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TAYNÁ MOURA MATOS

EFEITO DE DIFERENTES MEIOS NO CULTIVO ORGANOTÍPICO *IN VITRO* DE FRAGMENTOS TESTICULARES DE CATETOS (*Pecari tajacu Linnaeus*, 1758) ADULTOS

MOSSORÓ-RN

2026

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Dissertação apresentada à Universidade Federal Rural do Semi-Árido (UFERSA), como exigência final para obtenção do título de Mestre no Curso de Pós-Graduação em Ciência Animal

Linha de Pesquisa: Morfofisiologia da Reprodução e Pós-Graduação em Ciência Animal

Orientador: Prof. Dr. Alexandre Rodrigues Silva

MOSSORÓ, RN

2026

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Orientador: Prof. Dr. Alexandre Rodrigues Silva

Defendida em: 25/02/2026

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MOSSORÓ, RN

2026

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RESUMO

Esta dissertação objetivou definir um protocolo eficiente para o cultivo *in vitro* de tecido testicular de catetos adultos, de modo a viabilizar a utilização e a avaliação do germoplasma masculino previamente armazenado em biobancos. No primeiro capítulo foram investigados os efeitos de meios de cultura suplementados sobre a morfologia, viabilidade, apoptose e integridade do DNA de fragmentos testiculares de catetos adultos *in vitro*. Cinco testículos foram coletados, dissecados e cultivados em meios DMEM/F12, DMEM ou MEM. Esses meios foram suplementados igualmente com 10% de soro fetal bovino, 25 mM de HEPES, 2 µL/mL de insulina-transferrina-selênio (ITS), 1% de solução de aminoácidos não essenciais (NEAA), 1% de solução vitamínica, 0,2% de albumina sérica bovina, 1% de solução antibiótico-antifúngica, 10 ng/mL de LH/FSH, 20 ng/mL de mEGF, 10 ng/mL de GDNF e 10 ng/mL de bFGF. Os fragmentos foram cultivados em um sistema organotípico em gel de agarose a 0,75% por 7 e 14 dias a 34 °C e 5% de CO_2. O tempo de cultivo reduziu os escores morfológicos em comparação com o controle fresco ($P < 0,05$). O meio MEM apresentou melhor preservação morfológica da vacuolização da membrana em 7 dias e de todos os parâmetros em 14 dias. Por outro lado, o meio DMEM apresentou maior estabilidade temporal, garantindo um microambiente mais consistente. A viabilidade celular foi mantida, sem diferenças entre os tratamentos ($P > 0,05$). As taxas de apoptose mantiveram-se constantes, sugerindo equilíbrio celular ao longo do experimento. O meio MEM preservou melhor a integridade do DNA por até 7 dias, enquanto o meio DMEM manteve a estabilidade por 14 dias. Em conclusão, os meios MEM e DMEM, quando adequadamente suplementados, são adequados para o cultivo organotípico de tecido testicular de catetos adultos por até 14 dias. No segundo capítulo, investigaram-se os efeitos de diferentes meios de cultura suplementados na morfologia, viabilidade celular e apoptose de fragmentos testiculares de catetos adultos previamente criopreservados e posteriormente cultivados *in vitro*. Cinco testículos foram coletados, dissecados e cultivados em meios DMEM/F12 e DMEM. Ambos os meios receberam suplementação idêntica, contendo 10% de soro fetal bovino, 25 mM de HEPES, 2 µL/mL de insulina-transferrina-selênio (ITS), 1% de aminoácidos não essenciais (NEAA), 1% de solução vitamínica, 0,2% de albumina sérica bovina, 1% de solução antibiótico-antifúngica, 10 ng/mL de LH/FSH, 20 ng/mL de mEGF, 10 ng/mL de GDNF e 10 ng/mL de bFGF. Fragmentos testiculares foram cultivados em um sistema organotípico utilizando gel de agarose a 0,75% por períodos de 7 e 14 dias, sob condições controladas de 34 °C e atmosfera contendo 5% de CO_2. O tempo de cultivo reduziu significativamente os escores morfológicos em comparação com o controle fresco e criopreservado ($P < 0,05$). Após 7 dias de cultivo, o meio DMEM proporcionou melhor preservação morfológica, especialmente nos parâmetros de separação da membrana basal e estrutura tubular; entretanto, após 14 dias, não foram encontradas diferenças estatisticamente significativas entre as avaliações dos meios. A predição celular permaneceu estável ao longo do experimento, sem diferenças entre os tratamentos ($P > 0,05$). Da mesma forma, as proporções de células apoptóticas permaneceram constantes durante todo o período experimental, manutendo a homeostase celular. Em conclusão, os meios DMEM/F12 e DMEM, quando suplementados adequadamente, mostraram-se adequados para o cultivo organotípico de tecido testicular de catetos adultos, a partir de amostras previamente criopreservadas por até 14 dias. Palavras-chave: Biobanco; Criopreservação de tecido testicular; Cultivo *in vitro*

ABSTRACT

This dissertation aimed to define an efficient protocol for the in vitro culture of testicular tissue from adult peccaries, in order to enable the use and evaluation of male germplasm previously stored in biobanks. The first chapter investigated the effects of supplemented culture media on the morphology, prediction, apoptosis, and DNA integrity of testicular fragments from adult peccaries in vitro. Five testes were collected, dissected, and cultured in DMEM/F12, DMEM, or MEM media. These media were equally supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 µL/mL insulin-transferrin-selenium (ITS), 1% non-essential amino acid solution (NEAA), 1% vitamin solution, 0.2% bovine serum albumin, 1% antibiotic-antifungal solution, 10 ng/mL LH/FSH, 20 ng/mL mEGF, 10 ng/mL GDNF, and 10 ng/mL bFGF. The fragments were cultured in an organotypic system on 0.75% agarose gel for 7 and 14 days at 34 °C and 5% CO_2. The study time reduced the morphological scores compared to the fresh control ($P < 0.05$). The MEM medium showed better morphological preservation of membrane vacuolization at 7 days and of all parameters at 14 days. On the other hand, the DMEM medium showed greater temporal stability, ensuring a more consistent microenvironment. Cell protection was maintained, with no differences between treatments ($P > 0.05$). Apoptosis rates remained constant, increasing cellular equilibrium throughout the experiment. The MEM medium better preserved DNA integrity for up to 7 days, while the DMEM medium maintained stability for 14 days. In conclusion, MEM and DMEM media, when developed with supplementation, are suitable for the organotypic culture of testicular tissue from adult peccaries for up to 14 days. In the second chapter, we investigated the effects of different supplemented culture media on the morphology, viable cells, and apoptosis of testicular fragments from adult peccaries previously cryopreserved and subsequently cultured in vitro. Five testes were collected, dissected, and cultured in DMEM/F12 and DMEM media. Both media received identical supplementation, containing 10% fetal bovine serum, 25 mM HEPES, 2 µL/mL insulin-transferrin-selenium (ITS), 1% non-essential amino acids (NEAA), 1% vitamin solution, 0.2% bovine serum albumin, 1% antibiotic-antifungal solution, 10 ng/mL LH/FSH, 20 ng/mL mEGF, 10 ng/mL GDNF, and 10 ng/mL bFGF. Testicular fragments were cultured in an organotypic system using 0.75% agarose gel for periods of 7 and 14 days, under controlled conditions of 34 °C and an atmosphere containing 5% CO_2. Culture time significantly altered morphological scores compared to fresh and cryopreserved controls ($P < 0.05$). After 7 days of culture, DMEM medium provided better morphological preservation, especially in the separation of the basement membrane and tubular structure; however, after 14 days, no statistically significant differences were found between the media evaluations. Cell prediction remained stable throughout the experiment, with no differences between treatments ($P > 0.05$). Similarly, the proportions of apoptotic cells remained constant throughout the experimental period, maintaining cellular homeostasis. In conclusion, DMEM/F12 and DMEM media, when supplemented with specific treatments, proved suitable for the organotypic culture of testicular tissue from adult peccaries, from samples previously cryopreserved for up to 14 days.

Keywords: Biobank; Testicular tissue cryopreservation; In vitro culture

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1. INTRODUÇÃO

O desequilíbrio climático constitui um dos principais desafios contemporâneos, promovendo alterações profundas nos ecossistemas (Braga e Laurini., 2024). Nesse contexto, a Caatinga destaca-se pela elevada vulnerabilidade à desertificação, às mudanças climáticas e à expansão agropecuária (Tabarelli et al., 2018), intensificando impactos sobre espécies nativas essenciais para o equilíbrio e funcionamento ambiental.

O cateto (*Pecari tajacu* Linnaeus, 1758), também conhecido como caititu ou porco-do-mato, é um representante da família Tayassuidae amplamente distribuído no Brasil, desempenhando papel relevante na recomposição florestal e no equilíbrio das cadeias alimentares (Silva et al., 2017; Vieira et al., 2023). Entretanto, segundo o Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), a espécie encontra-se ameaçada em biomas como a Mata Atlântica e a Caatinga, o que evidencia a necessidade de estratégias inovadoras para a conservação da biodiversidade e a preservação de genomas de espécies vulneráveis (Hagedorn et al., 2024).

Nessa conjuntura, os biobancos surgem como instrumentos essenciais para a conservação de material genético, oferecendo suporte tanto para a medicina reprodutiva quanto para a preservação de espécies silvestres (Comizzoli, 2018). Esses bancos utilizam tecidos reprodutivos e possibilitam a manutenção da variabilidade celular necessária para pesquisas e aplicações biotecnológicas. No entanto, o aproveitamento desses tecidos exige protocolos adequados, pois diferenças próprias, da espermatogênese e da histologia testicular podem influenciar diretamente o sucesso da criopreservação e do cultivo (Silva et al., 2022).

A manutenção da viabilidade celular pós-descongelamento é particularmente desafiadora, uma vez que taxas inadequadas de resfriamento comprometem a integridade dos espermatozoides (Pereira et al., 2025a). Além disso, diferentes tipos de células germinativas respondem de modo variável ao processo de congelamento (Unni et al., 2012).

Diante dessas limitações, o cultivo *in vitro* (CIV) de tecido testicular tem se destacado como uma alternativa promissora, especialmente para programas de conservação e multiplicação de catetos (Silva et al., 2024). Além de auxiliar na preservação da fertilidade, a integração entre criopreservação e CIV permite aprofundar a

compreensão da espermatogênese. Embora o cultivo de tecidos adultos ainda representa um desafio (Sato et al., 2015), o CIV oferece um ambiente controlado capaz de preservar aspectos estruturais do testículo e explorar fatores essenciais para o desenvolvimento germinativo (Sato et al., 2012; Ibtisham e Honaramooz, 2020).

A aplicação do CIV também possibilita a retomada da espermatogênese em tecidos previamente criopreservados, favorecendo a recuperação de células germinativas em diferentes estágios (Silva et al., 2024) e potencialmente, a produção de espermatozoides maduros para aplicação em biotecnologias reprodutivas. Assim, além de ampliar o aproveitamento do germoplasma armazenado, o CIV fortalece pesquisas voltadas à reprodução assistida e à conservação de espécies silvestres.

Para catetos pré-púberes, dois protocolos de cultivo já foram descritos. Silva et al. (2024) demonstraram que tanto o DMEM quanto o StemPro-34 SFM, suplementados com fator neurotrófico derivado da glia (GDNF, 10 ng/mL), são adequados para promover a espermatogênese *in vitro*. Pereira et al. (2025), por sua vez, observaram que o cultivo organotípico é mais indicado para a manutenção prolongada dos fragmentos testiculares e que a suplementação com FGF não é essencial para essa espécie.

Apesar dos avanços, a preservação e o cultivo do tecido testicular adulto ainda representam desafios significativos. Após o término da espermatogênese, os túbulos seminíferos apresentam elevada complexidade estrutural e diversidade celular, o que os torna mais suscetíveis a danos físicos, isquêmicos e metabólicos (Da Silva et al., 2019). A isquemia pós-coleta reduz rapidamente a oxigenação, induz a apoptose de células germinativas e compromete a integridade do epitélio seminífero (Farias et al., 2005). Ademais, durante o cultivo prolongado, fragmentos adultos tendem a perder organização histológica e funcionalidade, refletindo sua baixa adaptabilidade ao ambiente *in vitro* (Sato et al., 2015).

Diferenças biológicas fundamentais entre testículos imaturos e adultos também explicam essa discrepância. A maturação testicular envolve remodelação do nicho das células-tronco espermatogônias e formação da barreira hemato-testicular, tornando o microambiente adulto mais complexo e rigidamente regulado (Voigt et al., 2023). Em contraste, o testículo pré-púbere apresenta células de Sertoli ainda proliferativas e menor organização estrutural, condições que favorecem a manutenção em CIV. Além disso,

como a espermatogênese ainda não está estabelecida em indivíduos jovens, suas gônadas exibem túbulos seminíferos imaturos, uniformes e sem lúmen (Ibtisham et al., 2022).

Diante desse cenário, torna-se evidente que protocolos desenvolvidos para tecidos juvenis não são totalmente válidos para o tecido adulto, reforçando a necessidade de metodologias que reproduzam de forma mais fiel as condições microambientais *in vivo* e possibilitem o avanço da conservação reprodutiva em espécies ameaçadas.

2. REVISÃO DE LITERATURA

2.1. Aspectos ecológicos dos catetos

Os catetos *(Pecari tajacu*) pertencem à família Tayassuidae, ordem Cetartiodactyla, subordem Suiformes e superfamília Suoidea. São mamíferos silvestres conhecidos como caititu ou porco-do-mato, em razão de sua semelhança com o javali e o suíno doméstico. Apresentam ampla distribuição geográfica, ocorrendo desde o sudoeste dos Estados Unidos e América Central até grande parte da América do Sul, incluindo o Brasil (Gongora et al., 2011; Silveira et al., 2024), onde habitam biomas como Mata Atlântica, Cerrado, Amazônia, Caatinga e Pantanal (De Sousa et al., 2021).

Possuem hábito diurno-crepuscular e apresentam comportamento gregário, formando grupos sociais, que podem variar de 5 a 25 indivíduos (Desbiez et al., 2012), distribuindo-se em habitats fixos com áreas estimadas de 24 a 800 hectares (Sowls, 1997). Arranjam-se em unidade social permanente, onde todos os indivíduos movimentam-se, alimentam-se e dormem juntos, além disso, os grupos são frequentemente estáveis e os membros permanecem no seu grupo de nascimento até a morte. Segundo (Nogueira Filho et al. 1999; Silva, 2023).

Esses ungulados apresentam hábito alimentar onívoro, baseado em uma dieta que inclui o consumo de uma variedade de alimentos como frutas, vegetais, carnes e tubérculos (Prado et al., 2013). Atuam também como importantes agentes ecológicos, pois, apesar de não serem considerados substancialmente dispersores, acabam por dispersar algumas espécies vegetais devido à alimentação variável (Marinho et al., 2019). Ao longo de milênios, a espécie tem desempenhado papel significativo na cultura de diversos povos indígenas, que a empregam como fonte de alimento, além de utilizá-la em práticas medicinais e rituais tradicionais (Alves et al., 2016).

Dematteo et al (2025) destacam que a presença desta espécie nos biomas pode funcionar como um indicador de qualidade ambiental, visto que sua ausência do meio sugere um alto grau de perturbação do habitat, já que são animais com alta capacidade de adaptação. Devido à sua ampla distribuição e ocorrência em uma variedade de habitats, incluindo ambientes relativamente perturbados, *Pecari tajacu* é classificada globalmente pela IUCN como espécie de “Menos preocupante”, indicando que, até o momento, não é considerada ameaçada em nível mundial (IUCN, 2025). No entanto, a

degradação dos habitats decorre sobretudo do avanço da agricultura, da extração de madeira e do crescimento urbano, fatores que diminuem os espaços adequados para a sobrevivência e a alimentação dos catetos (Silveira et al., 2024) aumentaram a conscientização sobre a necessidade de monitorar essas populações. Assim, em diversos biomas, essa classificação é vista como defasada e não representativa (Nagy Reis et al., 2019).

Uma pesquisa realizada pelo Instituto Chico Mendes de Biologia (ICMBIO) revelou que, no Brasil, a espécie possui um risco de extinção menor em praticamente todos os biomas, com exceção da Mata Atlântica e Caatinga, onde é considerada quase ameaçada, respectivamente. Diante disso, faz-se necessário garantir a conservação do material genético dessa espécie e, para tal, é necessário aplicar programas de reprodução em cativeiro e técnicas de reprodução assistida (Santos et al., 2004). E, utilizando o cateto como modelo experimental, esses estudos seriam vitais não apenas para a formação de bancos de germoplasma da espécie, mas também para espécies filogeneticamente semelhantes aos taguás (Catagonus wagneri) e queixadas (Tayassu pecari) (Silva et al., 2020). Conforme apresentado na Figura 1, o cateto pode ser observado em ambiente natural/semi-cativo.

Figura 1 – Exemplar de cateto (Pecari tajacu) em ambiente natural/semi-cativo.

Fonte: Arquivo pessoal (2025).

2.2. Fisiologia da Reprodução Aplicada aos Catetos Machos

Para contribuir com a conservação da espécie, executaram-se diversos estudos em catetos para entender melhor sua biologia reprodutiva, tanto masculina quanto feminina (Peixoto et al., 2016). O primeiro passo para o desenvolvimento de biotecnologias reprodutivas é o conhecimento, que permite identificar semelhanças com espécies domésticas que seguem protocolos pré-estabelecidos.

Identificar essas semelhanças é crucial para adaptar técnicas reprodutivas de forma eficaz e segura para novas espécies. No caso dos catetos, a espécie doméstica que mais se aproxima filogeneticamente é a suína (Silva et al., 2024). Embora pertença a uma família diferente, a espécie suína pode ser usada como modelo inicial de comparação devido às suas similaridades fisiológicas e reprodutivas com os catetos.

Os machos de catetos apresentam um escroto localizado entre a região inguinal e perineal com alguns pelos finos e curtos, e atingem a puberdade em torno de 11 e 12 meses (Guimarães et al., 2013). Durante a cópula, o cortejo é iniciado pela fêmea. (Castelo, 2010). Os testículos dessa espécie têm em média 2,8 cm de largura, 2,6 cm de espessura, 5,4 cm de comprimento e 22,5 cm^3 de volume, sendo ambos simétricos. Segundo Costa et al. (2010), os testículos possuem um peso médio de 23,7±1,8 g, e as células de Leydig ocupam 12,8±1,8% do parênquima testicular.

O ciclo espermatogênico e o processo completo da espermatogênese (com base em 4,5 ciclos) têm duração de cerca de 12,3±0,2 e 55,1±0,7 dias, respectivamente. A produção diária de espermatozoides por grama de testículo em catetos é de aproximadamente 23,4±2 x 10^6, semelhante à de suínos domésticos e selvagens (Costa et al., 2010). É importante ressaltar que os catetos apresentam uma arquitetura histológica testicular peculiar, com nichos isolados de células de Leydig no tecido intersticial (Costa et al., 2010). Além disso, sua biometria testicular e o perímetro escrotal não são bons indicadores da produção espermática, pois não há relação entre esses parâmetros e os parâmetros seminais (Peixoto et al., 2012). A arquitetura e organização dos túbulos seminíferos pode ser observada na Figura 2.

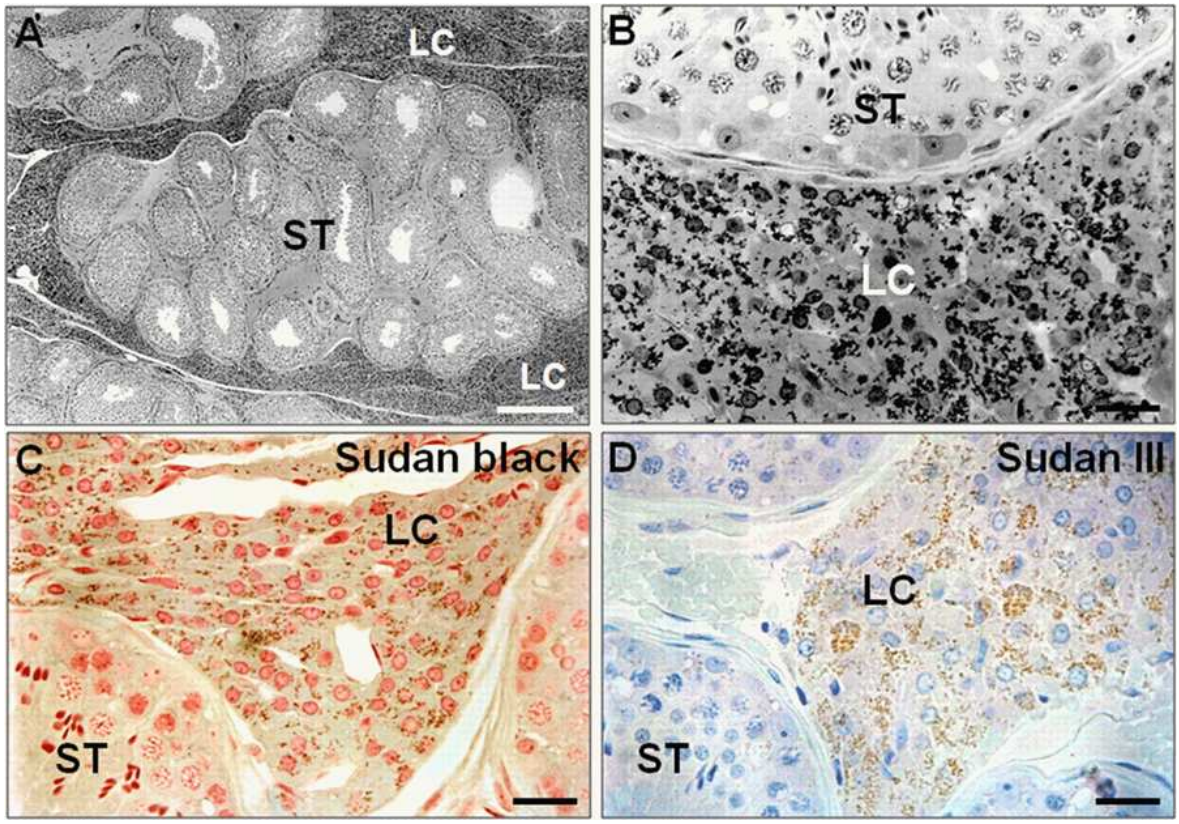

Figura 2. Duração do ciclo espermatogênico e produção de espermatozoides em uma espécie de porco selvagem (cateto, Pecari tajacu).

