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ERICA CAMILA GURGEL PRAXEDES

OTIMIZAÇÃO DO PROTOCOLO DE VITRIFICAÇÃO E ESTABELECIMENTO DO SISTEMA DE CULTIVO IN VITRO PARA O TECIDO OVARIANO DE CUTIAS (*Dasyprocta leporina*)

MOSSORÓ
2021

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

Linha de pesquisa: Morfofisiologia e Biotecnologia Animal

Orientador: Prof. Dr. Alexandre Rodrigues Silva - UFERSA

Co-orientadora: Profa. Dra. Alexsandra Fernandes Pereira.

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Érica Camila Gurgel Praxedes (discente)

Dedicatória,

A Josimary (mamis) por ser o meu MAIOR símbolo de força e coragem.

(In memorian)

TE AMO MÃE!

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A DEUS pelos 731 dias de oportunidades durante esses 4 anos de doutorado, e por toda a força e a coragem a mim concedidos.

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“Nós somos aquilo que fazemos repetidamente. Excelência, então, não é um modo de agir, mas um hábito”.

Prof. Dr. Alexandre Rodrigues Silva

RESUMO

As cutias são mamíferos silvestres que apresentam importantes funções ecológicas por serem dispersadoras de sementes, além de contribuírem para o equilíbrio da cadeia alimentar. Esses animais são utilizados como modelo experimental tanto para o aperfeiçoamento de técnicas reprodutivas, quanto para a formação de bancos de germoplasma. Nesse contexto, o objetivo do presente estudo foi utilizar a manipulação de oócitos inclusos em folículos ovarianos pré-antrais (MOIFOPA) como ferramenta para o resgate e conservação do uso de gametas femininos de cutias (*Dasyprocta leporina*). A tese foi dividida em dois capítulos experimentais. No primeiro experimento foi analisado o uso do TCM-199 suplementado com diferentes concentrações de pFSH (10 e 50 ng/mL) sobre a morfologia, desenvolvimento e viabilidade de folículos ovarianos pré-antrais (FOPAS) inclusos em tecido ovariano. Em seguida, verificou-se o efeito desse sistema de cultivo *in vitro* (CIV) sobre os mesmos parâmetros dos FOPAS previamente à vitrificação. Após o cultivo, o grupo FSH50 apresentou maior porcentagem de folículos morfologicamente normais quando comparado ao grupo FSH10 ($P < 0,05$). Além disso, essa mesma resposta foi observada para os folículos primordiais. Independentemente das concentrações de FSH utilizadas durante o CIV, não foi observada diferença em relação ao percentual de folículos viáveis ($P > 0,05$). Assim, o grupo FSH50 foi utilizado para experimento posterior, no qual um total de 76,2 ± 7,2% de PAFs normais previamente vitrificados foi observado após a cultivo de 6 dias, apresentando os maiores valores ($P < 0,05$) para a morfologia dos folículos primordiais (95,2 ± 4,7 %). Contudo, o cultivo manteve a viabilidade dos FOPAS derivados de tecidos criopreservados ($P > 0,05$). No segundo experimento, os ovários de oito fêmeas foram recuperados e fragmentados, com quatro fragmentos de córtex imediatamente fixados e avaliados (grupo fresco). Os demais fragmentos foram processados pelo método de vitrificação de superfície sólida (SSV) ou ovarian tissue cryosystem (OTC) usando soro fetal bovino (SFB), etilenoglicol (EG) e sacarose (SAC) como crioprotetores, armazenados por duas semanas a -196 ºC e aquecidos. Posteriormente, os fragmentos foram submetidos a um cultivo *in vitro* de 24 h e avaliados quanto à carga microbiológica, morfologia dos FOPAS e integridade do DNA. Não houve contaminação fúngica; entretanto, as amostras vitrificadas de dois indivíduos apresentaram contaminação bacteriana de 79.200 unidades formadoras de colônias por mililitro (UFC) / mL para SSV e 3.120 UFC / mL para OTC. Em relação à morfologia dos FOPAS, ambos os sistemas proporcionaram preservação adequada da morfologia, com valores superiores a 70% dos folículos normais observados antes e após a cultivo. O TUNEL revelou que tanto a SSV (52,39%) quanto o OTC (41,67%) podem preservar a integridade do DNA após a vitrificação e após 24 h de cultivo. Para a proliferação celular, baixas taxas foram detectadas para amostras frescas (11,53%) e SSV (29,87%); contudo, nenhuma evidência de proliferação foi encontrada quando OTC foi usado. Assim, o uso da MOIFOPA em cutias permitiu a conservação através da vitrificação em superfície sólida e utilizando o sistema OTC, bem como apresentou resultados promissores para o cultivo *in vitro* de FOPAS inclusos em tecido ovariano previamente vitrificados.

Palavras-chaves: folículos, germoplasma, cultivo *in vitro*, cutia.

ABSTRACT

The agouti are wild mammals that have important ecological functions for being seed dispersers, in addition to contributing to the balance of the food chain. These animals are used as an experimental model both for the improvement of reproductive techniques and for the formation of germplasm banks. In this context, the aim of the present study was to use the manipulation of oocytes included in preantral ovarian follicles (MOIFOPA) as a tool for the rescue and conservation of the use of female agouti gametes (*Dasyprocta leporina*). The thesis was divided into two experimental chapters. In the first experiment, the use of TCM-199 supplemented with different concentrations of pFSH (10 and 50 ng/mL) was analyzed on the morphology, development and viability of preantral ovarian follicles (PAFs) included in ovarian tissue. Then, the effect of this in vitro culture system (IVC) on the same PAFs parameters prior to vitrification was verified. After cultivation, the FSH50 group had a higher percentage of morphologically normal follicles when compared to the FSH10 group ($P < 0.05$). Furthermore, this same response was observed for primordial follicles. Regardless of the FSH concentrations used during the IVC, no difference was observed in relation to the percentage of viable follicles ($P > 0.05$). Thus, the FSH50 group was used for a further experiment, in which a total of 76.2 ± 7.2% of previously vitrified normal PAFs was observed after 6 days of cultivation, with the highest values ($P < 0.05$) for the morphology of the primordial follicles (95.2 ± 4.7%). However, the culture maintained the viability of FOPAS derived from cryopreserved tissues ($P > 0.05$). In the second experiment, the ovaries of eight females were retrieved and fragmented, with four cortex fragments immediately fixed and evaluated (fresh group). The remaining fragments were processed by the solid surface vitrification (SSV) or ovarian tissue cryosystem (OTC) method using fetal bovine serum (SFB), ethylene glycol (EG) and sucrose (SAC) as cryoprotectants, stored for two weeks at -196 ºC and heated. Afterwards, the fragments were submitted to an in vitro culture for 24 h and evaluated for microbiological load, PAFs morphology and DNA integrity. There was no fungal contamination; however, vitrified samples from two individuals showed bacterial contamination of 79,200 colony-forming units per milliliter (CFU) / mL for SSV and 3.120 CFU / mL for OTC. Regarding the morphology of PAFs, both systems provided adequate preservation of morphology, with values above 70% of normal follicles observed before and after cultivation. TUNEL revealed that both SSV (52.39%) and OTC (41.67%) can preserve DNA integrity after vitrification and after 24 h of culture. For cell proliferation, low rates were detected for fresh samples (11.53%) and SSV (29.87%); however, no evidence of proliferation was found when OTC was used. Thus, the use of manipulation of oocytes included in preantral ovarian follicles in agouti allowed conservation through vitrification on solid surface and using the OTC system, as well as presented promising results for the in vitro culture of PAFs included in previously vitrified ovarian tissue.

