population doubling

In the ultrastructural analysis, fibroblasts after cryopreservation had less translucent characteristics, and the cytoplasmic membrane presented more significant irregularities and structural damage under its surface, probably due to the effects of cryopreservation (Fig. 7A-A'). Furthermore, no significant differences ($P > 0.05$) were observed in the cell growth curves (Fig. 7B). However, cryopreserved cells (24.4 h) were negatively affected compared with non-cryopreserved cells (17.2 h) after slow freezing and PDT analysis (Fig. 7C).

3.2.3. Effect of cryopreservation on oxidative stress, $\Delta\Psi_m$, and apoptotic levels

A significant increase ($P < 0.05$) was observed after evaluating oxidative stress (1.49 ± 0.7) and $\Delta\Psi_m$ (1.34 ± 0.7) in cryopreserved fibroblasts compared to non-cryopreserved cells (1.00 ± 0.5 ; 1.00 ± 0.4) (Fig. 8A-A'; Fig. 8B-B'), respectively.

After apoptotic analysis, a reduction in the number of viable cells was verified after cryopreservation ($75.0 \% \pm 1.1$) compared to the non-cryopreserved group ($88.2 \% \pm 1.1$) (Fig. 8C'). Furthermore, an increase in the number of cells during initial ($16.4 \% \pm 0.7$) and late ($5.7 \% \pm 0.5$) apoptosis was observed after cryopreservation and when comparing non-cryopreserved cells ($7.6 \% \pm 0.7$; $2.1 \% \pm 0.5$) (Fig. 8C-C').

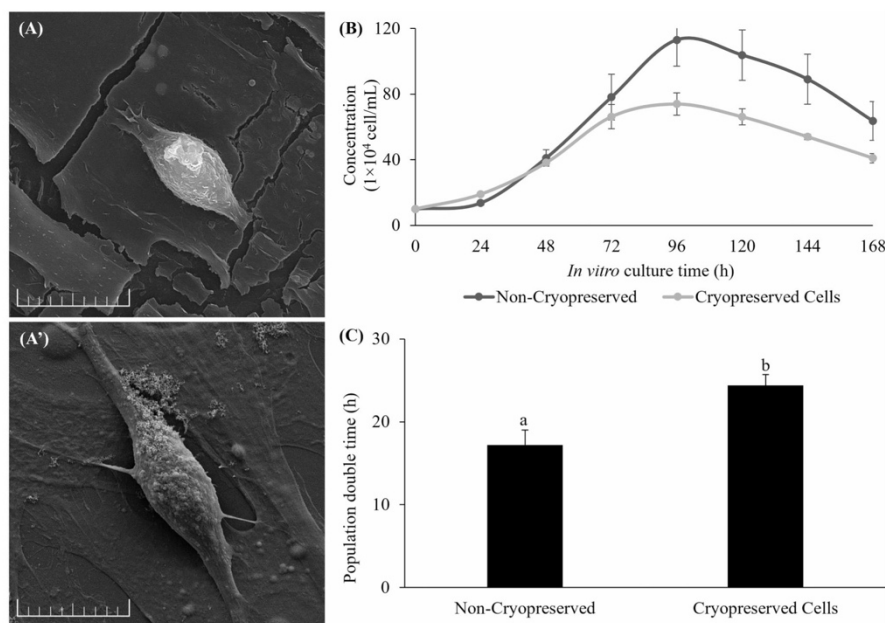


Fig. 7. Ultrastructural evaluation and proliferative rate of *G. spixii* fibroblasts after cryopreservation. (A-A') Ultrastructural analysis of cells before and after cryopreservation. Bar scale = 10 μ m. (B) Cell concentration after evaluating the growth curve. (C) Population doubling time of fibroblasts before and after slow freezing. $P < 0.05$.

4. Discussion

In our study, we established and physiologically characterized, over ten passages, fibroblastic lines from the ear skin of Spix's yellow-toothed cavy, a rodent with a stable population (Catzeflis et al., 2016), which is a study model for developing *in vitro* strategies (Ryder and Onuma, 2018). We also used a cryopreservation protocol for *G. spixii* fibroblasts. Although cellular damage was observed, the tool can introduce cryopreserved materials into reproductive techniques.

Initially, we chose cell recovery through direct culture of somatic explants instead of enzymatic methodologies for the primary culture of fibroblasts originating from *G. spixii* from the ear skin. This was because good sample fixation (94 %) after the beginning of the *in vitro* culture was observed, and the technique has already been used efficiently in other species (Seluanov et al., 2010; Durkin et al., 2013). Unlike in mice, a low rate of cell recovery was observed after using biopsies with irregular edges, contributing to poor fixation owing to tissue thickness in culture and causing damage to cellular structures after treatment using enzymatic methodologies (Khan and Gasser, 2016).

In addition, biopsies recovered from the ear apical region caused minimal damage to the animal, requiring a short time for adhesion to the culture dishes (4 days), favoring a good initial cell growth rate (85 %) up to the eighth day, and guaranteeing maximum confluence in up to 15 days. Although these results differed from those of a culture of *C. porcellus* skin fibroblasts (Mehrabani et al., 2014), which required up to 30 days for an adequate rate of cell subconfluence, they were similar to *D. leporina*, in which cells reached their maximum subconfluency within 15 days (Praxedes et al., 2021). With respect to morphological aspects, the somatic cells exhibited typical characteristics of fibroblasts from the second to the fifth passage, that is, they showed spindle-shaped cytoplasm, elongation, and very adherent growth to the cell culture dish, corroborating the findings observed in *D. leporina* (Praxedes et al., 2021)

and *M. auratus* (Mehrabani et al., 2016) when fibroblasts were isolated from the skin. Our results were similar when comparing the morphology of fibroblasts from the skin, tail, and lungs of adult and neonatal murine animals (Walmsley et al., 2016; Fuentes-Mateos et al., 2023).

However, we observed that the fibroblast morphology changed after morphological analysis from the eighth passage, with the cytoplasm losing its fusiform aspect and becoming slender. These data are associated with the onset of replicative senescence or spontaneous mutations that immortalize cells during this passage. This is justified by the fact that cells derived from primary and secondary cultures may present functional limitations owing to the number of passages during culture. This is also observed in spiny mice (*Acomys cahirinus*), where cultures of primary and secondary cells present morphological and functional alterations that limit their cells to early passages, which are necessary for their immortalization (Dill et al., 2023). In addition to morphological evaluation, fifth-passage *G. spixii* fibroblasts were identified using a vimentin antibody immunostaining assay. This technique is routinely used when establishing new fibroblastic lines (Praxedes et al., 2021; Van Vuuren et al., 2023) because, unlike epithelial cells, these cells express type III intermediate filament proteins, allowing for distinction between the two cell types.

Through karyotyping, we evaluated fibroblast chromosomal stability at the second, fifth, eighth, and tenth passages. The karyotype observed in this study was similar to that described in a report by Maia (1984), where the cell chromosome of the *G. spixii* fibroblast line corresponded to $2n = 64$ from 8–10 metaphases observed on the slides in each passage. However, this resulted in more significant cell genomic instability in the eighth and tenth passages, as shortening of the chromosomal length was observed, possibly due to the wear of replicative senescence (Harley et al., 1990; Bodnar et al., 1998), which presents as a shortening of telomeres caused by the number of passages in *G. spixii* cultures, unlike in mouse cells (Itahana et al., 2004; Parrinello et al., 2003).

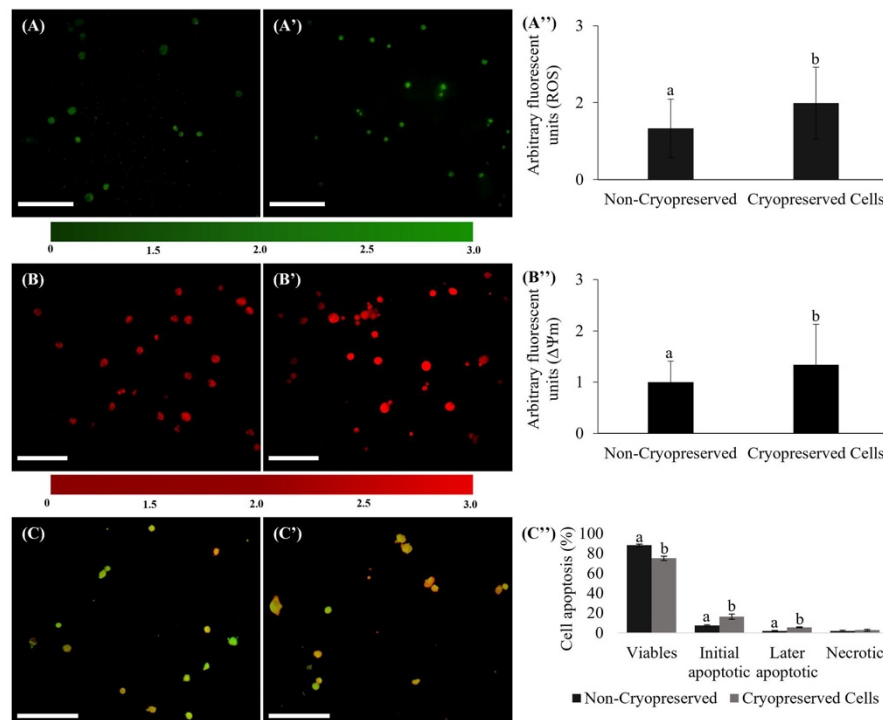


Fig. 8. Quantification of oxidative stress, mitochondrial membrane potential ($\Delta\Psi_m$), and apoptotic levels of *G. spixii* fibroblasts before and after slow freezing. (A-A') Representative image and arbitrary units of fluorescence obtained in each cell evaluated after oxidative stress analysis. Bar scale = 50 μm , mag. 20 $\times$ (A-A'). (B-B') Representative image and arbitrary unit of fluorescence obtained in each cell evaluated after the $\Delta\Psi_m$ evaluation. Bar scale = 50 μm , mag. 20 $\times$ (B-B'). (C-C') Representative image and percentage of the number of fibroblasts in cellular apoptosis. Bar scale = 50 μm , mag. 20 $\times$ (C-C'). $P < 0.05$.

Body mass and metabolic rate of *G. spixii* are other reasons for the shortening of chromosomes observed in this study, with smaller and larger values, respectively, compared to other wild rodents (Rowe and Honeycutt, 2002). According to Gorbunova and Seluanov (2009), rodent body mass is associated with transcriptional telomerase activity. Although 90 % of captive mice die of cancer because of failure to utilize the telomerase mechanism (Lipman et al., 2004; Seluanov et al., 2007), *G. spixii* may have evolved a genetic system to set rigid patterns and boundaries in cell proliferation (Wright and Shay, 2000), as observed in primates, deer, and humans (Steinert et al., 2002; Zou et al., 2002). This explains the chromosomal shortening of cells in the eighth and tenth passages of cell culture. The *G. spixii* fibroblastic lines remained superior to 80 % after evaluating their viability and metabolic rate using trypan blue and MTT assays, respectively, throughout the passages.

With respect to the proliferation rate, although the cells presented a sigmoid curve, the cell density from the eighth passage was lower than those from the second and fifth passages. However, the cells required only a longer time for PDT at the tenth passage (32.5 h). This pattern of variation has been observed in other laboratory and wild rodent species, such as *C. porcellus* (Mehrabani et al., 2014), *Acomys cahirinus* (Dill et al., 2023), and *D. leporina* (Praxedes et al., 2021), and may be associated with culture time and lifetime (Seluanov et al., 2010).

With respect to the *in vitro* culture system, production of ROS and mitochondrial activity during passaging were evaluated in an *in vitro* culture system. Thus, oxidative stress significantly increased during the fifth passage. However, no correlation was observed with mitochondrial

activity, which was similar in the second passage however differed only in the eighth and tenth passages. Many factors may be responsible for the increased synthesis of intracellular free radicals observed during the passages. We observed the cellular environment, medium composition, light, temperature, and metabolic activity when cell passage was performed (López-Díazguerrero et al., 2005). This is acceptable because fibroblasts in the eighth and tenth passages presented the highest ROS index owing to greater mitochondrial activity, which is one of the organelles responsible for greater ROS synthesis (Turrens, 2003). Consequently, DNA damage occurs. Although cells have repair systems for oxidative damage from p53 and glutathione (Liu et al., 2008; Voudsen and Prives, 2009), excess ROS can cause pro-oxidant effects, eventually leading to irreversible dysfunction such as cell death (López-Díazguerrero et al., 2005). Despite this, no significant difference was observed after evaluating the apoptotic levels, where the cells maintained a viability greater than 80 %, as observed in our study.

After establishing the fibroblastic lines, *G. spixii* cells in the fifth passage were cryopreserved to evaluate their cryotolerance in the cryopreservation medium most commonly used for laboratory and wild rodents (Mehrabani et al., 2016; Praxedes et al., 2023). No changes were observed in the cell morphology after cryopreservation. However, after ultrastructural analysis, the *G. spixii* fibroblasts showed irregularities on the surface of their plasma membranes. This may have occurred because of cell dehydration during slow freezing, during which cryoprotective solutions remove water from the cell interior through osmosis to prevent the formation of ice crystals (Meryman, 2007). Therefore, this

mechanism is essential when using this technique because cells require an adequate cooling rate for the ideal use of slow freezing so that their molecules and ultrastructural components remain protected by the cryoprotectants used in this study (Mazur, 2004). This may be explained by the recovery of this characteristic during *in vitro* culture following thawing. However, a reduction in cell viability was observed immediately after cryopreservation, where the cells maintained metabolic rates above 90 %, with no difference in cell quantity after analyzing the growth curve.

We also observed that fibroblasts required a longer time (24.4 ± 1.3 h) to duplicate after slow freezing compared to the non-cryopreserved group (17.2 ± 1.8 h) after the PDT assay. A variation in PDT before and after slow freezing has been reported in other rodent species, such as *D. leporina* (16.5 ± 1.3 – 23.4 ± 3.5 h) (Praxedes et al., 2021). This may have occurred because of the need to optimize the concentration of cryoprotectants used, as reported for the cryopreservation of *D. leporina* skin fibroblasts (Praxedes et al., 2023). Furthermore, the biochemical and physiological conditions of the cells may be compromised during cryopreservation because of the cooling rate and exposure to hypertonic solutions, as observed in our study, where the cells required a longer time for duplication.

Other factors that could be associated with the proliferative physiology observed in *G. spixii* skin fibroblasts after cryopreservation were oxidative stress and mitochondrial activity, which were significantly higher than those in the non-cryopreserved group. This may have occurred because the intracellular water stress caused by cryopreservation and after culture can continuously increase the number of ROS molecules (Martindale and Holbrook, 2002) owing to the byproducts of the aerobic metabolism of fibroblast mitochondria from the skin of this small rodent, corroborating our findings (Malpique et al., 2009). Accumulation of free radicals, such as $O_2^{\bullet-}$, H_2O_2 , and $\bullet OH$, may damage the lipids of biological membranes, proteins, core acids, and other biological macromolecules (Lee et al., 2014), causing or inducing cellular apoptosis (Lee et al., 2014; Sastre et al., 2000). This explains the reduction in the number of viable cells and the increase in the number of cells during early and late apoptosis, as observed after cryopreservation of the fibroblastic lines of *G. spixii*.

5. Conclusions

This is the first study to describe the establishment, physiological characterization, and cryopreservation of new cell lines from *G. spixii*. Wild rodents play significant roles in both ecology and science. Therefore, cells up to the fifth passage are most suitable for cellular and molecular studies, as enhanced genetic, morphological, and physiological stability has been observed. Although we efficiently cryopreserved the fibroblastic line of *G. spixii*, optimizing the development of protocols and cryopreservation solutions for this species is still necessary. Finally, our results offer an opportunity to manage this somatic resource, providing cellular, molecular, and genetic knowledge of this species. This information can help in actions related to the conservation of this species and others phylogenetically close to *G. spixii* since this small wild rodent stands out as an ideal model for scientific study, enabling an increase in *in vitro* techniques, such as nuclear reprogramming.

CRedit authorship contribution statement

Leonardo VC Aquino: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, and writing the original draft. **Samara L Olindo:** Methodology. **Yara Leticia F Silva:** Methodology. **Lhara RM Oliveira:** Methodology. **Yasmin BF Moura:** Methodology. **Ana Livia R Rodrigues:** Methodology. **Erika A Praxedes:** Methodology. **Moacir F Oliveira:** Methodology. **Alexandre R Silva:** Formal Analysis and Investigation. **Alexsandra F Pereira:** Conceptualization, Formal Analysis, Investigation, Resources, Data Curation, Writing – Review and Editing, Supervision, and Funding acquisition.

Declaration of Competing Interest

The authors declare having no conflict of interest.

Acknowledgments

The authors thank the Center for Wild Animals Multiplication (CEMAS/UFERSA) for providing the biological samples used in this study. This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil (CNPq, nº 309078/2021–0).

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ANEXO D – VERSÃO PUBLICADA DO ARTIGO EXPERIMENTAL II PUBLICADO NA BIOPRESERVATION AND BIOBANKING

BIOPRESERVATION AND BIOBANKING
Volume 23, Number 2, 2025
© Mary Ann Liebert, Inc.
DOI: 10.1089/bio.2024.0006

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L-Proline as a Cryoprotective Agent for the Preservation of *Galea spixii* Skin Fibroblasts

Leonardo V.C. Aquino, Luanna L.V. Rodrigues, Samara L. Olindo, Yara L.F. Silva, Lhara R.M. Oliveira, Yasmin B.F. Moura, and Alessandra F. Pereira

Somatic cell biobanking is a promising strategy for developing reproductive techniques. Although cryopreservation, a technique used for creating biobanks, has been performed on *Galea spixii*, structural and physiological damage to its cells highlight the need to optimize the cryoprotective solution being used. Therefore, the osmoprotective activity of 5 mM L-proline was evaluated as an alternative cryoprotectant for *G. spixii* fibroblast conservation. The concentration was defined based on previous studies conducted on mammalian cells. Cells derived from the skin of six individuals were cultured until the fifth passage were cryopreserved under the following treatments: (i) control (non-cryopreserved); (ii) a solution with 10% dimethyl sulfoxide (Me₂SO), 10% fetal bovine serum (FBS), and 0.2 M sucrose; (iii) a solution with 10% Me₂SO, 10% FBS, and 5 mM L-proline; and (iv) a solution with 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. Tests were conducted to analyze cell morphology, viability, metabolism, proliferation, and apoptosis; reactive oxygen species (ROS) levels; and mitochondrial membrane activity ($\Delta\Psi$ m). A reduction in the number of viable cells ($72.3\% \pm 1.2\%$) was observed in the sucrose-containing group compared to the control ($86.7\% \pm 2.0\%$) and L-proline ($88.4\% \pm 1.8\%$ and $87.8\% \pm 2.1\%$) groups. After apoptotic analysis, a reduction in the number of viable cells was observed in the group with sucrose alone ($74.6\% \pm 4.1\%$) compared to the control group ($88.2\% \pm 1.1\%$). The ROS levels (1.03 ± 0.5 and 1.07 ± 0.5 , respectively) and $\Delta\Psi$ m values (0.99 ± 0.42 and 1.22 ± 0.73 , respectively) observed in the groups with L-proline were similar to that observed in the control group (1.00 ± 0.5 and 1.00 ± 0.4 , respectively). Moreover, no difference was observed between groups for cell morphology, metabolism, or proliferation. Thus, L-proline is a cryoprotectant agent that can be used during *G. spixii* fibroblast cryopreservation, alone or with sucrose. In addition, we developed an adequate biobank for *G. spixii*, whereby stored cells could be used for reproductive techniques.