Fonte: adaptado de Costa et al. (2010).

Parênquima testicular de Pecari Em baixa ampliação: (A) organização peculiar das células de Leydig (CL) ao redor do lóbulo do túbulo seminífero (TS). Aparentemente, não há células de Leydig presentes no túbulo seminífero que constitui um lóbulo. Em maior ampliação: (B) o citoplasma das células de Leydig está densamente preenchido com pigmentos escuros, que denotam lipídios, de acordo com a coloração com Sudan Black e Sudan III (C, D) . Barra de escala branca = 250 μm; barra de escala preta = 30 μm.

2.3 Biotecnologia da Reprodução Aplicada aos Catetos Machos

Desde 2010, protocolos para a criopreservação de sêmen de cateto têm sido desenvolvidos (Castelo et al., 2010) visando melhorar a eficiência e a viabilidade dos espermatozoides após o descongelamento. É possível coletar sêmen nesta espécie por meio de eletroejaculação, usando uma variedade de protocolos anestésicos (Castelo, 2010). A aplicação desses protocolos têm sido bem-sucedida, proporcionando material genético de alta qualidade para estudos e reprodução assistida (Vieira, 2021) e após a descongelação, é possível recuperar até 48,3% de espermatozoides móveis (Silva, 2023).

Diversas substâncias têm sido estudadas como aditivos ao meio de criopreservação de espermatozoides, como detergentes, (Moreira et al., 2023), antioxidantes (Moreira et al., 2023) e antimicrobianos (Moreira et al., 2022), e mais recentemente Santos et al., (2024) verificaram o efeito do diluente e tempo de armazenamento em recipiente de transporte em sêmen de cateto por 4, 24 e 48 h antes da criopreservação, utilizando diferentes meios de diluição (Tris + gema de ovo (20%) e Primxcell Ultra). Nesse estudo, os autores concluíram que o diluente Tris + gema de ovo (20%) é uma opção eficaz, permitindo que o sêmen fique resfriado por 24 ou 48 h em um recipiente de transporte antes da criopreservação.

No intuito de contribuir com a conservação de material genético dos catetos, Bezerra et al. (2018) demonstraram ser possível preservar material genético em animais que foram recentemente mortos devido a causas diversas. Os autores obtiveram espermatozoides do epidídimo por meio de lavagem retrógrada e os processam por meio da criopreservação, utilizando o diluente à base de Tris acrescido de gema de ovo e glicerol, obtendo 54,1% de motilidade pós-descongelação. No entanto, nesse tipo de estudo, apenas os epidídimos são aproveitados, sendo realizado o descarte dos testículos, resultando em uma perda significativa de material genético valioso. Com isso, era evidente a perda de germoplasma a partir dessas amostras, o que fomentou o início das pesquisas a respeito de métodos de conservação e cultivo de tecido testicular. Diante disso, o estabelecimento de protocolos de criopreservação associados a protocolos de cultivo *in vitro* que proporcionem a ocorrência da

espermatogênese é fundamental para a formação de biobancos. Além de que, o cateto pode ser usado como um modelo experimental não apenas para os taiaçuídeos selvagens (Da Silva et al., 2019), mas também para outros ungulados.

Para a criopreservação de tecido testicular de catetos, já foram conduzidos diversos estudos ao longo dos últimos anos. O trabalho de Da Silva et al. (2019) representou o primeiro registro dessa técnica aplicada a ungulados silvestres, marcando um avanço importante para a conservação de espécies nativas. Nesse estudo, os autores avaliaram a vitrificação por superfície sólida (SSV) de tecido testicular adulto, expondo os fragmentos a três diferentes soluções crioprotetoras: 3,0 M de dimetilsulfóxido (DMSO), 3,0 M de etilenoglicol (EG) ou a combinação de 1,5 M DMSO + 1,5 M EG. Após o descongelamento, observaram que somente a solução combinada de DMSO + EG foi capaz de preservar de forma satisfatória a arquitetura dos túbulos seminíferos, a integridade da membrana basal, as junções celulares e a viabilidade nuclear, resultados comparáveis aos obtidos com tecidos não congelados.

Dando continuidade às investigações sobre a espécie, o mesmo grupo de pesquisa publicou, em 2021, um estudo no qual comparou diferentes técnicas de criopreservação do tecido testicular de adultos, avaliando o congelamento lento (SF), a vitrificação convencional (CV) e a vitrificação por superfície sólida (SSV). Além disso, foram testadas diferentes combinações de crioprotetores DMSO + EG, DMSO + glicerol e EG + glicerol a fim de identificar qual associação e qual método seriam mais eficazes na preservação da estrutura e da viabilidade tecidual após o descongelamento. Com base nesses resultados, os autores concluíram que, para o tecido testicular adulto de cateto, os métodos mais adequados foram o congelamento lento (SF) e a vitrificação convencional (CV), ambos utilizando a combinação de DMSO + EG. As combinações contendo glicerol não foram recomendadas.

Mais recentemente, ampliando o conhecimento sobre a eficiência das soluções crioprotetoras, Silva et al. (2025) compararam dois esquemas de vitrificação por superfície sólida (SSV): uma solução com 3 M (1,5 M de DMSO + 1,5 M de EG) e outra com 6 M (3 M DMSO + 3 M EG). Os resultados foram promissores e mostraram que a vitrificação por SSV utilizando a solução de 6 M foi superior ao congelamento lento para a preservação a longo prazo dos tecidos testiculares da espécie. Além disso,

os autores concluíram que, mesmo dentro de uma mesma espécie, é fundamental diferenciar machos pré-púberes de machos sexualmente maduros ao definir um protocolo eficiente de criopreservação de tecido testicular

2.3. Criopreservação de tecido testicular

A criopreservação é uma técnica que permite a conservação do material genético em nitrogênio líquido, possibilitando a manutenção da integridade celular e das funções endócrinas dos testículos (Yildiz et al., 2013). O material criopreservado pode ser posteriormente empregado em programas de reprodução assistida (Poels et al., 2012). Além disso, a manutenção do tecido em temperaturas criogênicas possibilita sua associação a sistemas de cultivo de células gonadais, favorecendo a retomada e a progressão da espermatogênese completa *in vitro* (Hu et al., 2015).

Essa técnica mostra-se especialmente útil em situações em que a obtenção de espermatozoides não é viável, como em animais que morrem de forma inesperada, em espécies com forte sazonalidade reprodutiva que venham a óbito fora do período fértil, ou ainda em pacientes que necessitam de tratamentos gonadotóxicos (Santiago-Moreno; Blesbois, 2022)

Vale ressaltar que não é apenas crucial considerar a sobrevivência de células individuais, mas também a preservação da capacidade de interações célula a célula, o que torna desafiador para a maioria dos tecidos e órgãos complexos atender aos requisitos de aplicação clínica após a criopreservação (Liu et al., 2022). Além disso, é extremamente importante a manutenção da matriz extracelular e das células de sustentação para a sobrevivência e o funcionamento das células germinativas.

Nesse sentido, a idade do mamífero é um fator crucial que afeta diversos parâmetros como a morfologia, a dinâmica do transcriptoma e as mudanças de resiliência do tecido (Zou et al., 2023), podendo afetar também a adaptação *in vitro*, bem como os protocolos de criopreservação. Os tecidos testiculares são compostos por células somáticas, e diversos tipos de células germinativas, que abrangem desde espermatogônias e espermatócitos e espermátides e espermatozoides (Patra et al., 2021).

Após a criopreservação, os fragmentos testiculares podem ser transplantados para

um hospedeiro compatível, ou as células germinativas imaturas, como as espermatogônias, podem ser isoladas e cultivadas *in vitro*, permitindo, em ambos os casos, a retomada da espermatogênese e a produção de espermatozoides maduros para uso em técnicas de reprodução assistida (Eugeni et al., 2022).

Estudos recentes demonstram avanços na criopreservação de tecido testicular de catetos, com destaque para o trabalho pioneiro de Da Silva et al. (2019), que identificou a combinação DMSO + EG como a mais eficaz na vitrificação por superfície sólida. Em 2021, o mesmo grupo confirmou que o congelamento lento e a vitrificação convencional, ambos com DMSO + EG, são os métodos mais adequados para tecidos adultos. Em 2025 os mesmos autores, observaram que a vitrificação por superfície sólida com solução de 6 M supera o congelamento lento, reforçando a importância de ajustar protocolos conforme a maturidade sexual dos machos.

2.4. Cultivo *in vitro* de germoplasma testicular

As pesquisas realizadas *in vitro* são fundamentais para elucidar mecanismos biológicos em um ambiente controlado e isolado (Giordano & Costa, 2011). O cultivo de células e tecidos teve início no final do século XIX e, com o passar do tempo, tornou-se uma ferramenta essencial nas ciências biológicas, servindo de base para o estudo dos mecanismos de regulação, proliferação e diferenciação celular (Chang et al., 2011). Nesse cenário, destaca-se a relevância do desenvolvimento de protocolos eficazes para o cultivo *in vitro* de fragmentos ou células oriundos das gônadas masculinas de mamíferos, capazes de permitir a ocorrência completa da espermatogênese (Hu et al., 2015).

Sato et al. (2011) apresentaram um sistema *in vitro* inovador capaz de sustentar todo o processo de espermatogênese, resultando na formação de espermatozoides funcionalmente férteis. Por utilizar fragmentos inteiros de testículos de camundongos neonatos, esse modelo mantém a presença integral dos tipos celulares presentes no testículo *in vivo*, incluindo não apenas as células germinativas, mas também as células de Sertoli e de Leydig.

Dentre os diferentes sistemas, o cultivo organotípico tem sido bastante relatado na literatura e consiste na acomodação de um fragmento de tecido sobre um gel de agarose, cercado por um meio que dará aporte nutricional à multiplicação celular (Wahab et al., 2020). Paralelo ao sistema, o meio de cultivo utiliza-se de uma importante fonte de energia para as células e componentes que controlam o ciclo celular, devendo suportar a sobrevivência, proliferação e funções celulares, pois a sua qualidade afeta diretamente a progressão da espermatogênese (Yao & Asayama, 2017). Dessa forma, destacam-se os benefícios do cultivo *in vitro*, que oferece um ambiente controlado capaz de preservar a microarquitetura do tecido testicular. Essa abordagem permite investigar, em nível molecular, os processos que influenciam a espermatogênese, já que possibilita analisar de forma isolada o efeito de distintos fatores sobre esse processo (Sato et al., 2012).

Diversos estudos têm empregado o cultivo organotípico de tecido testicular como estratégia para preservar a arquitetura dos túbulos seminíferos e permitir a progressão da espermatogênese em condições *ex vivo*. O marco inicial dessa abordagem foi estabelecido por Sato et al. (2011), que demonstraram a geração de espermatozoides funcionais a partir de fragmentos testiculares de camundongos neonatos cultivados na interface ar–líquido. Em 2013, o mesmo grupo consolidou esse avanço ao publicar um protocolo detalhado capaz de sustentar a espermatogênese completa *in vitro*, confirmando que o ambiente organotípico, quando mantido sob condições controladas, é suficiente para conduzir todas as etapas do desenvolvimento das células germinativas.

Posteriormente, em 2015, os autores ampliaram ainda mais a aplicabilidade da técnica ao demonstrar que o método anteriormente estabelecido para testículos de camundongos jovens também pode ser empregado em animais adultos. Nesse estudo, fragmentos de tecido testicular de camundongos entre 4 e 29 semanas foram mantidos em cultivo, evidenciando que a abordagem organotípica é robusta e adaptável a diferentes faixas etárias. Mais recentemente, outros autores também utilizaram essa forma de cultivo, como Sanjo et al. (2018), que demonstraram que o uso de um meio quimicamente definido foi capaz de sustentar a progressão das células germinativas através das etapas meióticas até a formação de células haploides. Esses resultados reforçam que a organocultivo mantém a capacidade de promover a diferenciação espermatogênica mesmo na ausência de suplementos tradicionalmente utilizados, evidenciando a robustez do sistema organotípico.

Avançando além dos modelos baseados em roedores, Kanbar et al. (2022) aplicaram a organocultivo em uma espécie filogeneticamente mais próxima dos catetos, o suíno. Esse estudo representa um dos primeiros relatos envolvendo um mamífero de grande porte e ampliou significativamente o uso de cultivos organotípicas. Os autores demonstraram a manutenção da viabilidade tecidual, o progresso da espermatogênese com inclusão das fases meióticas e pós-meióticas e a atividade endócrina, evidenciada pela produção de testosterona. Embora os autores relatem que a eficiência global ainda seja limitada, os resultados indicam que a

maturação *ex vivo* de tecido testicular imaturo também é possível em espécies de maior porte.

Seguindo essa mesma linha de expansão para modelos biológicos, Silva et al. (2024) também empregaram o cultivo organotípico em fragmentos testiculares de catetos pré-púberes, contribuindo para a compreensão da aplicabilidade da técnica em espécies silvestres. Nesse estudo, os autores avaliaram não apenas um meio de cultivo adequado para essa espécie, mas também investigaram a resposta das células germinativas à suplementação com o Fator Neurotrófico Derivado da Linhagem de Células Gliais (GDNF). Os resultados reforçam o potencial do modelo organotípico para aplicações em conservação, reprodução assistida e estudos comparativos sobre os mecanismos da espermatogênese.

Pereira et al. (2025) avançaram ainda mais na aplicação da organocultivo testicular em espécies silvestres ao estabelecerem um protocolo de cultivo de longo prazo para fragmentos testiculares de catetos pré-púberes. Neste estudo, autores compararam diferentes sistemas de cultivo organotípico e tridimensional e testaram a suplementação com Fatores de Crescimento de Fibroblastos (FGF), avaliando sua influência sobre a sobrevivência tecidual e a manutenção da viabilidade dos fragmentos testiculares durante o cultivo *in vitro*. Portanto, o conjunto desses estudos demonstra o avanço contínuo e a crescente versatilidade dos sistemas de cultivo organotípico aplicados ao tecido testicular.

2.5. Meios de cultivo:

Nos últimos anos, os avanços na biologia têm se sustentado fortemente na tecnologia de cultivo celular, que se tornou uma ferramenta essencial para o desenvolvimento científico. Essa tecnologia tem viabilizado importantes aplicações práticas, como a avaliação da eficácia e toxicidade de novos fármacos (Ryoo et al., 2024), a produção de vacinas (Zinnecker et al., 2024) e biofármacos (Dong et al., 2024), além de contribuir significativamente para as técnicas de reprodução assistida (Tan et al., 2024). Nesse cenário, ressalta-se a relevância do aprimoramento contínuo das técnicas de cultivo celular, uma vez que o meio de cultivo é amplamente reconhecido como o fator mais decisivo para o sucesso dessa tecnologia.

Um meio de cultivo é uma solução fluida que garante a sobrevivência, a proliferação e o funcionamento celular. Dessa forma, a qualidade desse meio influencia diretamente os resultados das pesquisas, a eficiência da produção biofarmacêutica e o sucesso dos tratamentos em técnicas de reprodução assistida (Yao e Asayama, 2017). Portanto, a escolha de um meio adequado para cada tipo de célula ou tecido, bem como a sua modificação quando necessário, é essencial para o êxito dos cultivos celulares (Yao & Asayama, 2017). Tais meios devem suportar altas densidades celulares viáveis, ao mesmo tempo que estimulam a síntese e o transporte extracelular de produtos biológicos. (Ritacco et al., 2018)

Pesquisas sobre meios de cultivo celular evidenciam avanços importantes no entendimento de sua composição e limitações. Atualmente, os meios sintéticos podem ser classificados em diversos grupos, com base no tipo de suplementos adicionados; por exemplo, meios contendo soro, meios sem soro, meios sem proteínas e meios quimicamente definidos (Yao e Asayama, 2017). Os meios suplementados com soro apresentam uma composição intrinsecamente indefinida, pois incorporam diversos componentes provenientes do próprio soro, cujas concentrações podem variar entre diferentes lotes. Essa característica aumenta o risco de contaminação por bactérias e compromete a reprodutibilidade experimental. Por outro lado, os meios isentos de soro possuem composição bem definida, o que favorece maior precisão e reprodutibilidade nos cultivos celulares.

No caso de tecidos ou células testiculares, cada meio básico é utilizado levando em consideração as necessidades específicas dessas células, de acordo com sua espécie e o objetivo do cultivo (Yao & Asayama, 2017). Observa-se que a escolha do meio de cultivo varia conforme a espécie estudada. Até o momento, apenas meios de cultivo convencionais, como o meio essencial mínimo modificado de Dulbecco (DMEM), o meio F12 e o meio essencial mínimo (MEM), têm sido utilizados no cultivo de células testiculares (Sato et al., 2011).

O meio essencial mínimo modificado de Dulbecco (DMEM) tem sido utilizado em cultivos de fragmentos de tecido testicular de ratos (Reda et al., 2014), cães (Lee et al., 2016), cabras (Heidari et al., 2012) e catetos pré-púberes (Silva et al., 2024). O meio de cultivo Dulbecco's Modified Eagle Medium F12 (DMEM F12) é uma formulação básica amplamente empregada no cultivo de diferentes tipos de células de mamíferos e já foi empregado em cultivo de gônadas em caprinos (Salimi et al., 2024), suínos (Fayaz et al., 2022), bovinos (Tang et al., 2023). O Meio Essencial Mínimo (MEM), desenvolvido por Harry Eagle, é considerado uma formulação mais simples, porém capaz de fornecer os nutrientes essenciais como vitaminas e aminoácidos necessários para a manutenção e proliferação celular em condições laboratoriais. Apesar de sua composição básica, o MEM também tem sido utilizado com êxito em diversos modelos biológicos, incluindo o cultivo de células testiculares de ratos (Reda et al., 2016), o cultivo de tecido ovariano de cabras (Andrade et al., 2018) e o cultivo de células de peixes (Higaki et al., 2013).

Alguns meios de cultivo já foram adaptados e testados para a espécie abordada nesta revisão. No estudo de Silva et al. (2024), foram avaliados dois meios DMEM e StemPro-34™ SFM, livre de soro é utilizado para células-tronco combinados com diferentes concentrações do fator neurotrófico derivado de células gliais (GDNF: 0, 10 ou 20 ng/mL) no cultivo *in vitro* de fragmentos testiculares de catetos pré-púberes por 28 dias. Os autores concluíram que tanto o DMEM quanto o StemPro-34 SFM são adequados como meios base para o CIV do tecido testicular da espécie. Além disso, os resultados mostram que a suplementação com 10 ng/mL de GDNF é especialmente eficaz para manter a viabilidade e estimular a proliferação celular ao longo do período de cultivo.

Por fim, o mesmo grupo, em Pereira et al. (2025), também empregou o DMEM para cultivar fragmentos de testículo de catetos pré-púberes. Nesse estudo, foram comparados dois sistemas de cultivo: o método organotípico (fragmentos posicionados sobre agarose) e o sistema tridimensional (3D), com os fragmentos imersos na matriz além da avaliação da suplementação com o fator de crescimento FGF. Os autores concluíram que o sistema organotípico, sem a necessidade de adição de FGF, foi o mais eficiente na preservação da estrutura, viabilidade e integridade do tecido ao longo do cultivo prolongado, sendo atualmente o método mais indicado para o CIV do tecido testicular de catetos pré-púberes. Portanto, as pesquisas realizadas com tecido testicular de catetos fornecem avanços metodológicos essenciais em criopreservação e CIV.