Keywords: follicles, germplasm, *in vitro* culture, agouti.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

% Porcentagem
± Mais ou menos
+ Mais
- Menos
< Menor
> Maior
~ Semelhante
nº número
µ Micrometro
ºC Graus Celsius
°C/min Graus Celsius por minuto
ACP Agente crioprotetor
ANOVA Análise de Variância
CEMAS Centro de multiplicação de animais silvestres
CIV Cultivo *in vitro*
cm Centímetros
CPA Crioprotetor
DMSO Dimetilsulfóxido
DNA ácido desoxirribonucleico
EG Etilenoglicol
FCS fetal calf serum
FIV Fecundação *in vitro*
FOPA Folículos ovarianos pré–antrais
g Grama
g/mol gramas/mol
h Hora
ICMbio Instituto Chico Mendes de Conservação da Biodiversidade
IUCN (International Union for Conservation of Nature) União Internacional de Conservação da Natureza
Kg Kilograma
kV Quilovolt
LN2 Nitrogênio Líquido
M Molar
µg/mL Micrograma por mililitro
µL microlitro
mL Mililitro
mm Milímetros
mm^3 Milímetros cúbico
m^2 Metro quadrado
min Minutos
Mm Milimolar

mol/L Mol por litro
mL/ml Miligrama por mililitros
mL/kg Mililitro por quilo
mg/l Miligrama por litro
MEM Meio Essencial mínimo
MIV Maturação *in vitro*
MOIFOPA Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais
PIVE Produção *in vitro* de embriões
PBS Phosphate Buffered Saline
PFs Folículos pré – antrais
pg/ml Picograma por mililitro
RN Rio Grande do Norte
s Segundo
SE Erro Padrão
SEM Erro Padrão e Média
SCID Imunodeficiência severa combinada
SFB soro fetal bovino
SSV vitrificação em superfície sólida
SV solução de vitrificação
SUC Sacarose
TdT Enzima terminal deoxinucleotidil transferase
UFERSA Universidade Federal Rural do Semi-árido
VS Vitrification solution
x Vezes

SUMÁRIO

1. INTRODUÇÃO

O Brasil apresenta-se como um dos principais alvos do desmatamento e caça predatória, uma vez que está entre os países mais ricos em biodiversidade (MITTERMEIER et al., 2005). Dentre os biomas mais afetados encontra-se o bioma Caatinga, no qual já foram catalogadas diversas espécies animais, incluindo as cutias *(Dasyprocta leporina)* as quais têm sido caçadas pela população para serem utilizadas como uma fonte alternativa de proteína animal, assim como para a produção de pele, couro e pelos (SANTOS et al., 2007). Ecologicamente, estas espécies são de suma importância para o equilíbrio da cadeia alimentar, contribuindo para a manutenção de seus predadores (HOSKEN; SILVEIRA, 2001), além de atuarem diretamente na dinâmica das populações vegetais e como dispersoras de sementes (FONTANA et al., 2003).

Além de sua importância econômica e ecológica, esses roedores silvestres, são utilizados como modelos experimentais para outras espécies vulneráveis e ameaçadas de extinção por apresentarem baixo custo de manutenção, curto período de gestação e pequeno porte (PUKAZHENTHI et al., 2006). Por serem considerados como uma fonte de renda para a população, vem sofrendo com a crescente caça predatória, e a criação em cativeiro surge como uma alternativa para a sua conservação (SANTOS et al., 2007).

Dessa forma, a criação em cativeiro representa uma alternativa tanto para a oferta regular de produtos como a carne e a pele, como para a conservação da espécie (BONAUDO et al., 2005). Para tanto, faz-se necessária a aplicação de programas de reprodução em cativeiro e técnicas de reprodução assistida como formas de garantir a conservação do material genético feminino (SOUZA et al., 2003), merecendo destaque a criação de bancos de germoplasma. Entretanto, mesmo a maioria dos trabalhos estando voltados para a manipulação e conservação de gametas masculinos (Criopreservação de espermatozóides epididimários - CASTELO et al., 2010; Método de coleta de espermatozóides - BEZERRA et al., 2014; Eletroejaculação - COSTA et al., 2014; Diferentes métodos de coloração - SILVA et al., 2014; Congelação e descongelação - CAMPOS et al., 2014), já se têm algumas informações a respeito do desenvolvimento e aplicações de biotécnicas relacionadas às fêmeas (Congelação lenta de tecido ovariano - WANDERLEY et al., 2012; Estimativa da população folicular - SANTOS et al., 2018; Vitrificação de tecido ovariano e xenotransplante PRAXEDES et al., 2015, 2018).

Nesse sentido, visando a otimização do uso desse material genético vem sendo desenvolvida a biotécnica de manipulação de oócitos inclusos em folículos ovarianos pré antrais (MOIFOPA), a qual consiste no isolamento ou resgate de folículos ovarianos pré-antrais

(FOPAS) e na criopreservação de tecido ovariano (SANTOS et al., 2008), sendo está uma etapa importante, uma vez que poderá manter a viabilidade folicular até que seja realizado o cultivo *in vitro* (CIV) ou transplante ovariano. A criopreservação pode ser realizada por meio de dois métodos como a congelação lenta e a vitrificação. Este último consiste na solidificação de uma solução utilizando baixas temperaturas (FAUSTINO et al., 2011), com taxas ultra rápidas de congelação e altas concentrações de agentes crioprotetores (ACPs) proporcionando a solidificação da célula, sem cristalização (MUKAIDA; OKA 2012). Dentre as diversas técnicas de vitrificação, a que utiliza a superfície sólida e o *ovarian cryosystem tissue* (OTC) vem se destacando nos últimos anos, por utilizar uma superfície metálica e de aço inoxidável, respectivamente, com boa condução de calor, o que proporciona o rápido resfriamento da amostra, condição essa fundamental para uma vitrificação eficiente (MOAWAD et al., 2012; CARVALHO et al., 2013).