Keywords: Rodentia, Spix's yellow-toothed cavy, cryoinjury, osmolyte cryoprotectant, somatic cells

Introduction

Cellular biobanks are a collection of isolated *in vitro*-cultured somatic cells. They enable the formation of an organized and searchable collection of biological samples,¹ and provide relevant information for the management, optimization, and development of protocols for wild species. With respect to laboratory and wild rodents, biobanks offer applications for the treatment of genetic diseases and development of regenerative therapies.² Furthermore, somatic cell banks offer an opportunity for species conservation,³ and genetic

reintroduction of populations threatened with extinction through assisted reproduction techniques,⁴ as observed in cloning by somatic cell nuclear transfer⁵ and the induction of cells to pluripotency.⁶ In addition, with the formation of cellular banks for wild rodents, it is possible to implement other techniques irrespective of limiting factors, such as reproductive dysfunction, unexpected deaths, geographical isolation, and the age and sex of the animal.⁷

Spix's yellow-toothed cavy (*Galea spixii*) is a wild rodent that was initially used to develop a biobank derived from fibroblastic lines.⁸ *G. spixii* is an herbivorous rodent native to the

Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, Brazil.

Caatinga biome, but is located in other biomes, such as in Brazil, Paraguay, Bolivia, and east of the Andes Mountains.⁹ This species is 22.5–23.5 cm long,¹⁰ with uniform-colored fur, a gray back, white belly, and no tail.¹¹ *G. spixii* contributes to the maintenance of local fauna and flora.¹² Despite environmental disturbances associated with hunting this rodent,¹³ its population is considered stable¹⁴ because of its adaptive, physiological, reproductive, and genetic characteristics.¹⁵ Therefore, it stands out as an ideal study model for developing protocols involving biobank formation at scientific breeding sites, and among other wild rodents.

For *G. spixii*, a biobank of somatic tissues has already been developed and used to observe the structural and morphological characteristics of the skin, which is an organ from which somatic cells can be isolated in a minimally invasive, inexpensive, and rapid manner.¹⁶ The establishment and cryopreservation of fibroblastic lines is another technique implemented for wild rodents.⁸ After cell cryopreservation, changes are observed in the cell viability and proliferation rate, owing to an imbalance in their cellular redox system and an increased number of apoptotic cells, probably caused by osmotic stress and ice crystal formation during cryopreservation. This may have caused lipid peroxidation, which is an important event in cell membrane damage during cryopreservation.¹⁷ Therefore, it is necessary to optimize this process.

The first parameter evaluated was the selection of the cryoprotective solution. The first protocol established for *G. spixii* was based on protocols used for other phylogenetically close species,^{3,18} in which the cryoprotectant solution comprised 10% dimethyl sulfoxide (Me₂SO), 10% fetal bovine serum (FBS), and 0.2 M sucrose.⁸ However, after *in vitro* culturing of the cryopreserved fibroblastic lines,⁸ their viability, metabolism, and proliferative rate were affected, probably due to the osmotic stress generated by intracellular and extracellular ice crystal formation.¹⁷ Herein, only the cryopreservation mechanisms of the cryoprotectants used were studied, such as Me₂SO, which promotes the osmotic dehydration of cells by penetrating into the cytosol and preventing intracellular ice crystal formation.¹⁹ Moreover, sucrose, a disaccharide, promotes the stabilization of the lipid membrane because of direct interactions with the polar components of the cell bilayer, preventing the aggregation of intracellular molecules and osmotic stress.¹⁷

However, it is also necessary to consider species-specific characteristics, as cryopreservation protocols are dependent on these characteristics. This is true for *G. spixii*, in which physiological characteristics, such as size, weight, and metabolic rate, differ from other laboratory and wild rodents.¹⁵ Therefore, considering the damage observed after *G. spixii* fibroblast cryopreservation, and the need to optimize the cellular cryopreservation protocol for *G. spixii*, selecting new cryoprotective agents facilitates the promotion of greater membrane stability and controls osmotic stress. Thus, L-proline may be used as an alternative cryopreserving agent, because in red blood cells,¹⁹ lung carcinoma cells,²⁰ endothelial cells,²¹ and mouse oocytes,²² it has been found to have multifunctionality after cryopreservation.

L-proline, a natural amino acid, is structurally formed by a non-polar cyclic pyrrolidine side chain with an amino group.¹⁹ In endothelial cells, L-proline has been implicated as a cell survival factor at a concentration of 5 mM,²¹ as it protects cells against osmotic stress and maintains cell cycle progression through the binding and transmission of signal transporters

that participate in cell control and death.^{20,23,24} Furthermore, studies have shown that L-proline balances cell turgor pressure, reduces water stress, and controls the acidity of the cell culture medium, as it stabilizes proteins in the cytosol, preventing cryopreservation stress-induced denaturation, aggregation, or dissociation.^{23,25}

Considering the positive characteristics of 5 mM L-proline, we hypothesized that such an agent could improve the viability and physiological quality of *G. spixii* fibroblasts. However, the isolated effect of L-proline and its synergistic effect with sucrose need to be investigated. Therefore, this study aimed to evaluate the synergistic interaction of L-proline with sucrose and isolated effect of L-proline on the viability and physiology of *G. spixii* fibroblasts.

Materials and Methods

Reagents and solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco BRL (Carlsbad, CA, USA). The study protocol was approved by the Animal Use Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, No. 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, No. 60428-1).

Animals, collection, and processing skin biopsies

Biopsies from the peripheral ear region were recovered from six adult male *G. spixii* individuals (1-year old) that were kept in scientific breeding facilities located at the Wild Animal Multiplication Center (CEMAS/UFERSA, Mossoró, RN, Brazil 5° 10'S, 37° 10' W, No. 1478912). To simultaneously collect all skin biopsies, samples sterilized with 70% alcohol with sizes ranging from 1.0 to 2.0 cm² were obtained using surgical pliers. The samples were then transported to the laboratory in Dulbecco's modified minimal essential medium (DMEM) with 10% FBS and 2% antibiotic-antimycotic (ATB-ATM) solution at 4°C for up to 1 hour. In the laboratory, skin biopsies were processed by first removing the hair, and then cutting them into sections of 9.0 mm³ (3.0 × 3.0 × 1.0 mm) for *in vitro* culture.

Primary culture of skin biopsies and cell subculture

Subsequently, a primary culture was set up with incubation at 38.5°C and 6.5% CO₂.⁸ When the cells reached confluency ranging 70%–80%, they were subcultured and cell lines were established.⁸ Trypsinization was then performed using 0.25% trypsin with 0.05% EDTA. This was considered the first cell passage. After the fifth passage, the cells were cryopreserved as described in the Experimental design section.

Experimental design

To evaluate the effect of L-proline during cryopreservation, fibroblasts were stored at a low temperature (−196°C) using the slow freezing technique.^{26,27} The fibroblasts were evaluated under the following experimental conditions: (i) control (non-cryopreserved); (ii) cryopreserved with a solution comprising 10% Me₂SO, 10% FBS, and 0.2 M sucrose; (iii) cryopreserved with a solution comprising 10% Me₂SO, 10% FBS, and 5 mM L-proline; or (iv) cryopreserved with a solution comprising 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. The L-proline concentration used in this study was

L-PROLINE PRESERVES CELL QUALITY

111

based on the cryopreservation of human endothelial cell lines.²² In this study, 5 mM L-proline did not cause negative effects after the cells were recultured and stored at low temperatures (-196°C); however, higher concentrations generated high levels of osmotic stress.

We evaluated standard cryopreservation medium with the presence and absence of L-proline and synergism of L-proline.^{3,18} This allowed us to visualize its effects on reducing or controlling osmotic stress during the cryopreservation of fibroblastic lines derived from *G. spixii*. Finally, non-cryopreserved and cryopreserved cells were evaluated for their morphology and physiological characteristics, such as viability, metabolism, proliferation, apoptosis, levels of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi\text{m}$).

Slow freezing and thawing of fibroblasts

A concentration of 1.0×10^5 cells/mL was recovered from *in vitro* cultures, and the cells were counted using a Neubauer chamber.⁸ The cells were then added to cryotubes containing DMEM, 10% Me_2SO , and 10% FBS, and were maintained for 15 minutes at 4°C . After incubation, 0.2 M sucrose and 5 mM L-proline were added and the cells were further incubated for 15 minutes at 4°C .^{3,22} The Mr. Frosty system[®] (Thermo Scientific Nalgene, Rochester, NY, USA), a programmable freezer, was then used to freeze the cells held in a container for 12 hours at -80°C ($1^{\circ}\text{C}/\text{min}$). The samples were stored in cryotubes in liquid nitrogen (-196°C) for up to 2 weeks.

The cryovials were thawed by initially incubating at 25°C for 1 minute, followed by 4 minutes in a temperature-controlled double boiler (35°C – 37°C). DMEM containing 0.2 M sucrose or 5 mM L-proline was added to the thawed cells, according to the experimental groups,^{3,22} and they were maintained at 4°C for 15 minutes. After incubation, the cell suspension was centrifuged for 10 minutes at $600\times g$. The supernatant was removed, cells were resuspended in DMEM, 10% FBS, and 2% ATB–ATM solution, incubated for 15 minutes at 25°C , followed by centrifugation (10 minutes; $600\times g$). After centrifugation, the cells were recovered and subjected to *in vitro* culture using a previously described medium (DMEM, 10% FBS, and 2% ATB–ATM solution) and evaluated according to the experimental design.

Morphological evaluation and cell viability assay using trypan blue

Fibroblast morphology, before and after cryopreservation, nuclear shape, and cytoplasmic extension of the cells were evaluated using an inverted microscope (Nikon TS100, Tokyo, Japan).²⁸

A cell suspension (20 μL) of fibroblasts was recovered before and after cryopreservation (immediately after thawing and *in vitro* culturing) to determine the percentage of viable cells. For this, the cells were stained using trypan blue (20 μL ; 0.4%), where non-viable cells (dead; stained blue) were those with ruptured membranes and viable cells (alive; colorless) were those with intact cell membranes.²⁸ A Neubauer chamber was used for cell counting and quantification was performed under an optical light microscope (CX23, Olympus Tokyo, Japan; magnification $10\times$) using the following formula: number of viable cells/number of total quantified cells.

Metabolic and proliferative activity of cells

The metabolic activity of cells was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.²⁷ A concentration of 5.0×10^4 cells/mL was recovered and seeded in 12-well dishes. The cells were incubated for 5 days in an incubator (38.5°C with 6.5% CO_2). After incubation, the MTT solution (5 mg/mL) was added, and after 3 hours under modified temperature and atmosphere (38.5°C and 6.5% CO_2), the cells were analyzed at 595 nm using a spectrophotometer (UV-mini-1240; Shimadzu[®], Kyoto, Japan).

To evaluate the rate of proliferation, cells were seeded in 24-well dishes at an initial concentration of 1.0×10^4 cells/mL for 7 days (168 hours). At intervals of 24 hours, after trypsinization, the average cell count was obtained, and used to generate a growth curve and calculate the population doubling time (PDT),²⁶ which was estimated using the following mathematical calculation:

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

The PDT is expressed in hours (h), where T is the incubation time, $\ln$ is Napier's logarithm, X_e is the number of cells at the end of the incubation period, and X_b is the number of cells at the start of the incubation period.

Assessment of apoptotic levels

The apoptotic levels of fibroblasts were evaluated using a cell suspension (35 μL) in which the cells were stained with ethidium bromide (10 $\mu\text{g}/\text{mL}$) and acridine orange (2 $\mu\text{g}/\text{mL}$) diluted in 8 μL of phosphate-buffered saline.²⁹ Analysis was performed using a fluorescence microscope (Olympus BX51TF) at an absorbance of 480 nm. Cells were considered to be (i) viable if they had a uniform green nucleus; (ii) at the initial stage of apoptosis if they had non-uniform green nucleus; (iii) at a late stage of apoptosis if they had a uniform orange nucleus; and (iv) undergoing necrosis if they had a uniform red nucleus.²⁶ Images were captured at a magnification of $20\times$, and 300 cells were quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Quantification of ROS and $\Delta\Psi\text{m}$ levels

Oxidative stress mediated by ROS molecules and $\Delta\Psi\text{m}$ was quantified using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; 10 μM ; Invitrogen, Carlsbad, CA, USA) and MitoTracker Red[®] (CMXRos; 500 nM; Invitrogen). Approximately 50 μL of cell suspension was labeled with fluorescent probes. After 30 minutes of incubation (38.5°C and 6.5% CO_2), 100 cells per group were processed and the probes were analyzed using a fluorescence microscope (Olympus BX51TF).⁸

Statistical analysis

Data obtained from six adult male *G. spixii* individuals were expressed as the mean $\pm$ standard error (one animal/one replicate) and analyzed using GraphPad Prism software (Version 10.2.3, Graph-Pad Prism Software Incorporation, La Jolla, CA, USA). Normality was assessed using the Shapiro–Wilk test, and homoscedasticity was assessed using Levene's test. Data that were not normally distributed were transformed

using arcsine. Data were compared using analysis of variance, followed by Tukey's test or an unpaired Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

No phenotypic change was observed in cellular morphology between the experimental groups (Fig. 1A–D). The cells maintained their fusiform shape, were elongated, and had a centralized nucleus. Moreover, we observed that, after cryopreservation and *in vitro* culture, fibroblasts exposed to the cryoprotectant solution containing 10% Me₂SO, 10% FBS, and 5 mM L-proline or 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline were more adherent to the surface than those exposed to only 10% Me₂SO, 10% FBS, and 0.2 M sucrose. This may explain the lower viability observed after the trypan blue assay (Fig. 2A), which demonstrated that, immediately after cryopreservation, the cell viability rate was lower for cells in the solution with 10% Me₂SO, 10% FBS, and 0.2 M sucrose ($72.3\% \pm 1.2\%$) than for non-cryopreserved cells ($86.7\% \pm 2.0\%$). Moreover, cell viability was similar between cells cryopreserved in 10% Me₂SO, 10% FBS, and 5 mM L-proline ($88.4\% \pm 1.8\%$) and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline ($87.8\% \pm 2.1\%$) and non-cryopreserved cells (Fig. 2A). However, no difference was observed among the experimental groups after *in vitro* culture (Fig. 2A).

After cryopreservation, it was initially evident that cell metabolism was $>90\%$ in all experimental groups (Fig. 2B; $p > 0.05$), demonstrating the cell physiological functionality per group. Similar results were obtained when comparing the experimental groups after PDT analysis, in which the proliferation rate varied between 17 and 20 hours (Fig. 2C; $p > 0.05$). Although the metabolism and proliferation rate between experimental groups did not differ, after 24 hours of cell growth curve analysis (Fig. 2D), only the groups containing 10% Me₂SO, 10% FBS, and 5 mM L-proline (22.3 ± 1.3 cells/mL) and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline (25.3 ± 1.6 cells/mL) had a higher concentration of cells than the non-cryopreserved group (13.7 ± 1.4 cells/mL). Furthermore, after 168 hours of evaluation, only the group containing 10% Me₂SO, 10% FBS, and 5 mM L-proline (41.1 ± 3.5 cells/mL) and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline (50.0 ± 2.7 cells/mL) were similar to the non-cryopreserved (63.6 ± 11.8 cells/mL) group, while the group containing 10% Me₂SO, 10% FBS, and 0.2 M sucrose (36.0 ± 3.0 cells/mL) had a lower cell concentration (Fig. 2D).

After apoptotic analysis of the cryopreserved cells (Fig. 3A–D), only the groups with 10% Me₂SO, 10% FBS, and 5 mM L-proline ($78.4\% \pm 3.4$) and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline ($78.8\% \pm 3.1$) were similar to the non-cryopreserved group ($88.2\% \pm 1.1$, Fig. 3E).

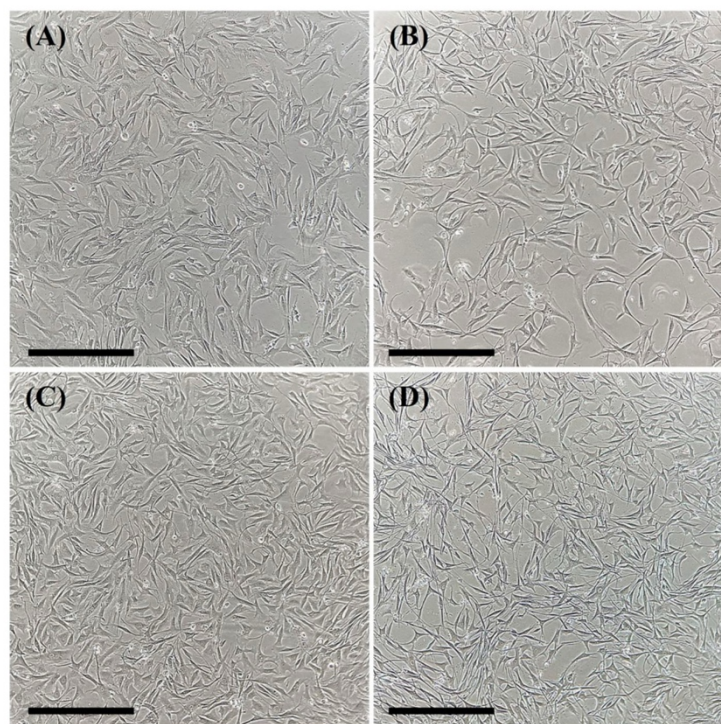


FIG. 1. Representative images of groups of fibroblasts isolated from *Galea spixii* ear skin before and after slow freezing. (A) Control (non-cryopreserved). (B) 10% Me₂SO, 10% FBS, and 0.2 M sucrose. (C) 10% Me₂SO, 10% FBS, and 5 mM L-proline. (D) 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. Bar scale = 50 μ m, magnitude 40 $\times$.