I. Meio Dulbecco's Modified Eagle Medium F12 (DMEM F12)

O DMEM/F12 é um meio basal amplamente empregado no cultivo de células de mamíferos devido à sua formulação combinada, que integra a elevada concentração de aminoácidos, vitaminas e glicose do DMEM aos componentes reguladores característicos do meio Ham's F-12. Ham (1965) descreveu o F-12 como um meio quimicamente definido enriquecido com putrescina, biotina, ácido linoleico e traços de minerais, elementos que possibilitam o crescimento clonal de células de ovário de Hamster Chinês (CHO) em condições livres de proteínas. Yao e Asayama (2017) destacam que esses componentes conferem ao F-12 a capacidade de sustentar células com elevadas exigências nutricionais.

A formulação DMEM/F12 corresponde à combinação em proporção 1:1 do DMEM com o meio F-12, resultando em uma mistura complexa e nutricionalmente enriquecida. Essa integração reúne a ampla oferta de nutrientes do DMEM com a diversidade de moléculas reguladoras do F-12, garantindo versatilidade e estabilidade metabólica em diferentes condições experimentais. Como observado por Inoue et al. (2020), essa composição favorece tanto o crescimento quanto a manutenção de uma grande variedade de células de mamíferos, podendo ser empregada tanto em sistemas convencionais suplementados com soro quanto em formulações quimicamente definidas.

Originalmente desenvolvido para permitir o crescimento clonal de células CHO em meio totalmente definido, o F-12 mostrou-se posteriormente capaz de sustentar outros

tipos celulares de mamíferos, com desempenho variável conforme a concentração de soro adicionada (Maun et al., 2019). Quando associado ao DMEM, esse potencial é ampliado, resultando em um meio robusto, adequado a diferentes modelos celulares e a aplicações que requerem maior controle dos componentes químicos presentes (Arora, 2013).

Além disso, o DMEM/F12 tem sido amplamente utilizado na área da reprodução animal, sobretudo em protocolos envolvendo espécies de interesse zootécnico. Estudos relatam sua aplicação em sistemas *in vitro* para bovinos (Suyatno et al., 2018) e suínos (Sakib et al., 2019), destacando sua capacidade de oferecer suporte nutricional elevado e ambiente adequado ao desenvolvimento de células e tecidos de alta exigência fisiológica. Apesar de sua composição rica poder influenciar determinadas respostas experimentais, o balanço entre sua complexidade e aplicabilidade torna o DMEM/F12 um dos meios mais empregados em pesquisas com células de mamíferos.

II. Meio essencial mínimo modificado de Dulbecco (DMEM)

O Dulbecco's Modified Eagle Medium (DMEM) é um dos meios basais mais utilizados no cultivo de células de mamíferos, devido à sua composição nutricional enriquecida, capaz de sustentar a manutenção, proliferação e manipulação de diversas linhagens celulares em estudos em biologia celular e reprodutiva. Desenvolvido inicialmente para avaliar a formação de placas do vírus polioma em células embrionárias de camundongo (Dulbecco e Freeman, 1959), passou a ser amplamente empregado no cultivo de células primárias de mamíferos e aves, além de ser utilizado em estudos sobre inibição por contato celular. O DMEM também demonstra elevada eficiência no cultivo de linhagens híbridas, como hibridomas (Inoue et al., 2020), e tem sido aplicado em pesquisas envolvendo células testiculares, inclusive em catetos pré-púberes (Silva et al., 2022; Pereira et al., 2025).

Por ser um meio basal isento de proteínas e fatores de crescimento, necessita de suplementação adequada, como soro fetal bovino para se tornar um meio completo (Arora, 2013). Sua formulação original contém glicose a 5,6 mmol L^{-1}, mas versões com alta concentração (25 mmol L^{-1}) estão disponíveis para linhagens com maior demanda energética (Yao e Asayama, 2017). Além disso, o DMEM é facilmente preparado e

amplamente distribuído, favorecendo a obtenção de resultados reprodutíveis (Lee et al., 2013).

Desenvolvido a partir do Eagle's MEM, o DMEM se destaca por apresentar concentrações mais elevadas de aminoácidos e vitaminas. Diferentes formulações encontram-se disponíveis, variando conforme a concentração de glicose e a presença de piruvato de sódio (Thermo Fisher Scientific, 2025). O meio utiliza um sistema tampão baseado em bicarbonato, permitindo a manutenção do pH fisiológico em atmosferas controladas de CO_2.

O trabalho clássico de Eagle demonstrou que a adição de aminoácidos, vitaminas e sais específicos aumentava significativamente o crescimento celular, resultando em uma formulação padronizada amplamente adotada em pesquisas (Eagle, 1955). O DMEM contém o dobro da quantidade de aminoácidos não essenciais em comparação ao MEM (Arora, 2013). Sua composição equilibrada contribui para a estabilidade do pH, da pressão osmótica e das condições do cultivo, especialmente em ensaios prolongados (Thermo Fisher Scientific, 2025).

Por ser amplamente difundido, o DMEM possui boa disponibilidade comercial, custo acessível e estabilidade de estoque. Sua formulação padronizada globalmente confere maior consistência experimental e reduz a variabilidade entre ensaios (Yao e Asayama, 2017).

III. Meio essencial mínimo (MEM)

O meio de cultivo MEM (Minimum Essential medium) é um dos meios basais mais clássicos e amplamente utilizados no cultivo de células de mamíferos. Desenvolvido por Harry Eagle na década de 1950 para atender às necessidades nutricionais mínimas de células como fibroblastos normais e linhagens HeLa, o MEM foi proposto com o objetivo de estabelecer uma formulação mínima capaz de sustentar o crescimento celular *in vitro*, possibilitando maior controle experimental e melhor compreensão das exigências metabólicas das células (Eagle, 1955; Akiyama et al., 2017;).

Trata-se de um meio de baixa complexidade, composto por uma solução salina balanceada, 13 aminoácidos, oito vitaminas hidrossolúveis, glicose, seis sais inorgânicos e soro dialisado, além de piruvato de sódio e uma concentração reduzida de bicarbonato de

sódio (Yao e Asayama, 2017). Inicialmente, o MEM não incluía aminoácidos não essenciais, uma vez que as próprias células são capazes de sintetizá-los; no entanto, sua adição é frequentemente realizada de acordo com a necessidade celular para reduzir o esforço biossintético celular e favorecer o crescimento (Eagle, 1959).

Por apresentar uma formulação básica e pouco complexa, o MEM geralmente necessita de suplementação adicional, como soro fetal bovino e outros fatores de crescimento, para garantir uma adequada capacidade proliferativa das células cultivadas (Arora, 2013). Além disso, o meio foi desenvolvido a partir de modificações do BME (Basal Medium Eagle), no qual as concentrações de aminoácidos foram ajustadas para atender melhor às demandas celulares, resultando em níveis aproximadamente duas vezes superiores em comparação ao BME original. (Dulbecco e Freeman 1959). Segundo o manual da Thermo Fisher, o MEM com Earle's salts contém 2,2 g/L de bicarbonato de sódio, o que exige ambiente com CO_2 para manter o pH fisiológico. Já o MEM com Hanks' salts tem apenas ~0,35 g/L de bicarbonato, conferindo menor capacidade de tamponamento, e por isso é projetado para uso sem CO_2. Tais diferenças impactam diretamente o tamponamento do meio e, consequentemente, a estabilidade do pH durante o cultivo.

Entre as principais vantagens do MEM destaca-se sua baixa complexidade nutricional, o que facilita o controle experimental e contribui para a redução de custos, além de apresentar ampla compatibilidade com diferentes linhagens celulares (Arora, 2013; Yao e Asayama, 2017). Entretanto, suas limitações incluem a menor concentração de nutrientes em comparação a meios mais enriquecidos, o que pode restringir sua aplicação em células com metabolismo acelerado ou com maiores exigências para proliferação e diferenciação. Nesses casos, o meio é frequentemente suplementado com aditivos específicos ou maiores concentrações de soro, tornando-se mais adequado para o cultivo de uma ampla gama de células de mamíferos (Arora, 2013). De modo geral, o MEM é considerado um meio eficiente para experimentos que demandam menor complexidade nutricional, além de servir como base para o desenvolvimento de formulações mais complexas.

Estudos na literatura frequentemente comparam o MEM a outros meios de cultivo, analisando sua capacidade de manter viabilidade celular e estimular proliferação

(Arodin Selenius et al., 2019). Assim, o MEM permanece um recurso fundamental na biologia celular, desempenhando papel relevante tanto em estudos básicos quanto em aplicações aplicadas, como virologia, biotecnologia e testes farmacológicos. Sua composição controlada e versatilidade garantem sua utilidade contínua em inúmeras pesquisas ao redor do mundo.

3. ANÁLISE DO DESENVOLVIMENTO *IN VITRO* DO TECIDO TESTICULAR

Morfologia:

Para a avaliação das características morfológicas do tecido fresco, criopreservado e cultivado, as amostras devem ser preparadas de modo a preservar ao máximo a arquitetura original. A integridade morfológica do tecido testicular é essencial, pois fornece um ambiente funcional para o ciclo espermatogênico (Song et al., 2010). Nesse sentido, diversas condições fisiológicas e patológicas podem alterar a morfologia testicular, refletindo-se diretamente na capacidade reprodutiva (Noorafshan, 2014).

A escolha de fixadores adequados é um aspecto determinante nessas análises, visto que a integridade estrutural e a correlação entre o tecido conjuntivo e os túbulos seminíferos dependem diretamente do método de fixação empregado (Tian et al., 2024). Em termos gerais, os fixadores são substâncias químicas utilizadas para manter a citoarquitetura o mais próxima possível das condições *in vivo*, evitando a autólise do tecido (Cabrera et al., 2017).

Esses compostos podem atuar de duas formas principais: coagulando proteínas ou promovendo ligações cruzadas entre suas cadeias de aminoácidos. Assim, dividem-se em duas categorias: fixadores coagulantes (ou desnaturantes) e fixadores de reticulação (Fournie et al., 2000). Enquanto os fixadores coagulantes rompem a estrutura nativa das proteínas, os reticulantes formam pontes covalentes entre suas cadeias, preservando a organização geral do tecido (Cabrera et al., 2017). Tradicionalmente, a solução de Bouin é recomendada para biópsias testiculares, sendo composta por 1,5% de ácido pícrico, 9% de formaldeído e 5% de ácido acético em solução aquosa (Ellenburg et al., 2020).

Após os processos de fixação, inclusão, corte e coloração, ocorre geralmente uma redução no volume testicular. Por isso, é importante estimar a deformação tecidual resultante (Mühlfeld, 2021). Estudos recentes têm se voltado à análise morfofuncional dos testículos e ao processo espermatogênico em diferentes espécies, buscando compreender como essas alterações estruturais impactam a função (Martins et al., 2024).

A coloração de hematoxilina e eosina (HE) é uma das técnicas mais utilizadas para avaliar essas alterações. Segundo Bancroft e Layton (2018), os corantes de hematoxilina e eosina são amplamente empregados em tecidos de origem animal: a hematoxilina cora os núcleos em tons de roxo, enquanto a eosina confere tonalidade rosada ao citoplasma e aos espaços intercelulares (Junqueira & Carneiro, 2013), permitindo a visualização geral dos elementos teciduais.

Os principais parâmetros avaliados na coloração HE incluem a integridade da membrana celular, a arquitetura dos túbulos seminíferos, a identificação dos tipos celulares, a presença de vacuolização citoplasmática e a organização estrutural geral (Meistrich et al., 2012). Essa análise possibilita identificar danos morfológicos decorrentes dos métodos de criopreservação, que podem comprometer a estrutura testicular (Lima et al., 2018), bem como comparar tecidos frescos, criopreservados e cultivados, determinando o impacto de cada procedimento.

Campos-Júnior et al. (2011) descrevem que os catetos (*Pecari tajacu*) possuem uma organização testicular distinta, na qual as células de Leydig se distribuem ao redor dos lóbulos dos túbulos seminíferos, formando cordões no espaço interlobular. Em suínos, espécie filogeneticamente próxima, essas células apresentam uma proporção volumétrica semelhante e também se agrupam em cordões compactos que preenchem quase todo o interstício testicular (Fawcett et al., 1973). Tal organização é facilmente identificada em lâminas histológicas coradas com HE.

Viabilidade

As técnicas empregadas para o cultivo e a criopreservação de tecidos apresentam, intrinsecamente, o potencial de desencadear alterações estruturais e funcionais nas células, o que pode comprometer de maneira significativa sua viabilidade (Silva, 2022). Durante o período de adaptação ao microambiente *in vitro*, diversos fatores presentes nos compartimentos extra- e intracelulares podem induzir rupturas químicas ou físicas na membrana plasmática (Ammendolia et al., 2021). A conservação da integridade dessa membrana constitui um aspecto central nesses processos, considerando que ela desempenha papel fundamental na manutenção da homeostase

celular, regulando o transporte seletivo de moléculas e assegurando condições adequadas para a sobrevivência e o desempenho funcional das células.

No contexto do cultivo, destaca-se a peroxidação lipídica como um dos principais mecanismos de estresse oxidativo em diferentes ambientes teciduais. A origem das espécies reativas de oxigênio (ROS) pode variar substancialmente conforme o tipo de estressor; entretanto, em condições de hipóxia ou durante eventos de lesão por isquemia/reperfusão, observa-se que o suprimento sanguíneo insuficiente resulta na disfunção mitocondrial e no aumento da produção de ROS, fenômeno que se intensifica com a reoxigenação subsequente (Zhou et al., 2018).

No âmbito da criopreservação, a redução progressiva da temperatura induz modificações estruturais nos lipídios de membrana, promovendo a transição da fase líquida para a fase gel. Essa alteração físico-química favorece a formação de cristais de gelo, o rompimento da membrana plasmática e a perda de componentes intracelulares, processos que culminam, frequentemente, em morte celular (Pegg, 2010). Nesse sentido, a avaliação da integridade das membranas após a descongelação configura etapa imprescindível para aferir a eficácia dos protocolos utilizados.

Diversos estudos têm demonstrado a possibilidade de preservação da viabilidade do tecido testicular submetido à criopreservação (Zarandi et al., 2018). Todavia, assegurar não apenas a viabilidade, mas também a funcionalidade do tecido requer uma seleção criteriosa da metodologia empregada, uma vez que parâmetros como o tamanho do fragmento testicular e o objetivo do uso pós-descongelamento ou reaquecimento influenciam diretamente a eficiência do processo (Gomes et al., 2023).

A integridade funcional da membrana plasmática pode ser mensurada por meio de sondas fluorescentes anfipáticas, as quais penetram exclusivamente células com membranas preservadas, possibilitando a distinção entre células viáveis e inviáveis (Harrison et al., 1990). Dentre os fluorocromos amplamente empregados, destacam-se o iodeto de propídio (PI) e o Hoechst. O PI, por não atravessar a membrana de células íntegras, marca apenas aquelas que apresentam perda de integridade, funcionando como um indicador direto de danos celulares (Riccardi et al., 2019). O Hoechst, utilizado como contracorante, permeia a membrana e apresenta elevada afinidade pelo DNA nuclear (Bastos et al., 2005). A utilização combinada destes fluorocromos constitui abordagem

Apoptose

O epitélio seminífero testicular é um tecido de elevada taxa proliferativa, no qual as células germinativas são continuamente produzidas de forma clonal por meio de sucessivas divisões mitóticas e meióticas. Alterações nesse processo podem gerar desequilíbrios na produção celular; por essa razão, o epitélio possui mecanismos específicos para regular e manter o número adequado de células germinativas (Rojas et al., 2017). Nesse contexto, a apoptose constitui um componente essencial desse sistema de controle, atuando na eliminação seletiva de células que não devem prosseguir no processo espermatogênico. Estima-se que até 75% das células germinativas sejam removidas ao longo da espermatogênese, reduzindo consideravelmente o número de espermatozoides que atingiram a maturação. A literatura indica que a indução da apoptose nas células germinativas masculinas está relacionada a três fatores principais: deficiência hormonal, especialmente de gonadotrofinas e andrógenos; o estágio do ciclo espermatogênico; e a fase de desenvolvimento do animal (Dunkel et al., 1997).

Considerando a relevância desses eventos na dinâmica do tecido testicular, torna-se essencial avaliar a integridade do material genético das células germinativas e espermáticas. Entre os métodos utilizados para esse fim, destaca-se a sonda laranja de acridina (LA), um marcador fluorescente metacromático capaz de emitir fluorescência em dois comprimentos de onda distintos: aproximadamente 530 nm, correspondente à coloração verde, e cerca de 640 nm, correspondente à coloração vermelha. Quando o DNA encontra-se íntegro, a LA se intercala entre as bases nitrogenadas e emite fluorescência verde. Em contrapartida, na presença de fragmentação do DNA, a sonda se liga aos grupos fosfato das fitas simples, resultando em fluorescência vermelha (De Albuquerque et al., 2024).

A utilização de marcadores fluorescentes, como a LA, torna-se, portanto, uma ferramenta essencial para compreender de forma mais precisa os efeitos de diferentes condições experimentais sobre a estabilidade do material genético das células germinativas, permintindo não apenas avaliar a eficiência de protocolos de cultivo e criopreservação, mas também aprofundar o entendimento dos mecanismos fisiológicos que regulam a dinâmica celular no epitélio seminífero.

Integridade do DNA

Diversos estudos têm demonstrado que o cultivo e a criopreservação podem causar danos ao material genético das células germinativas (Paasch et al., 2004). Tais danos estão frequentemente associados a alterações no microambiente testicular, que podem reduzir o potencial fértil masculino (Lacerda et al., 2011). Entre os principais fatores envolvidos nesse processo destacam-se variações de temperatura, alterações na composição físico-química do meio, contaminações e desequilíbrios moleculares, todos capazes de comprometer a integridade genômica dessas células (Love et al., 2002).

Para rastrear essas alterações, atualmente, existe uma variedade de testes comercialmente disponíveis que permitem avaliar, de maneira precisa, a integridade do DNA. Entre eles, destaca-se o ensaio TUNEL (Terminal deoxynucleotidyl Transferase–mediated dUTP Nick End Labeling), um método comercial amplamente empregado para a detecção e quantificação da quebra de fitas de DNA, característica de apoptose tardia em nível celular. Esse ensaio baseia-se na marcação de quebras nas fitas de DNA, permitindo sua visualização por meio de microscopia de fluorescência (Da Silva et al., 2019).

4. JUSTIFICATIVA

Apesar da elevada diversidade dos biomas brasileiros, ainda são escassas as estratégias científicas e tecnológicas voltadas à conservação sustentável do germoplasma de espécies nativas. Nesse contexto, o cateto, uma espécie de significativa importância ecológica e econômica, destaca-se como modelo relevante, reforçando a necessidade de desenvolver técnicas que permitam o aproveitamento e a preservação de seu material genético.

Assim, o aprofundamento no conhecimento sobre protocolos de cultivo *in vitro*, especialmente aqueles que possam ser integrados à criopreservação, torna-se essencial. A associação dessas duas abordagens permitiria otimizar o aproveitamento do material genético e fortalecer programas de conservação e multiplicação de genótipos valiosos. Apesar de já existirem protocolos de criopreservação bem estabelecidos para catetos adultos, ainda não há um método padronizado para o cultivo *in vitro* de tecido testicular nessa espécie. Ademais, a integração entre cultivo e criopreservação possibilitaria avaliar a funcionalidade dos bancos de germoplasma em curto, médio e longo prazo.

É importante destacar que o tecido testicular de animais adultos apresenta maior sensibilidade e é mais complexo de manter viável em condições ex situ, devido à elevada diferenciação e à diversidade de tipos celulares presentes. Apesar dessas limitações, o uso de tecido adulto em cultivos *in vitro* permanece fundamental, pois permite avaliar a manutenção da espermatogênese completa, validar a funcionalidade do material criopreservado armazenado em bancos de germoplasma e ampliar as possibilidades de conservação. Além disso, em muitas espécies silvestres, como o cateto, o acesso às amostras ocorre majoritariamente a partir de animais adultos, o que reforça a necessidade de desenvolver protocolos aplicáveis a esse tipo de tecido.

Além disso, permite-nos estabelecer e testar sistemas diversos, no intuito de torná-los funcionais e de que futuramente possam ser utilizados como modelo experimental, para espécies correlatas filogeneticamente como o queixada (Tayassu pecari) e o taguá (Catagonus wagneri) e outros ungulados selvagens ou domésticos.

5. HIPÓTESE CIENTÍFICA

I- O meio DMEM/F12 é capaz de manter de forma mais eficiente a viabilidade e a integridade estrutural do tecido testicular adulto de *Pecari tajacu* durante 14 dias de cultivo *in vitro,* quando comparado aos demais meios avaliados.

II- O meio DMEM/F12 é capaz de manter de forma eficiente a viabilidade e a integridade do tecido testicular adulto de *Pecari tajacu*, previamente criopreservado, durante 14 dias de cultivo *in vitro*.

6. OBJETIVOS

6.1. Objetivo geral

- Estabelecer um protocolo eficiente para o cultivo *in vitro* de tecido testicular de catetos adultos, de modo a viabilizar a utilização e a avaliação do germoplasma masculino previamente armazenado em biobancos.