Dessa forma, estudos relacionados à conservação de material genético feminino são de grande importância para espécies silvestres, como a cutia, pois além de possibilitar o estudo de suas particularidades fisiológicas, permitem a aplicação da biotécnica de MOIFOPA como estratégia para uma maior compreensão dos mecanismos envolvidos na foliculogênese inicial e uso do material genético para a aplicação em outras biotécnicas, visando a conservação e multiplicação dentro de um sistema produtivo.

2. REVISÃO DE LITERATURA

2.1. Biotécnicas reprodutivas aplicadas a *Dasyprocta leporina*

A cutia (*Dasyprocta leporina*) é um roedor mamífero silvestre pertencente a classe *Mammalia*, ordem *Rodentia*, subordem *Hystricomorpha*, infraordem *Hystricognathi* com ampla distribuição na floresta Amazônia, Atlântica, Cerrado e Caatinga (KAISER et al., 2011; SILVA et al., 2003d). Este animal tem hábito crepuscular e atividades noturnas, são naturalmente herbívoros, alimentando-se de folhas, raízes, flores, fungos, sementes e, especialmente, de frutos encontrados no solo (BOSSO, 2007). É um roedor bastante sociável que se reproduz em qualquer época do ano, até mesmo nas épocas secas e na ausência de abundância de alimento (LANGER, 1998).

Esta espécie, assim como outros roedores apresenta um potencial para ser modelo experimental para outras espécies que estão vulneráveis a extinção. O IUCN lista a *Dasyprocta ruatanica*, *Dasyprocta mexicana*, *Dasyprocta coibae*, o rato-candango (*Juscelinomys candango*), e duas espécies de rato-do-mato (*Kunsia fronto; Wilfredomys oenax*) como considerados criticamente ameaçados e o ouriço-preto (*Chaetomys subspinosus*) considerável vulnerável a extinção.

A população das cutias é considerada estável e não preocupante, mas vem sofrendo com a crescente caça predatória (CATZEFLIS et al., 2014). Devido à ameaça iminente de extinção, estratégias de conservação destas espécies são requeridas, bem como a possibilidade de criação desses animais em cativeiro, com o intuito de diminuir a caça predatória e também fornecer uma fonte alternativa de proteína para a população (NOGUEIRA; NOGUEIRA-FILHO, 2011), bem como o desenvolvimento de biotécnicas reprodutivas que permitam a obtenção de maiores informações específicas sobre os gametas femininos da *D. leporina* permitiria a otimização do seu uso, aumentando seu potencial produtivo e reprodutivo.

Entretanto, a maioria dos estudos estão voltados para os machos desta espécie. Estudos acerca da morfofisiologia das fêmeas poderia elucidar quais são os mecanismos envolvidos na foliculogênese inicial, bem como otimizar as diversas biotécnicas reprodutivas a partir de um único ovário e obtenção de um grande número de oócitos. Desse modo, nos capítulos 1 e 2 serão abordadas, em detalhes, as biotécnicas reprodutivas desenvolvidas e aplicadas para a espécie silvestre em estudo.

2.2. Vitrificação de tecido ovariano

A criopreservação de germoplasma é uma alternativa que diminui as limitações impostas pelo tempo e pela distância para a conservação de diversas espécies silvestres (COSTA; MARTINS, 2008). Essa biotécnica consiste na conservação de material biológico sob baixas temperaturas, a temperatura criobiológica do nitrogênio líquido (-196ºC). Essa técnica possibilita à célula ou tecido permanecerem viáveis por tempos indeterminados, podendo serem recuperados viáveis após descongelação (BAKHACH, 2009) devido à redução do seu metabolismo o qual permanece em fase de anabiose. Estudos por meio desse método em espécies silvestres apresentaram resultados promissores em cutias (WANDERLEY et al., 2012; PRAXEDES et al., 2020); preás (PRAXEDES et al., 2015) e catetos (CAMPOS et al., 2019; LIMA et al., 2019).

Independente de utilizar tecido ou oócito isolado, a criopreservação envolve as etapas de exposição ao agente crioprotetor ou período de equilíbrio; resfriamento; armazenamento; descongelação ou aquecimento e remoção do agente crioprotetor (SANTOS et al., 2008). A adição deste último é indispensável, sendo um fator crucial para o seu sucesso, sua presença é necessária para que as células resistam às injúrias resultantes da criopreservação (MYCOCK et al., 1995). Contudo, os metabólitos resultantes da sua degradação pela célula podem ser tóxicos, sendo este um fator limitante para o sucesso da utilização dos mesmos (FAHY, 2010).

De acordo com Amorim et al. (2011), vários fatores estão envolvidos para o sucesso da criopreservação, incluindo o tipo e concentração de ACPs, temperatura de exposição ao ACP, taxa de resfriamento e taxa de aquecimento. O tempo de exposição determina a permeação do ACP no tecido e nas células, afetando assim a vitrificação e a sobrevivência dos folículos (AMORIM et al., 2011). Existem vários métodos de conservação celular, entretanto, não há um método ideal que garanta a conservação perfeita das características morfofisiológicas e genéticas do material a ser criopreservado. Portanto, é imprescindível primeiramente avaliar o material biológico a ser conservado para realizar a escolha do método de conservação, considerando as vantagens e desvantagens de cada método (SOLA, 2012).

A criopreservação pode ser realizada por dois métodos distintos: congelação lenta ou vitrificação. A congelação lenta ou congelação convencional baseia-se na redução gradual da temperatura sob a utilização de um freezer programável, com a utilização de baixas concentrações de ACPs, promovendo o aumento na concentração de solutos extracelulares, levando a cristalização da água no interior da célula (PAYNTER, 2000). Característica

diretamente relacionada com o equilíbrio osmótico determinando a velocidade com que a água sai da célula (MAZUR, 1984; LUVONI, 2006). Na cutia (*Dasyprocta leporina*), este método já foi realizado com sucesso na criopreservação de tecido ovariano, e possibilitou a obtenção de até 64% de FOPAS morfologicamente normais após aquecimento das amostras (WANDERLEY et al., 2012).