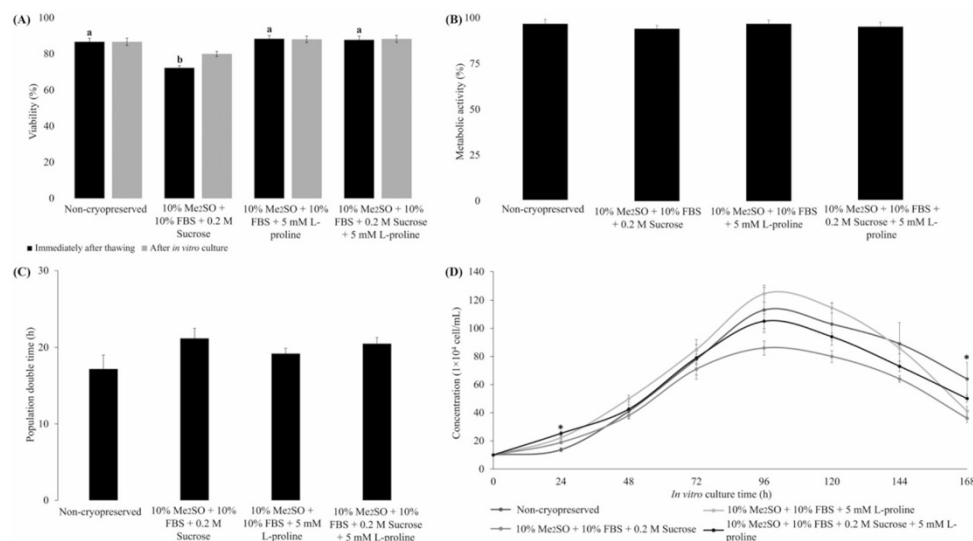


FIG. 2. Viability analysis (immediately after cryopreservation and after *in vitro* culture) and physiological characteristics of *G. spixii* skin fibroblasts before and after slow freezing. **(A)** Quantification of cell viability by trypan blue assay. **(B)** Percentage of metabolic activity mediated by MTT assay. **(C)** Analysis of population doubling time. **(D)** Evaluation of the cell growth curve. ^{a,b,*}Subscripts indicate difference between the experimental groups ($p < 0.05$).

Furthermore, the 10% Me₂SO, 10% FBS, and 0.2 M sucrose group ($74.6\% \pm 4.1$) showed a lower number of viable cells than the non-cryopreserved group ($88.2\% \pm 1.1$, Fig. 3E). An identical result was observed after evaluating the levels of ROS and $\Delta\Psi_m$ (Fig. 4), where only the group containing 10% Me₂SO, 10% FBS, and 0.2 M sucrose showed an increase in ROS levels (2.31 ± 0.9) and $\Delta\Psi_m$ (1.27 ± 0.5) compared to the non-cryopreserved group (1.00 ± 0.5 ; 1.00 ± 0.4), whereas the groups containing 10% Me₂SO, 10% FBS, and 5 mM L-proline (1.03 ± 0.5 and 0.99 ± 0.42) and Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline (1.07 ± 0.5 and 1.22 ± 0.73) had similar results.

Discussion

The formation of cell biobanks for wild rodents through cryopreservation significantly contributes to studies aimed at conservation,³ as it maintains genetic resources. The samples can be used for basic and applied studies, to gain information regarding the physiological, reproductive, biological, and molecular nature of *G. spixii*.³⁰ For *G. spixii*, cryopreservation of somatic cells occurred for the first time using fibroblastic lines isolated from the skin by slow freezing.⁸ Although this is a promising technique, cryopreservation by slow freezing results in morphological, structural, and physiological damage to the fibroblasts. In

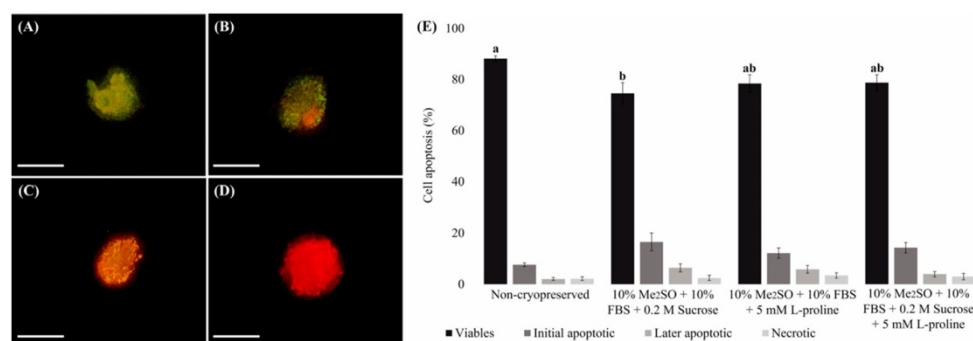


FIG. 3. Assessment of the percentage of apoptotic levels after *in vitro* culture of *G. spixii* skin fibroblast lines. **(A)** Viable cell. **(B)** Initial apoptosis. **(C)** Late apoptosis. **(D)** Necrotic cell. **(E)** Percentage after assessment of apoptotic levels. Bar scale = 100 μ m, magnitude 100 $\times$. ^{a,b}Subscripts indicate difference between the experimental groups ($p < 0.05$).

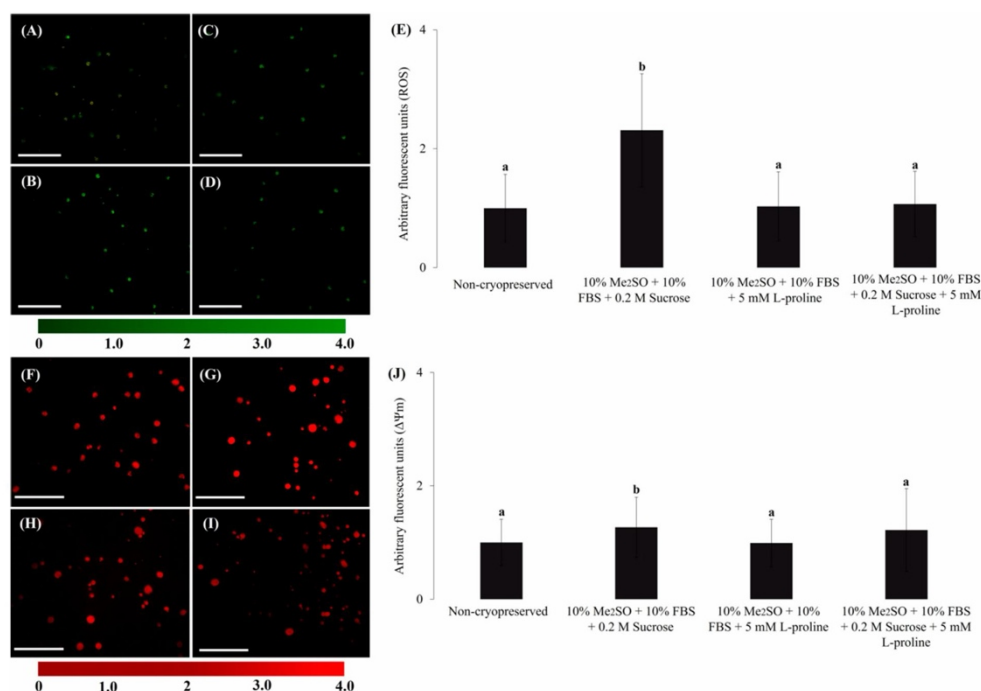


FIG. 4. Assessment of intracellular levels of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) of *G. spixii* fibroblasts before and after slow freezing. (A–D) Representative image of the ROS assessment of groups (control [non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 μ m, magnitude 20 $\times$. (E) Arbitrary fluorescence units obtained after evaluating cellular oxidative stress. (F–I) Representative image of the $\Delta\Psi_m$ assessment of groups (control [non-cryopreserved]; 10% Me₂SO, 10% FBS, and 0.2 M sucrose; 10% Me₂SO, 10% FBS, and 5 mM L-proline; and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline) of samples before and after slow freezing. Bar scale = 50 μ m, magnitude 20 $\times$. (J) Arbitrary fluorescence units obtained after evaluating the mitochondrial activity of fibroblasts. ^{a,b}Subscripts indicate difference between the experimental groups ($p < 0.05$).

addition, cryopreservation causes an imbalance in the cellular redox system, altering ROS levels and $\Delta\Psi_m$. In other laboratory and wild rodents, such as *Cavia porcellus*,¹⁸ *Mesocricetus auratus*,²⁶ and *Dasyprocta leporina*,²⁷ characteristics similar to those of *G. spixii* have been verified, leading to a reduction in the quality, viability, and stability of cells stored in cellular biobanks.

Due to the damage generated in the fibroblastic lines derived from *G. spixii*, we searched for alternatives to improve the quality of this cell type after cryopreservation. As the first parameter evaluated, the selection of the cryoprotectant solution components was an essential factor to be improved during slow freezing³¹ as this guarantees greater cell viability and stability during *in vitro* culture and cryopreservation. We used a protocol usually applied to the cryopreservation of wild mammalian cells,³ involving the use of 10% Me₂SO, which is an intracellular cryoprotectant that promotes multiple effects and actions on specific and non-specific targets.²¹ Furthermore, Me₂SO acts through the osmotic dehydration of cells, reducing ice crystal formation during nucleation and thawing events, which may lead to cell death.³²

In this same cryopreservation protocol, 0.2 M sucrose was used to stabilize the lipid membrane. Sucrose interacts directly

with the cellular components present in the lipid bilayer, avoiding intracellular ice aggregation and providing cytoprotection.²¹ Although cryoprotectants are widely used in the cryopreservation of wild rodents cells,³³ many studies have demonstrated that these agents are not always efficient. For instance, when Me₂SO reaches different cellular compartments, it can cause negative effects that are required for the physiological activity of cells, causing changes in the enzymatic reactions of the mitochondria, modifications in transport mechanisms, and ionic exchange.³³ Therefore, an increase in the release of electrons increases ROS synthesis, leading to oxidative stress, as observed in some experimental groups in our study.

To optimize slow freezing during *G. spixii* fibroblast cryopreservation, with the aim of minimizing the deleterious effects caused by these cryoprotective solutions, L-proline was used either alone or in combination with sucrose as an alternative to synthetic congeners and their limitations, and its effects in terms of toxicity and cytoprotective function on cryopreserved *G. spixii* fibroblastic lines were studied. L-proline is a natural amino acid synthesized by microorganisms in response to cold exposure. It aids in eliminating oxidizing molecules³⁴ and involves certain biological functions, such as protein synthesis,³⁵ metabolism,³⁶

cellular structure maintenance,²⁵ and intracellular redox state regulation.³⁷

Using this approach, we initially observed no phenotypic difference between the fibroblasts of the experimental groups after morphological evaluation; this result was consistent with those of other studies involving somatic cells from laboratory and wild rodents.^{3,18} However, after analyzing the viability of these cells using the membrane exclusion test, we observed that, immediately after cryopreservation, cells in the group cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose had lower viability than those in the non-cryopreserved group and group cryopreserved with 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline. A similar result was observed in the study carried out by Jesus et al.,³⁸ where superior viability was observed for HaCaT cells cryopreserved with L-proline compared to cells cryopreserved with only Me₂SO. According to Bojic et al.,³¹ this may have occurred because of the close interactions between the cells and cryopreservation medium, causing the cells to be markedly more susceptible to damage caused by changes during the freezing and thawing phases.

In *M. auratus*,²⁶ a reduction in cell viability was observed immediately after cryopreservation due to the association between cell osmotic limits and the cryoprotective solutions used in the study (10% Me₂SO and 90% FBS). In our study, L-proline guaranteed a greater interaction between the molecules of cell cytosol and extracellular environment to which this natural amino acid was subjected. Pi et al. corroborated this response,³⁹ by combining and observing the synergistic effect of L-proline during T-cell cryopreservation.

The results previously obtained are important because in our study, we also observed that, after cryopreservation and *in vitro* culturing of *G. spixii* fibroblastic lines, the groups cryopreserved in 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline had superior cell growth curves (cells/mL) than non-cryopreserved cells in the first 24 hours of evaluation. However, after 168 hours, the group cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose showed a lower number of cells (cells/mL) than the non-cryopreserved group. This may have occurred because after reculturing, the structural integrity of *G. spixii* fibroblasts was not positively supported when they were cryopreserved in a solution containing 10% Me₂SO, 10% FBS, and 0.2 M sucrose only; however, the cells that were cryopreserved with 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline were less affected by slow freezing.

Therefore, cryopreserved cells containing L-proline recover more rapidly from injuries caused by ice crystal formation during freezing and thawing. In contrast, *G. spixii* fibroblasts cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose lost their biochemical activity, although they maintained intact cell membranes. These results corroborated those of a study on endothelial cells.²² When compared with the control group (Me₂SO) and cells treated with the other osmolytes evaluated, cells cryopreserved in a medium containing L-proline were more adherent to the *in vitro* culture dishes.

Although 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline promoted an increase in cell viability and proliferation rate, after analyzing the cell growth curve in comparison with cells

cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose, we did not observe differences in PDT or metabolism. These results suggested that the cryoprotective agents tested, at the appropriate concentrations, are not toxic to fibroblastic lines isolated from the skin of *G. spixii*. Furthermore, cryoprotective solutions act positively in reducing and controlling ice crystal formation, and in processes related to recrystallization after cell thawing, especially in the presence of L-proline, to allow greater cell viability and proliferation after cryopreservation.²¹

In addition, the apoptotic index of *G. spixii* fibroblasts was analyzed to predict cell death, which has been associated with osmotic stress events during the slow freezing of cells.⁴⁰ This assay also allowed cell quality and stability to be observed during *in vitro* culturing.²⁸ The number of viable cells was lower in the group cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose than in the non-cryopreserved group. However, the number of viable cryopreserved fibroblasts in the groups cryopreserved with 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline was identical. This may be because the group containing only sucrose may have initiated biochemical pathways related to programmed cell death, such as caspase-3 and caspase-7 pathways, which are highly active during cell death.^{41,42}

Moreover, unlike L-proline, sucrose is a disaccharide with a mechanism of action similar to that of trehalose, allowing lipid membrane stabilization only because of direct interactions between the polar components of the cell bilayer.¹⁷ L-proline exerts different biological functions, such as inducing the control of the *p53* gene and enzyme synthesis, for example, proline oxidase (POX/PRODH), which is located in the inner mitochondrial membrane and catalyzes the transfer of electrons as well as regulates intracellular redox reactions,³⁷ thereby regulating the *in vitro* culture of cells.

This justification was verified in our study, as cells cryopreserved with 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline maintained ROS and $\Delta\Psi_m$ levels similar to their levels in non-cryopreserved cells, whereas their levels were higher in cells cryopreserved with 10% Me₂SO, 10% FBS, and 0.2 M sucrose. This may have occurred because free and polypeptide-bound proline can react with hydrogen peroxide (H₂O₂) and hydroxyl groups to form stable free radicals.²⁵ For instance, a study of the antioxidant action of 5 mM L-proline⁴³ found that, after 30 minutes, the H₂O₂ levels were reduced by >90% when compared to those of the control.

In addition, L-proline was added to a culture of human keratinocytes, and it was found to protect cells against induced apoptosis.⁴⁴ Therefore, this molecule reduces oxidative stress, ROS levels, and $\Delta\Psi_m$ levels in the experimental groups containing 10% Me₂SO, 10% FBS, and 5 mM L-proline and 10% Me₂SO, 10% FBS, 0.2 M sucrose, and 5 mM L-proline, ensuring greater osmotic control as well as balance of deplasmolysis pressure and cell turgidity.^{20,44}

Conclusions

This is the first study to optimize a technique for the cryopreservation of fibroblasts derived from *G. spixii* using L-proline. In our study, *G. spixii* cells stored at low temperatures, after thawing, L-proline alone or with sucrose remained viable and functional. Moreover, the cells cryopreserved with L-proline showed better maintenance of viability and proliferative rate, as

well as control of ROS and $\Delta\Psi_m$ levels. Therefore, L-proline, either alone or with sucrose, is an alternative for the cryopreservation of *G. spixii*-derived fibroblasts. Thus, it is possible to develop an adequate cellular biobank for *G. spixii*, in which the stored cells could be used to obtain biological, genetic, and reproductive knowledge, by somatic cell nuclear transfer cloning and inducing pluripotency.

Acknowledgments

The authors thank the Center for Wild Animals Multiplication (UFERSA) for providing biological material.

Authors' Contributions

L.V.C.A.: Conceptualization, methodology, writing, original draft, and investigation. L.L.V.R.: Methodology and investigation. S.L.O.: Methodology and investigation. Y.L.F.S.: Methodology and investigation. L.R.M.O.: Methodology and investigation. Y.B.F.M.: Methodology and investigation. A.F.P.: Conceptualization, methodology, writing, original draft, supervision, project administration, and funding acquisition.

Author Disclosure Statement

The authors report no potential conflicts of interest.

Funding Information

This study was partially funded by the Coordination of Improvement of Higher Education Personnel, Brazil (CAPES, Financial Code 001), and the National Council for Scientific and Technological Development (CNPq, No. 309078/2021-0).

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Address correspondence to:
 Alessandra F. Pereira, PhD
 Laboratory of Animal Biotechnology
 Federal Rural University of Semi-Arid
 Av. Francisco Mota, 572
 Costa e Silva
 Mossoró 59625900
 Brazil

E-mail: alexsandra.pereira@ufersa.edu.br

ANEXO E – VERSÃO PUBLICADA DO ARTIGO EXPERIMENTAL V PUBLICADO NA ANIMALS



Article

Effect of Epidermal Growth Factor and 6-Dimethylaminopurine on In Vitro Maturation and Artificial Activation of Spix's Yellow-Toothed Cavy (*Galea spixii* Wagler, 1831) Oocytes

Leonardo V. C. Aquino ¹, Samara L. Olindo ¹, Yara L. F. Silva ¹, Vinicius D. Silva ¹, Lhara R. M. Oliveira ¹, Moacir F. Oliveira ² and Alessandra F. Pereira ^{1,*}

- ¹ Laboratory of Animal Biotechnology, Federal Rural University of the Semi-Arid, Mossoró 59625-900, RN, Brazil; leonardovt@live.com (L.V.C.A.); samara.lima@alunos.ufersa.edu.br (S.L.O.); yara.silva@alunos.ufersa.edu.br (Y.L.F.S.); viniciusd647@gmail.com (V.D.S.); lhara@hotmial.com (L.R.M.O.)
² Center of Multiplication of Wild Animals, Federal Rural University of the Semi-Arid, Mossoró 59625-900, RN, Brazil; moacir@ufersa.edu.br
 * Correspondence: alexsandra.pereira@ufersa.edu.br

Simple Summary

In vitro maturation (IVM) and artificial oocyte activation are crucial steps in the development of assisted reproductive technologies (ARTs), including somatic cell nuclear transfer. While these techniques are well-established in laboratory rodents, such as mice and rats, they remain underdeveloped in wild species, like Spix's yellow-toothed cavy. Advancing ARTs in this species could support its conservation and help preserve biodiversity. This study evaluated the effects of two concentrations of epidermal growth factor (EGF; 10 or 50 ng/mL) in IVM medium, as well as the use of 6-dimethylaminopurine (6-DMAP) during artificial oocyte activation. Results showed that 10 ng/mL EGF enhanced *cumulus* cell viability and improved oocyte morphometric quality compared to 50 ng/mL. Although 6-DMAP positively influenced cleavage rates, it did not significantly affect morula formation. In conclusion, 10 ng/mL EGF is recommended for IVM in Spix's yellow-toothed cavy oocytes, and 6-DMAP shows potential to support early embryonic development, contributing to the establishment of ART protocols for this species.