6.2. Objetivos específicos

- Comparar diferentes meios de cultivo (DMEM/12, DMEM e MEM) para a manutenção do desenvolvimento *in vitro* da espermatogênese do tecido fresco em catetos adultos;
- Comparar diferentes meios de cultivo (DMEM/12, DMEM) para a manutenção do desenvolvimento *in vitro* da espermatogênese do tecido previamente criopreservado em catetos adultos;
- Avaliar as modificações estruturais e funcionais nos túbulos seminíferos em fragmentos testiculares de catetos adultos cultivados por 14 dias;
- Utilizar o cultivo *in vitro* como ferramenta de análise da eficiência dos protocolos de criopreservação de tecido testicular de catetos adultos.

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CAPÍTULO I

Influence of supplemented commercial media on *in vitro* culture outcomes of testicular tissues from adult collared peccaries

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Influence of supplemented commercial media on *in vitro* culture outcomes of testicular tissues from adult collared peccaries

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ABSTRACT

This study evaluated the effects of supplemented media on the histomorphology, viability, apoptosis, and DNA integrity of testicular fragments from adult peccaries in vitro. Five testes were collected, dissected, and cultured in DMEM/F12, DMEM, or MEM media. These media were supplemented equally with 10% fetal bovine serum, 25 mM HEPES, 2

μL/mL insulin-transferrin-selenium (ITS), 1% non-essential amino acid solution (NEAA), 1% vitamin solution, 0.2% bovine serum albumin, 1% antibiotic-antifungal solution, 10 ng/mL LH/FSH, 20 ng/mL mEGF, 10 ng/mL GDNF, and 10 ng/mL bFGF. The fragments were cultured in an organotypic system on 0.75% agarose gel for 7 and 14 days at 34 °C and 5% CO_2. The culture time significantly reduced the morphological scores compared to the fresh control ($P < 0.05$). MEM showed better morphological preservation of membrane vacuolization at 7 days and of all parameters at 14 days. On the other hand, DMEM showed greater temporal stability, ensuring a more consistent microenvironment. Cell viability was maintained, with no differences between treatments ($P > 0.05$). The percentages of apoptotic cells remained stable during the experimental period, indicating the maintenance of cellular homeostasis. MEM better preserved DNA integrity up to 7 days, while DMEM maintained stability for 14 days. In conclusion, MEM and DMEM, when adequately supplemented, are suitable for the organotypic culture of testicular tissue from adult peccaries for up to 14 days. The study provides a basis for the development of protocols in peccary conservation and reproduction biotechnologies.

Key words: Testicular tissue, in vitro culture, Conservation of wild species; Culture media; Organotypic cultivation

1. INTRODUCTION

In recent decades, culture techniques have evolved from exploratory approaches to multidisciplinary integration, consolidating themselves as practical tools for simulating the in vivo environment (Klausner et al., 2021). In this scenario, organotypic testicular tissue culture stands out as an in vitro model that best mimics testicular physiology, since it reproduces the architecture of the seminiferous tubules with high fidelity and preserves the testicular cell niche (Li et al., 2025). Although not yet fully optimized, especially in adult individuals, this technique shows relevant advances and promising results in areas such as reproductive developmental biology, toxicology, fertility preservation, and regenerative medicine (Mecca et al., 2025). However, despite these advances, intrinsic biological factors of the tissue still limit the efficiency of the culture, mainly in adult individuals.

Adult testicular tissue harbors germ cells at different stages of development, and spermatogenesis depends on a highly organized and functional microenvironment (Lima et al., 2018; Kaya et al., 2024). This complex tissue architecture is particularly sensitive to environmental variations and difficult to maintain under conditions different from those found in vivo, such as those imposed by in vitro culture (Sato et al., 2015). Therefore, the reconstitution of this microenvironment under in vitro conditions still represents a significant challenge. In this context, the age of the animal stands out as one of the most determining factors for the preservation of testicular fragments, since the testes present marked morphological differences between individuals at different stages of sexual maturity (Pereira et al., 2023).

Preserving testicular tissue from adult individuals who have already initiated spermatogenesis presents a significant challenge, since the seminiferous tubules exhibit high cellular diversity and a more complex metabolism, making this tissue more susceptible to manipulation compared to that of pre-pubertal animals (Sato et al., 2015). Furthermore, adult tissue shows greater sensitivity to ischemia, compromising its survival ex vivo and ex situ. Conversely, it is plausible that the immature testis exhibits greater plasticity, favoring its adaptation to different environmental conditions (Silva et al., 2024).

Once efficient protocols for the culture of adult tissues are established, they can be used to preserve the genetic material of individuals with valuable characteristics,

especially in situations of sudden death (Kaneko et al., 2019) or when sperm collection is not feasible (Peris-Frau et al., 2023). This approach presents a particularly promising potential for preserving male fertility, especially when associated with cryopreservation (Yokonishi et al., 2014). Furthermore, it enables advances in studies on spermatogenesis and assisted reproduction, contributing significantly to wildlife conservation programs (Silva et al., 2022).

Organotypic culture of testicular tissue has been widely used to preserve the architecture of the seminiferous tubules and allow the progression of spermatogenesis under ex vivo conditions. The initial milestone of this approach was established by Sato et al. (2011), who demonstrated, for the first time, the production of functional spermatozoa and the maintenance of complete spermatogenesis in vitro from testicular fragments of mice. Subsequently, the same authors confirmed the effectiveness of the technique also in testes of adult individuals (Sato et al., 2015).

Subsequent studies reinforced the robustness of the organotypic system, including the use of chemically defined media capable of sustaining meiotic progression and the formation of haploid cells (Sanjo et al., 2018). The successful expansion of this model to larger mammals was reported by Kanbar et al. (2022), who applied organotypic culture to porcine testicular tissue, demonstrating the maintenance of tissue viability, spermatogenic progression, and endocrine activity. In wild species, Silva et al. (2024) and Pereira et al. (2025) confirmed the applicability of the same technique in testicular fragments of pre-pubertal peccaries, highlighting its potential for species conservation, assisted reproduction, and comparative studies of spermatogenesis.

The success of these strategies is directly related to the development of culture media capable of supporting cell growth and development. In this sense, rich and complex media containing growth factors, hormones, small molecules, antioxidants, and lysophospholipids have been employed (Matsumura et al., 2021). Recent studies show that the composition of the medium can significantly influence cell metabolism and behavior, highlighting its physiological relevance (Wijerathna-Yapa et al., 2025). Thus, the choice of culture medium should take into account the specific needs of the cells, the species studied, and the experimental objective (Yao & Asayama, 2017).

Currently, the culture of testicular cells and tissues predominantly uses conventional media, such as Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, and Eagle's Minimum Essential Medium (MEM) (Sato et al., 2011). DMEM/F12 combines the high concentration of amino acids, vitamins, and glucose of DMEM with the regulatory components of Ham's F-12 medium and has been successfully applied in the culture of goat gonads (Salimi et al., 2024), pigs (Fayaz et al., 2022), and cattle (Tang et al., 2023). DMEM stands out for its enriched nutritional composition, capable of supporting cell maintenance and proliferation, and is used in the culture of testicular fragments from rats (Reda et al., 2014), dogs (Lee et al., 2016a), goats (Heidari et al., 2012), and pre-pubertal peccaries (Silva et al., 2024). In turn, Eagle's MEM was developed to support the growth of different cell types, providing essential nutrients, vitamins, and amino acids, and has been used in the cultivation of rat testicular cells (Reda et al., 2016), goat ovarian tissue (Andrade et al., 2018), and fish cells (Higaki et al., 2013). Despite the different methods used, comparisons between them are scarce, but these approaches would be interesting to highlight the methods best suited to the specificities of testicular cells in each species.

In recent years, the collared peccary (Pecari tajacu Linnaeus, 1758) has been widely used as an interesting wild ungulate model both for establishing cryopreservation protocols for testicular tissues (Da Silva et al., 2019; 2021) and for in vitro cultivation (Silva et al., 2024; Pereira et al., 2025). The relevance of the collared peccary as a model is reinforced by its reproductive particularities, such as the specific distribution of Leydig cells and the unique architecture of the seminiferous tubules, distinct from that observed in other ungulates and in phylogenetically close species, such as pigs (Costa et al., 2010). Furthermore, the collared peccary stands out among species of ecological relevance, playing a fundamental role in forest restoration (Vieira et al., 2023); however, its populations have suffered sharp regional declines, such as in the Pampas and the Atlantic Forest, due to intense hunting and continuous habitat loss, which reinforces the need for specific and regionally adapted conservation strategies (Garbino et al., 2018), including the development of assisted reproductive technologies. Despite advancements, limitations remain regarding the optimization of organotypic culture systems in adult wild species,

particularly concerning the choice of culture medium and the maintenance of the testicular microenvironment in vitro.

Therefore, this study aimed to characterize the effects of DMEM, DMEM/F12, and MEM culture media on the histological structure, observation, cell apoptosis, and DNA integrity of testicular tissue fragments from adult peccaries cultured in vitro for up to 14 days. Our hypothesis is that more enriched culture media, such as DMEM/F12, would promote better preservation of the structural and cellular integrity of testicular tissue compared to MEM.

2. MATERIALS AND METHODS

2.1. Animal ethics and husbandry

All experimental procedures were performed in accordance with ethical standards, with approval from the Ethics Committee on the Use of Animals of the Federal Rural University of the Semi-Arid Region (UFERSA; protocol no. 24/2023) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio; no. 72170). The males used belonged to the Wildlife Multiplication Center (CEMAS–UFERSA), located in Mossoró, RN, Brazil, duly registered with the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding center (registration no. 1478912). The center is located at coordinates 5°10′ S and 37°10′ W, at an altitude of 16 m, in a semi-arid tropical climate region.

2.2 Testis collection and experimental design

The gonads of five adult males, aged between 3 and 6 years, were collected. The pairs of testes were washed in 0.9% saline solution (NaCl) and transported for 5 minutes to the to the laboratory at 22 °C in a solution composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotic-antifungal solution. Each animal was considered an independent reproduction, with collections performed at different times.

After removal of adjacent tissues, the testes were subjected to three successive washes in the transport solution and then sectioned into fragments of approximately 1.0 mm^3 (1 × 1 × 1 mm). A total of 72 fragments per pair of testes were randomly distributed between the experimental groups of fresh, uncultured tissue and in vitro culture (IVC), using different culture media: DMEM/F12, DMEM, and MEM (Figure 1).

All treatments were allocated to 24-well cell culture plates (four wells per pair of testes) and incubated in a humidified atmosphere containing 5% CO_2 at 34 °C for a total period of 14 days. The in vitro culture time was defined based on the study by Sato et al. (2015), who cultured testicular fragments from adult rats under similar conditions. The fragments from all groups were evaluated at seven-day intervals, as illustrated in Figure 1.

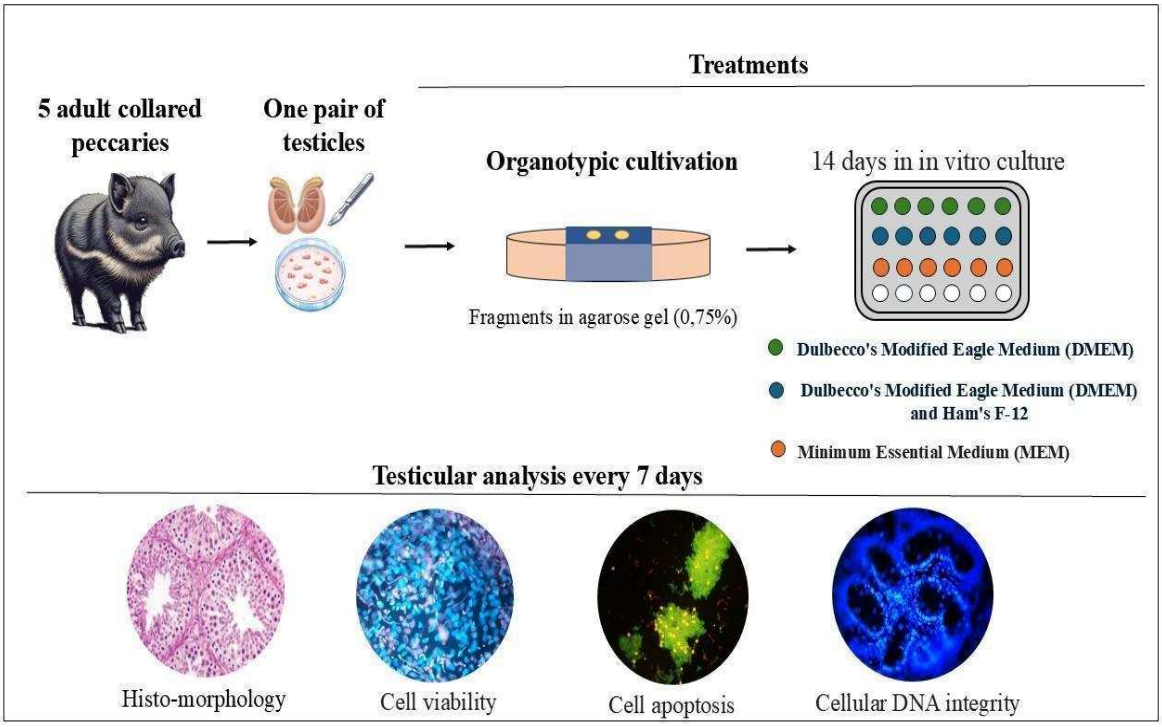

Figure 1. Schematic representation of the experimental design adopted for the evaluation of different in vitro culture media of testicular fragments from adult peccaries.

2.3 *In vitro* organotypic culture of testicular fragments

The protocol employed for organotypic culture was based on the methodology described by Silva et al. (2024). Initially, a solution containing 0.75% agarose powder (Sigma, A013) dissolved in sterile distilled water was prepared. This solution was spread in 10 cm diameter Petri dishes and kept at room temperature until the gel completely solidified. After solidification, the gel was sectioned into 1 × 1 × 1 cm cubes, which were distributed into the wells of the culture plates, using one cube per well. Then, 500 µL of culture medium was added to each well, approximately 12 hours before the start of the culture.

After 12 hours, the culture medium was replaced again, and two testicular fragments were added to each well. The 24-well plates were then incubated for 14 days at 34 °C in a humidified atmosphere containing 5% CO_2. Each well received 500 µL of culture medium composed of DMEM/F12 (Sigma-Aldrich, D0697), DMEM (Gibco, 11885084), and MEM (Sigma, M8042). All media were supplemented with 10% fetal bovine serum (FBS; ThermoFisher, 12657029), 25 mM HEPES (Sigma, H6147), 2 µL/mL insulin-transferrin-selenium solution (ITS; Gibco, 41400-045), 1% non-essential amino acid solution (NEAA; Sigma, M7145), 1% vitamin solution (Gibco, 11120), 0.2% bovine serum albumin (BSA; Sigma-Aldrich, A7906), 1% antibiotic-antifungal solution (Gibco, 15240), 10 ng/mL LH/FSH (Pluset®, Hertape Calier), 20 ng/mL mEGF (Sigma, E4127), and 10 ng/mL GDNF (PehproTech, 512-GF) was combined with 10 ng/mL of bFGF (PehproTech, 100-18B).

The culture medium was completely changed every 72 hours, totaling four replacements over the 14 days of cultivation. On days 7 and 14, fragments were collected for later analysis.

2.4 Testicular fragment histomorphology

Testicular fragments, both fresh and cultured, were fixed in Bouin's solution for 12 hours, then embedded in paraffin blocks, sectioned into 5.0 µm sections, stained with hematoxylin and eosin, and evaluated using optical criteria (Olympus CX 31 RBSFA) at 400× magnification. Table 1 presents the parameters and scoring systems adopted for the histological evaluation of testicular tissue, as described by Silva et al., (2024).

For each parameter, five seminiferous tubules were analyzed in six distinct fields, totaling 30 tubules per treatment (Da Silva et al., 2019). Fragments with a total score of 3 were classified as morphologically normal, while those with a score of 1 were considered degenerated. Table 1 below describes the evaluated parameters in detail.

Table 1. Histo‑morphological parameters of seminiferous tubules in testicular fragments from collared peccaries.

Parameter	Scores		
	3	2	1
Shrinkage from basal membrane	No shrinkange	Partly shrinkange (<50%)	Mostly shrinkange (>50%)
Tubular cell swelling	No swelling	>50% cells without swelling	>50% cells with swelling
Rupture from the basal membrane	No rupture	Partly ruptured (<50%)	Mostly ruptured (>50%)
Tubular cell loss	No cell loss	<75% cell type loss	>75% cell type loss
Tubular structure	Structure intact	All cell types of presents although slightly disordered structure	Random distribution of remaining cells

Silva et al., 2024.

2.5 Cell viability in testicular fragments

Testicular cells were obtained from fragments by enzymatic digestion, according to the methodology described by Pereira et al. (2025). In summary, uncultured fragments were incubated with 0.2% type IV collagenase for 10 minutes at 37 °C. Then, an equivalent volume of DMEM supplemented with fetal bovine serum (FCS) was added to inactivate the enzyme, followed by centrifugation of the samples at 114 × g for 5 minutes. For previously cultured fragments, enzymatic digestion was not necessary, as spontaneous tissue disaggregation occurred.

The cell pellets or fragments were then incubated with a solution containing 3 µL of propidium iodide (0.5 mg/mL in PBS) and 5 µL of Hoechst 33342 (40 µg/mL in PBS)

for 10 minutes at 37 °C, allowing the identification of non-viable cells, marked in red, and viable cells, with nuclei stained in blue, respectively. The samples were specifically tested for epifluorescence (Leica, Kista, Sweden) at 400× magnification, with 100 cells counted per experimental group.

2.5 Cell DNA integrity in testicular fragments

Testicular fragments were fixed overnight in a 4% paraformaldehyde solution, subsequently embedded in paraffin, sectioned into serial 5 μm thick sections, mounted on slides, and analyzed using the biotin-dUTP terminal deoxynucleotidyl transferase-mediated DNA end labeling kit (TUNEL Assay Kit – Roche, 11684809910), according to the methodology described by Da Silva et al. (2021). The TUNEL assay reaction mixture was prepared from an enzymatic solution containing 5 μL of terminal deoxynucleotidyl transferase (TdT) and 45 μL of the labeling solution composed of nucleotide polymers.

The sections were incubated with 50 μL of the TUNEL reaction mixture for 1 hour at 37 °C in a humid chamber protected from light. As a negative control, only 50 μL of the labeling solution was used, without the addition of the TdT enzyme. The positive control was obtained by incubating the sections with 5 μL of recombinant DNase I (Sigma-Aldrich) and 45 μL of 0.05% Triton X-100 in PBS for 10 minutes before the labeling procedure, in order to induce DNA breaks.

Cell nuclei were counterstained with Hoechst 33342 (1:100; Sigma-Aldrich). Ten images per experimental group were evaluated, obtained using an epifluorescence microscope. The images were analyzed using ImageJ software, and the cells present in ten images per treatment were quantified. To determine the level of nuclear DNA fragmentation, at least 1,000 cells were analyzed per sample. TUNEL-positive cells, characterized by green fluorescence, were classified as damaged, while TUNEL-negative cells, exhibiting blue fluorescence, were considered normal (Pereira et al., 2025).

2.6 Assessment of cellular apoptosis

Approximately 10 µl of a suspension of fresh, cultured testicular cells were placed on previously sanitized glass slides. Then, the same volume of a freshly prepared solution composed of ethidium bromide (0.1% w/v in distilled water) and acridine orange (0.1% w/v in distilled water), mixed in a 1:1 ratio, was added. The solution was gently inserted into the cell protection, and the preparation was covered with a coverslip, remaining in contact with the stains for approximately five minutes. The cells were then examined using epifluorescence staining (Leica, Kista, Sweden) at 400× magnification, classifying 100 cells per experimental group. According to the observed staining, the cells were categorized as: non-apoptotic (NA), exhibiting a uniform light green nucleus; early apoptotic (AP), with a heterogeneous green nucleus; late apoptotic (AT), exhibiting a bright orange and irregular nucleus; or necrotic (N), characterized by a uniform orange core (Kalia et al., 2009).

2.7 Statistical analysis

Each of the five animals used was considered an independent replicate. The results are expressed as mean and standard error. The normality of the residuals and the homogeneity of the variance were initially analyzed. Morphology values were compared using the Wilcoxon test for paired samples (comparison of culture periods) and Kruskal–Wallis for comparison of the groups of media used.

Viability, apoptosis, and DNA integrity parameters were analyzed using a mixed-effects analysis procedure with repeated measures, including the different culture media, culture time, and their interactions as the main effects. Tukey's and Dunnett's tests were used for comparison of means when significant effects were found in the F-test of the analysis of variance. The analyses were performed using GraphPad Prism® software version 9.3 (GraphPad Software Inc., San Diego, CA, USA), with $p < 0.05$.