Embora seja um método bastante difundido, seu uso leva a formação de cristais de gelo intracelulares, responsáveis por danos celulares irreversíveis durante a criopreservação (MORATÓ et al., 2008). A crioinjúria ocorre em decorrência da alta instabilidade provocada pelo super-resfriamento da água intracelular, sendo este fator principal para o rompimento das membranas plasmáticas celulares e consequentemente perda da integridade das mesmas, podendo induzir a morte celular por apoptose (LIEBERMANN et al., 2003).

Com o intuito de evitar estes danos, protocolos de vitrificação têm sido empregados surgindo como uma alternativa ao processo de congelação lenta (BANDEIRA et al., 2015). Devido, principalmente, a não formação de cristais de gelo, permitindo uma maior preservação da morfologia celular (WIEDEMANN et al., 2012). Além de não necessitar de máquina de congelação, levando a redução no tempo de processamento (OLIVEIRA, 2013).

Esse método consiste na redução ultra-rápida da temperatura, em uma velocidade média de 20.000 a 40.000ºC/min (WOWK, 2000). São utilizadas altas concentrações de ACPs associadas a outros constituintes na solução de vitrificação (SV) por curtos períodos (25 segundos a 8 min), como consequência, a água passa do estado líquido para um estado vítreo, considerado um sólido amorfo, sem exposição ao estágio cristalino (RALL e FAHY, 1985). Neste estado, partes das cadeias moleculares encontram-se desorganizadas, promovendo certa mobilidade as moléculas. Esta particularidade permite uma melhor acomodação das estruturas celulares (CARVALHO et al., 2011).

Muitos estudos vêm demostrando com sucesso a conservação de material genético feminino incluso em tecido ovariano utilizando o método de vitrificação em animais selvagens. O uso da vitrificação em ovários de cutias (*Dasyprocta leporina*) preservou 76% de FOPAS morfologicamente normais após vitrificação, bem como em preás (*Galea spixii* - PRAXEDES et al., 2015) onde foi observada mais de 70% dos FOPAS com morfologia normal após aquecimento das amostras. Estudos com veados (*Cervus elaphus hispanicu*) (GARCÍA-ÁLVAREZ et al., 2011) e cangurus (*Macropus giganteus*) (RICHINGS et al., 2006) evidenciaram que os FOPAS se mantiveram viáveis quando criopreservado inclusos em tecido ovariano. Em cutias (*Dasyprocta leporina* – PRAXEDES te al., 2018), e babuínos (AMORIM

et al., 2013) os autores relataram a sobrevivência folicular, crescimento e ovulação após autotransplante de tecido ovariano previamente vitrificado.

2.2.1. Técnicas de vitrificação

Diversas técnicas de vitrificação têm sido desenvolvidas objetivando a redução da solução de vitrificação (SANTOS et al., 2007). Dentre as principais técnicas, destaca-se a vitrificação em superfície sólida (TAKAHASHI et al., 2001; MIGISHIMA et al., SOMFAI et al., 2007; SANTOS et al., 2007; MOAWAD et al., 2012; PRAXEDES et al., 2015, 2018) a qual pode, inclusive, ser confeccionada artesanalmente com o uso de papel alumínio (SANTOS et al., 2007). Esta superfície, posicionada acima do nitrogênio líquido (N2), proporciona um rápido resfriamento da amostra, por ser boa condutora de calor, condição necessária para uma criopreservação eficiente (CAMPOS et al., 2019).

Desse modo a vitrificação em superfície sólida tem se tornado uma alternativa bastante promissora para a criopreservação de tecido ovariano em animais domésticos e silvestres, como bovinos (CELESTINO et al., 2010); ratos (XING et al., 2010), macacos (TING et al., 2011), caprinos (CARVALHO et al 2011), humanos (KAWAMURA et al., 2013), camundongos (FATEHI et al., 2014), preás (PRAXEDES et al., 2015), cutias (PRAXEXES et al., 2018; 2020) e catetos (LIMA et al., 2019; CAMPOS et al., 2019). Em murinos, foi possível obter nascimentos de crias viáveis de amostras previamente vitrificadas (MIGISHIMA et al., 2003). Além disso, em cutias (*Dasyprocta leporina*), foi observado o retorno da atividade ovariana em camundongas imunossuprimidas após o xenotransplante de amostras vitrificadas utilizando a superfície sólida (PRAXEDES et al., 2018). Entretanto, vitrificar tecido ovariano ainda é um desafio devido à diversidade dos componentes do tecido (HAVOTTA, 2005). E em animais silvestres, não é uma técnica bem estabelecida, sendo necessário o seu aprimoramento, principalmente quanto a concentração dos ACPs e a constituição da solução de vitrificação. Sabe-se que para se alcançar o sucesso em um protocolo de vitrificação algumas propriedades principais precisam ser consideradas: a taxa de resfriamento, resultando em queda da temperatura de centenas para dezenas de milhares de graus Celsius por minuto; a viscosidade do meio, adquirida através dos constituintes da solução de vitrificação e o volume, quanto menor o volume celular, mais rápido a transferência de calor e consequentemente maior taxa de resfriamento (SARAGUSTY; ARAV, 2011).

Outro método de vitrificação faz uso de um sistema específico, fechado, denominado *ovarian tissue cryosystem* (OTC). Esse sistema segue o mesmo princípio da vitrificação em superfície sólida, embora apresente a vantagem de facilitar o processo de remoção de ACPs, não havendo a necessidade do uso de criotubos ou palhetas e ausência do contato do operador com a solução de vitrificação.

O OTC é uma estrutura cilíndrica formada por três estruturas: uma base (2,1 cm de altura; 2,8 cm de diâmetro e 0,2 cm de espessura), em que as amostras são colocadas; uma inserção (2,8 cm de altura; 2,3 cm diâmetro e 0,1 cm de espessura) contendo 20 perfurações, para permitir a exposição e remoção da solução de vitrificação, e uma tampa (2,0 cm de altura; 2,8 cm de diâmetro e 0,2 cm de espessura), para fechar hermeticamente o dispositivo. Esse dispositivo é confeccionado em aço inoxidável, e pode suportar temperaturas inferiores a -196° C e superior a 200° C, até mesmo sob alta pressão, permitindo a sua esterilização e reutilização (CARVALHO et al., 2013a).

Além dessas características, também possibilita uma rápida troca de calor, acelerando os processos de redução de temperatura e posterior aquecimento do material vitrificado. Por apresentar forma cilíndrica, garante a uniformidade da redução de temperatura por toda a amostra, oferecendo assim maior segurança durante a realização do procedimento (CARVALHO et al., 2013).