Abstract

The successful application of assisted reproductive technologies (ARTs), such as in vitro maturation (IVM) and artificial oocyte activation, requires species-specific adaptations. Although these methods are routinely used in laboratory rodents, their use in wild or non-model species remains limited, such as the Spix's yellow-toothed cavy, a Neotropical species of ecological and reproductive interest. This study evaluated the effects of different concentrations of epidermal growth factor (EGF; 10 or 50 ng/mL) on IVM (Experiment 1) and of 6-dimethylaminopurine (6-DMAP) on artificial oocyte activation (Experiment 2). EGF at 10 ng/mL ($93.8\% \pm 1.6$; $84.9\% \pm 0.7$) promoted greater viability and less apoptosis in *cumulus* cells, compared to 50 ng/mL ($83.0\% \pm 1.6$; $78.9\% \pm 2.7$), maintaining *cumulus* expansion, ultrastructural integrity, and better morphometric quality of oocytes. Thus, this concentration was used in the next step, where oocytes were activated with or without 6-DMAP. After five days, the presence of 6-DMAP increased cleavage rates ($69.3\% \pm 5.0$) compared to activation without the compound ($53.5\% \pm 3.5$), without significantly affecting morula formation ($13.2\% \pm 3.1$ to $17.3\% \pm 2.9$). It is concluded that EGF improves the oocyte microenvironment, while 6-DMAP enhances cleavage, with these being the initial steps in the development of ARTs for Spix's yellow-toothed cavy.



Received: 7 July 2025
 Revised: 10 August 2025
 Accepted: 14 August 2025
 Published: 15 August 2025

Citation: Aquino, L.V.C.; Olindo, S.L.; Silva, Y.L.F.; Silva, V.D.; Oliveira, L.R.M.; Oliveira, M.F.; Pereira, A.F. Effect of Epidermal Growth Factor and 6-Dimethylaminopurine on In Vitro Maturation and Artificial Activation of Spix's Yellow-Toothed Cavy (*Galea spixii* Wagler, 1831) Oocytes. *Animals* **2025**, *15*, 2403. <https://doi.org/10.3390/ani15162403>

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Keywords: wildlife; neotropical rodents; ex situ conservation; in vitro embryo production; oocyte viability; embryonic development

1. Introduction

The Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) is a Neotropical rodent with predominantly diurnal and crepuscular habits, whose diet is based on a diverse range of plant species [1]. Its natural geographic distribution includes the Caatinga biome, extending to other regions of Brazil, Paraguay, and the eastern slopes of the Andes Mountains [2]. This broad distribution reflects its remarkable ecological adaptability, granting it a relevant functional role in dry forest ecosystems, particularly through its contributions to seed dispersal, soil aeration, and trophic chain regulation [3,4].

In addition to its ecological importance, Spix's yellow-toothed cavy exhibits reproductive traits that qualify it as a promising experimental model for the development of assisted reproductive technologies (ARTs). The species has a continuous polyestrous cycle with an average duration of approximately 14 days, as well as a gestation period of around 59 days, typically producing litters of one to two offspring [3]. These parameters, combined with its ease of management in captivity, due to its small body size, high metabolic rate, physiological adaptability, and consistent reproductive performance [5], reinforce its potential as a surrogate model for the development and standardization of protocols applicable to the conservation of phylogenetically related species, particularly those classified as threatened with extinction.

Among these vulnerable species are representatives of the suborder Hystricomorph, such as *Ctenomys ibicuiensis* and *Ctenomys flamarioni*, both classified as endangered and whose populations present significant logistical challenges for conducting reproductive studies [6]. In this context, the use of Spix's yellow-toothed cavy as a model species represents a viable, ethical, and translational alternative, providing a solid foundation for the development of reproductive approaches that can later be adapted to these more sensitive taxa.

Although Spix's yellow-toothed cavy is still considered a species with a stable population according to the International Union for Conservation of Nature (IUCN) [7], its increasing exposure to anthropogenic pressures, such as natural habitat fragmentation and illegal hunting, also raises concerns about its medium- and long-term sustainability and resilience. In this context, investing in research focused on its reproductive biology while populations remain stable represents a preventive and proactive strategy, enabling the early development of reproductive biotechnologies and genetic management protocols that may be more effectively applied in future scenarios of population decline or extinction risk.

Among wildlife conservation strategies, ARTs have gained significant attention [8]. One such ART is in vitro maturation (IVM) combined with artificial oocyte activation, both of which are essential steps in the development of techniques, such as somatic cell nuclear transfer (SCNT) [9]. With the advent of nuclear reprogramming—in which the epigenetic landscape of a somatic cell is altered by the remarkable reprogramming capacity of an enucleated oocyte to initiate embryonic development [10]—SCNT has emerged as a powerful biotechnological tool for the multiplication, conservation, and scientific study of reproductive biology in both laboratory and wild rodents [11].

However, the successful application of SCNT, including IVM and artificial oocyte activation, depends on species-specific adaptations [12]. Although these techniques are well established in laboratory rodents, their application in wild or non-model species remains limited and underexplored. Regarding IVM, it is clear that optimized protocols are

essential, as oocyte development depends on factors such as temperature [13], duration [14], and the composition and supplementation of the culture medium [9] to acquire meiotic competence. In *in vitro* culture systems for both laboratory and wild rodents, epidermal growth factor (EGF) is frequently included due to its role as 1 of the 13 families of secondary proteins that regulate *cumulus* cell expansion [15,16]. These *cumulus* cells are crucial for signaling the resumption of meiosis I, facilitating first polar body extrusion, aligning the metaphase plate, and promoting cytoplasmic maturation [17,18]. Studies in other rodent species have demonstrated that EGF concentrations ranging from 10 to 50 ng/mL increase IVM efficiency [9,19], whereas higher doses may negatively affect oocyte quality and maturation success. Therefore, evaluating the effects of EGF concentration during IVM of Spix's yellow-toothed cavy oocytes appears to be an important step in establishing this technical stage in the species.

It is essential to emphasize that, in addition to the oocyte undergoing IVM, the sensitivity of embryonic development during artificial activation is a crucial factor [10]. In both laboratory and wild rodents, a protocol combining 10 mM strontium chloride (SrCl₂) with 5 µg/mL cytochalasin B (CB) for 6 h has been used to artificially activate oocytes of the red-rumped agouti, resulting in a morula formation rate of 25% [9]. Although blastocysts were not observed, these results suggest the potential for optimizing embryo production protocols for this genus. In contrast, artificial activation protocols in laboratory rodents have demonstrated greater efficiency, with blastocyst rates varying depending on the methods applied. For example, Ma et al. [20] reported two commonly used murine protocols: the first, using 10 mM SrCl₂ and 5 µg/mL CB for 6 h, achieved a blastocyst rate of 56.3%; the second, a more effective protocol, applied 10 mM SrCl₂ for 2.5 h followed by 2 mM 6-dimethylaminopurine (6-DMAP) and 5 µg/mL CB for 3.5 h, achieving a blastocyst rate of 60.1%. These findings highlight that artificial activation efficiency may be species-dependent, which reinforces the need to adapt and validate these protocols specifically for Spix's yellow-toothed cavy in the context of its reproductive technologies.

Therefore, this study aimed to evaluate the effects of two EGF concentrations (10 or 50 ng/mL) in IVM medium and the use of 6-DMAP during the artificial activation of Spix's yellow-toothed cavy oocytes. To this end, we assessed the quality of the microenvironment and matured oocytes by analyzing *cumulus* cell expansion, ultrastructure, viability, and apoptosis levels, as well as morphometric parameters, first polar body extrusion, nuclear stage, and embryonic development kinetics.

2. Materials and Methods

2.1. Chemicals and Media

Unless otherwise stated, all chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gibco-BRL (Carlsbad, CA, USA).

2.2. Bioethics and Animals

The Animal Use Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.010566/2017-20) and the Chico Mendes Institute for Biodiversity Conservation (no. 60428-1) approved all experimental procedures. Nineteen healthy adult females, aged one year and weighing 250–350 g, were used in the study. They were housed in a controlled environment with access to fruit and water at the Center of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10' S, 37°10' W), which is registered in Brazil in the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912).

2.3. Collection, Transport of Ovaries, and Recovery of the Cumulus–Oocyte Complexes

Females were subjected to a 12 h fasting period prior to ovary collection. Anesthesia was induced using a combination of xylazine hydrochloride (Xilazin[®] 2%, 1 mg/kg, Manufacturer Syntec, São Paulo, SP, Brazil) and ketamine hydrochloride (Ketamine[®] 10%, 15 mg/kg, Vetnil, São Paulo, SP, Brazil). Euthanasia was then carried out via intravenous administration of potassium chloride (Chlorure de potassium[®] 19.1%, 2.56 mEq/kg, Halexistar, Goiânia, GO, Brazil) following established protocols [21].

After ovary collection using surgical scissors, the ovaries were transported to the laboratory in a pre-warmed saline solution (0.9% NaCl, *w/v*) at 30 °C, supplemented with 0.05 mg/mL penicillin (*w/v*), and maintained for up to 30 min [13]. For cumulus–oocyte complex (COC) retrieval, the ovaries were transferred to in vitro culture plates (60 × 15 mm) containing oocyte recovery medium (ORM). The ORM consisted of tissue culture medium (TCM-199) supplemented with 0.2 mM sodium pyruvate (*w/v*), 1% antibiotic-antimycotic (*v/v*), and 10% fetal bovine serum (FBS, *v/v*).

Following ovarian slicing, oocytes were recovered and morphologically evaluated under a stereomicroscope based on cumulus cell compaction, the number of surrounding cell layers, and ooplasm homogeneity. COCs were classified into three categories [14]: Type A—oocytes surrounded by three or more layers of cumulus cells; Type B—oocytes with one to three layers of cumulus cells; and Type C—oocytes with few or no cumulus cells, including denuded oocytes. Only COCs classified as Type A and Type B were selected for use in the experiments.

2.4. Experimental Design

Two experiments were conducted to evaluate the effects of different EGF concentrations (10 or 50 ng/mL) in the IVM medium (Experiment I) and the influence of 6-DMAP on artificial oocyte activation (Experiment II) (Figure 1). In Experiment I, seven females were used across three replicates (with two to three animals per replicate) to evaluate the effect of EGF on the oocyte microenvironment and nuclear maturation. Recovered oocytes were randomly allocated to IVM media supplemented with one of two EGF concentrations: 10 ng/mL (EGF10 group) and 50 ng/mL (EGF50 group). The concentrations selected for this study were based on prior research in other South American rodent models, including guinea pigs (*Cavia porcellus*) and red-rumped agoutis (*Dasyprocta leporina*). These studies demonstrated that EGF supplementation at concentrations between 10 and 50 ng/mL effectively promoted oocyte maturation and improved subsequent embryonic development. Conversely, doses exceeding this range were associated with reduced developmental competence and lower overall efficiency in vitro [9,19]. Analyses focused on the expansion of cumulus cells and their ultrastructural characteristics using scanning electron microscopy (SEM). Additionally, the viability and apoptosis levels of cumulus cells were evaluated to assess the quality of the in vitro culture environment. Oocytes were also examined for morphometric parameters and nuclear maturation, the latter determined by the extrusion rate of the first polar body (1PB) and classification of the nuclear stage in mature structures.

Following the selection of the optimal EGF concentration, Experiment II was conducted to evaluate the developmental competence of oocytes based on their ability to progress to early embryonic stages after artificial activation. Twelve females were used across four replicates (three animals per replicate). Recovered oocytes were matured in vitro using the previously determined optimal EGF concentration, and their quality was assessed according to the parameters previously described. Oocytes exhibiting 1PB extrusion were selected, subdivided into groups, and subjected to one of two artificial activation protocols: 10 mM SrCl₂ + 5 µg/mL CB for 6 h (SrCl₂ + CB group), or 10 mM SrCl₂ for 2.5 h followed by 5 µg/mL CB + 2 mM 6-DMAP for 3.5 h (SrCl₂ + CB + 6-DMAP group). In this study,

6-DMAP was evaluated during artificial activation using a standard protocol employed in the artificial activation of murine oocytes [20]. Finally, embryonic culture was conducted in a simple, optimized, and potassium-enriched medium (KSOM) for five days [19,22]. During this period, we monitored the kinetics of embryonic development and performed ultrastructural analyses to assess cleavage and embryo production rates.

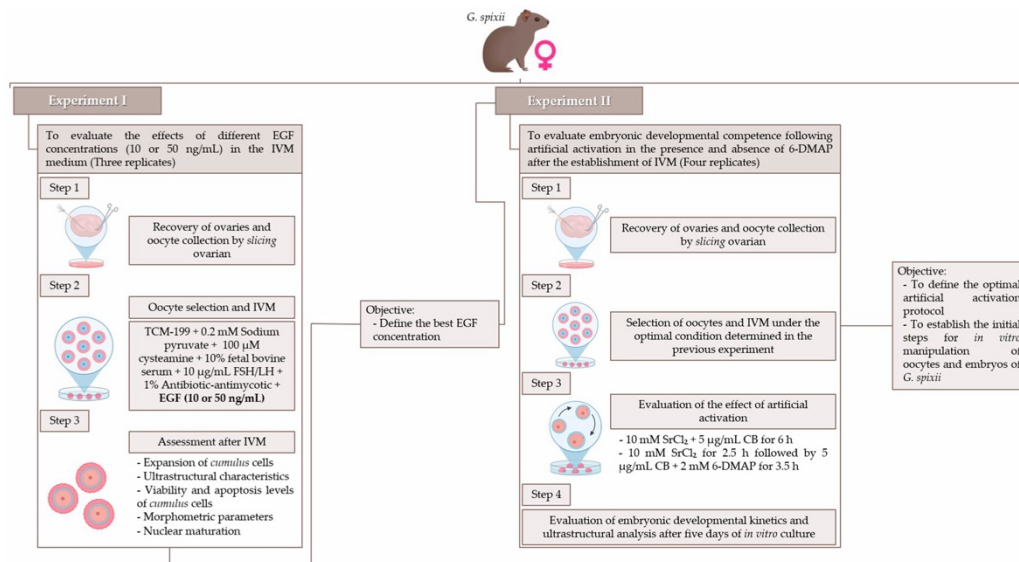


Figure 1. Experimental design of the steps involved in establishing in vitro manipulation conditions for oocytes and embryos of *G. spixii*.

2.5. In Vitro Maturation of Oocytes

For the oocyte IVM, recovered COCs were initially washed three times in ORM, followed by two additional washes in the IVM medium. Groups of approximately 20–25 oocytes were then cultured in 100 µL droplets of maturation medium, covered with mineral oil, and incubated at 38.5 °C in an atmosphere of 6.5% CO₂ [23]. The maturation medium consisted of ORM supplemented with 0.1 mM cysteamine and 10 mIU/mL FSH/LH (Pluset®, Hertape-Calier, Barcelona, Spain) [9]. EGF was added to the IVM medium at concentrations of 10 or 50 ng/mL. After 24 h, mature structures were evaluated according to the parameters described in the experimental design.

2.6. Assessments of the Microenvironment and Oocyte Maturation

2.6.1. Expansion of Cumulus Cells

Matured oocytes were evaluated for cumulus cell expansion under a stereomicroscope, following the classification method described by Zhao et al. [24]. COCs were scored on a scale from 0 to 4, as follows: Score 0—no observable expansion; Score 1—minimal expansion, with rounded cumulus cells predominantly adhered to the substrate; Score 2—expansion observed in the outer layers of cumulus cells, forming two distinct layers; Score 3—expansion of all layers except the corona radiata, forming more than three layers; Score 4—full expansion of both the cumulus oophorus and corona radiata cell complexes.

Moreover, the cumulus expansion index (CEI) was calculated for each EGF concentration tested. This index represents the mean expansion score within each group and was determined using the formula proposed by Fagbohun and Downs [25]. Groups with a

CEI value approaching 0 were indicative of minimal *cumulus* expansion and suboptimal in vitro culture conditions. In contrast, values approaching 4 reflected complete expansion and were associated with higher oocyte quality [25].

2.6.2. Ultrastructure Assessment by SEM

Matured oocytes previously exposed to different concentrations of EGF, as well as zygotes, were analyzed by scanning electron microscopy (SEM) to assess their cellular quality. Sample preparation followed the protocol described by Li et al. [26], with minor modifications. Briefly, the oocytes were initially fixed in 2.5% glutaraldehyde diluted in phosphate-buffered saline (PBS; pH 7.2) at 4 °C for 2 h.

Specifically for the zygotes, a preliminary step was performed to remove the zona pellucida prior to fixation. To accomplish this, the zygotes were washed in ORM-HEPES medium (25 mM HEPES; pH 2.5) until complete digestion of the zona pellucida and then fixed in glutaraldehyde, as previously described. After fixation, all samples (oocytes or zygotes) were washed twice for 5 min each in 2% cacodylate buffer and subsequently post-fixed in 1% osmium tetroxide for 2 h at room temperature (25 °C).

Following fixation, the cells (oocytes or zygotes) were washed again in PBS and dehydrated in a graded ethanol series (7.5%, 15%, 30%, 50%, 70%, 90%, and 100%) for 5 min at each concentration. After dehydration, the structures (oocytes or zygotes) were mounted on glass slides, gold-sputtered, and examined under a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France). This approach enabled a detailed analysis of the surface morphology of the evaluated structures, allowing for the observation of the structural integrity of oocytes and zygotes, the arrangement of *cumulus* cells, the texture and continuity of the zona pellucida, as well as the characteristics of the plasma membrane. These parameters provided relevant insights for assessing cellular quality and identifying possible morphological alterations resulting from the different experimental treatments applied.

2.6.3. Viability and Apoptotic Levels of Cumulus Cells

Matured oocytes were denuded by mechanical pipetting in ORM containing 0.1% hyaluronidase (*w/v*). Next, 50 µL of the cell suspension was collected and stained with Trypan blue (0.2%) diluted in PBS [9]. The *cumulus* cell suspension was then analyzed for viability using a Neubauer chamber. Non-viable cells (dead; stained blue) were distinguished from viable cells (alive; colorless), based on the rupture of the cell membrane. The percentage of viable cells was calculated using the formula: number of viable cells/total number of cells quantified $\times$ 100.