3. RESULTS

3.1 Testicular histomorphology

The values for histomorphological evaluations are presented as mean ± standard error in Table 2, while the main structural changes observed are illustrated in Figure 2. Overall, a significant decrease in all parameters was evaluated when comparing fresh control to the cultured groups ($P < 0.05$). In an overview, when analyzing the culture over time (7 and 14 days), a significant interaction ($P < 0.05$) was noted between the culture time and the media evaluated, demonstrating that there was an effect of time on the maintenance of testicular morphology that varied according to the culture medium. The MEM medium showed better performance in terms of morphological maintenance, compared to DMEM/F12 with ($P < 0.05$), while DMEM without remained stable over time. Regarding DMEM/F12, it showed greater sensitivity to the prolonged cultivation period, with a significant reduction in morphological parameters at 14 days.

On the 7th day of culture, no significant differences were observed between the media in the parameters of basement membrane separation, basement membrane rupture, tubular cell loss, and tubular structure. For the membrane vacuolization parameter, MEM showed better performance, with significantly higher scores compared to DMEM/F12 ($P < 0.05$), while DMEM did not differ statistically from the other media.

On the 14th day of culture, statistically significant differences were observed between the culture media for the membrane separation parameter. The DMEM/F12 medium showed significantly lower scores ($P < 0.05$), differing from the MEM and DMEM media, which did not show a statistical difference between themselves. Regarding the membrane vascularization parameter, a statistically significant difference was also observed ($P < 0.05$), with the DMEM medium differing from the other media. On the other hand, DMEM/F12 and MEM did not differ statistically from each other. Regarding the membrane rupture parameter, a statistically significant difference was observed ($P < 0.05$), in which the DMEM/F12 and MEM media differed from each other, while DMEM and MEM showed statistically similar results. Regarding the tubular cell loss parameter, a statistically significant difference was also observed ($P < 0.05$), with the DMEM/F12 medium differing from MEM, while DMEM and DMEM/F12 did not show a statistical difference between themselves.

Table 2. Mean values (± SEM) for morphological evaluation scores (from 3 – normal - to 1 – degenerated) in adult peccary testicular tissues (n = 5) cultured *in vitro* for up to 14 days using an organotypic *in vitro* culture system in different culture media: Dulbecco's modified Eagle medium and Ham's (DMEM/F12) Dulbecco's modified Eagle medium (DMEM) and minimum essential medium (MEM).

	Days of culture	Shrinkage from basal membrane	Tubular cell swelling	Rupture from the basal membrane	Tubular cell loss	Tubular structure
Controls (non-cultured)	0	2.02 ± 0.04*	2.35 ± 0.05*	2.38 ± 0.05*	2.43 ± 0.06*	2.15 ± 0.06*
DMEM/F12	7D	1.68 ± 0.04 Aa	1.29 ± 0.04 Aa	1.67 ± 0.06 Aa	1.64 ± 0.06Aa	1.26 ± 0.04Aa
	14D	1.49 ± 0.05 Ba	1.39 ± 0.06 Ba	1.31 ± 0.06 Ba	1.31 ± 0.06 Ba	1.13 ± 0.03 Ba
DMEM	7D	1.66 ± 0.04 Aa	1.41 ± 0.04 Aa	1.54 ± 0.05 Aa	1.52 ± 0.05 Aa	1.27 ± 0.03 Aa
	14D	1.65 ± 0.05 Aa	1.36 ± 0.04 Aa	1.64 ± 0.05 Aa	1.51 ± 0.05 Aa	1.26 ± 0.03 Aa
MEM	7D	1.59 ± 0.04 Ab	1.59 ± 0.05 Ab	1.52 ± 0.05 Ab	1.51 ± 0.05 Ab	1.15 ± 0.03 Ab
	14D	1.65 ± 0.05 Aa	1.36 ± 0.05 Aa	1.64 ± 0.06 Aa	1.51 ± 0.06 Aa	1.26 ± 0.04 Aa

* Indicates a statistically significant difference compared with the fresh (non-cultured) control group ($p < 0.05$). Different superscript uppercase letters (A,B) indicate statistically significant differences between culture times (7 and 14 days) within the same culture medium ($p < 0.05$). Different superscript lowercase letters (a,b) indicate statistically significant differences among culture media within columns ($p < 0.05$)

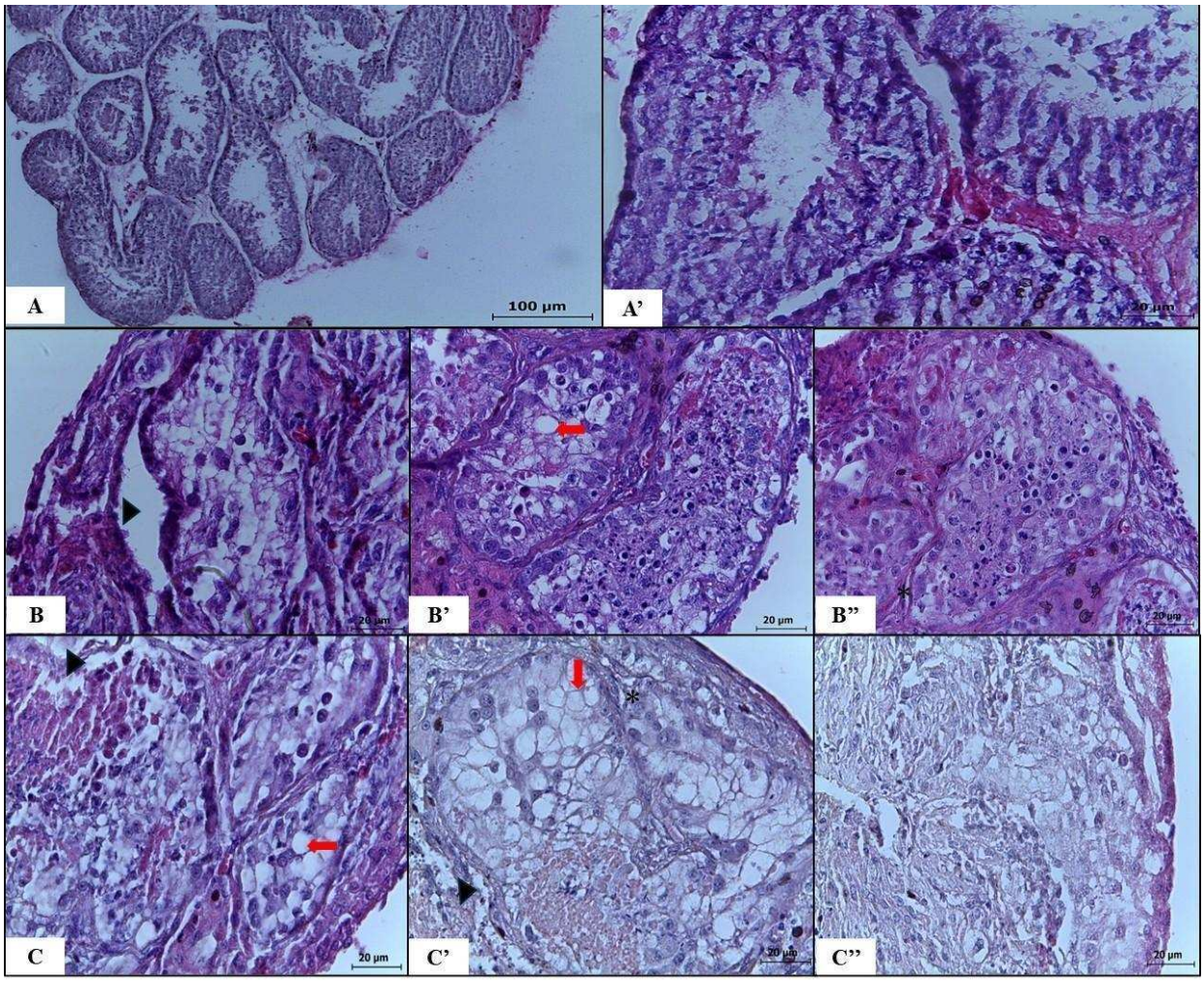

Figure 2. Histomorphological evaluation of testicular fragments from adult peccaries (*Pecari tajacu*) (n = 5). (A) and (A') Fresh control group, with maintenance of the normal histological architecture of the seminiferous tubules. (B–B″) Testicular fragments cultured in vitro for 7 days: (B) DMEM/F-12, (B′) DMEM and (B″) MEM. (C–C″) Testicular fragments cultured in vitro for 14 days: (C) DMEM/F-12, (C′) DMEM and (C″) MEM. Arrowheads indicate basement membrane separation; red arrows indicate cellular vacuolization; and asterisks indicate basement membrane rupture. Scale bar: 100 µm (A) and 20 µm (A'–C″).

Figure 3 shows testicular fragments cultured in vitro for 14 days in an organotypic culture system, in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12), Dulbecco's Modified Eagle Medium (DMEM), and Minimum Essential Medium (MEM). The presence and migration of testicular cells from the fragments are observed, with the

formation of cell clusters around the tissue and their aggregation in the agarose gel matrix throughout the culture period.

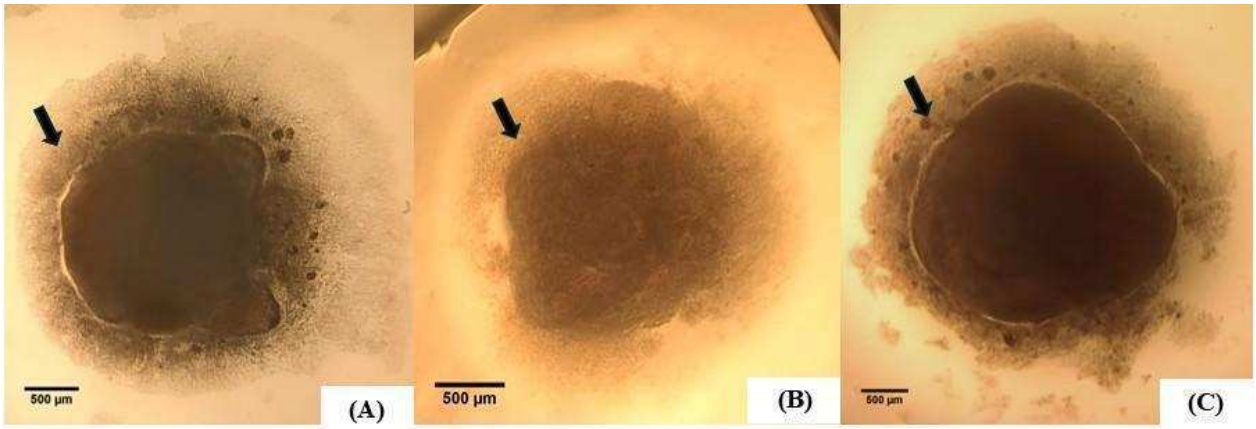

Figure 3. Micrographs of testicular fragments from adult peccaries cultured in vitro for 14 days in different culture media: (A) DMEM/F12, (B) DMEM and (C) MEM. Testicular cells can be observed around the fragments and aggregated in the agarose gel matrix. Scale bar: 500 µm.

3.2 Testicular cells viability

A significant decrease ($P < 0.05$) in testicular cell viability was observed when the fresh control group was compared to groups subjected to different periods and culture media. The variations can be seen in Figure 4. Regarding the test groups in cell viability, there was no significant difference between the culture media, nor between the interaction of the variables Group x Time ($p < 0.05$).

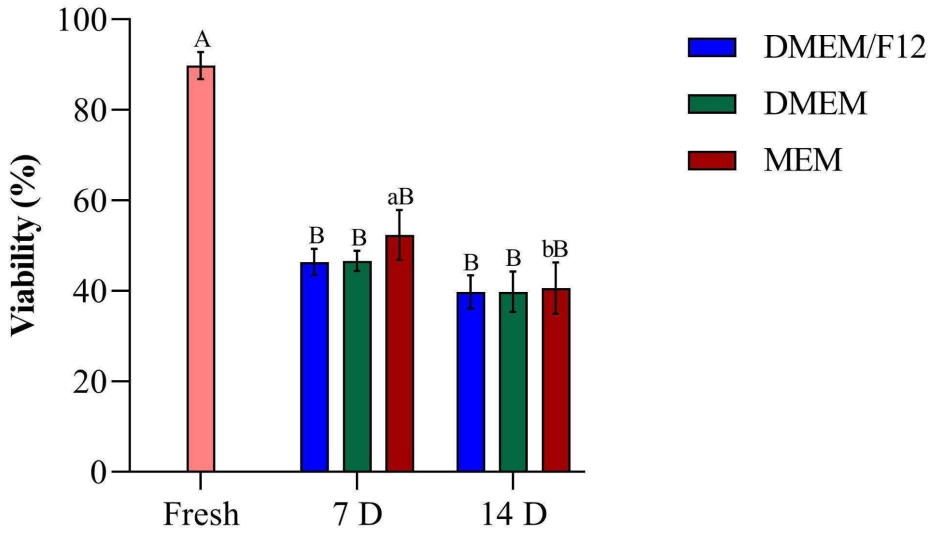

Figure 4. Mean values (± SEM) of germ cell viability from adult peccary testes (n=5) cultured in vitro for 14 days in organotypic systems with different culture media: DMEM/F12, DMEM, and MEM. Capital letters above bars (A,B) indicate statistical differences between the fresh group (reference group) and the other experimental groups (Dunnett's test; $p < 0.05$). Lowercase letters above bars (a,b) indicate a significant difference in measurements over time (7 and 14 days) (Tukey's test; $p < 0.05$) for each specific medium.

3.3 Testicular cells apoptosis

The analysis of parameters related to the evaluation of cellular apoptosis is presented in Figure 5. A significant decrease ($P < 0.05$) was observed in all parameters related to apoptosis in testicular cells in relation to the fresh control group. When compared to the groups subjected to different periods and culture media, no significant differences were observed between the culture media, nor was there an interaction between medium and time in the early and late apoptosis indices ($p < 0.05$).

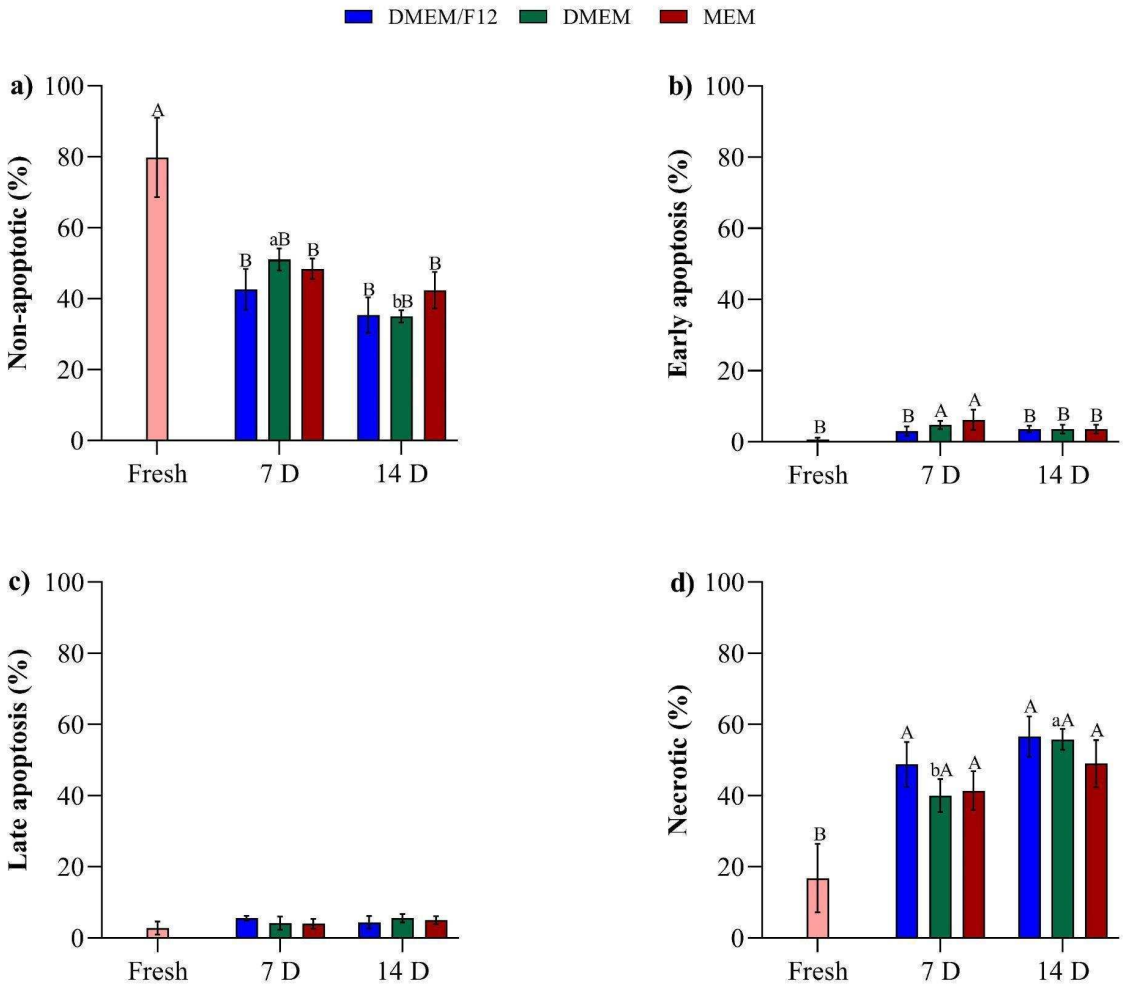

Figure 5. Mean values (±SEM) of the evaluation of apoptotic cells from testicular tissue of adult peccaries (n=5) cultured in vitro for 14 days in organotypic systems with different culture media (DMEM/F12, DMEM, and MEM). Capital letters above bars (A,B) indicate statistical differences between the fresh group (reference group) and the other experimental groups (Dunnett's test; $p < 0.05$). Lowercase letters above bars (a,b) indicate a significant difference in measurements over time (7 and 14 days) (Tukey's test; $p < 0.05$) for each specific medium.

3.4 Cell DNA integrity in testicular fragments

When evaluating the DNA integrity (Figure 6), a statistically significant reduction was observed between the fresh control group and the cultured groups ($p <$

0.05). The MEM at 7 days does not differ from the control, nor does the DMEM at 14 days. Culture time significantly influenced the percentage of intact DNA, and an interaction between the culture media over time was observed. In this context, the MEM medium showed better preservation of DNA integrity at 7 days of culture, while the DMEM medium demonstrated greater stability over time, maintaining high values at 14 days, without statistically differing from the fresh control group. However, when evaluating the isolated effect of the culture media, no statistically significant differences were observed between them ($p > 0.05$).

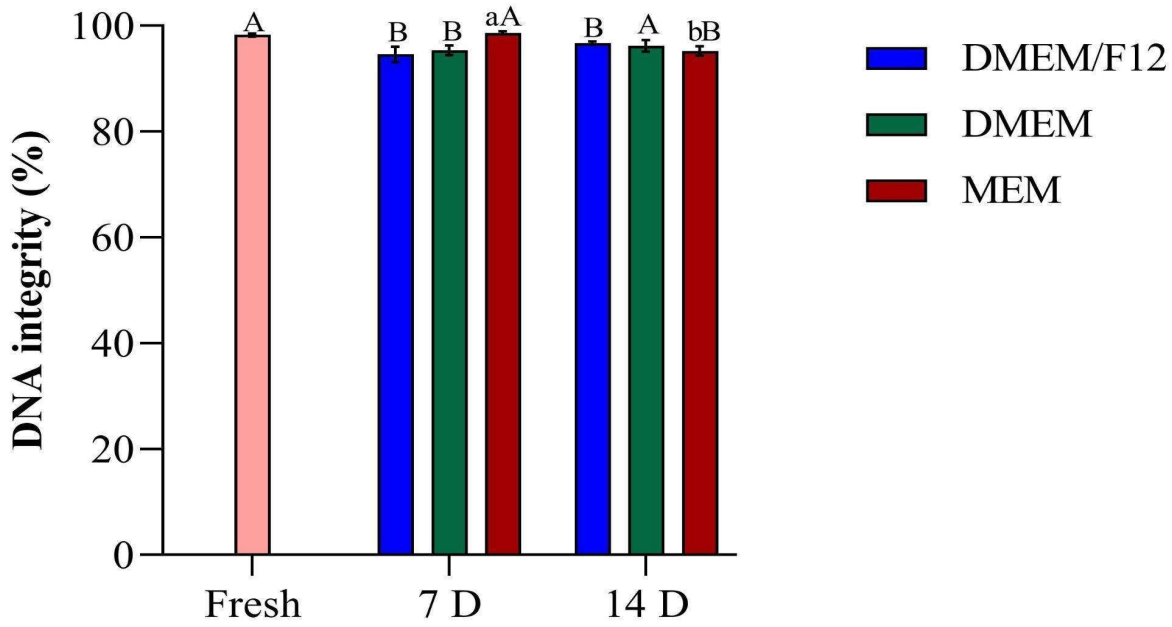

Figure 6. Superscript uppercase letters (A, B) in the column indicate statistical differences between the fresh group (reference group) and the other experimental groups (Dunnett's test; $p < 0.05$). Capital letters above bars (A,B) indicate statistical differences between the fresh group (reference group) and the other experimental groups (Dunnett's test; $p < 0.05$). Lowercase letters above bars (a,b) indicate a significant difference in measurements over time (7 and 14 days) (Tukey's test; $p < 0.05$) for each specific medium.