Essa técnica já foi utilizada com sucesso na vitrificação de tecidos ovarianos caprinos (CARVALHO et al., 2014). Bandeira et al. (2015) comparam a técnica de OTC com a superfície sólida sobre a matriz extracelular e o desenvolvimento folicular ovariano *in vitro* em ovinos, foi verificado que o OTC preservou a viabilidade e a morfologia folicular, mantendo a integridade dos componentes da matriz extracelular. Recentemente, em catetos (*Pecari tajacu*), foi relatado que o OTC melhor preservou a viabilidade e morfologia folicular, bem como manteve a capacidade proliferativa, diminuindo a apoptose folicular quando comparada a SSV (CAMPOS et al., 2019).

No entanto, a grande maioria, por ser considerado um sistema aberto, não evita os riscos de contaminação da amostra pelo contato direto com o nitrogênio líquido (CARVALHO et al., 2013). Mas, mesmo o OTC sendo considerado um sistema fechado, é necessário análises microbiológicas, pois até o momento, não foi quantificado e identificado o grau de contaminação por microrganismos nas amostras previamente vitrificadas por esse método. A contaminação pode ocorrer em todos os processos de manipulação do tecido, que vão desde a coleta e processamento, até o armazenamento, visto que o nitrogênio líquido pode ser uma fonte

de contaminação. Os microrganismos podem estar presentes no ambiente (SANTOS et al., 2020), bem como em substâncias constitutivas de meios de CIV quando não manipuladas em ambientes assépticos e/ou no fluxo laminar.

2.4. Cultivo *in vitro* de tecido ovariano

O cultivo *in vitro* de FOPAS inclusos em tecido ovariano é uma técnica que vem sendo largamente empregada com o objetivo de avaliar o efeito de diferentes substâncias, em diferentes concentrações e em distintas fases do desenvolvimento folicular (CAMPOS et al., 2019). Além disso, possibilita o estudo dos fatores que controlam a foliculogênese, e pode ser utilizado como importante ferramenta para avaliar a eficiência da criopreservação, uma vez que lesões foliculares não detectadas pós-descongelação podem evoluir e serem manifestadas durante o CIV (FAUSTINO et al., 2011).

Nesse contexto, a eficiência do CIV pode ser afetada por fatores como o tipo de sistema de cultivo, a composição do meio base, a concentração e associação de fatores adicionados ao meio (FIGUEIREDO et al., 2007). No sistema *in situ*, é possível estudar principalmente os fatores que podem afetar o início do crescimento de folículos primordiais quiescentes e o crescimento de folículos primários (SILVA et al., 2004). Além da praticidade, este sistema tem a vantagem de manter o contato celular e a integridade tridimensional dos folículos (ABIR et al., 2011) fornecendo um complexo sistema de suporte que se assemelha ao ovário *in vivo* (PICTON et al., 2008).

A composição do meio é outro aspecto de extrema importância (CORTVRINDT SMITZ; 2002). Estes podem ser simples, contendo apenas sais inorgânicos e substratos energéticos ou em sua maioria, ser complexos contendo aminoácidos e vitaminas. Entre os meios mais utilizados, destacam-se o meio de cultivo celular 199 (TCM199) (CAMPOS et al., 2019; LIMA et al., 2019), meio essencial mínimo (MEM) (MATOS et al., 2007); meio essencial mínimo alfa (α-MEM+) (SARAIVA et al, 2010b; FATEHI et al., 2014; ZHANG et al., 2017) e meio McCoy's (TELFER et al., 2000; MCLAUGHLIN et al., 2010). Várias substâncias adicionadas podem influenciar a sobrevivência e o crescimento folicular, incluindo antibióticos, tampões, substratos nutricionais, diferentes fontes proteicas, antioxidantes, hormônios e fatores de crescimento (FIGUEIREDO et al., 2007; CHAVES et al., 2010).

Nesse sentido, vários estudos demonstraram que hormônios, fatores de crescimento e peptídeos estão envolvidos no controle do desenvolvimento de FOPAS, destacando-se o hormônio folículo estimulante (FSH).

O hormônio gonadotrófico FSH é uma glicoproteína produzida na adenohipófise e que, em fêmeas, age primeiramente no folículo ovariano. É essencial para a promoção da esteroidogênese através da estimulação da atividade da enzima aromatase, promove a proliferação e diferenciação das células da granulosa e a conexão entre estas células e o oócito (ALBERTINI et al., 2001). *In vitro*, foi demonstrado que níveis basais de FSH são necessários para o desenvolvimento de pequenos folículos, mas ainda não está claro de que maneira o FSH afeta o desenvolvimento de pequenos FOPA. Tem sido sugerido que este hormônio excerce um efeito indireto sobre o desenvolvimento folicular inicial através da liberação de fatores parácrinos produzidos por folículos maiores ou pelas células do estroma ovariano (VAN DEN HURK; ZHAO, 2005). Estudos demonstraram que o FSH regula a expressão de vários fatores de crescimento, como Kit Ligand (KL), Fator de Diferenciação e Crescimento 9 (GDF-9) e Proteína Morfogenética Óssea 15 (BMP-15), os quais possuem papéis importantes na ativação e crescimento folicular (JOYCE et al., 1999, THOMAS et al., 2005).

Dentre os métodos de cultivo comumente utilizados, destaca-se o cultivo do ovário inteiro, de fragmentos do córtex ovariano e de folículos isolados. O cultivo do ovário inteiro é bastante útil para o estudo da foliculogênese inicial em pequenos mamíferos, uma vez que o pequeno tamanho do ovário possibilita este tipo de cultivo (JIN et al., 2010), entretanto, em animais de médio e grande porte é preferível o cultivo de fragmentos do ovário. Assim, fragmentos de córtex ovariano tem a vantagem de manter o contato celular e facilitar a perfusão do meio para o tecido ovariano (LIMA et al., 2019).

Dessa forma, estudos relacionados a conservação e CIV são de grande importância para espécies silvestres, gerando conhecimentos fundamentais para a aplicação de outras biotécnicas com o objetivo de se conservar estas espécies e outras próximas filogeneticamente a estas que estejam ameaçadas de extinção (LIMA et al., 2019).