The apoptotic levels of *cumulus* cells were determined using fluorescent dyes, following the method described by Rodrigues et al. [27] with modifications. For this analysis, a 50 µL aliquot of the suspension containing *cumulus* cells from previously denuded oocytes was collected. The cells were stained with ethidium bromide (5 µg/mL) and acridine orange (1 µg/mL), diluted in 8 µL of PBS. After the staining procedure, the sample was transferred to a glass slide and examined under a fluorescence microscope to assess the stages of cellular apoptosis. Through differential dye uptake, chromatin condensation in the cell nucleus was identified [28]. Based on fluorescence patterns, cells were classified into four categories: (i) viable cells, exhibiting uniform green fluorescence; (ii) early apoptosis, indicated by non-uniform green staining of the nucleus; (iii) late apoptosis, characterized by uniform orange nuclear fluorescence; and (iv) necrosis, marked by intense red fluorescence in the nucleus. This classification followed the criteria described by Aquino et al. [29]. For this, a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) was used with an absorbance of 480 nm. Finally, images were saved, and 300 cells were counted for each

replicate using Image J 7.1 (National Institutes of Health, Wellesley, MA, USA) software at 20× magnification.

2.6.4. Morphometric and 1PB Evaluation of Oocytes

Morphometric analyses involved the morphometric evaluation of oocytes with and without the presence of 1PB after 24 h of IVM. Images were captured at 20× magnification using an inverted microscope (Leipzig IMx, Phoenix Optics, Leipzig, Germany). Pixel distances were converted using ImageJ 1.54 software to determine the outer oocyte diameter (OOD), inner oocyte diameter (IOD), zona pellucida thickness (ZPT), and ooplasm diameter (OD) [30]. Based on these parameters, the following measurements were calculated using mathematical formulas: space perivitelline diameter (SPD; $OID - OD$), internal oocyte area (IOA; $\pi \times (IOD/2)^2$), ooplasm area (OA; $\pi \times (OD/2)^2$), and perivitelline space area (PSA; $IOA - OA$) [31].

2.6.5. Nuclear Stage Assessment

The nuclear stage of the oocytes was determined by observing the extrusion of the first polar body (1PB), followed by fluorescent staining. Initially, the oocytes were fixed in 4% paraformaldehyde solution for 15 min. After fixation, they were washed twice in phosphate-buffered saline (PBS) supplemented with 0.4% bovine serum albumin (PBS-BSA). Subsequently, nuclear staining was performed using 10 µg/mL of Hoechst 33,342 for 15 min. For this, a stock solution of Hoechst (5 mg/mL) was diluted in 1.5 mL of ORM to reach the desired final concentration. After staining, the oocytes were washed twice again in PBS-BSA to remove excess Hoechst 33,342 and mounted on glass slides. Fluorescence analysis was performed using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and oocytes were considered mature when observed at the metaphase II stage [23]. The excitation wavelength for the fluorophore was 510 nm, and the emission was detected at 590 nm.

2.7. Artificial Activation, Ultrastructure Analysis, and Embryonic Kinetic Patterns

For artificial activation, denuded oocytes that had shown 1PB extrusion were used. The oocytes were first washed three times in ORM. Then, they were artificially activated in calcium-free Tyrode's albumin lactate pyruvate (TALP) medium, supplemented with 10 mM SrCl₂ and 5 µg/mL CB for 6 h at 38.5 °C and 6.5% CO₂. For the 6-DMAP group, oocytes were initially incubated at 38.5 °C and 6.5% CO₂ in TALP medium containing 10 mM SrCl₂ for 2.5 h. After this period, the oocytes were washed three times in ORM and transferred to drops containing TALP medium with 5 µg/mL CB and 2 mM 6-DMAP for 3.5 h at 38.5 °C and 6.5% CO₂, following the protocol described by Ma et al. [20]. The TALP medium was composed of salts, 10 mM sodium lactate (*v/v*), 3 mg/mL bovine serum albumin fraction V (*w/v*), and 1% antibiotic-antimycotic (*v/v*).

After artificial activation, presumed zygotes were rewashed in ORM and placed in 60 µL drops containing KSOM, which was supplemented with 1% essential amino acids, 0.5% non-essential amino acids, and 15 mM BSA. The structures were cultured for five days, with D0 defined as the day of artificial activation. On D2, cleavages were identified, and the rate for each group was recorded. Additionally, on D4 and D5, the production rates of morulae and blastocysts were observed.

Furthermore, during embryonic culture, one structure at each stage of embryonic development was recovered, fixed, and processed as previously described for ultrastructural evaluation by SEM of oocytes.

2.8. Statistical Analysis

All data were expressed as the mean $\pm$ standard error and were analyzed using StatView 5.0 (SAS Institute Inc, Cary, NC, USA). The normality of the data was verified using the Shapiro–Wilk test, while homoscedasticity was verified using Levene’s test. After confirming the normality and homoscedasticity of the data, variables related to *cumulus* cell expansion, viability and apoptotic levels of *cumulus* cells, presence of the 1PB, nuclear stage, and embryonic development rates were analyzed using Fisher’s exact test. CEI data and oocyte morphometric analyses were evaluated by analysis of variance (ANOVA), followed by Tukey’s test. Differences were considered statistically significant when the $p < 0.05$.

3. Results

After slicing 38 ovaries (Figure 2A), the number of oocytes recovered and their morphological classification are detailed in Table 1. This table shows the proportion of each oocyte type obtained from the ovaries used in this study.

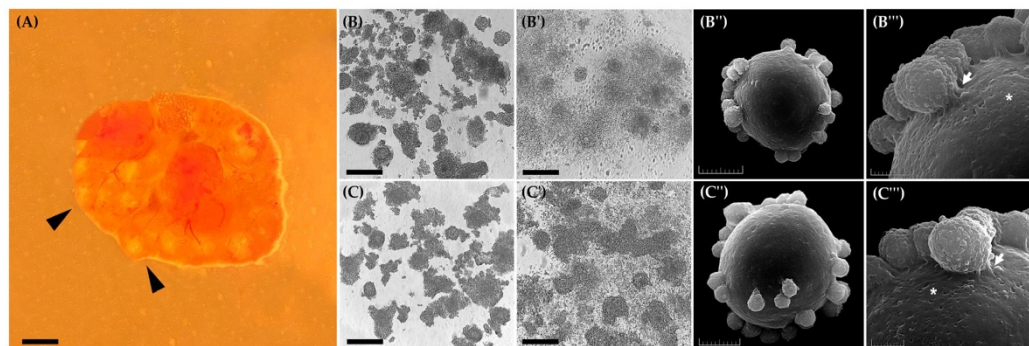


Figure 2. Representation of Spix’s yellow-toothed cavy ovaries and oocytes before and after IVM with EGF as a supplement to evaluate oocyte competence acquisition. (A) Ovary containing follicles (arrowhead) before processing by slicing. (B,B’) Immature Type I and II oocytes and *cumulus* cell expansion after 24 h of IVM with 10 ng/mL of EGF. (B’’,B’’’) Ultrastructural analysis of oocyte after IVM with 10 ng/mL EGF, showing transzonal projections from *cumulus* cells (arrow) and zona pellucida pores (asterisk). (C,C’) Immature Type I and II oocytes and *cumulus* cell expansion after 24 h of IVM with 50 ng/mL of EGF. (C’’,C’’’) Ultrastructural analysis of oocyte after IVM with 50 ng/mL EGF, showing transzonal projections from *cumulus* cells (arrow) and zona pellucida pores (asterisk). Scale bar = 100 μ m (A); 50 μ m, mag. 20 $\times$ (B,B’,C,C’); 20 μ m (B’’,C’’); 5 μ m (B’’’,C’’’).

Table 1. Number and classification of oocytes recovered from Spix’s yellow-toothed cavy ovaries.

Experiment	No. Ovaries	Classification of Immature Oocytes			Oocytes Type A/No. Ovaries	Oocytes Type B/No. Ovaries	Oocytes Type C/No. Ovaries	Total Oocytes/Ovaries
		Type A (%)	Type B (%)	Type C (%)				
I	14	52.7 $\pm$ 5.5 (78/148)	18.9 $\pm$ 3.7 (28/148)	28.4 $\pm$ 2.1 (42/148)	5.6 $\pm$ 1.6	2.0 $\pm$ 0.0	3.0 $\pm$ 0.5	10.7 $\pm$ 2.0
II	24	44.6 $\pm$ 5.0 (107/240)	50.0 $\pm$ 2.3 (120/240)	5.4 $\pm$ 3.2 (13/240)	4.5 $\pm$ 0.2	5.0 $\pm$ 0.8	0.5 $\pm$ 0.3	10.0 $\pm$ 0.4

Note: The results are expressed as mean $\pm$ standard error. Type (A): Oocytes that had three or more layers of *cumulus* cells; Type (B): Oocytes that had between 1 and 3 layers of *cumulus* cells; Type (C): Oocytes that had few or no *cumulus* cells or were naked, as described by Yao et al. [14]. Experiment I: three repetitions; Experiment II: four repetitions.

3.1. Experiment I: Effect of EGF on Cumulus Cells Expansion and Ultrastructural Visualization of Oocytes

Figure 2 shows immature oocytes before (Figure 2B,C) and after (Figure 2B',C') IVM with 10 and 50 ng/mL of EGF. It was observed that after the IVM process, neither concentration of EGF significantly influenced the different degrees of *cumulus* cell expansion (Table 2; $p > 0.05$). However, it is essential to note that 10 ng/mL and 50 ng/mL EGF resulted in more than 40% of the evaluated oocytes achieving a score of 4. Additionally, this result was considered positive, as the CEI analysis revealed oocytes with indices of up to 3.4, where a score of 4 represents the maximum value on the scale used to determine oocyte quality in this study (Table 2).

Table 2. Evaluation of the grade of *cumulus* cell expansion and CEI of Spix's yellow-toothed cavy oocytes matured in IVM medium supplemented with two concentrations of EGF after 24 h.

Groups	No. Oocytes	Grade of Cumulus Cell Expansion (%)					CEI
		Score 0	Score 1	Score 2	Score 3	Score 4	
EGF10	54	-	1.8 ± 1.5 (1/54)	13.0 ± 1.2 (7/54)	29.6 ± 4.2 (16/54)	55.6 ± 2.6 (30/54)	3.4 ± 0.1
EGF50	52	-	9.6 ± 0.3 (5/52)	11.5 ± 3.5 (6/52)	34.6 ± 9.0 (18/52)	44.2 ± 7.5 (23/52)	3.1 ± 0.1
P	-	-	0.1093	1.000	0.6784	0.3314	0.120

Note: The results are expressed as mean ± standard error. The CEI (*cumulus* expansion index) was assessed according to a subjective scoring system, ranging from 0 to 4, where 0 indicated the absence of expansion and 4 indicated the expansion of all layers. The calculation of the average expansion was performed as described by Fagbohun and Downs [25]. According to the example—of 55 COCs, 20 COCs had a score of 0; 20 COCs had a score of 1; 10 COCs had a score of 2; 5 COCs had a score of 3; and 0 COCs had a score of 4—this group would present the CEI calculated: $[(0 \times 20) + (1 \times 20) + (2 \times 10) + (3 \times 5) + (4 \times 0)]/55 = 1.0$. Three repetitions.

The ultrastructural analysis of oocytes matured in vitro with 10 ng/mL or 50 ng/mL of EGF (Figure 2B'',C'') revealed a clear adhesion of *cumulus* cells to the oocyte surface, forming densely organized layers around the zona pellucida (Figure 2B''',C'''). In both groups, transzonal cytoplasmic projections crossing the zona pellucida were observed, indicating the maintenance of bidirectional communication pathways between the oocyte and *cumulus* cells, an essential feature for the exchange of signals and nutrients during maturation. Furthermore, no relevant morphological differences were identified between the treated groups, as the oocytes preserved the integrity of the zona pellucida, without signs of structural or surface disorganization. Additionally, the presence of small depressions or pores on the zona pellucida surface was observed, which may be associated with dynamic processes occurring during oocyte maturation (Figure 2B''',C''').

3.2. Experiment I: EGF Effect on Viability and Apoptotic Levels of Cumulus Cells

Cell viability was assessed using the Trypan blue assay (Figure 3A). It was observed that the IVM medium supplemented with 10 ng/mL EGF ($93.8\% \pm 1.6$; 982/1047) ensured greater *cumulus* cell viability compared to the medium with 50 ng/mL EGF ($83.0\% \pm 1.6$; 643/775). In addition to cell viability, the apoptotic levels of *cumulus* cells were analyzed (Figure 3B,C). It was observed that in the EGF10 group ($84.9\% \pm 0.7$; 764/900), the cells were more viable compared to the EGF50 group ($78.9\% \pm 2.7$; 710/900) (Figure 3D). This difference may be attributed to the higher number of cells in early apoptosis observed in the EGF50 group ($15.1\% \pm 2.4$; 136/900) compared to the EGF10 group ($7.9\% \pm 1.1$; 71/900) after 24 h of IVM (Figure 3D). No significant differences were observed later between the groups regarding the number of cells in later stages of apoptosis and necrosis (Figure 3D).

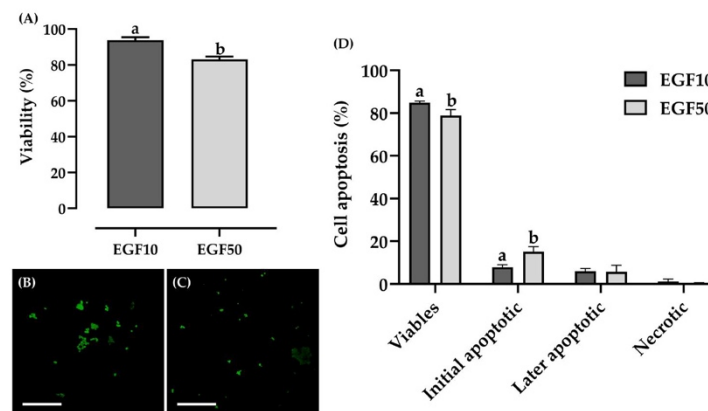


Figure 3. Assessment of viability and apoptotic levels of *cumulus* cells after 24 h of IVM of Spix's yellow-toothed cavy oocytes. (A) Percentage of cell viability by Trypan blue assay. (B) Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 10 ng/mL EGF. (C) Representation of the apoptotic assay in *cumulus* cells under IVM medium supplemented with 50 ng/mL EGF. (D) Rate of apoptotic levels of *cumulus* cells under 10 and 50 ng/mL EGF. The results are expressed as mean $\pm$ standard error. a,b Values with different lowercase letters within columns significantly differ ($p < 0.05$). Scale bar = 50 μ m (B,C), mag. 20 $\times$. Three repetitions.

3.3. Experiment I: Effect of EGF on Morphometric Parameters and Nuclear Maturation

After 24 h of IVM, a morphometric evaluation was performed on immature oocytes and those showing extrusion of the 1PB (Figure 4A,B). The results are presented in Table 3. It was observed that oocytes matured in IVM medium supplemented with 10 ng/mL EGF had a larger diameter compared to those supplemented with 50 ng/mL EGF, regarding IOD ($78.8 \pm 5.2 \mu\text{m}$ vs. $74.9 \pm 2.6 \mu\text{m}$), ZPT ($9.4 \pm 0.8 \mu\text{m}$ vs. $8.5 \pm 0.9 \mu\text{m}$), and OD ($69.6 \pm 4.2 \mu\text{m}$ vs. $64.8 \pm 2.8 \mu\text{m}$). Consequently, oocytes matured with 10 ng/mL EGF exhibited a larger IOA ($15,502 \pm 2466 \mu\text{m}^2$ vs. $13,877 \pm 957 \mu\text{m}^2$) and OA ($12,058 \pm 1630 \mu\text{m}^2$ vs. $10,412 \pm 901 \mu\text{m}^2$) compared to those with 50 ng/mL EGF.

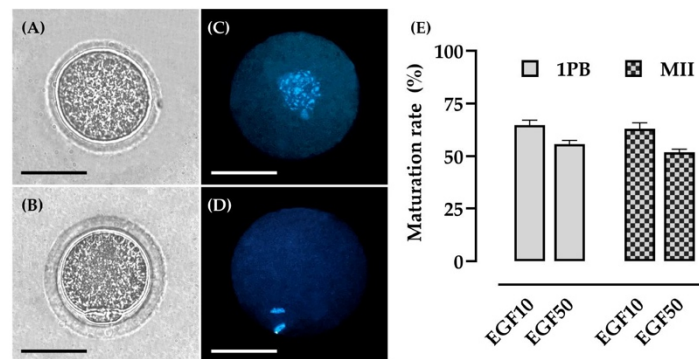


Figure 4. Representative image of Spix's yellow-toothed cavy oocytes before and after 24 h of IVM containing different concentrations of EGF. (A) Immature oocyte. (B) Oocyte after 1PB extrusion. (C) Oocyte in germinal vesicle breakdown (GVBD) configuration. (D) Oocyte in metaphase II. (E) Percentage of oocyte rate after nuclear maturation assessment. The results are expressed as mean $\pm$ standard error. Scale bar = 100 μ m, mag. 20 $\times$ (A,B,C,D). Three repetitions.

Table 3. Morphometric evaluation of immature and mature *Spix's* yellow-toothed cavy oocytes after 24 h of IVM supplemented with EGF.

Groups	1PB	No. Oocytes	OOD (μm)	IOD (μm)	ZPT (μm)	OD (μm)	SPD (μm)	IOA (μm ²)	OA (μm ²)	PSA (μm ²)
EGF10	—	19	92.8 ± 4.5 ^a	76.4 ± 2.9 ^{ab}	9.3 ± 0.5 ^{ab}	69.2 ± 2.4 ^a	7.2 ± 2.3 ^a	14,431 ± 1087 ^{ab}	11,833 ± 805 ^{ab}	2598 ± 840 ^a
	+	35	95.7 ± 6.9 ^a	78.8 ± 5.2 ^a	9.4 ± 0.8 ^a	69.6 ± 4.2 ^a	9.2 ± 3.4 ^a	15,502 ± 2466 ^a	12,058 ± 1630 ^a	3443 ± 1437 ^a
EGF50	—	23	93.9 ± 0.2 ^a	76.7 ± 2.6 ^{ab}	8.9 ± 0.8 ^{ab}	69.0 ± 3.3 ^a	7.7 ± 2.7 ^a	14,564 ± 1021 ^{ab}	11,813 ± 1101 ^{ab}	2751 ± 941 ^a
	+	29	94.3 ± 1.7 ^a	74.9 ± 2.6 ^b	8.5 ± 0.9 ^b	64.8 ± 2.8 ^b	10.1 ± 2.7 ^a	13,877 ± 957 ^b	10,412 ± 901 ^b	3464 ± 926 ^a

Note: The results are expressed as mean ± standard error. ^{a,b} Values with different superscript letters within columns significantly differ ($p < 0.05$). OOD, outer oocyte diameter; IOD, inner oocyte diameter; ZPT, zona pellucida thickness; OD, ooplasm diameter; SPD, space perivitelline diameter; IOA, internal oocyte area; OA, ooplasm area; PSA, perivitelline space area. The morphometric calculation for each parameter evaluated on the oocytes was performed as described by Saadeldin et al. [31]. Three repetitions.