4. DISCUSSION

In general, throughout the culture period, the testicular fragments presented morphological alterations, an expected finding in adult tissues maintained under in vitro conditions. Among the media evaluated, MEM stood out for promoting better morphological preservation, reducing structural alterations such as basement membrane separation, vascularization, tubular cell loss, and disorganization of the seminiferous tubule architecture. The DMEM medium, in turn, demonstrated greater stability over time, consistently maintaining the evaluated cellular parameters, both being superior to DMEM/F12.

Despite the observed structural alterations, cell viability was maintained throughout the experimental period, regardless of the culture medium used. Similarly, the rates of early and late apoptosis remained stable throughout the culture, indicating the maintenance of cellular homeostasis in the experimental system. Regarding DNA integrity, a progressive increase in genomic damage was observed throughout the culture period; However, the MEM medium showed better preservation of DNA integrity, while DMEM maintained levels of intact DNA comparable to the fresh control group at the end of the study.

Silva et al. (2024) demonstrated, for the first time, the viability of organotypic culture of testicular fragments from pre-pubertal peccaries for 28 days, evaluating the most effective culture medium and the response of germ cells to supplementation with glial cell line-derived neurotrophic factor (GDNF). Complementarily, Pereira et al. (2025) advanced the development of these systems by establishing a long-term culture protocol (56 days) for testicular fragments from pre-pubertal peccaries, comparing organotypic and three-dimensional culture systems, as well as investigating the effects of fibroblast growth factor (FGF) supplementation on cell viability and tissue survival. Together, these studies demonstrate the increasing applicability and versatility of organotypic culture systems in wild species.

Despite advances in this technology in pre-pubertal animals, there is still a scarcity of information related to testicular culture in adult individuals of this species. In this context, the present study represents an initial milestone for the establishment of specific protocols aimed at this reproductive phase. To this end, a 14-day organotypic culture

period was adopted, defined based on the study by Sato et al. (2015), which performed testicular tissue culture in mature mice. Furthermore, this work aimed to identify the most suitable culture medium for maintaining cellular and tissue integrity. The composition of the culture medium is recognized as a critical factor for the success of cell and tissue culture (Gat et al., 2017); however, to date, there are no studies that directly compare DMEM/F12, DMEM, and MEM media in the culture of testicular cells from adult peccaries.

In our study, the morphological results obtained demonstrated a significant decrease in the scores of all parameters evaluated in the cultured tissues. These findings are in accordance with the literature, which describes that, although there are reports of adult tissue culture for prolonged periods, such as weeks or months, such tissues often present marked changes in their histological architecture and cellular composition over time (Sato et al., 2015). Furthermore, adult tissues, regardless of the organ's origin, are generally more vulnerable to in vivo conditions, which makes their structural and functional maintenance difficult in in vitro culture systems (Summerlin et al., 1970).

Regarding histomorphology, the treatments submitted to culture showed better structural preservation when using MEM (Minimum Essential Medium), which proved capable of significantly reducing the incidence of vascularization at 7 days of culture, while the other media did not show significant differences during this period. At 14 days, MEM also demonstrated superior performance, minimizing basement membrane separation, vascularization, tubular cell loss, and alterations in tubular structure. DMEM medium, in turn, showed greater stability over time. Overall, both media showed superior performance when compared to DMEM/F12.

The superior performance of MEM medium may be related to its characteristics, as it has a basic and low-complexity formulation, often associated with the need for supplementation with fetal bovine serum and additional growth factors to ensure adequate proliferative capacity and cell maintenance (Arora, 2013). This simpler composition allows the researcher to modulate the medium according to the specific requirements of the cultured cells. Under these conditions, the medium can be enriched with related additives or higher serum concentrations, making it more suitable for the culture of a wide

variety of mammalian cells (Arora, 2013). In addition, this supplementation contributes to greater nutritional stability of the medium, enabling the maintenance of cultures for slightly longer periods without the need for frequent refeeding (Cantor, 2019). This medium has already been successfully used in several studies, including the culture of goat ovarian tissue (Andrade et al., 2018) and the culture of fish cells (Higaki et al., 2013).

Dulbecco's Modified Minimum Essential Medium (DMEM) is one of the most widely used basal media in mammalian cell culture, having been successfully employed in studies involving rats (Reda et al., 2014), dogs (Lee et al., 2016a), goats (Heidari et al., 2012), pigs (Wang et al., 2015), and pre-pubertal peccaries (Silva et al., 2024; Pereira et al., 2025). In these last two studies with pre-pubertal peccaries, DMEM demonstrated the ability to stably maintain cellular and tissue parameters throughout the in vitro culture period, using supplements similar to those employed in the present study, which contribute to the maintenance, proliferation, and manipulation of different cell lines.

During peccary tissue culture, intense cell migration was also observed, characterized by the detachment of cells from the seminiferous tubules and their subsequent adhesion to the agarose gel matrix. A similar phenomenon was described in the first report of in vitro culture (IVC) of testicular fragments from pre-pubertal peccaries carried out by our group, in which cell detachment from the seminiferous tubules and migration of these cells to the agarose matrix were observed over 28 days of culture (Silva et al., 2024). Consistently, in another study by our group, Pereira et al. (2025) also reported this cell detachment process during a prolonged culture of 56 days in testicular fragments from pre-pubertal peccaries. Experimental evidence indicates that testicular cells isolated from domestic pig testes cultured in vitro have a remarkable capacity for self-organization, reassociating to form organoids that largely recapitulate the structural architecture of the testis (Sakib et al., 2019). It is worth highlighting that, in the present study, when evaluating the cells adhered to the gel, it was also possible to observe the migration of viable spermatozoa to the matrix, reinforcing the tendency of the cells to migrate to the gel. This finding further highlights the potential of the culture system to support ex situ cell survival and differentiation.

The preservation of plasma membrane integrity is essential for proper cell viability and function (Dias et al., 2021), since this structure selectively controls the transport of molecules between the intra- and extracellular environment. Furthermore, their organization and composition can undergo adaptive adjustments, conferring greater resistance to damage in different cellular contexts (Ammendolia et al., 2021). In the present study, regardless of the culture medium used, the testicular fragments maintained cell viability throughout the 14 days of culture. Similar results were described by Pereira et al. (2025) in pre-pubertal peccaries. Complementarily, Silva et al. (2024) reported an initial reduction in cell viability in the first weeks of culture of testicular fragments of this species; however, throughout the experimental period, the tissue demonstrated the ability to adapt to ex situ conditions, culminating in the maintenance of cell viability as the culture time progressed.

The relevance of apoptosis becomes even more evident during spermatogenesis, since the control of cell number is fundamental for the correct development of spermatozoa (Jahnukainen et al., 2004), playing a key role in controlling cell number and eliminating defective germ cells throughout testicular development and the spermatogenic process (Ibtisham et al., 2023). In this study, there was stability in early and late apoptosis rates throughout the 14 days of culture, suggesting that the system used was able to maintain cellular homeostasis, avoiding the progressive activation of programmed cell death pathways. According to Buarpung et al. (2012), apoptosis should be avoided to ensure the preservation of fertility.

Several studies have shown that in vitro culture processes and cryopreservation can induce damage to the genetic material of germ cells (Paasch et al., 2004). In the present study, an increase in the percentage of testicular cells with damaged DNA was observed throughout the culture period, when compared to the fresh control group. These findings are consistent with the results of Ibtisham et al. (2023), who also reported an increase in TUNEL-positive cells over the culture time in testicular tissue from pre-pubertal pigs.

However, the group cultivated in MEM medium showed better preservation of DNA integrity, evidenced by the reduction in the number of damaged cells throughout the

evaluated period. In turn, DMEM medium maintained DNA integrity in a stable manner and, at 14 days of culture, the percentage of intact DNA did not differ statistically from the fresh control group. Corroborating these results, Ibtisham et al. (2023) also reported that groups supplemented with GDNF tend to show better maintenance of DNA integrity in testicular tissues, especially in more advanced stages of culture. In addition, the presence of the growth factor FGF may be associated with this preservation, since similar results were described by Kurokawa et al. (2005) in rats with cryptorchidism in the presence of this factor. These exogenous growth factors may exert a protective effect against cell death through activation of the mitogen-activated protein kinase signaling pathway (Gantner et al. 2020).

In conclusion, this study demonstrates that in vitro culture of testicular tissue from adult peccaries, using supplemented MEM and DMEM culture media, can be viable for up to 14 days. Furthermore, the results obtained provide initial information and fundamental findings that are significantly important for the development and standardization of protocols applicable to testicular culture from adult peccaries.

Conflict of interest

The authors declare that they have no conflict of interest.

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CAPÍTULO II

In vitro culture of cryopreserved testicular tissue from adult collared pecccaries using different commercial culture media

***In vitro* culture of cryopreserved testicular tissue from adult collared pecccaries using different commercial culture media**

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ABSTRACT

This study investigated the effects of different supplemented culture media on the histomorphology, cell prediction, and apoptosis of testicular fragments from adult peccaries maintained in vitro. Five testes were collected, dissected, and cultured in DMEM/F12 and DMEM media. Both media received identical supplementation, containing 10% fetal bovine serum, 25 mM HEPES, 2 µL/mL insulin-transferrin-selenium (ITS), 1% non-essential amino acids (NEAA), 1% vitamin solution, 0.2% bovine serum albumin, 1% antibiotic-antifungal solution, 10 ng/mL LH/FSH, 20 ng/mL mEGF, 10 ng/mL GDNF, and 10 ng/mL bFGF. Testicular fragments were cultured in an organotypic system using 0.75% agarose gel for periods of 7 and 14 days under controlled conditions of 34 °C and an atmosphere containing 5% CO_2. The culture time significantly reduced the morphological scores compared to the fresh and cryopreserved control ($P < 0.05$). At 7 days of culture, the DMEM medium provided better morphological preservation, especially in the parameters of basement membrane separation and tubular structure; however, after 14 days, no statistically significant differences were found between the media evaluated. Cell prediction is stable throughout the experiment, with no differences between treatments ($P > 0.05$). Similarly, The proportions of apoptotic cells remain constant throughout the experimental period, maintaining cellular homeostasis.. In conclusion, DMEM/F12 and DMEM media, when properly supplemented, proved suitable for the organotypic culture of testicular tissue from adult peccaries from samples previously cryopreserved for up to 14 days. The results of this study are considered as an initial basis for the improvement and standardization of protocols applicable to the conservation and reproduction biotechnologies of this species.

Key words: Conservation of wild species; Organotypic culture; Testicular cryopreservation

1. Introduction

Biobanks constitute important repositories of genetic resources, playing a fundamental role in the advancement of reproductive medicine and in the development of strategies aimed at the conservation of wildlife (Comizzoli, 2018). Traditionally, these repositories have prioritized the storage of seminal samples; however, in recent decades, there has been a growing interest in the implementation of gonadal biobanks, especially given the limitations associated with obtaining gametes under certain biological and environmental conditions (Pereira et al., 2025).

However, the use of testicular tissue for conservation purposes requires the establishment of specific and rigorously standardized protocols, since the particularities of spermatogenesis and testicular histology among species can directly influence the efficiency of cryopreservation and cell maintenance during in vitro culture (Silva et al., 2022). In this context, it becomes essential to consider the morphophysiological differences that exist between the different age groups of individuals.

While immature testicular tissues have a more homogeneous cell population, tissues from adult individuals contain germ cells at different stages of development, including spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa (Kaya et al., 2024). This cellular heterogeneity gives adult tissue a more complex metabolism, making it potentially more sensitive to manipulation steps, such as cryopreservation and in vitro culture, when compared to tissues from pre-pubertal animals (Sato et al., 2015; Da Silva et al., 2021).

Considering that spermatogenesis is a highly dynamic process dependent on a structurally and functionally organized testicular microenvironment (Lima et al., 2018), restoring these conditions in in vitro systems still represents one of the main challenges of reproductive biotechnology. This limitation has already been evidenced in classic organ culture studies, in which significant difficulties were observed in the maintenance and progression of spermatogenesis in adult testicular tissues subjected to different experimental systems (Davis, 1967; Summerlin et al., 1970; Sato et al., 2015). These findings reinforce the need for the development of specific approaches aimed at the culture of germ tissues from adult individuals.

In addition to the age factor, another relevant aspect concerns the history of samples originating from biobanks, since these tissues have generally been previously cryopreserved. In this sense, it is important to consider that testicular tissue may present different responses to in vitro culture when comparing fresh and previously cryopreserved samples. One of the main obstacles in this process is maintaining cell viability after thawing, since exposing the tissue to high cooling rates can cause structural and functional damage to germ cells (Pereira et al., 2025).

The literature shows that culturing fragments of testicular tissue is more efficient for assisted reproduction purposes when compared to culturing isolated cells, since the fragments preserve, albeit partially, the intrinsic microenvironment of the testicle (Ibtisham et al., 2020; Ibtisham et al., 2022). For this environment to be sustained in vitro, culture media must ensure high densities of viable cells, in addition to favoring the synthesis and release of biological products essential to the functional maintenance of the tissue (Ritacco et al., 2018). At the same time, it is essential to preserve the integrity of nuclear DNA and minimize the occurrence of apoptosis, ensuring the reproductive potential of germ cells (Buarpung et al., 2012).

In organotypic culture systems, different traditional media have been employed, notably Dulbecco's modified Eagle medium (DMEM), F12 medium, and minimum essential medium (MEM) (Sato et al., 2011; Movahedin et al., 2024). Both the composition of the medium and the type of serum used have a direct influence on survival, proliferation, and maintenance of cellular characteristics (Yao e Asayama, 2017). Therefore, variations in formulations can significantly impact the proliferation rate, tissue organization, colony formation, and cell differentiation potential.

Given this, the comparative evaluation of different culture media becomes essential, since the in vitro testicular microenvironment needs to be adequately mimicked to sustain the survival of the multiple cell types involved in spermatogenesis and, consequently, allow their functional progression (Cho et al., 2023), especially in previously cryopreserved tissues. Each medium presents specific combinations of nutrients, vitamins, amino acids, salts, and regulatory factors (Yao e Asayama, 2017),

which may or may not adequately reproduce the testicular niche. Thus, the comparison between different formulations allows the identification of more efficient conditions for maintaining cell viability and for optimizing assisted reproduction protocols, particularly in wild species, in which knowledge about in vitro testicular physiology is still limite

In parallel with the technical challenges, the intensification of anthropogenic actions has severely compromised the survival of numerous species of wild fauna in different ecosystems (Comizzoli & Holt, 2024). The population decline of threatened species makes the genetic material of remaining individuals highly valuable and, in many cases, irreplaceable (Ibtisham et al., 2020). In this context, the collared peccary (Pecari tajacu Linnaeus, 1758) stands out as a species of high ecological importance, especially for its contribution to forest restoration processes (Vieira et al., 2023).

Natural populations of this species have shown a marked decline in different regions of Brazil, including the Pampas (Gongora et al., 2011) and remnants of the Atlantic Forest in the Northeast, where the collared peccary is considered locally extinct (Garbino et al., 2018). This scenario is mainly attributed to indiscriminate hunting and the continuous loss of habitat (Gongora et al., 2011). Given this reality, the peccary emerges as a promising biological model for the development and application of reproductive biotechnologies, including cryopreservation of testicular tissue (Da Silva et al., 2019; 2021) and in vitro culture (Silva et al., 2024; Pereira et al., 2025).

These strategies are especially relevant in situations where obtaining spermatozoa is not feasible, such as in cases of sudden death, pronounced reproductive seasonality, pathologies associated with azoospermia, or exposure to gonadotoxic agents (Santiago-Moreno & Blesbois, 2022). Furthermore, the peccary presents striking reproductive particularities, including a specific organization of Leydig cells and a differentiated architecture of the seminiferous tubules, distinguishing it from other ungulates and even from phylogenetically close species, such as pigs (Costa et al., 2010).

Considering that the cultivation of cryopreserved adult testicular tissue still presents significant limitations, that there is no consensus regarding the ideal culture medium for maintaining this tissue in adult wild species, and that there are no long-term

cultivation studies in this age group, especially using previously cryopreserved cell structures, it becomes essential to investigate how different culture media influence the structure, cell viability, and functional integrity parameters of testicular tissue.

Therefore, the objective of this study was to evaluate the effect of different culture media (DMEM/F12 and DMEM) on the morphology, cell viability, and functional integrity parameters of cryopreserved testicular tissue fragments subsequently cultured in adult peccaries in vitro for 14 days.

2. Materials and methods

2.1. Animal ethics and husbandry

All experiments were conducted following the Animal Ethics Committee of the Federal Rural University of Semi‑Arid - UFERSA (no. 24/2023) and Chico Mendes Institute for Biodiversity Conservation (no. 72170). All males belong to the Wild Animal Multiplication Center (CEMAS—UFERSA, Mossoró, RN, Brazil), which is registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding center (no. 1478912) and located at 5°10′ S and 37°10′ W at an altitude of 16 m, characterized by a semiarid tropical climate.

2.2 Testis collection and experimental design

The gonads of five adult males, aged between 3 and 6 years, were collected. The tests were cleaned in 0.9% saline solution (NaCl) and subsequently transported to the laboratory in a solution composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotic and antifungal, maintained at a temperature of 22 °C. Each animal was considered an independent biological replication, since the collections occurred at different times.

In the laboratory, the tests were carefully dissected from adjacent tissues and subjected to three consecutive washes with the transport solution. They were then sectioned into small fragments with approximate dimensions of 1.0 mm^3 (1 × 1 × 1 mm).

A portion of the samples was designated for the fresh control group. For the vitrification procedure, 120 fragments were used, which remained stored for a period of one week; subsequently, the samples were heated 37°C. Following this process, the fragments were randomly distributed among the in vitro culture (IVC) experimental groups, using different culture media: DMEM and DMEM/F12 (Figure 1).

All treatments were conducted in 24-well cell culture plates, with four wells used per pair of tests, and maintained in a humidified atmosphere incubator containing 5% CO_2 at 34 °C for a total period of 14 days. The in vitro culture time was defined based on the study by Sato et al. (2015), who cultured testicular fragments from adult rats under similar conditions. Evaluations of the fragments in all experimental groups were performed at seven-day intervals (Figure 1).

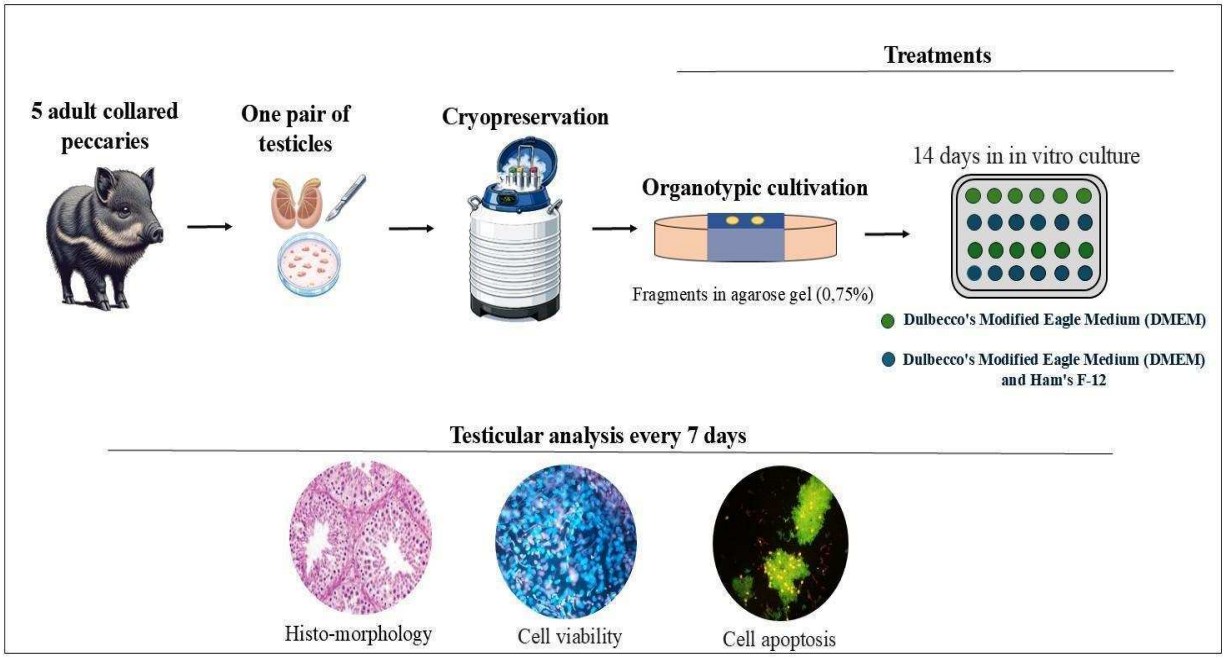

Figure 1. Schematic representation of the experimental design adopted in the evaluation of cryopreserved adult peccary testicular tissue fragments subjected to in vitro culture in different culture media.