3. JUSTIFICATIVA

As cutias desempenham uma importante função ecológica no equilíbrio e na composição de cadeias alimentares, contribuindo substancialmente para a manutenção de seus predadores. Como se adapta facilmente às condições de cativeiro e apresentar baixo custo de manutenção, curto período de gestação e pequeno porte é considerada um excelente modelo experimental para outras espécies ameaçadas de extinção. Dente as espécies silvestres, são as mais indicadas para serem exploradas comercialmente, através da venda de sua carne e pele no mercado internacional, bem como, de animais machos e fêmeas para servirem como reprodutores em criatórios comerciais.

Nesse sentido, estratégias de conservação *in situ*, como a preservação de habitats, em associação com as biotécnicas reprodutivas são de grande importância na preservação desta espécie. Dentre estas biotécnicas, destaca-se a MOIFOPA, a qual visa o aumento do potencial reprodutivo de fêmeas, a partir do resgate, conservação, CIV e maturação *in vitro* (MIV) de FOPA. Desde modo, com o aprimoramento da biotécnica de MOIFOPA, há a possibilidade de um maior aproveitamento deste material genético, possibilitando seu uso em bancos de germoplasma e em outras biotécnicas como a FIV.

Adicionalmente, na busca por um eficiente sistema de cultivo de folículos pré-antrais, diversos pesquisadores têm aprofundado as investigações sobre o efeito e a concentração ideal de diferentes fatores de crescimento, que atuem diretamente no desenvolvimento folicular. Já é bem estabelecido que o FSH estimula o desenvolvimento de folículos pré-antrais *in vitro* em diferentes espécies, mas em cuitas, o papel desta substância sobre a foliculogênese *in vitro* ainda continua desconhecido. Diante disso, constatou-se a necessidade de se estudar os efeitos de diferentes concentrações de FSH sobre o cultivo *in vitro* de folículos ovarianos pré-antrais, permitindo assim o estabelecimento de estratégias para melhorar a ativação e o crescimento folicular *in vitro*.

Um dos principais obstáculos na adaptação e adoção de técnicas de reprodução assistida (ATRs) em animais silvestres é a escassez de informações sobre sua fisiologia reprodutiva. Dessa forma, a MOIFOPA consiste em importante ferramenta na aquisição destas informações, permitindo não só o resgate dos FOPA do ambiente ovariano antes que estes iniciem a atresia, mas ao se avaliar a influência de diversos componentes sobre o desenvolvimento e sobrevivência folicular *in vitro*, permite uma melhor compreensão sobre os fatores que regulam estes eventos no ambiente ovariano.

Em fêmeas de cutias, já existem estudos iniciais acerca da estimativa e caracterização da população folicular, tendo sido descrita, inclusive, a sua conservação utilizando o método de vitrificação em superfície solida. Dessa forma, com base nestas informações, foi possível realizar o cultivo *in vivo* através do xenotransplante de tecido ovariano o que representou um passo importante para os estudos acerca das suas particularidades morfofisiológicas.

Diante do exposto, sugerem-se novas pesquisas priorizando o aprimoramento de protocolos de CIV e vitrificação, visando, melhores taxas de desenvolvimento e conservação de FOPAS, no intuito de obter estruturas viáveis para aplicabilidade futura. A aplicação da biotécnica de MOIFOPA como estratégia para o maior conhecimento e uso do material genético feminino permitiria não só o aumento do potencial reprodutivo, mas também a preservação de espécies silvestres, como cutias.

4. HIPÓTESES CIENTÍFICAS

- O TCM199 adicionado ao meio de cultivo *in vitro* suplementado com 50 ng/ml de pFSH é eficiente para manter a morfologia, desenvolvimento e viabilidade de folículos ovarianos pré-antrais de cutias;

- A vitrificação de tecido ovariano em superfície sólida e o ovarian tissue cryosystem permitem a efetiva conservação de gametas femininos de cutias (*Dasyprocta leporina*);

- O OTC permite um melhor controle da contaminação bacteriana do que o método de superfície sólida na vitrificação de tecido ovariano de cutias (*Dasyprocta leporina*).

5. OBJETIVOS

5.1.OBJETIVO GERAL

- Otimizar a técnica de vitrificação e o desenvolver um sistema de cultivo *in vitro* para o tecido ovariano de cutias (*Dasyprocta leporina*).

5.2. OBJETIVOS ESPECÍFICOS

- Testar um sistema de cultivo *in vitro* utilizando TCM199 suplementado com diferentes concentrações de pFSH sobre a morfologia, desenvolvimento e viabilidade de folículos ovarianos preantrais de cutias submetidas ao cultivo *in vitro* por 6 dias;

- Verificar as interações entre o sistema de cultivo *in vitro* e a técnica de vitrificação do tecido ovariano de cutias;

- Comparar o efeito da técnica de vitrificação em SSV e OTC sobre a contaminação microbiológica e preservação de folículos ovarianos pré antrais de cutias

REFERÊNCIAS BIBLIOGRÁFICAS

ABIR, R.; FISCH, B.; JESSEL, S.; FELZ, C.; BEN-HAROUSH, A,; ORVIETO, R. Improving posttransplantation survival of human ovarian tissue by treating the host and graft. **Fertility and Sterility**, v.95, p.1205–10, 2011.

AMORIM, C.A.; CURABA, M.; VAN LANGENDONCKT, A.; DOLMANS, M.M.; AND DONNEZ, J. Vitrification as an alternative means of cryopreserving ovarian tissue. **Reproduction Biology Medicine Online.** v.23, p.160– 186, 2011.

AMORIM, C.A.; JACOBS, S.; DEVIREDDY, R.V.; VAN LANGENDONCKT, A.; VANACKER, J.; JAEGER, J.; LUYCKX, V.; DONNEZ, J.; DOLMANS, M.M. Successful vitrification and autografting of baboon (*Papio anubis*) ovarian tissue. **Human Reproduction,** v.28, n.8, p.2146–56, 2013.

MORATÓ, R.; IZQUIERDO, D.; PARAMIO, M.T.; MOGAS, T. Embryo development and structural analysis of in vitro matured bovine oocytes vitrified in flexipet denuding pipettes**. Theriogenology** v.70, n.9, 1536–1543, 2008.

BAKHACH, J. The cryopreservation of composite tissues: Principles and recent advancement on cryopreservation of different type of tissues. **Organogenesis**, v.5, n.3, p.119–126, 2009.

BANDEIRA, F.T.; CARVALHO, A.A.; CASTRO, S.V.; LIMA, L.F.; VIANA, D.A.; EVANGELISTA, J. Two methods of vitrification followed by *in vitro* culture of the ovine ovary: evaluation of the follicular development and ovarian extracellular matrix. **Reproduction in Domestical Animal,** v.50, p.177–185, 2015.