Regarding the evaluation of nuclear maturation, the extrusion rate of the 1PB was observed, as well as the analysis of the nuclear stage of oocytes matured in vitro (Figure 4B–D). In this context, it was observed that there was no difference between oocytes subjected to in vitro maturation with 10 ng/mL EGF ($64.8\% \pm 2.4$; 35/54) and 50 ng/mL EGF ($55.8\% \pm 1.7$; 29/52) after 1PB extrusion. Additionally, no differences were observed between the groups after evaluating the nuclear stage ($63.0\% \pm 2.9$; 34/54 vs. $51.9\% \pm 1.5$; 27/52) (Figure 4D). Although this was the case, it is important to note that the results obtained in this study were promising and represent a significant step towards implementing in vitro techniques in this species.

3.4. Experiment II: Assessment of the Microenvironment and Quality of Oocytes After IVM

Considering the benefits of EGF10 observed in the previous experiment, oocytes used in the second experiment were subjected to IVM in medium supplemented with 10 ng/mL EGF. To ensure the appropriate microenvironment and nuclear maturation of the oocytes that were artificially activated, cells were analyzed across four replications for expansion grade, viability, and apoptotic levels of *cumulus* cells. Additionally, 1PB extrusion was observed, and cells exhibiting this structure were subjected to artificial activation to evaluate embryonic development rates over a five-day period.

The results regarding the grade of expansion of *cumulus* cells and CEI are presented in Table 4. Concerning viability, as assessed by the trypan blue assay, oocytes matured in the presence of 10 ng/mL EGF exhibited a viability rate greater than 90% (2970/3279). Additionally, 97% (878/900) of *cumulus* cells were viable following the apoptotic assay. Finally, regarding 1PB extrusion, oocytes matured with EGF10 showed a rate of 64% (146/227). These oocytes were then randomly subdivided to evaluate the effect of the presence or absence of 6-DMAP, as per the artificial activation protocol previously described in the experimental design.

Table 4. Rate of expansion of *cumulus* cells and CEI of *G. spixii* oocytes after 24 h of IVM in medium supplemented with 10 ng/mL of EGF.

Group	No. Oocytes	Grade of Cumulus Cell Expansion (%)					CEI
		Score 0	Score 1	Score 2	Score 3	Score 4	
EGF10	227	1.3 ± 0.9 (3/227)	6.6 ± 1.2 (15/227)	21.1 ± 3.9 (48/227)	27.1 ± 3.4 (66/227)	41.9 ± 2.9 (95/227)	3.0 ± 0.03

Note: The results are expressed as mean ± standard error. The CEI (*cumulus* expansion index) was assessed according to a subjective scoring system, ranging from 0 to 4, where 0 indicated the absence of expansion and 4 indicated the expansion of all layers. The calculation of the average expansion was performed as described by Fagbohun and Downs [25]. According to the example—of 55 COCs, 20 COCs had a score of 0; 20 COCs had a score of 1; 10 COCs had a score of 2; 5 COCs had a score of 3; and 0 COCs had a score of 4—this group would present the CEI calculated: $[(0 \times 20) + (1 \times 20) + (2 \times 10) + (3 \times 5) + (4 \times 0)]/55 = 1.0$. Four repetitions.

3.5. Experiment II: Effect of 6-DMAP on Activation and Kinetics of Embryonic Development

After artificial activation, analysis of embryonic development kinetics and ultrastructure (Figure 5) revealed that the zygotes exhibited cleavage stages ranging from two cells to three to seven cells (Figure 5A,B). In addition, zona pellucida digestion in the zygotes was observed following SEM sample processing (Figure 5A'). The ultrastructural analysis of the zygotes revealed the outer surfaces of the blastomeres, which appeared spherical and showed clear signs of cell division (Figure 5A',B').

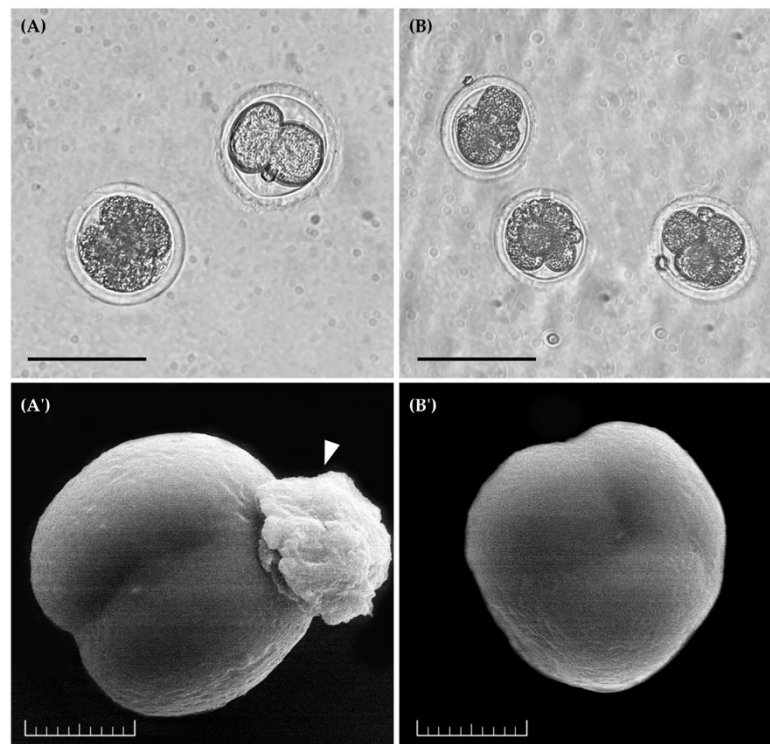


Figure 5. Representative image of cleaved zygotes after artificial activation and two days of embryonic culture. (A) Zygotes with 2 cleavages. (B) Zygotes with 3–7 cleavages. (A') Ultrastructure of zygotes with two cleavages. The arrowhead indicates remains of the degraded zona pellucida after processing for visualization in SEM. (B') Ultrastructure of cleavages containing three blastomeres. Scale bar = 100 μ m, mag. 20 $\times$ (A,B); 20 μ m (A',B'). Four repetitions.

During the evaluation of embryonic development kinetics over five days, artificially activated oocytes achieved cleavages beyond eight cells (Figure 6A). For the first time in this species, compacted morulae were also obtained (Figure 6B). Regarding embryonic development kinetics, 6-DMAP did not affect the number of cleavages in two-cell, three- to seven-cell, and >eight-cell zygotes (Figure 6C). However, the presence of 6-DMAP ($69.3\% \pm 5.0$; 52/75) positively influenced the total number of cleaved zygotes compared to the group without the secondary activator ($53.5\% \pm 3.5$; 38/71) (Figure 6D), resulting in the production of compacted morulae at rates of $13\% \pm 3.1$ (5/38) in the group without 6-DMAP and $17\% \pm 2.9$ (9/52) in the group supplemented with 6-DMAP (Figure 6D).

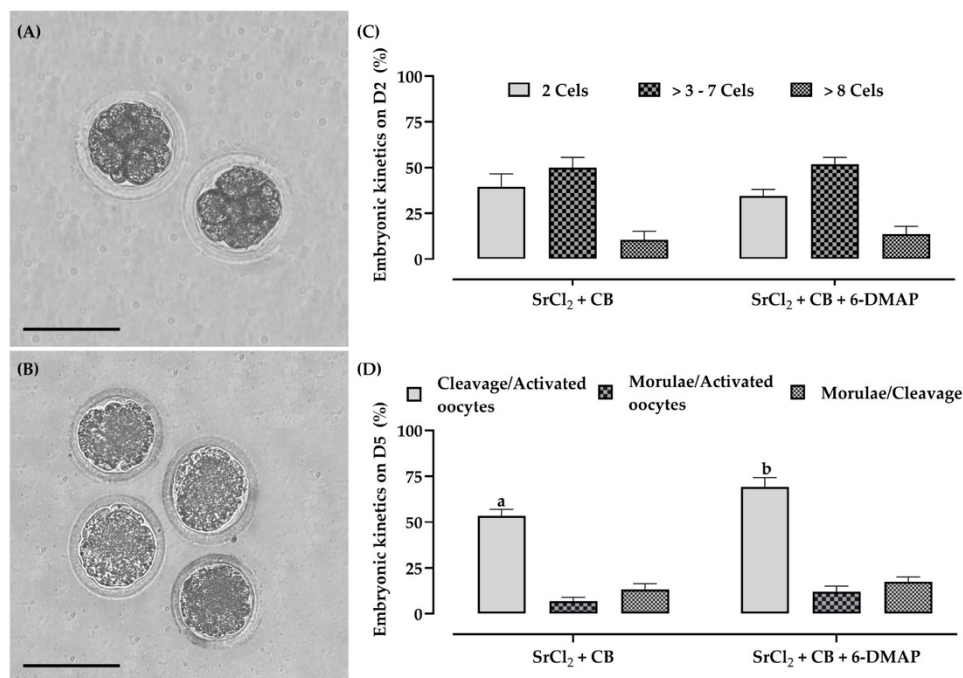


Figure 6. Embryonic development rate of artificially activated *G. spixii* oocytes. (A) Zygotes with cleavages > 8 cells after two days of embryonic culture. (B) Compacted morulae after five days of embryonic culture. (C) Embryonic cleavage rates after 48 h in vitro culture. (D) Cleavage and morulae formation rates after 120 h of embryonic development. Scale bar = 100 µm, mag. 20× (A,B). The results are expressed as mean ± standard error. a,b Values with different lowercase letters within columns significantly differ ($p < 0.05$).

4. Discussion

We developed and refined in vitro manipulation strategies for Spix's yellow-toothed cavy oocytes and embryos over seven experimental replicates, aiming to evaluate the effects of EGF supplementation at two concentrations (10 and 50 ng/mL) during the IVM process, as well as the impact of 6-DMAP during artificial activation. We analyzed the influence of EGF on the quality of the microenvironment and nuclear maturation of oocytes. EGF interacts with specific receptors that play essential roles in the endocrine, paracrine, and autocrine processes of oocytes and surrounding cells, positively influencing differentiation, communication, and nuclear and cytoplasmic maturation [17,18]. After selecting the optimal EGF concentration, we assessed the mature structures using an established artificial activation protocol for red-rumped agouti, mice, and rats [9,20]. At this stage, we analyzed the influence of the presence or absence of 6-DMAP. This molecule inhibits serine/threonine kinases and seems to enhance the efficiency of artificial activation for greater in vitro embryo production [32,33].

Regarding *cumulus* cell expansion, our study observed that both EGF concentrations, 10 ng/mL and 50 ng/mL, resulted in more than 40% expansion of the *cumulus oophorus* complex and the corona radiata, achieving a Score of 4. This was subsequently justified by the CEI values, which ranged between 3.1 and 3.4. In contrast, a study by Praxedes et al. [9] with red-rumped agouti demonstrated that 10 ng/mL EGF in conjunction with 10 mIU/mL

FSH resulted in a $31.8\% \pm 1.3$ rate of oocytes with Score 3 and $15.9\% \pm 0.9$ with Score 4, reaching a CEI of up to 3.6. This discrepancy may be attributed to the fact that, although phylogenetically close, there are intrinsic biological and reproductive differences between these species [34,35]. In Spix's yellow-toothed cavy, EGF may be more efficiently utilized for *cumulus* cell expansion without affecting the CEI. In contrast, in red-rumped agouti, there may be lower efficiency in the activation of these molecules at this concentration. For example, in mice, when paracrine and juxtacrine components of the ovulatory signal were inactivated by knockout of EGF-like transmembrane peptides AREG and EREG [36,37], reduced efficiency in *cumulus* cell expansion and oocyte maturation was observed.

Another justification is that while FSH exerts an inhibitory effect on the initial resumption of meiosis, it induces the final stages of meiotic maturation [24]. This process is regulated by the expression levels of natriuretic peptide precursor type C (NPPC) in *cumulus* cells and the positive modulation of natriuretic peptide receptor 2 (NPR2), both of which are influenced by the actions of FSH and EGF. The oocytes in these species may respond differently to the treatments observed in each species [38], which could affect *cumulus* cell expansion. For example, in red-rumped agouti (*D. leporina*), a higher rate of Score 3 was observed compared to Score 4, and when compared to Spix's yellow-toothed cavy. For this reason, we evaluated Spix's yellow-toothed cavy oocytes in our study for their ultrastructural characteristics, viability, and apoptotic levels as quality indicators of the *in vitro* environment.

Regarding viability after Trypan blue staining, oocytes in medium supplemented with 10 ng/mL EGF had a higher rate of viable cells compared to 50 ng/mL EGF. Consequently, we performed an analysis of apoptotic levels of these cells using ethidium bromide and acridine orange assays. In this context, we observed that the medium supplemented with 50 ng/mL EGF showed a higher initial apoptosis index in *cumulus* cells compared to 10 ng/mL EGF. This result may reflect the metabolic activity of oocyte mitochondria, which are involved in the production of reactive oxygen species (ROS), leading to a higher incidence of apoptosis in *cumulus* cells [39]. Therefore, a more effective antioxidant supplementation than the cysteamine used in our study may be necessary.

Additionally, during the acquisition of meiotic competence of mouse oocytes *in vitro*, EGF promotes a significant and rapid increase in phosphorylation within the first 30 min of the event compared to oocytes matured *in vivo* [40]. This suggests that this metabolic activity may lead to increased ATP production, which in turn negatively affects the quality of *cumulus* cells through the subsequent synthesis of ROS molecules [41]. Consequently, this results in a lower cellular viability rate, as observed after Trypan blue staining and apoptosis assays. Another associated reason is that it has been demonstrated *in vitro* that *cumulus* cells can also be affected by the accumulation of glycosylations derived from transmembrane proteins (AREG, EREG, and BTC) in response to EGF [42,43]. Therefore, it is possible that an excess of these factors could cause significant damage to the *cumulus* cells of Spix's yellow-toothed cavy oocytes.

Although supplementation with 50 ng/mL EGF reduced the viability rate of *cumulus* cells, our study did not observe any morphological changes from the SEM analysis. Our results revealed that oocytes matured under both EGF supplementation conditions maintained the necessary machinery for development and oocyte competence. This is justifiable as we could observe the highly specialized transzonal cytoplasmic projections of *cumulus* cells penetrating the zona pellucida of Spix's yellow-toothed cavy oocytes [17]. This likely ensures the interface and intricate bidirectional communication between the microenvironment and the oocyte, which are crucial for providing the necessary nutrition and support for meiotic acquisition [17]. This result is corroborated by our study, where, in the initial attempt to perform IVM of Spix's yellow-toothed cavy oocytes using EGF

as a supplement, we observed nuclear maturation rates ranging from 55% to 64% for the extrusion of the 1PB, while 51% to 63% corresponded to the metaphase II nuclear stage. These rates are similar when compared to guinea pigs [13,19], paca (*Cuniculus paca*) [44], *D. prymnolopha* [45], and red-rumped agouti (*D. leporina*) [9].

In addition to the parameters analyzed, we performed, for the first time, morphometric evaluations on Spix's yellow-toothed cavy oocytes regarding structures that exhibited or did not exhibit the extrusion of the 1PB after IVM with 10 or 50 ng/mL EGF. According to Saadeldin et al. [31], assessing oocyte morphometry can also be a decisive step in evaluating the quality and meiotic competence for adequate embryonic development following artificial activation. Thus, a larger ooplasm diameter (OD) and a smaller space perivitelline diameter (SPD) are expected to indicate better oocyte quality [31]. In our study, we initially observed that Spix's yellow-toothed cavy oocytes exhibited a diameter ranging from 92.8 to 94.3 μm . Additionally, after IVM, oocytes matured in media containing 10 ng/mL EGF demonstrated a larger OD compared to those matured with 50 ng/mL EGF. However, no significant difference was observed in SPD. Notably, this result is interesting because, in in vivo systems, oocytes from small antral follicles are usually not responsive to EGF signaling cascades [46]. In contrast, COCs from Graafian follicles can respond to progressively increasing EGF levels, thereby enabling better embryonic development [47,48]. Therefore, the acquisition of EGF signaling capability by cumulus cells in relation to oocytes aligns with the mechanisms governing oocyte meiotic competence, and these processes are likely interrelated [48,49].

Based on the previous justifications, we selected 10 ng/mL EGF as the ideal concentration for use during the IVM of Spix's yellow-toothed cavy oocytes. We considered this concentration for evaluating artificial activation in the presence or absence of 6-DMAP. Initially, regarding the artificial activation protocol for embryonic development, we chose 10 mM SrCl_2 , which can induce repetitive intracellular calcium releases, similar to what occurs during in vitro fertilization in mice and rats, which are laboratory rodents phylogenetically close to Spix's yellow-toothed cavy. In these species, 10 mM SrCl_2 mimics natural conditions, similar to the role of spermatozoa on the oocyte, providing an opportunity for the reconstruction of cloned embryos or in physiological studies [50,51]. Additionally, in our study, we also used 5 $\mu\text{g/mL}$ CB. This compound is included during cloning and prevents the formation and extrusion of the second polar body, resulting in diploid embryos. In mice, its addition has significantly enhanced blastocyst development compared to artificial activation without this compound [20]. This occurs because, in diploid mouse embryos, a lower incidence of apoptosis is observed compared to haploid embryos [52]. We therefore hypothesized that its addition could enhance embryo quality in our experiment.

In our study, we also evaluated the presence or absence of 6-DMAP as a secondary activator. The addition of this compound was based on previous evidence showing that its inclusion in activation protocols in mice can significantly enhance artificial activation efficiency, achieving blastocyst rates of up to 60%. This effect is likely due to the reduction in meiosis-promoting factor (MPF) activity, thereby improving cell cycle progression efficiency. In our protocol, we combined 10 mM SrCl_2 for 2.5 h followed by exposure to 5 $\mu\text{g/mL}$ CB and 2 mM 6-DMAP for 3.5 h (SrCl_2 + CB + 6-DMAP). We observed a higher overall cleavage rate compared to the control group (without 6-DMAP), although the compact morulae formation rate remained limited to 13% to 17%. This result may be related to the fact that, although the Spix's yellow-toothed cavy is phylogenetically close to other laboratory and wild rodents, important gaps remain in our understanding of reproductive technologies applied to this species.