2.3 Cryopreservation

The tissues were vitrified using Minimum Essential Medium (MEM) supplemented with 0.25 M sucrose, 10% fetal bovine serum (FBS), and the cryoprotectants DMSO and ethylene glycol (EG), both at a concentration of 1.5 M. The fragments were exposed to the vitrifying solution for 5 minutes at 25 °C, according to the methodology previously described by Da Silva et al. (2021). After this period, the tissues were placed on sterile filter paper to remove excess solution and then subjected to the conventional vitrification (CV) process. The fragments (n = 24) were placed in cryotubes, immediately immersed in liquid nitrogen, and kept in cryobiological storage for one week, as described by Da Silva et al. (2021).

After the storage period, the samples were heated, initially keeping the cryotubes at room temperature (25 °C) for 1 minute, followed by immersion in a water bath at 37 °C to reheat the testicular tissue fragments. For the gradual removal of cryoprotectants, the fragments were washed three times, for 5 minutes each time, in MEM supplemented with 10% FBS, using decreasing concentrations of sucrose (0.50 M, 0.25 M and absence of sucrose).

2.4 In vitro organotypic culture of cryopreserved testicular tissue fragments

The protocol adopted for organotypic culture was based on the methodology described by Silva et al. (2024). Initially, a solution containing 0.75% agarose powder (Sigma, A013), dissolved in sterile distilled water, was prepared. This solution was distributed into 10 cm diameter Petri dishes and kept at room temperature until the gel completely solidified. Subsequently, agarose blocks measuring approximately 1 × 1 × 1 cm were cut and positioned in the wells of the culture plates, using one cube per well.

Then, 500 µL of culture medium was added to each well, approximately 12 hours before the start of the culture. After this period, the medium was replaced again, and two testicular fragments were placed in each well. The 24-well plates were then incubated for 14 days at 34 °C under a humidified atmosphere containing 5% CO_2. Each well received 500 µL of culture medium composed of DMEM/F12 (Sigma-Aldrich, D0697) and DMEM (Gibco, 11885084). All media were supplemented with 10% fetal bovine serum (FBS; ThermoFisher, 12657029), 25 mM HEPES (Sigma, H6147), 2 µL/mL

insulin-transferrin-selenium solution (ITS; Gibco, 41400-045), 1% non-essential amino acids (NEAA; Sigma, M7145), 1% vitamin solution (Gibco, 11120), 0.2% bovine serum albumin (BSA; Sigma-Aldrich, A7906), 1% antibiotic-antifungal solution (Gibco, 15240), 10 ng/mL LH/FSH (Pluset®, Hertape Calier), 20 ng/mL mEGF (Sigma, E4127), and 10 ng/mL of GDNF (Pethotech, 512-GF), in the presence of 10 ng/mL of bFGF (Pethotech, 100-18B).

Complete replacement of the culture medium was performed every 72 hours, totaling four changes over the 14-day experimental period. On days 7 and 14 of cultivation, fragments were collected for later analysis.

2.5 Testicular fragment histomorphology

Testicular fragments, both fresh and cultured, were fixed in Bouin's solution for 12 hours, embedded in paraffin blocks, cut into 5.0 μm sections, stained with hematoxylin and eosin, and applied to a specific optical microscope (Olympus CX 31 RBSFA) at 400x magnification. Table 1 presents the parameters and scoring systems used for the histological evaluation of testicular tissue, as described by Silva et al. (2024). For each parameter, five seminiferous tubules were analyzed in six distinct fields, totaling 30 tubules per treatment (Da Silva et al., 2019). Fragments that obtained a total score equal to 3 were classified as morphologically normal, while those with a score equal to 1 were considered degenerated. Table 1 below presents a description of the parameters evaluated.

Table 1. Histo‑morphological parameters of seminiferous tubules in testicular fragments from collared peccaries.

Parameter	Scores		
	3	2	1
Shrinkage from basal membrane	No shrinkange	Partly shrinkange (<50%)	Mostly shrinkange (>50%)
Tubular cell swelling	No swelling	>50% cells without swelling	>50% cells with swelling
Rupture from the basal membrane	No rupture	Partly ruptured (<50%)	Mostly ruptured (>50%)
Tubular cell loss	No cell loss	<75% cell type loss	>75% cell type loss

Tubular structure	Structure intact	All cell types of presents although slightly disordered structure	Random distribution of remaining cells

Silva et al., 2024.

2.6 Cell viability in testicular fragments

Testicular cells were obtained from fragments through enzymatic digestion, as described by Abrishami et al. (2010). Briefly, uncultured fragments were incubated with 0.2% collagenase type IV for 10 minutes at 37°C. Then, an equal volume of DMEM supplemented with FCS was added to inactivate the enzyme, and the samples were centrifuged at 114 × g for 5 minutes. For previously cultured fragments, enzymatic digestion was not necessary due to their spontaneous disaggregation.

The pellets or fragments were incubated with a solution containing 3 µL of propidium iodide (0.5 mg/mL in PBS) and 5 µL of Hoechst 33342 (40 µg/mL in PBS) for 10 minutes at 37 °C, allowing the identification of nonviable cells (stained in red) and viable cells (golden nuclei in blue), respectively. The samples were stained using an epifluorescence stain (Leica, Kista, Sweden) at 400× magnification, with 100 cells counted per experimental group.

2.7 Cell DNA integrity in testicular fragments

Testicular fragments were fixed overnight in 4% paraformaldehyde solution, embedded in paraffin, serially sectioned (5 µm thick), mounted on slides, and evaluated using the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling kit (TUNEL Assay Kit – Roche, 11684809910) as proposed by Da Silva et al. (2021). The TUNEL reaction mixture was prepared using the enzymatic solution containing 5 µL of terminal deoxynucleotidyl transferase (TdT) and 45 µL of the labeling solution containing nucleotide polymers. The sections were incubated with 50 µL of the TUNEL reaction mixture for 1 h at 37 °C inside a humidified and dark container. A negative control was used, in which only 50 µL of labeling solution, without terminal transferase, was used instead of the TUNEL reaction mixture. The positive control was performed by incubating the cells with 5 µL of recombinant DNase I (Sigma-Aldrich) and 45 µL of 0.05% Triton X-100 in PBS for 10 minutes before labeling procedures to induce DNA breaks. The

nuclei of all cells were counterstained with Hoechst 33342 (1:100, Sigma-Aldrich). We evaluated 10 images per experimental group, captured under an epifluorescence microscope. The images were evaluated using ImageJ software, and the cells from 10 images per treatment were counted.

A minimum of 1,000 cells was analyzed per sample to determine the level of nuclear DNA fragmentation damage. TUNEL-positive cells displayed green fluorescence and were classified as damaged, while TUNEL-negative cells, counterstained with blue fluorescence, were classified as normal (Pereira et al 2025).

2.8 Assessment of cellular apoptosis

Approximately 10 µl of a suspension of fresh, cultured testicular cells were placed on previously sanitized glass slides. Then, the same volume of a freshly prepared solution composed of ethidium bromide (0.1% w/v in distilled water) and acridine orange (0.1% w/v in distilled water), mixed in a 1:1 ratio, was added. The solution was gently inserted into the cell protection, and the preparation was covered with a coverslip, remaining in contact with the stains for approximately five minutes. The cells were then examined using epifluorescence staining (Leica, Kista, Sweden) at 400× magnification, classifying 100 cells per experimental group. According to the observed staining, the cells were categorized as: non-apoptotic (NA), exhibiting a uniform light green nucleus; early apoptotic (AP), with a heterogeneous green nucleus; late apoptotic (AT), exhibiting a bright orange and irregular nucleus; or necrotic (N), characterized by a uniform orange core (Kalia et al., 2009).

2.9 Statistical analysis

Each of the five animals used was considered an independent biological replica. The results were expressed as mean ± standard error of the mean. Statistical analyses were performed using Jamovi software (SAS Institute Inc.), adopting a significance level of $P < 0.05$. Initially, the normality of the residuals and the homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene tests, respectively. Viability and apoptosis parameters were analyzed by two-way analysis of variance (ANOVA) with repeated measures over time, and comparisons between treatments were performed using

the Tukey and Holm tests. For the evaluation of morphological variables, the existence of differences between groups was verified using the non-parametric Kruskal–Wallis test.

3. RESULTS

3.1 Testicular histomorphology

The results of the testicular histomorphological evaluation are presented in Table 2. In general, a significant reduction was observed in most of the parameters analyzed when comparing the fresh control group with the cryopreserved group ($P < 0.05$). However, in the cryopreserved group, the parameters related to basement membrane separation and tubular structure did not differ statistically from the fresh control. Although cryopreservation caused changes in the other morphological parameters, the preservation of basement membrane integrity and the structural organization of the seminiferous tubules suggests the maintenance of essential histological characteristics of the testicular tissue, indicating its potential viability for material use.

When compared to the fresh control group, the tissue cultured for 7 days in DMEM/F12 medium did not show statistically significant differences ($P < 0.05$) in the parameters of vacuolization, basement membrane rupture, and tubular cell loss, indicating that the culture period did not promote an increase in cell degeneration in these morphological aspects. Regarding the cryopreserved group, culturing for 7 days in DMEM/F12 medium resulted in a significant reduction ($P < 0.05$) in the scores related to basement membrane separation and tubular structure, evidencing a worsening of these morphological parameters throughout the culture period. Similarly, in DMEM medium, during the same culture interval, statistically significant differences were observed compared to the cryopreserved group in the parameters of tubular cell loss and tubular structure ($P < 0.05$), also indicating morphological impairment of the tissue. At 7 days of culture, when the media were compared to each other, DMEM showed better preservation of basement membrane separation and tubular structure compared to DMEM/F12 ($P < 0.05$), reflecting greater integrity of the seminiferous tubules, while the other parameters did not show statistically significant differences between the media ($P < 0.05$).

When compared to the fresh control group, tissue cultured for 14 days in DMEM/F12 medium showed a statistically significant reduction ($P < 0.05$) in the parameters of basement membrane separation, vacuolization, tubular cell loss, and tubular structure, with no difference observed for basement membrane rupture.

Similarly, culture for 14 days in DMEM medium resulted in a statistically significant reduction compared to the fresh control in all histomorphological parameters evaluated ($P < 0.05$).

When compared to the cryopreserved group, tissues cultured for 14 days showed statistically significant differences ($P < 0.05$) in several histomorphological parameters. In DMEM/F12 medium, significant reductions were observed in the parameters of basement membrane separation, vacuolization, and tubular structure, while no differences were detected for basement membrane rupture and tubular cell loss. In the DMEM medium, 14-day culture resulted in statistically significant differences in all parameters evaluated when compared to the cryopreserved group ($P < 0.05$). Although both media showed morphological impairment after prolonged culture, DMEM presented higher scores than those observed in DMEM/F12, indicating less intense structural changes.

In a comparison between the culture media over 14 days of culture, no statistically significant differences were observed between the DMEM and DMEM/F12 media for the histomorphological parameters evaluated ($P < 0.05$), indicating similar cellular behavior between the media during this period in the structural integrity of the testicular tissue even after prolonged culture.

Table 2 - Mean values (± SEM) for morphological assessment scores (from 3 – normal – to 1 – degenerated) in cryopreserved testicular tissues from adult peccaries (n = 5) and subsequently cultured for 14 days using an in vitro organotypic culture system in different culture media: Dulbecco and Ham modified Eagle medium (DMEM/F12), Dulbecco modified Eagle medium (DMEM).

	Days of culture	Shrinkage from basal membrane	Tubular cell swelling	Rupture from the basal membrane	Tubular cell loss	Tubular structure
Controls (Fresh)	0	2.02 ± 0.0 ^{A}	2.35 ± 0.1 ^{A}	2.38 ± 0.1 ^{A}	2.43 ± 0.1 ^{A}	2.15 ± 0.1 ^{A}

	Days of culture	Shrinkage from basal membrane	Tubular cell swelling	Rupture from the basal membrane	Tubular cell loss	Tubular structure
Post cryopreservation	0	2.15 ± 0.0 ^{A}	1.72 ± 0.0 ^{B}	2.59 ± 0.1 ^{B}	2.16 ± 0.1 ^{B}	2.17 ± 0.0 ^{A}
DMEM/F12	7D	1.34 ± 0.0 ^{Ba}	2.19 ± 0.0 ^{Aa}	2.33 ± 0.0 ^{Aa}	2.31 ± 0.0 ^{ABa}	1.19 ± 0.0 ^{Ba}
	14D	1.65 ± 0.0 ^{Ca}	1.41 ± 0.0 ^{Ba}	1.84 ± 0.1 ^{Aa}	1.75 ± 0.1 ^{BaB}	1.69 ± 0.0 ^{Ca}
DMEM	7D	1.63± 0.0 ^{Cb}	1.78 ± 0.0 ^{Cb}	2.16 ± 0.1 ^{Cb}	2.14 ± 0.1 ^{Cb}	1.83 ± 0.0 ^{Db}
	14D	1.59 ± 0.1 ^{Cb}	1.52 ± 0.0 ^{BCb}	1.77 ± 0.1^{Cb}	1.70 ± 0.0 ^{Cb}	1.63± 0.0 ^{Db}

Table 2. Histomorphological evaluation of testicular tissue after cryopreservation and organotypic culture for 7 and 14 days in different culture media. Data are presented as mean ± standard error. Different uppercase letters (AB) indicate a statistically significant difference ($P < 0.05$) between treatments at the same culture period (7 days compared to 7 days and 14 days compared to 14 days). Different lowercase letters (ab) indicate a statistically significant difference ($P < 0.05$) between culture periods within the same medium. Data normality was assessed using the Kolmogorov–Smirnov test and homogeneity of variances using Levene's test. Differences between groups were analyzed using the Kruskal–Wallis test.

Figure 2. shows testicular fragments that were cultured, cryopreserved, and then cultured in vitro for 14 days in an organotypic culture system, in DMEM/F-12 (Dulbecco's Modified Eagle Medium/F-12) and DMEM (Dulbecco's Modified Eagle Medium). The presence and displacement of testicular cells from the fragments is observed, with the consequent formation of cell aggregates around the tissue and their incorporation into the agarose gel matrix throughout the culture period.

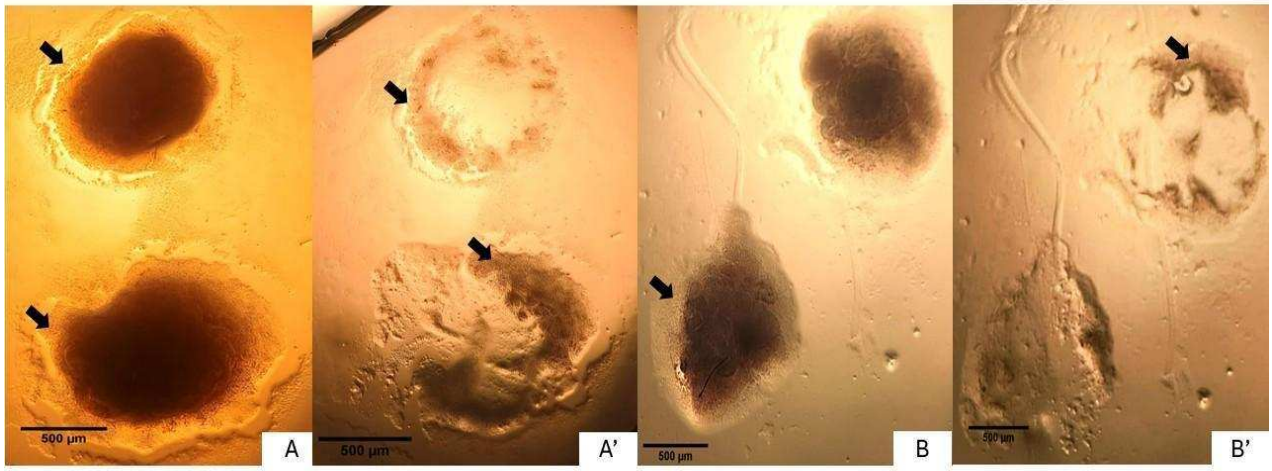

Figure 2. Photomicrographs of cryopreserved adult peccary testicular fragments cultured in vitro for 14 days in different culture media: (A) DMEM/F12 and (B) DMEM. The corresponding enlarged images (A' and B') show the persistence of testicular cells after removal of the fragments, which remain aggregated to the agarose gel matrix, indicating cellular interaction with the culture substrate. Scale bar: 500 µm.

3.2 Testicular cells viability

Testicular cell viability showed a significant reduction ($P < 0.05$) in the cryopreserved and cultured groups in different media and time periods compared to the fresh control group. However, among the experimental groups, cell viability did not differ statistically as a function of the culture medium used or the interaction between group and culture time ($P > 0.05$). These differences can be seen in Figure 2.

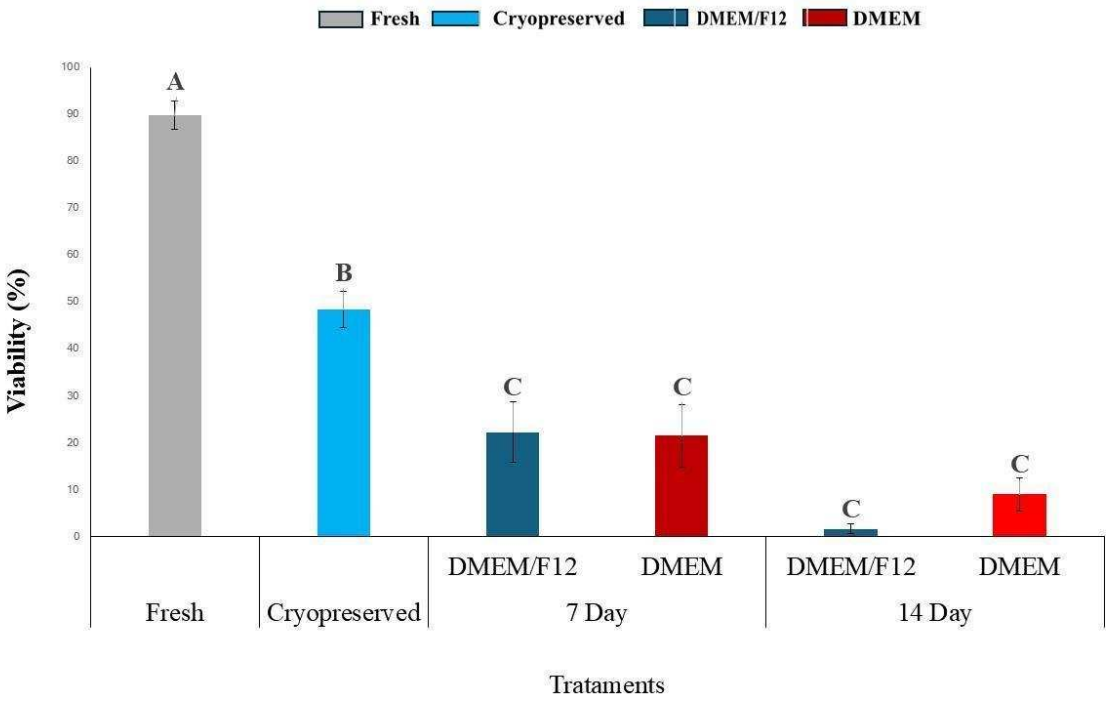

Figure 3. Mean values (±SEM) of germ cell viability from adult peccary testes (n=5) in cryopreserved samples cultured in vitro for 14 days in organotypic systems with different culture media: DMEM/F12 and DMEM. The superscript capital letters (A, B and C) in the column indicate statistical differences between the fresh group (reference group) and the other experimental groups (Tukey and Holm test $p < 0.05$).

3.3 Testicular cells apoptosis

Figure 3 presents the results of the analysis of parameters related to cell apoptosis. Statistically significant differences ($P < 0.05$) were observed in the percentages of non-apoptotic and necrotic cells when comparing the fresh control group with the cryopreserved group and with the groups subjected to different culture treatments. However, no significant differences were identified between the culture media used, nor was there an effect of the interaction between medium and time on the indices of non-apoptotic and necrotic cells ($P > 0.05$). Regarding the parameters of early and late apoptosis, no statistically significant differences ($P > 0.05$) were observed between the groups evaluated.