BEZERRA, J.A.B.; SILVA, A.M.; PEIXOTO, G.C.X.; SILVA, M.A.; OLIVEIRA, M.F.; SILVA, A.R. Influence of Recovery Method and Centrifugation on Epididymal Sperm from Collared Peccaries (*Pecari tajacu*, Linnaeus, 1758). **Zoological Science,** v.31, p.338–342, 2014.

BONAUDO, T.; LE PENDU, Y.; FAURE, J.F.; QUANTZ, D. The effects of deforestation on wildlife along the transamazon highway. **European Journal of Wildlife Research,** v.51, n.3, p.199–206, 2005.

BOSSO, P.L. **Zoológico de São Paulo.** Disponível em: http://www.zoologico.sp.gov.br/peca7.htm.

BOUTELLE, S.; LENAHAN, K.; KRISHER, R.; BAUMAN, K.L.; ASA, C.S.; SILBER, S. Vitrification of oocytes from endangered Mexican gray wolves (*Canis lupus baileyi*). **Theriogenology,** v.75, p.647–654, 2011.

CAMPOS, L.B.; SILVA, M.A.; BEZERRA, J.A.B.; CASTELO, T.S.; PRAXEDES, E.C.G.; BEZERRA, L.G.P.; LINS, T.L.B.; MENEZES, V.G.; SILVA, A.R. Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants. **Cryobiology,** v.91, p.77-83, 2019.

CAMPOS, L.B.; SILVA, M.A.; BEZERRA, J.A.B.; CASTELO, T.S.; PEIXOTO, G.C.X.; SILVA, A.R. Sobrevivência de espermatozoides de catetos (*Pecari tajacu*) após congelação-descongelação no uso de diferentes diluentes. **Acta Scientiae Veterinariae** (Online), v.42, p.1217, 2014.

CARVALHO, A.A.; FAUSTINO, L.R.; SILVA, C.M.; CASTRO, S.V.; LOPES, C.A.; SANTOS, R.R. Novel wide-capacity method for vitrification of caprine ovaries: Ovarian Tissue Cryosystem (OTC). **Animal Reproduction Science,** v.138, p.220–7, 2013.

CARVALHO, A.A.; FAUSTINO, L.R.; SILVA, C.M.; CASTRO, S.V.; LUZ, H.K.; ROSSETTO, R.; LOPES, C.A.; CAMPELLO, C.C.; FIGUEIREDO, J.R.; RODRIGUES, A.P.; COSTA, A.P. Influence of vitrification techniques and solutions on the morphology and survival of preantral follicles after *in vitro* culture of caprine ovarian tissue. **Theriogenology**, v.76, p.933–941, 2011.

CARVALHO, A.A.; FAUSTINO, L.R.; SILVA, C.M.G.; CASTRO, S.V.; LOBO, C.H.; SANTOS, F.W.; SANTOS, R.R.; CAMPELLO, C.C.; BORDIGNON, V.; FIGUEIREDO, J.R.; RODRIGUES, A.P.R. Catalase addition to vitrification solutions maintains goat ovarian preantral follicles stability. **Research in Veterinary Science,** v.97, p.140–147, 2014.

CASTELO, T.S.; SILVA, A.M.; BEZERRA, L.G.P.; COSTA, C.Y.M.; LAGO, A.E.A.; BEZERRA, J.A.B. Comparison among different cryoprotectants for cryopreservation of epididymal sperm from agouti *(Dasyprocta leporina).* **Cryobiology,** v.71, p.442–447, 2015.

CATZEFLIS, F.; PATTON, J.; PERCEQUILLO, A.; BONVICINO, C.; WEKSLER, M. 2008. **Galea spixii.** The IUCN Red List of Threatened Species. Version 2014.2. Disponível em <www.iucnredlist.org> Acessado em 17 de outubro de 2016.

CELESTINO, J.J.D.; DOS SANTOS, R.R.; LOPES, C.A.P.; MARTINS, F.S.; MATOS, M.H.T.; MELO, M.A.P.; BAO, S.N.; RODRIGUES, A.P.R.; SILVA, J.R.V.; FIGUEIREDO, J.R. Preservation of bovine preantral follicle viability and ultra-structure after cooling and freezing of ovarian tissue. **Animal Reproduction Science,** v.108, p.309–318, 2008.

CORTVRINDT, R.; SMITZ, J.E.J. *In vitro* follicle growth: Achievements in mammalian species. **Reproduction in Domestic Animals,** v. 36, p. 3-9, 2001.

COSTA, C.Y.M.; BEZERRA, L.G.P.; CASTELO, T.S.; SOUZA, A.L.P.; PEIXOTO, G.C.X.; SILVA, A.R. Características espermáticas de cutia *(Dasyprocta azarae)* coletadas por eletroejaculação. In: VII Congresso Norte Nordeste de Reprodução Animal, Mossoró - RN. **Revista Acta Veterinaria Brasilica,** Sup.2, Mossoró-RN: UFERSA, v.8, p.35-36, 2014.

COSTA, P.M.; MARTINS, C.F. Conservação de recursos genéticos animais através de biotécnicas de reprodução. **Universitas Ciências da Saúde,** v.6, p.39–55, 2008.

FAHY, G.M. Cryoprotectant toxicity neutralization. **Cryobiology,** v.60, p.45-53, 2010.

FATEHI, R.; EBRAHIMI, B.; SHAHHOSSEINI, M.; FARROKHI, A.; FATHI, R. Effect of ovarian tissue vitrification method on mice preantral follicular development and gene expression. **Theriogenology,** v.15, p.302–8, 2014.

FAUSTINO L.R.; SILVA, C.M.G.; ROSSETTO, R.; RODRIGUES, G.Q.; FIGUEIREDO, J.R.; RODRIGUES. A.P.R. Estado atual e desafios da criopreservação de tecido ovariano em mamíferos. **Revista Brasileira de Reprodução Animal,** v.35, n.1, p.3-15, 2011.

FIGUEIREDO, J.R.; CELESTINO, J.J.H.; RODRIGUES, A.P.R.; SILVA, J.R.V. Importância da biotécnica de MOIFOPA para o estudo da foliculogênese e produção *in vitro* de embriões em larga escala. **Revista Brasileira de Reprodução Animal**, v.31, p.143–152, 2007.

FONTANA CS, BENCKE GA, REIS RE. **Livro vermelho de fauna ameaçada de extinção no Rio Grande do Sul.** Porto Alegre: Edipucrs; 2003;632.