Therefore, implementing optimized protocols by adjusting gas composition, temperature, medium supplementation, and IVM duration could represent a viable alternative

to improve this developmental step. This observation underscores the need for further investigations, especially considering that studies in mice have shown that treatment with chorionic gonadotropin (hCG), administered between days 13 and 18, can lead to artificial activation rates of 30% to 60% in mature oocytes [20].

Thus, future studies are essential to enable embryonic development beyond the morula stage, potentially reaching blastocyst formation and hatching. Although blastocysts were not observed under the *in vitro* conditions used in this study, the achievement of compact morulae represents a biologically relevant milestone for this species. This finding demonstrates that the combination of IVM and artificial activation was sufficient to support early key processes of embryonic development, such as cleavage, compaction, and cell polarization, steps that are tightly regulated and indicative of embryonic competence. Considering that there are no prior reports describing *in vitro* embryo production beyond early cleavage stages in *G. spixii*, our findings expand the application of reproductive biotechnologies in this Neotropical rodent.

Despite this limitation, the production of compact morulae represents a significant experimental advancement and provides a solid foundation for improving reproductive biotechnologies in wild rodents of the Hystricomorph suborder. Furthermore, this progress paves the way for future investigations involving nuclear transfer, embryo cryopreservation, and the development of embryo transfer protocols tailored to this species.

5. Conclusions

This was the first study to establish the complete sequence of *in vitro* manipulation steps for oocytes and embryos of Spix's yellow-toothed cavy, with the aim of promoting species multiplication, conservation, and reproductive research. Our findings indicate that supplementation with 10 ng/mL of EGF during IVM is the most suitable condition, as it enhances *cumulus* cell viability and reduces apoptosis rates. Additionally, EGF10 improved the morphometric quality of oocytes and *cumulus* structures, achieving 60% nuclear maturation.

We also demonstrated, for the first time, the production of compact morulae using an artificial activation protocol commonly applied to laboratory rodents. Although the protocol still requires optimization to reach later embryonic stages, this represents a significant advancement for this species.

These results provide a foundational framework for the development of assisted reproductive technologies (ARTs) tailored to *G. spixii*, and they highlight the potential of this species as a model for reproductive biotechnology in Neotropical hystricomorph rodents. In a broader context, the methodologies and outcomes presented here may directly contribute to *ex situ* conservation programs, support the establishment of genetic resource banking, and inform comparative studies on ART development in other wild rodent species. Future efforts should focus on refining embryo culture systems, extending embryonic development to the blastocyst stage, and evaluating the feasibility of embryo cryopreservation and transfer in this species.

Author Contributions: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing—Original Draft, L.V.C.A.; Methodology, S.L.O.; Methodology, Y.L.F.S.; Methodology, V.D.S.; Methodology, L.R.M.O.; Methodology, Investigation, M.F.O.; Conceptualization, Formal analysis, Investigation, Data Curation, Writing—Review and Editing, Supervision, Funding Acquisition, A.F.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES, Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil (CNPq, no. 309078/2021-0).

Institutional Review Board Statement: All procedures were approved by the UFERSA Animal Ethics Committee, Opinion no. 23091.010566/2017-20, and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The authors thank the Center for Wild Animals Multiplication (CEMAS/UFERSA) for providing the biological samples used in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

1PB	First polar body
6-DMAP	6-dimethylaminopurine
ARTs	Assisted reproductive technologies
BSA	BSA bovine serum albumin
CB	Cytochalasin B
CEI	<i>Cumulus</i> expansion index
CEMAS	Centre of Multiplication of Wild Animals
CEUA	Animal Use Ethics Committee
COCs	<i>Cumulus</i> –oocyte complexes
EGF	Epidermal growth factor
GVBD	Oocyte in the germinal vesicle breakdown
hCG	Chorionic gonadotropin hormones
IOA	Internal oocyte area
IOD	Inner oocyte diameter
IVM	In vitro maturation
KSOM	Simple, optimized, and potassium-enriched medium
MPF	Meiosis-promoting factor
OA	Ooplasm area
OD	Ooplasm diameter
OOD	Outer oocyte diameter
ORM	Oocyte recovery medium
PBS	Phosphate buffer solutions
PSA	Perivitelline space area
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
SEM	Scanning electron microscopy
SPD	Space perivitelline diameter
SrCl ₂	Strontium chloride
TALP	Tyrodé's albumin lactate pyruvate
UFERSA	Federal Rural University of Semi-Arid

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ANEXO F – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 2

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: Cryopreservation and passaging optimization for *Galea spixii* (Wagler, 1831) adult skin fibroblast lines: a step forward in species management and genetic studies

Authors: Leonardo Vitorino Costa de Aquino, Samara Lima Olindo, Yara Letícia Frutuoso e Silva, Lhara Ricarlany Medeiros de Oliveira, Yasmin Beatriz França Moura, Ana Lívia Rocha Rodrigues, Érika Almeida Praxedes, Alexandre Rodrigues Silva, Moacir Franco de Oliveira, Alessandra Fernandes 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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Mauro Cesar da Silveira Costa
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ANEXO G – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 3



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MANUSCRIPT TITLE
L-proline as a Multifunctional Agent for Cryopreservation of Galea spixii Skin Fibroblasts

AUTHORS
Leonardo V.C. Aquino, Luanna L.V. Rodrigues, Samara L. Olindo, Yara L.F. Silva, Lhara R.M. Oliveira, Yasmin B.F. Moura, Aleksandra F. Pereira

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ANEXO H – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 4

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: Induction of G₀/G₁ arrest in Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) fibroblasts through roscovitine and serum starvation for improved cellular reprogramming

Authors: Leonardo Vitorino Costa de Aquino, Luanna Lorena Vieira Rodrigues, Ana Beatriz Morais de Oliveira, Sueli de Oliveira Lima, Samara Lima Olindo, Sarah Sant'Anna Maranhão, Maria Claudia dos Santos Luciano, Claudia Pessoa, Alessandra Fernandes 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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September 19, 2025

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ANEXO I – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 6

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: Effect of Epidermal Growth Factor and 6-Dimethylaminopurine on In Vitro Maturation and Artificial Activation of Spix's Yel-low-Toothed Cavy (*Galea spixii* Wagler, 1831) Oocytes

Authors: Leonardo V. C. Aquino, Samara L. Olindo, Yara L. F. Silva, Vinícius D. Silva, Lhara R. M. Oliveira, Moacir F. Oliveira, 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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ANEXO J – RESUMO DA PUBLICAÇÃO NA REVISTA ELETRÔNICA DO SEMINÁRIO DE INICIAÇÃO CIENTÍFICA DA UFERSA – 2025



Núcleo de Avaliação: Núcleo I

Área temática: Ciências Agrárias

Área do Conhecimento: Medicina Veterinária

Avaliação do papel modulador do fator de crescimento epidermal sobre o microambiente das células do *cumulus* e da maturação nuclear de oócitos de *Galea spixii* Wagler, 1831 (Rodentia: Caviidae)

Dallila Nascimento Rebouças, Antônia Beatriz Mendonça Pereira, Leonardo Vitorino Costa de Aquino, Yara Letícia Frutuoso e Silva, Alexsandra Fernandes Pereira

O fator de crescimento epidermal (EGF) é uma proteína polipeptídica que regula a comunicação entre oócito e células do *cumulus*, estimulando a expansão celular e retomada da meiose. Sua incorporação à maturação *in vitro* (MIV) aproxima o microambiente das condições fisiológicas e favorece a competência meiótica. Em *Galea spixii*, espécie endêmica da Caatinga, o EGF é promissor para a padronização da MIV e produção *in vitro* de embriões. Portanto, o objetivo foi avaliar duas concentrações de EGF (10 e 50 ng/mL) sobre expansão, viabilidade e apoptose das células do *cumulus*, bem como taxas de maturação nuclear de oócitos. Todos procedimentos foram aprovados pelo CEUA/Ufersa (no. 23091.010566/2017-20) e ICMBio (no. 60428-1). Para isso, os animais foram anestesiados com 2% de xilazina (1 mg/kg) e cloridrato de cetamina (Ketamine® 10%, 15 mg/kg). Em seguida, realizou-se eutanásia por administração intravenosa de cloreto de potássio (19,1%, 2,56 mEq/kg). Os oócitos recuperados por *slicing*, classificados de acordo com número de camadas de células do *cumulus* e homogeneidade do citoplasma foram submetidos à MIV por 24 h, a 38,5 °C e 6,5% de CO₂. O meio MIV consistiu em TCM-199 suplementado com 0,2 mM piruvato de sódio, 10% soro fetal bovino, 1% solução de antibiótico-antimicótico, 100 µM cisteamina, 10 µg/mL FSH/LH e 10 ng/mL (grupo EGF10) ou 50 ng/mL (grupo EGF50). Imediatamente após a MIV, oócitos foram avaliados quanto ao grau de expansão das células do *cumulus* de acordo com cinco escores: 0 (sem expansão), I (mínima), II (parcial), III (moderada) e IV (máxima). Além disso, células do *cumulus* foram analisadas para viabilidade com azul de tripan e níveis de apoptose com laranja de acridina e brometo de etídio. A maturação nuclear foi verificada pela extrusão do primeiro corpúsculo polar. Todos resultados foram expressos como média ± erro padrão e comparados usando teste exato de Fisher (P < 0,05). Após três repetições (quatro-seis ovários por repetição), 106 oócitos imaturos viáveis foram recuperados, resultando em 7,6 oócitos/ovário, os quais foram distribuídos entre os grupos EGF10 e EGF50. Nenhuma diferença foi observada entre EGF10 (escore I: 1,8% ± 1,5 [1/54]; escore II: 13,0% ± 1,2 [7/54]; escore III 29,6% ± 4,2 [16/54]; escore IV: 55,6% ± 2,6 [30/54]) e EGF50 (escore I: 9,6% ± 0,3 [5/52]; escore II: 11,5% ± 3,5 [6/52]; escore III 34,6% ± 9,0 [18/52]; escore IV: 44,2% ± 7,5 [23/52]) em termos de expansão das células do *cumulus*. Contudo, oócitos derivados do grupo EGF10 apresentaram maior viabilidade [93,8% ± 1,6 (982/1047)] e menor apoptose [7,9% ± 1,1 (71/900)] das células do *cumulus* que oócitos do grupo EGF50 [83,0% ± 1,6 (643/775); 15,1% ± 2,4 (136/900), respectivamente]. Além disso, nenhuma diferença foi observada nas taxas de maturação nuclear entre os grupos [EGF10: 64,8% ± 2,4 (35/54) e EGF50: 55,8% ± 1,7 (29/52)]. Em conclusão, EGF é eficiente na MIV de oócitos de *G. spixii*, sendo 10 ng/mL a concentração mais indicada para preservar a integridade celular e reduzir a apoptose. Esses resultados reforçam o potencial do EGF como modulador da competência oocitária em *G. spixii*.





Palavras-chave: Roedores Silvestres, Maturação *in vitro*, Competência oocitária.

Agência financiadora: PIBIC-Marie Curie.

Campus: Mossoró.



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ANEXO K – RESUMO E CERTIFICADO DA PUBLICAÇÃO NA REVISTA ELETRÔNICA DO X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION – 2024

Effect of epidermal growth factor concentrations as a signal for the expansion of *cumulus* cells and *in vitro* maturation of Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) oocytes

Leonardo Vitorino Costa de Aquino¹, Samara Lima Olindo¹, Yara Letícia Frutuoso e Silva¹, Vinícius Dantas da Silva¹, Lhara Ricarliany Medeiros de Oliveira¹, Moacir Franco de Oliveira², Alessandra Fernandes Pereira¹

¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil. ²Centre of Multiplication of Wild Animals, UFERSA, Mossoró, RN, Brazil.

Epidermal growth factor (EGF) constitutes a family of polypeptide proteins that signal, through cellular receptors and gap junctions, the genetic activation of mural cells, the expansion of *cumulus* cells and assistance in events involving oocyte meiosis. In this sense, the use of EGF during *in vitro* maturation (IVM) of oocytes has been a fundamental approach in understanding the reproductive mechanisms of different wild rodents. In Spix's yellow-toothed cavy, a wild rodent adapted to the Caatinga biome, this possibility offers an opportunity to establish protocols involving the oocyte manipulation, to understand reproductive mechanisms related to embryo production, and to genetic conservation of the species. Therefore, we aimed to evaluate the effect of two concentrations of EGF (10 vs. 50 ng/mL) on the expansion of *cumulus* cells and nuclear maturation of Spix's yellow-toothed cavy oocytes. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1). Then, seven Spix's yellow-toothed cavy were used for ovarian recovery. The *cumulus*-oocyte complexes (COCs) were recovered by slicing, classified under stereomicroscope and only oocytes with more than one layer of *cumulus* cells and homogeneous cytoplasm were used for IVM. The COCs were transferred to the IVM medium composed by TCM-199 with 0.2 mM sodium pyruvate, 10% fetal bovine serum, 100 μ M cysteamine, 10 μ g/mL FSH/LH, 1% antibiotic/antimycotic solution, and 10 ng/mL (EGF10 group) or 50 ng/mL (EGF50 group). Oocytes were divided randomly in both groups and matured *in vitro* for 24 h at 38.5 °C in a humidified atmosphere with 6.5% CO₂. Immediately after the IVM, oocytes were evaluated for the degree of *cumulus* cell expansion under a stereomicroscope, where they were classified as: score I (no expansion), score II (minimal expansion), score III (intermediate expansion) and score IV (maximum expansion). Moreover, denuded oocytes were stained with the Hoechst 33342 probe to visualize their nuclear stage under a fluorescence microscope, where those in metaphase II were considered matured. All results were expressed as mean $\pm$ standard error and means were compared using Fisher exact test with $P < 0.05$. Thus, after three repetitions (four-six ovaries per repetition), 106 viable immature oocytes were recovered, resulting in 7.6 oocytes/ovary, which were randomly distributed between EGF10 and EGF50. No difference ($P > 0.05$) was observed between EGF10 (score I: 1.8% $\pm$ 1.5 [1/54]; score II: 13.0% $\pm$ 1.2 [7/54]; score III 29.6% $\pm$ 4.2 [16/54]; score IV: 55.6% $\pm$ 2.6 [30/54]) and EGF50 (score I: 9.6% $\pm$ 0.3 [5/52]; score II: 11.5% $\pm$ 3.5 [6/52]; score III 34.6% $\pm$ 9.0 [18/52]; score IV: 44.2% $\pm$ 7.5 [23/52]) in terms of *cumulus* cell expansion. This same characteristic was repeated after analyzing the nuclear stage (EGF10: 63.0% $\pm$ 2.9 [34/54]; EGF50: 51.9% $\pm$ 1.5 [27/52], $P > 0.05$). In conclusion, in terms of expansion of *cumulus* cells and *in vitro* maturation observed in the Spix's yellow-toothed cavy's COCs, both evaluated concentrations of EGF ensures rates maturation rates greater than 50%. These results represent the first steps towards understanding oocyte maturation in Spix's yellow-toothed cavies.

Financial support: CNPq and CAPES

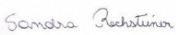
E-mail: leonardovt@live.com



We hereby certify that the abstract titled **Effect of epidermal growth factor concentrations as a signal for the expansion of cumulus cells and in vitro maturation of Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) oocytes** was presented as poster by **Leonardo Vitorino Costa de Aquino, Samara L. Olindo, Yara Leticia Frutuoso E Silva, Vinicius Dantas da Silva, Lhara Ricarlany Medeiros de Oliveira, Moacir Franco de Oliveira and Aleksandra Fernandes Pereira** at the X International Symposium on Animal Biology of Reproduction.

Fortaleza, Ceará, Brazil, July, 1-2, 2024.


DR. IVAN CUNHA BUSTAMANTE FILHO
COORDINATOR OF THE X ISABR
PRESIDENT OF THE CBRA


SANDRA FIALA RECHSTEINER
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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Morphometric evaluation of Spix's yellow toothed cavy (*Galea spixii* Wagler, 1831) oocytes after *in vitro* maturation in the presence of epidermal growth factor

Ericles Mateus Alencar da Silva¹, Leonardo Vitorino Costa de Aquino¹, Samara Lima Olindo¹, Yara Letícia Frutuoso e Silva¹, Vinícius Dantas da Silva¹, Lhara Ricarlany Medeiros de Oliveira¹, Moacir Franco de Oliveira², Alessandra Fernandes Pereira¹

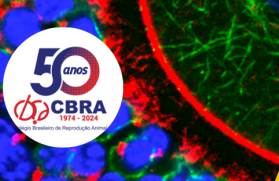
¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil

²Centre of Multiplication of Wild Animals, UFERSA, Mossoró, RN, Brazil

E-mail: ericles.silva@alunos.ufersa.edu.br

Financial support: CNPq and CAPES

Oocyte growth and ovulation are physiological processes initiated by the increase in luteinizing hormone (LH). Although this hormone is important for the acquisition of meiotic competence in oocytes, this cell does not express LH receptors, and this event is mediated by secondary molecules, such as epidermal growth factor (EGF). In this sense, using EGF during *in vitro* maturation (IVM) of Spix's yellow toothed cavy oocytes, a wild rodent with great ecological and scientific impact, can be a positive approach aimed at understanding mechanisms related to its reproductive physiology, as well as in technological applications, such as *in vitro* embryo production. Therefore, we evaluated morphometrically oocytes derived from Spix's yellow toothed cavy after IVM in the presence of EGF, comparing non-matured and matured oocytes after the IVM. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1). Then, viable oocytes were recovered from fourteen ovaries and matured *in vitro* in TCM-199 supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum, 1% antibiotic-antimycotic, 0.1 mM cysteamine, 10 µg/mL of FSH-LH and 50 ng/mL of EGF. All oocytes were cultured for 24 h, 38.5 °C and 6.5% CO₂. After IVM, matured oocytes were denuded using 0.1% hyaluronidase, and the structures were identified for the presence and absence of the first polar body (1PB) under a stereomicroscope. Subsequently, photomicrographs were obtained to carry out morphometric analysis using the Image J software, where it was observed the total oocyte diameter (OD), oocyte internal diameter (OID), thickness of the zona pellucida (TZP), ooplasm diameter (OPD), diameter of the perivitelline space (DPS), internal oocyte area (IOA), ooplasm area (OA), perivitelline space area (PSA). All results were expressed as mean ± standard error and means were compared using Fisher exact test with $P < 0.05$. After three repetitions, 52 oocytes were recovered (3.7 oocytes/ovary), of which 29 of these structures presented 1PB. No difference was observed between non-matured and matured oocytes, respectively, according to OD ($93.9 \pm 0.2 \mu\text{m}$ and $94.3 \pm 1.7 \mu\text{m}$), OID ($76.7 \pm 2.6 \mu\text{m}$ and $74.9 \pm 2.6 \mu\text{m}$), TZP ($8.9 \pm 0.6 \mu\text{m}$ and $8.5 \pm 0.9 \mu\text{m}$), DPS (7.7 ± 2.7 and $10.1 \pm 2.7 \mu\text{m}$), IOA ($14564.9 \pm 1021.4 \mu\text{m}^2$ and $13877.3 \pm 957.6 \mu\text{m}^2$), OA ($11813.3 \pm 1101.2 \mu\text{m}^2$ and $10412.9 \pm 901.7 \mu\text{m}^2$) and PSA ($2751.7 \pm 941.6 \mu\text{m}^2$ and $3464.9 \pm 926.5 \mu\text{m}^2$). These results are positive because it has already been demonstrated in other wild species that lower OD and higher DPS may indicate lower oocyte quality after IVM. However, OPD differed between non-matured and matured oocytes (69.0 ± 3.3 and $64.8 \pm 2.8 \mu\text{m}$), probably as a morphometric modification of the oocyte in response to the matured status. In summary, this is the first study that describes the morphometric parameters of matured and unmatured oocytes from Spix's yellow toothed cavy. These data are important to understand the events involved in the species' IVM.