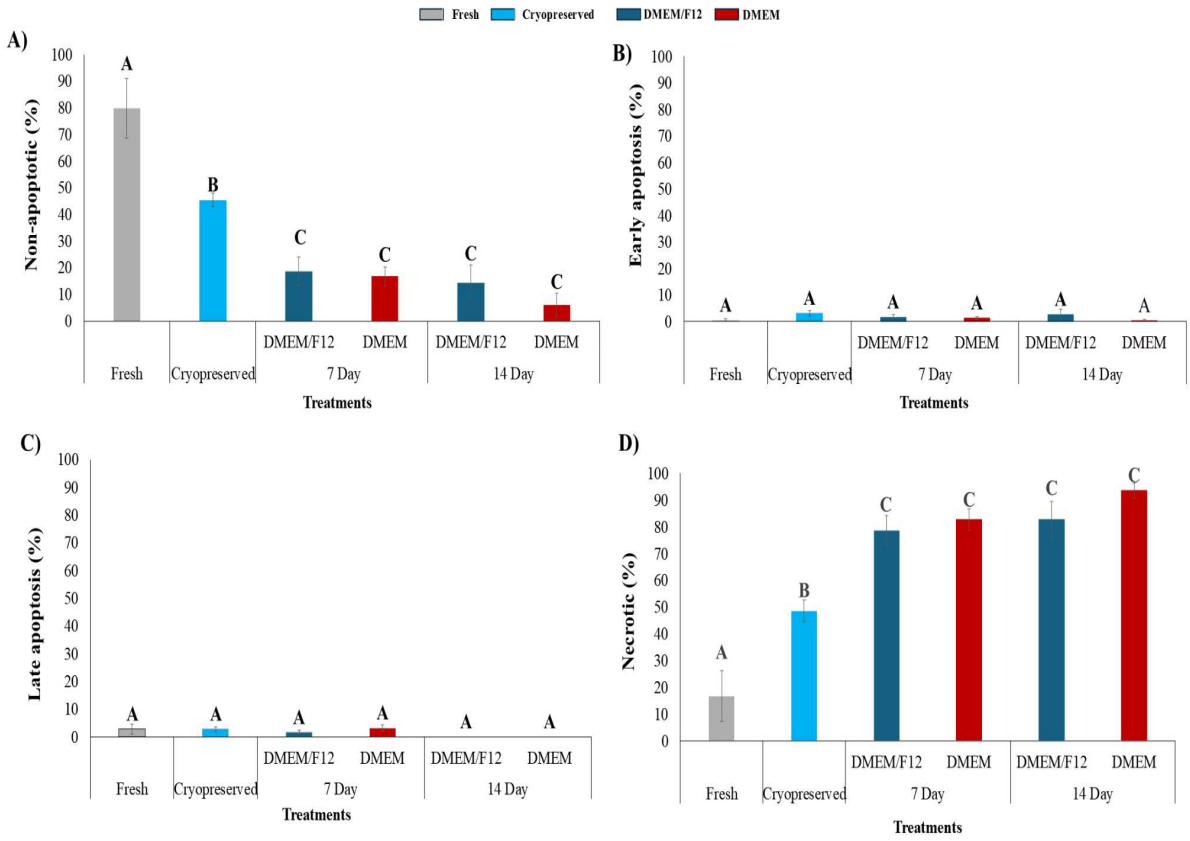

Figure 4. Mean values (±SEM) of the evaluation of apoptotic cells from the testicular tissue of adult peccaries (n=5) cryopreserved and cultured in vitro for 14 days in organotypic systems with different culture media (DMEM/F12 and DMEM).

4. DISCUSSION

Da Silva et al. (2019, 2021) established efficient protocols for the cryopreservation of genetic material from adult animals of this species. This information is fundamental for the development of strategies aimed at storing genetic material from genetically relevant categories that die unexpectedly or need to be sterilized for clinical reasons (Da Silva et al., 2019). However, one of the biggest obstacles remains the definition of conditions that allow the reactivation of spermatogenesis in tissues subjected to cryopreservation (Pereira et al., 2023). The previously stored material requires

complementary help that enables its in vitro development, thus ensuring the functionality and effectiveness of the biobank.

Silva et al. (2024) discovered, for the first time, the organotypic culture of testicular fragments from pre-pubertal peccaries for 28 days, evaluating the most effective culture medium and the response of germ cells to supplementation with GDNF. Additionally, Pereira et al. (2025) advanced this system, establishing a long-term culture protocol (56 days), comparing organotypic and three-dimensional systems and investigating the effects of FGF addition on cellular vitamins and tissue survival. These studies highlight the growing applicability and peculiarities of organotypic culture systems in wild species. However, there are still gaps in adult tissues; regardless of the organ's origin, these tissues exhibit high vulnerability to ex situ conditions and are difficult to maintain outside the organism (Sato et al., 2015).

In addition, for the organ culture method to be clinically viable, it needs to be successfully applied to cryopreserved testicular tissues, since tissue cryopreservation is a routine practice in fertility preservation strategies (Portela et al., 2019). To our knowledge, this is the first study conducted in this species using cryopreserved material for culture. Thus, the culture period was defined as 14 days, based on Yokonishi et al. (2014), who used cryopreserved testicular tissue from prepubertal mice in an organoculture system.

However, for proper development of the previously cryopreserved material to occur, the cryopreservation methods and the choice of cryoprotectant influence tissue integrity, resulting in differences in spermatogenic efficiency during the subsequent culture period (Yokonishi et al., 2014). Therefore, the culture medium must ensure cell survival and maintenance, as well as the maintenance of cell functions, so that its quality directly impacts the progression of spermatogenesis (Yao and Asayama, 2017). In our study, we evaluated the modified Eagle medium of Dulbecco and Ham's (DMEM/F12), which has already been used in the culture of goat gonads (Salimi et al., 2024), pigs (Fayaz et al., 2022), and cattle (Tang et al., 2023), and modified Eagle medium of Dulbecco (DMEM) (Reda et al., 2014), dogs (Lee et al., 2016a), goats (Heidari et al., 2012), pigs (Wang et al., 2015), and pre-pubertal peccaries (Silva et al., 2024; Pereira et al., 2025).

Regarding histomorphology, it was observed that, when comparing fresh and cryopreserved tissues, cryopreservation maintained the evaluated parameters. The integrity of the basement membrane and the maintenance of the tubular structure stood out, with no statistically significant difference between the fresh control groups. This is probably related to the intrinsic characteristics of adult testicular tissue, which has a thicker basement membrane than that of immature individuals (Richardson et al., 1995). Da Silva et al. (2021) also reported better preservation of the tubular structure when cryopreserving adult testicular tissue using the same protocol employed in the present study.

At 7 days, the DMEM medium showed significantly higher scores than DMEM/F12, differing statistically in the parameters of basement membrane separation and tubular structure, evidencing better preservation of the tubular architecture during this period. For the other parameters, no statistically significant differences were observed between the media.

In the comparison between the culture media over 14 days, DMEM showed better morphological preservation compared to DMEM/F12, with significantly higher values ($P < 0.05$) in the parameters of basement membrane separation, vacuolization, tubular cell loss, and tubular structure, demonstrating a greater capacity of DMEM to maintain the structural integrity of testicular tissue even after prolonged culture.

The better performance observed in the DMEM medium may be related to its enriched nutritional composition, which favors the maintenance, proliferation, and manipulation of different cell types, being widely used in cell and reproductive biology studies. Because it is a basal medium free of proteins and growth factors, DMEM requires adequate supplementation, such as fetal bovine serum, to become a complete medium (Arora, 2013). This characteristic allowed, in the present experiment, greater flexibility in the formulation of the medium, allowing its adaptation to the specific nutritional demands of testicular tissue.

Eagle demonstrated that supplementation with specific amino acids, vitamins, and inorganic salts significantly increases cell growth, culminating in the development of a standardized formulation widely adopted in biomedical research (Eagle, 1955). Additionally, DMEM has been successfully used in prolonged cultures of testicular tissue

from the same species, maintaining its morphological characteristics for up to 56 days, reinforcing its suitability for supporting testicular architecture and functionality in vitro.

Regarding the viability of testicular cells, it was significantly lower ($P < 0.05$) in the cryopreserved group when compared to that observed in the fresh group. This reduction was expected and was also reported by Da Silva et al. (2021) during cryopreservation of testicular tissue; however, the observed values remained within levels considered adequate, not compromising the quality or performance of the tissue during in vitro culture. When comparing the groups subjected to culture in different media and periods, no statistically significant differences were detected in cell viability as a function of the culture medium used or the interaction between group and culture time ($P > 0.05$). Pereira et al. (2025) also demonstrated the maintenance of testicular cell viability even after a prolonged culture period, extending up to 56 days.

The fresh control group of non-apoptotic cells differed statistically from all other groups, reflecting the ideal physiological condition of the tissue without manipulation. The cryopreserved group showed a significant reduction in this parameter, but maintained higher values than those observed in the cultured groups. After in vitro culture, at both 7 and 14 days, a further reduction in the percentages of non-apoptotic cells was observed, with no statistically significant differences between the DMEM and DMEM/F12 media, nor between the culture times.

The percentages of early apoptosis remained low and similar among all groups evaluated, with no statistically significant differences observed. This finding is particularly relevant, since apoptosis plays a fundamental role in spermatogenesis, and the control and maintenance of an adequate number of cells is essential for the correct development of spermatozoa (Hikim et al., 1999).

Similarly, the levels of late apoptosis also remained low in all groups, with no statistical differences between treatments. Considering that apoptosis is a specific type of cell death triggered by different signals and pathophysiological conditions, including oxidative stress (Hampton et al., 1998), the absence of a significant increase in these parameters indicates that neither in vitro culture nor cryopreservation promoted the progressive expression of programmed cell death. This result is consistent with evidence that the mode of cell death is directly related to the intensity of oxidative damage (Ozben

et al., 2007), suggesting that the procedures employed did not induce critical levels of stress capable of broadly activating apoptotic pathways.

In conclusion, this study demonstrates that testicular tissue from adult peccaries, after cryopreservation and subsequent in vitro culture in DMEM/F12 and supplemented DMEM media, can be viable for up to 14 days. Furthermore, the results obtained provide relevant initial information, contributing significantly to the advancement and standardization of specific protocols for testicular culture in this species.

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PERSPECTIVAS

A abordagem experimental delineada neste estudo teve como objetivo estabelecer um protocolo eficiente para o cultivo *in vitro* de tecido testicular de catetos adultos. Por meio desse protocolo, foi possível elucidar aspectos intrínsecos à espermatogênese com base nas respostas adaptativas do tecido testicular ao longo do período de cultivo, permitindo a análise comparativa entre o tecido fresco e aquele previamente submetido à criopreservação. Esses achados contribuem para o aprofundamento do conhecimento acerca da fisiologia reprodutiva dessa espécie, bem como para o potencial aproveitamento do germoplasma armazenado em biobancos.

Ademais, os resultados apresentados constituem uma base consistente para futuras investigações envolvendo o cultivo de tecido testicular adulto de catetos por períodos de até 14 dias. Esse avanço representa um passo relevante para o desenvolvimento de estratégias voltadas à obtenção de espermatozoides a partir de amostras testiculares criopreservadas em biobancos ou provenientes de indivíduos que venham a óbito de forma inesperada, fortalecendo, assim, abordagens biotecnológicas aplicadas à conservação da espécie.

ANEXOS

Anexo I: Trabalho publicado na IX Reunião Anual da ABRAA

Anexo II: MENÇÃO HONROSA na IX Reunião Anual da ABRAA

Anexo III: Trabalho publicado Conferência Anual da Sociedade Internacional de Tecnologia de Embriões (IETS)

Efeito de diferentes meios sobre a viabilidade celular e a integridade do DNA no tecido testicular de catetos adultos durante o cultivo organotípico

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Nos últimos anos, a tecnologia do tecido testicular tem surgido como uma alternativa efetiva para a conservação do germoplasma masculino, uma vez que permitiria um estoque ilimitado de espermatogônias e, consequentemente, de espermatozoides para aplicação em outras biotecnologias. Paralelamente aos avanços nos protocolos de criopreservação, é necessário o desenvolvimento de estratégias de utilização daquele germoplasma por meio do cultivo in vitro, cujo sucesso pode ser influenciado por diferentes fatores, tais como a idade do animal e o meio utilizado. No presente estudo, objetivou-se comparar diferentes meios (DMEM, MEM e DMEM/F12) utilizados no cultivo in vitro organotípico do tecido testicular de catetos (Pecari tajacu) adultos. Salienta-se que os catetos são animais selvagens de grande importância ecológica, cujas populações estão declinando em alguns biomas como a Caatinga e a Mata Atlântica. Os procedimentos foram autorizados pelo Comitê de Ética da UFERSA (Parecer nº 24/2023), com autorização SISBIO nº 72170-3. Assim, após eutanásia, foram coletadas as gônadas de cinco animais adultos, as quais foram dissecadas, lavadas em solução salina e fragmentadas (1 a 3 mm^3). Parte dos fragmentos foi destinada ao grupo controle fresco, e os demais aos grupos experimentais submetidos ao cultivo organotípico procedido por 14 dias a 34 °C, em atmosfera umidificada com 5% de CO_2. Foram testados três diferentes meios de cultivo: meio essencial modificado por Dulbecco (DMEM), meio essencial mínimo (MEM) e DMEM suplementado de nutrientes (DMEM/F12). A troca completa dos meios ocorreu a cada três dias, totalizando 5 trocas ao longo do experimento. Os fragmentos foram avaliados aos 7 e aos 14 dias quanto à viabilidade por sondas fluorescentes (Hoechst 33358 e iodeto de propídio), e quanto à integridade do DNA por meio do laranja de acridina em associação ao brometo de etídio. Os dados foram expressos como média ± erro padrão, avaliados pelos testes de Tukey e Sidak, utilizando o software GraphPad Prism® versão 9.3 (GraphPad Software Inc., San Diego, CA, EUA), com $p < 0,05$. No grupo fresco, observou-se viabilidade celular de 89,80 ± 3,01%, com 79,80 ± 11,20% de integridade do DNA. No 7º dia, os valores de viabilidade nos meios F12, MEM e DMEM foram de 46,40 ± 2,87%, 52,40 ± 5,49% e 46,60 ± 2,25%, enquanto a integridade do DNA foi de 42,60 ± 5,76%, 48,40 ± 2,86 e 51,00 ± 3,11 respectivamente. No 14º dia, a redução persistiu, com valores de viabilidade de 39,80 ± 3,68%, 40,60 ± 5,66% e 39,80 ± 4,47%, com integridade de DNA em torno de 35,40 ± 4,99%, 42,40 ± 5,18% e 35,00 ± 1,70%, respectivamente para F12, MEM e DMEM. Ao se comparar a viabilidade celular e a integridade do DNA do grupo fresco com as células cultivadas, verificou-se uma redução significativa ao longo do tempo em todos os meios de cultivo (P

< 0,05). Porém, não foram detectadas diferenças entre os três meios no tocante à manutenção dos parâmetros avaliados. De modo geral, acredita-se que a composição nutritiva dos três meios seja igualmente adequada à manutenção celular no decorrer do cultivo in vitro pelo período de 14 dias, porém outros parâmetros celulares necessitam ser melhor investigados. Ao momento, pode-se concluir que os meios MEM, DMEM e DMEM/F12 são igualmente adequados para o cultivo organotípico de tecido testicular de catetos adultos. No entanto, recomenda-se a utilização do meio essencial mínimo (MEM), uma vez que apresenta um custo de aquisição mais acessível.

Palavras-chave: biobanco, tecido gonadal, espermatogênese, meio de cultura.

Financiamento: CAPES, CNPq

Effect of different media on cell viability and DNA integrity in testicular tissue of adult collared peccaries during organotypic culture

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In recent years, testicular tissue technology has emerged as an effective alternative for the conservation of male germplasm, since it would allow an unlimited stock of spermatogonia and, consequently, spermatozoa for application in other biotechnologies. In parallel to the advances in cryopreservation protocols, it is necessary to develop strategies for using that germplasm through in vitro culture, the success of which can be influenced by different factors, such as the age of the animal and the medium used. The present study aimed to compare different media (DMEM, MEM and DMEM/F12) used in the in vitro organotypic culture of testicular tissue from adult collared peccaries (Pecari tajacu). It should be noted that collared peccaries are wild animals of great ecological importance, whose populations are declining in some biomes such as the Caatinga and the Atlantic Forest. The procedures were authorized by the UFERSA Ethics Committee (Opinion no. 24/2023), with SISBIO authorization no. 72170-3. Thus, after euthanasia, the gonads of five adult animals were collected, dissected, washed in saline solution and fragmented (1 to 3 mm^3). Part of the fragments was allocated to the fresh control group, and the rest to the experimental groups subjected to organotypic culture for 14 days at 34 °C, in a humidified atmosphere with 5% CO_2. Three different culture media were tested: Dulbecco's modified essential medium (DMEM), minimum essential medium (MEM) and DMEM supplemented with nutrients (DMEM/F12). The complete change of media occurred every three days, totaling 5 changes throughout the experiment. The fragments were evaluated at 7 and 14 days for viability using fluorescent probes (Hoechst 33358 and propidium iodide), and for DNA integrity using acridine orange in association with ethidium bromide. Data were expressed

as mean ± standard error, evaluated by Tukey and Sidak tests, using GraphPad Prism® software version 9.3 (GraphPad Software Inc., San Diego, CA, USA), with $p < 0.05$. In the fresh group, cell viability of 89.80 ± 3.01% was observed, with 79.80 ± 11.20% DNA integrity. On the 7th day, the viability values in the DEM/F12, MEM and DMEM media were 46.40 ± 2.87%, 52.40 ± 5.49% and 46.60 ± 2.25%, while DNA integrity was 42.60 ± 5.76%, 48.40 ± 2.86 and 51.00 ± 3.11 respectively. On the 14th day, the reduction persisted, with viability values of 39.80 ± 3.68%, 40.60 ± 5.66% and 39.80 ± 4.47%, with DNA integrity around 35.40 ± 4.99%, 42.40 ± 5.18% and 35.00 ± 1.70%, respectively for DEM/F12, MEM and DMEM. When comparing the cell viability and DNA integrity of the fresh group with the cultured cells, a significant reduction was observed over time in all culture media ($P < 0.05$). However, no differences were detected between the three media regarding the maintenance of the evaluated parameters. In general, it is believed that the nutritional composition of the three media is equally adequate for cell maintenance during in vitro culture for a period of 14 days, however other cellular parameters need to be further investigated. At present, it can be concluded that MEM, DMEM and DMEM/F12 media are equally suitable for the organotypic culture of testicular tissue from adult collared peccaries. However, the use of minimum essential medium (MEM) is recommended, since it has a more affordable acquisition cost.

Keywords: biobank, gonadal tissue, spermatogenesis, culture medium.

Financial support: CAPES, CNPq

Efficacy of different media on the organotypic *in vitro* culture for cryopreserved testicular tissue of adult collared peccaries

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Cell cultures are essential for recovering and maintaining the viability of genetic material stored in biobanks, enabling their effective use in assisted reproduction programs aimed at preserving valuable genotypes. However, testicular tissue from adult individuals presents a greater challenge, given the greater complexity and cellular heterogeneity of the tissue compared to immature animals. The objective was to compare a basic (DMEM) with an enriched medium (DMEM/F12) in the *in vitro* organotypic culture of cryopreserved testicular tissue from adult collared peccaries (*Pecari tajacu*). The procedures were approved by the UFERSA Ethics Committee (No. 24/2023) and by SISBIO (No. 72170-3). After euthanasia, gonads from five adult animals were collected, dissected, washed with sterile saline, and fragmented (1–3 mm^3). Part was used as a fresh control and the remainder was vitrified in Minimum Essential Medium (MEM) with 0.25 M sucrose, 10% FBS and DMSO/EG (1.5 M each). Finally, the samples were stored in cryovials and exposed to liquid nitrogen. After one week, the samples were warmed and cultured in vitro for 14 days at 34°C with 5% CO_2, using DMEM or DMEM/F12 media. Media were changed every three days, totaling five changes. Cell viability was assessed on days 7 and 14 by Hoechst 33358 and propidium iodide marking. DNA integrity was analyzed with acridine orange and ethidium bromide. Data are presented as mean ± standard error and analyzed by Tukey's and Holm's tests ($P < 0.05$). In the fresh control, 89.8±3.0% cell viability and 79.8±11.2% DNA integrity were observed. Immediately after cryopreservation and heating, there was a significant decrease ($P < 0.05$) in cell viability (48.4 ± 3.78%) and DNA integrity (45.4 ± 2.48%). On the 7th day of culture, the samples maintained in DMEM/F12 and DMEM media showed similar ($P > 0.05$) cell viability (22.2 ± 6.47% vs. 21.5 ± 6.67%) and DNA integrity (18.6 ± 5.45% and 16.8 ± 3.5). On the 14th day, a sharp drop in cell viability was observed ($P < 0.05$) for both groups, reaching values of 1.6±1.0% in DMEM/F12 and 9.00 ± 3.48% in DMEM. DNA integrity also followed this trend, with values of 14.4 ± 6.6% (DMEM/F12) and 6.00 ± 4.43% (DMEM). No significant differences between groups were detected in any time of the *in vitro* culture ($P > 0.05$). In conclusion, the media were similar during the organotypic culture of cryopreserved testicular tissue from adult collared peccaries for 14 days; however, due to the higher cost associated with the enriched medium, the use of DMEM is suggested for this purpose.

Keywords: gonadal tissue, cryopreservation, culture medium

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