X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION

July 1-2 2024 :: Fortaleza, CE, Brazil

We hereby certify that the abstract titled **Morphometric evaluation of Spix's yellow toothed cavy (*Galea spixii* Wagler, 1831) oocytes after in vitro maturation in the presence of epidermal growth factor** was presented as poster by **ericles mateus Alencar da Silva, Leonardo Vitorino Costa de Aquino, Samara L. Olindo, Yara Letícia Frutuoso E Silva, Vinicius Dantas da Silva, Lhara Ricarliany Medeiros de Oliveira, Moacir Franco de Oliveira and Alessandra Fernandes Pereira** at the X International Symposium on Animal Biology of Reproduction.

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ANEXO L – RESUMO E CERTIFICADO DA PUBLICAÇÃO NO I SIMPÓSIO POTIGUAR EM CIÊNCIA ANIMAL – 2023



CARACTERIZAÇÃO CROMOSSÔMICA DE FIBROBLASTOS ISOLADOS DA PELE AURICULAR DE *Galea spixii* (WAGLER, 1831)

CHROMOSOME CHARACTERIZATION OF FIBROBLASTS ISOLATED FROM THE EAR SKIN OF *Galea spixii* (WAGLER, 1831)

Morfofisiologia e Biotecnologia Animal

L.V.C. AQUINO^{1*}, A.F. PEREIRA¹

1 – Laboratório de Biotecnologia Animal (LBA), Universidade Federal Rural do Semi-Árido, UFERSA, Mossoró, RN.

*leonardovt@live.com

Objetivos: Caracterizar o cariótipo de fibroblastos isolados da pele auricular de seis machos adultos *Galea spixii*, por meio da coloração de Giemsa na segunda, oitava e décima passagem do cultivo celular. **Material e Métodos:** Biópsias da pele auricular foram recuperadas, e transportadas ao laboratório por até 1 h em meio essencial mínimo modificado por Dulbecco (DMEM), em associação a 10% de soro fetal bovino (SFB) e 2% de antibióticos-antimicóticos. No laboratório, amostras (9,0 mm³) foram cultivadas *in vitro* (38,5 °C; 6,5% de CO₂). A cada 24 h, foi realizado o monitoramento das células, e após atingirem 70% de confluência, foi realizada a primeira passagem celular. Na quinta passagem, fibroblastos foram identificados utilizando o anticorpo vimentina, enquanto na segunda, oitava e décima passagem um pool celular foi mantido em colchicina por 2 h. Em seguida, os fibroblastos foram fixados em solução de Carnoy, e submetidos à coloração por Giemsa. Posteriormente, lâminas foram montadas e 8–10 spreads metafásicos foram identificados, e registrados em microscópio de luz. Todos os dados foram apresentados de maneira descritiva. **Resultados:** Por meio da marcação dos filamentos intermediários, a marcação com vimentina confirmou as células como fibroblastos. Ainda, a morfologia celular confirmou um aspecto fusiforme com núcleo oval centralizado. Já na análise do cariótipo, fibroblastos a partir da oitava passagem apresentaram um encurtamento de seus cromossomos, resultado causado pelo desgaste da senescência replicativa das células. Tal resposta não foi observada em células avaliadas na segunda passagem do cultivo *in vitro*. **Conclusões:** Células somáticas de *G. spixii* a partir da oitava passagem apresentam alteração cromossômica, sendo sugerido passagens iniciais para uso destas células em estudos relacionados à reprogramação celular por transferência nuclear de célula somática e indução de células à pluripotência.

Palavras-chave: roedores silvestres, cultivo *in vitro*, linhagem somática, cariótipo.

Keywords: wild rodents, *in vitro* culture, somatic lines, karyotype.

Protocolo CEUA: no. 23091.010566/2017-20. **Protocolo ICMBio:** no. 60428-1.

Fonte de Fomento: CAPES, bolsa de doutorado, processo no. 88887.679884/2022-00; CNPq, auxílio à pesquisa, processo no. 309078/2021-0.



CERTIFICADO

Certificamos que o discente

Leonardo Vitorino Costa de Aquino

apresentou o trabalho intitulado **“Caracterização cromossômica de fibroblastos isolados da pele auricular de Galea spixii (Wagler, 1831)”** no I **Simpósio potiguar em ciência animal** da Universidade Federal Rural do Semi-Árido realizado nos dias 7 e 8 de dezembro de 2023.

Mossoró, 15 de dezembro de 2023.

FRANCISCO MARLON CARNEIRO FEIJÓ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL



ANEXO M – PRÊMIO DA 2ª MELHOR FOTO DO EVENTO NO I SIMPÓSIO
POTIGUAR EM CIÊNCIA ANIMAL – 2023



CERTIFICADO DE PREMIAÇÃO

Certificamos que

Leornado Vitorino Costa de Aquino

Foi premiado com o 2º lugar no I Concurso de Fotografia do PPGCA –
Ciência no Semi-Árido na categoria de imagem produzida por
instrumentos especiais, durante o **I Simpósio Potiguar em Ciência Animal**
da Universidade Federal Rural do Semi-Árido.

Mossoró, 15 de dezembro de 2023.

FRANCISCO MARLON CARNEIRO FEIJÓ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL



ANEXO N – RESUMO EXPANDIDO PUBLICADO NO CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL – 2022



XI CONGRESSO
NORTE E NORDESTE
DE REPRODUÇÃO
ANIMAL
17 a 19 de novembro de 2022

ANAIIS DO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL

ISOLAMENTO E CULTIVO *IN VITRO* DE CÉLULAS SOMÁTICAS DERIVADAS DE TECIDO SOMÁTICO DE PREÁS (*Galea spixii* WAGLER, 1831)

ISOLATION AND IN VITRO CULTURE OF SOMATIC CELLS DERIVED FROM SOMATIC TISSUE OF SPIX'S YELLOW-TOOTHED CAVY (*Galea spixii* WAGLER, 1831)

Leonardo Vitorino Costa de AQUINO^{1*}; Samara Lima OLINDO¹; Yasmin Beatriz França MOURA¹; Yara Letícia Frutuoso e SILVA¹; Ana Livia Rocha RODRIGUES¹; Vinicius Dantas da SILVA¹; Moacir Franco de OLIVEIRA²; Alessandra Fernandes PEREIRA¹

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

²Centro de Multiplicação de Animais Silvestres, Universidade Federal Rural do Semi-Árido, Mossoró, RN, Brasil.

* leonardovt@live.com

Resumo

O estabelecimento do cultivo *in vitro* de células a partir de fragmentos de pele tem sido proposto como uma técnica útil na conservação das espécies, especialmente quando associada à clonagem por transferência nuclear. Pertencente à ordem Rodentia, *G. spixii* se destaca como uma das espécies mais estudadas em virtude de seu papel ecológico, econômico e científico, uma vez que podem ser utilizadas em estudos para a conservação de roedores ameaçados de extinção. Portanto, o objetivo foi descrever o cultivo *in vitro* de células somáticas oriundas da pele de *G. spixii*, usando o ensaio de viabilidade celular por azul de tripan e atividade metabólica pelo ensaio de brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio ou MTT. Para tanto, biópsias de pele da região auricular periférica foram recuperadas de quatro machos adultos anestesiados. As amostras foram transportadas por 1 h em meio essencial mínimo modificado por Dulbecco (DMEM) suplementado com 10% de soro fetal bovino e 2% de solução de penicilina e estreptomicina, a 37°C. No laboratório, fragmentos (9,0 mm³) foram cultivados em atmosfera controlada (38,5°C, 5% de CO₂) e avaliados a cada 24 h. Após as células atingirem 70% de confluência, foi realizado o primeiro subcultivo celular para obtenção da concentração desejada (5,0 × 10⁴ células/mL) para o ensaio de MTT. Além deste, um pool de células tripsinizadas foram avaliadas quanto à viabilidade com azul de tripan. Todos os dados obtidos foram expressos como média ± erro padrão. Quanto ao ensaio de azul de tripan, foi obtida uma viabilidade de 93,8% ± 0,4. Já no ensaio de MTT foi obtido o percentual de 100% ± 6,2 de atividade metabólica. Em conclusão, biópsias da região auricular periférica de *G. spixii* permitiram o isolamento de células viáveis e com alta funcionalidade metabólica, fornecendo uma valiosa fonte celular para a transferência nuclear de célula somática.

Palavras-chave: Caviões, conservação, recursos somáticos, cultivo celular.

Abstract

The establishment of *in vitro* cell culture from skin fragments has been proposed as a useful technique in species conservation, especially when associated with cloning by nuclear transfer. Belonging to the order Rodentia, *G. spixii* stands out as one of the most studied species due to its ecological, economic and scientific role, since it can be used in studies for the conservation of endangered rodents. Therefore, the aim was to describe the *in vitro* culture of somatic cells from the skin of *G. spixii*, using the trypan blue cell viability assay and metabolic activity by 3-4,5-



XI CONGRESSO
NORTE E NORDESTE
DE REPRODUÇÃO
ANIMAL
17 a 19 de novembro de 2022

ANAIS DO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL

dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide or MTT assay. Then, skin biopsies from the peripheral ear region were retrieved from four anesthetized adult males. Samples were transported for 1 h in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin and streptomycin solution at 37°C. In the laboratory, fragments (9.0 mm³) were cultured in a controlled atmosphere (38.5°C, 5% CO₂) and evaluated every 24 h. After the cells reached 70% confluence, the first cell subculture was performed to obtain the desired concentration (5.0 × 10⁴ cells/mL) for the MTT assay. In addition to this, a pool of trypsinized cells were evaluated for viability with trypan blue. All data obtained were expressed as mean ± standard error. As for the trypan blue assay, a viability of 93.8% ± 0.4 was obtained. In the MTT assay, the percentage of 100% ± 6.2 of metabolic activity was obtained. In conclusion, biopsies from the peripheral ear region of *G. spixii* allowed the isolation of viable cells with high metabolic functionality, providing a valuable cellular source for somatic cell nuclear transfer.

Keywords: Cavids, conservation, somatic resources, cell culture.

Introdução

A ordem Rodentia é constituída por mais de 2000 roedores de pequeno a grande porte, e destes, o *G. spixii*, pertencente à família dos cavídeos, se destaca como uma das espécies mais estudadas em virtude de seu papel ecológico e econômico. Além disso, o *G. spixii* possui relevância científica, pois tem sido utilizado como modelo experimental para outros roedores ameaçados de extinção (SILVA *et al.*, 2016; PEREIRA *et al.*, 2021).

Nesse contexto, a formação de bancos de tecidos somáticos se destaca como uma ferramenta interessante na conservação do *G. spixii* (TUNSTALL *et al.*, 2018), uma vez que é possível empregar esses tecidos somáticos no cultivo *in vitro*, permitindo a recuperação de células que podem ser utilizadas em estudos relacionados a reprogramação nuclear por clonagem (PRAXEDES *et al.*, 2021) e indução de células à pluripotência para produção de gametas (TOYOOKA *et al.*, 2003). Portanto, o objetivo foi descrever o cultivo *in vitro* de células somáticas oriundas da pele de *G. spixii*, visando a formação de bancos de células para futuras biotecnologias.

Material e métodos

O experimento foi realizado em concordância com o Comitê de Ética de Uso de Animais (CEUA/UFERSA no. 23091.010566/2017-20), bem como pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, no. 60428-1). Um total de quatro espécimes de *G. spixii* machos adultos foi utilizado, provenientes do Centro de Multiplicação de Animais Silvestres (CEMAS/UFERSA).



XI CONGRESSO
NORTE E NORDESTE
DE REPRODUÇÃO
ANIMAL
17 a 19 de novembro de 2022

ANAIIS DO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL

Para as biópsias de pele, os animais foram anestesiados conforme descrito por PEREIRA *et al.* (2021). Após a confirmação do plano anestésico, biópsias do pavilhão auricular periférico com o auxílio de tesoura cirúrgica foram recuperadas. Em seguida, as amostras foram transportadas ao laboratório em meio essencial mínimo modificado por Dulbecco (DMEM) acrescido com 10% de soro fetal bovino (SFB) e 2% de solução de antibióticos-antimicóticos por 1 h a 37°C. No laboratório, as amostras foram tricotomizadas, fragmentadas em 9,0 mm³ e distribuídas aleatoriamente nas placas de cultivo.

Para o cultivo celular, fragmentos foram lavados em DMEM⁺ (10% de antibióticos-antimicóticos), álcool e DMEM⁻ (2% de solução de antibióticos-antimicóticos) (PRAXEDES *et al.*, 2021). Em seguida, foram encaminhados ao cultivo *in vitro*, onde permaneceram na incubadora em atmosfera úmida de 5% de CO₂, a 38,5°C. Diariamente, trocas de meios foram realizadas sob fluxo laminar e microscópio invertido. Após as células atingirem 70% de confluência, foi realizada a tripsinização e as células foram submetidas as análises de viabilidade e atividade metabólica.

Para avaliação da viabilidade celular, uma alíquota de células (20 µL) foi obtida e corada com 0,4% de azul de tripan, onde células viáveis foram as incolores, enquanto as não-viáveis foram as coradas em azuis (PRAXEDES *et al.*, 2021). Na atividade metabólica, o ensaio por brometo de (3- 572 (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio (MTT) foi empregado. Brevemente, células foram cultivadas durante 5 dias na concentração 5,0 × 10⁴ células/mL. Após o período, uma solução de MTT foi adicionada, e após 3 h foi realizado a análise das células somáticas a 595 nm (PRAXEDES *et al.*, 2021).

Finalmente, os dados foram expressos de forma descritiva, considerando a média ± erro padrão dos quatros indivíduos.

Resultados e discussão

O tempo de cultivo total foi de 23 dias para as amostras derivadas de todos os indivíduos. Dos dezesseis fragmentos submetidos ao cultivo (quatro fragmentos por animal, Fig. 1A) todos apresentaram aderência no segundo dia com crescimento celular ao redor dos explantes a partir do nono dia (6,0 ± 0,0). As células atingiram 70% de confluência formando monocamada no dia 20 ± 0,0. Em geral, a partir da análise morfológica, todas as células apresentaram morfologia fusiforme com núcleo oval centralizado, mostrando-se semelhante a fibroblastos (Fig. 1B).



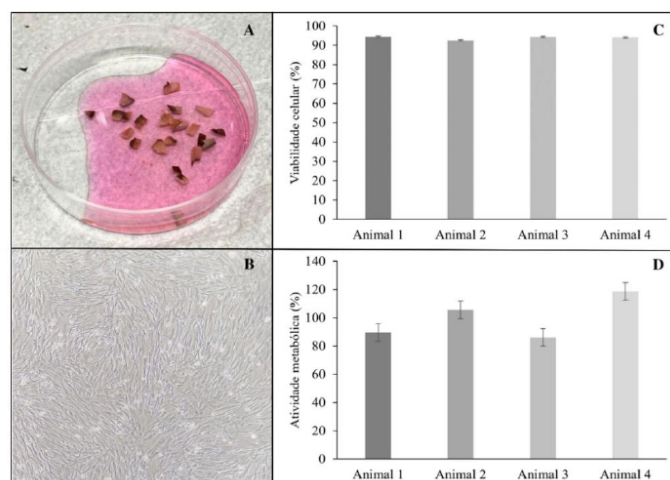
XI CONGRESSO
NORTE E NORDESTE
DE REPRODUÇÃO
ANIMAL
17 a 19 de novembro de 2022

ANAIS DO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL

Quanto ao ensaio de azul de tripan, foi obtida uma viabilidade de $93,8\% \pm 0,4$ [Animal 1, $94,41\% \pm 0,4$; Animal 2, $92,48\% \pm 0,4$; Animal 3, $94,33\% \pm 0,4$; Animal 4, $94,02\% \pm 0,4$ (Fig. 1C)]. Já no ensaio de MTT foi obtido o percentual de $100\% \pm 6,2$ de atividade metabólica [Animal 1, $89,6\% \pm 6,2$; Animal 2, $105,6\% \pm 6,2$; Animal 3, $86,1\% \pm 6,2$; Animal 4, $118,7\% \pm 6,2$] (Fig. 1D)].

Esses resultados foram similares quando comparado a outras espécies de roedores, como o porquinho-da-índia (MEHRABANI *et al.*, 2014), hamster (MEHRABANI *et al.*, 2016) e *Dasyprocta leporina* (PRAXEDES *et al.*, 2021), e demonstraram que células em condições de cultivo adequada são interessantes para estudos relacionados a biologia celular e molecular, podendo auxiliar ainda na conservação da espécie por meio do seu emprego em biotecnologias reprodutivas.

Figura 1 - Avaliação das condições de cultivo *in vitro* no estabelecimento do cultivo primário de células somáticas de *G. spixii*. **(A)** Amostras tricotomizadas e fragmentadas em $9,0 \text{ mm}^3$. **(B)** Células derivadas do cultivo primário, apresentando aspecto fusiforme. **(C)** Avaliação da viabilidade usando azul de tripan em células do cultivo primário. **(D)** Avaliação do metabolismo celular usando o ensaio de MTT.



Conclusão

As biópsias da região auricular periférica de *G. spixii* permitiram o isolamento de células viáveis com alta funcionalidade metabólica, fornecendo uma valiosa fonte celular para a transferência nuclear de célula somática, visando à conservação desta espécie.



XI CONGRESSO
NORTE E NORDESTE
DE REPRODUÇÃO
ANIMAL
17 a 19 de novembro de 2022

ANAIIS DO XI CONGRESSO NORTE E NORDESTE DE REPRODUÇÃO ANIMAL

Agradecimentos

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

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