GARCÍA-ÁLVAREZ, O.; MAROTO-MORALES, A.; BERLINGUER, F.; FERNÁNDEZ-SANTOS, MR.; ESESO, M.C.; MERMILLOD, P.; ORTIZ, J.A.; RAMON, M.; PÉREZ-GUZMÁN, M.D.; GARDE, J.J.; SOLER, A.J. Effect of storage temperature during transport of ovaries on *in vitro* embryo production in Iberian red deer (*Cervus elaphus hispanicus*). **Theriogenology,** v.75, p.65–72, 2011.

HOSKEN, F.M.; SILVEIRA, A.C. Criação de cutias. Viçosa, MG: **Aprenda fácil**, v.4, p.21-22, 2001.

HOVATTA, O. Methods for cryopreservation of human ovarian tissue. Reprod. Biomed. Online 10, 729–734,

IUCN - Red List of Threatened Animals. IUCN, Gland, Switzerland and Cambridge, UK. Versão 2011.2. Acesso em 22 de abril de 2021. **Disponível em <http://www.iucnredlist.org>.**

JIN S, Y.; LEI, L.; SHIKANOV, A.; SHEA, L.D.; WOODRUFF, D.T.K. A novel twostep strategy for in vitro culture of early-stage ovarian follicles in the mouse. **Fertility and Sterility**, v.93, n.8, 2633-9, 2010.

KAISER, S.K.; MARGARIDO T.C.C.; FISCHER. M.L. Avalia o do comportamento de cutias *Dasyprocta azarae* e *Dasyprocta leporina* (Rodentia: *Dasyproctidae*) em cativeiro e semicativeiro em parques urbanos de Curitiba, Parana, Brasil. **Revista de etologia**, v.10, n.2, p.68–82, 2011.

KAWAMURA, K. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. **Proceedings of the National Academy of Sciences of the United States of America**, v. 110, p. 17474-17479, 2013.

LANGE, R.R. Criação e Relocação de Cutias *Dasyprocta azarae* Lichtenstein, 1823 *(Dasyproctidae, Mammalia)* em Área Verde Urbana, Curitiba-PR. **Dissertação de mestrado**. Universidade Federal do Paraná, Curitiba, PR, 1998.

LIEBERMANN, J., DIET, P. VANDERZWALMEN, AND M. J. TUCKER. Recent developpments in human oocyte, embryo and blastocyst vitrification: where are we now? **Reproductive Biomedicine Online**, v.7, p.623-633, 2003.

LUVONI, G.C. Gamete cryopreservation in the domestic cat. **Theriogenology**, v.66, p.101–111, 2006.

MAZUR, P. Freezing of living cells: mechanisms and implications. **American Journal of Physiology-Cell Physiology**, v.247, p.125–142, 1984.

MCLAUGHLIN, M.; TELFER, D.E. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. **Society for Reproduction and Fertility**, v.139, p. 971-978, 2010.

MIGISHIMA, F.; SUZUKI-MIGISHIMA, R.; SONG, S.Y.; KURAMOCHI, T.; AZUMA, S.; NISHIJIMA, M.; YOKOYAMA, M. Successful cryopreservation of mouse ovaries by vitrification. **Biology Reproduction**, v.68, p.881–887, 2003.

MITTERMEIER, R.A.; FONSECA, G.A.B.; RYLANDS, A.B.; BRANDON. K. Uma breve história da conservação da biodiversidade no Brasil. **Megadiversidade**, v.1, n.1, 2005.

MOAWAD, A.R.; FISHER, P.; ZHU, J.; CHOI, I.; POLGAR, Z.; DINNYES, A.; CAMPBELL, K.H.S. *In vitro* fertilization of ovine oocytes vitrified by solid surface vitrification at germinal vesicle stage. **Cryobiology**, v.65, n.2, p.139–144, 2012.

MORATÓ, R.; IZQUIERDO, D.; PARAMIO, M.T.; MOGAS, T. Embryo development MUKAIDA, T.; OKA, C. Vitrification of oocytes, embryos and blastocysts. **Best Practice & Research Clinical Obstetrics and Gynaecology,** v.26, p.789–803, 2012.

MYCOCK, D.J.; WESLEY-SMITH, J.; BERJAK, P. Cryopreservation of somatic embryos of four species with and without cryoprotectant pre-tratment. **Annals of Botany**. 75, 331-336, 1995.

NOGUEIRA, S.S.C.; SÉRGIO NOGUEIRA-FILHO, L.G. Wildlife farming: an alternative to unsustainable hunting and deforestation in Neotropical forests? **Biodiversity and Conservation, v.**1, p.1-13, 2011.

OLIVEIRA, F.T.B. **Análise do desenvolvimento de folículos pré-antrais e da matriz extracelular ovariana após vitrificação de tecido ovariano ovino** — 70f. il. (algumas

color.) Dissertação (mestrado) – Universidade Estadual do Ceará, Faculdade de Veterinária, Mestrado Acadêmico em Ciências Veterinárias, Fortaleza, 2013.

PAYNTER, S.J. Current status of the cryopreservation of human unfertilized oocytes. **Human Reproduction,** v.6, p.449–456, 2000.

PICTON, HM.; HARRIS, S.E.; MURUVI, W.; CHAMBERS, E.L. The in vitro growth and maturation of follicles. **Reproduction**, *v.*136, p.703–715, 2008.

PRAXEDES, E.C.G.; LIMA, G.L.; BEZERRA, L.G.P.; SANTOS, F.A.; BEZERRA, M.B.; GUERREIRO, D.D.; RODRIGUES, A.P.R; DOMINGUES, S.F.S.; SILVA, A.R. Development of fresh and vitrified agouti ovarian tissue after xenografting to ovariectomised severe combined immunodeficiency (SCID) mice. **Reproduction, Fertility and Development,** v.30, p459–468, 2018.

PRAXEDES, É.C.G.; LIMA, G.L.; SILVA, A.M.; CAMPOS, L.B.; SOUZA, C.M.P.; MOREIRA, S.S.J.; OLIVEIRA, M.F.; SILVA, A.R. Comparison of different intracellular cryoprotectants on the solid surface vitrification of red-rumped agouti (*Dasyprocta Leporina* Lichtenstein, 1823) ovarian tissue, **Reproduction in Domestical Animal**, 00:1–8, 2020.

PRAXEDES, É.C.; LIMA, G.L.; SILVA, A.M.; APOLINÁRIO, C.A.; BEZERRA, J.A.; SOUZA, A.L.P.; OLIVEIRA, M.F.; RODRIGUES, A.P.; SILVA, A.R. Characterisation and cryopreservation of the ovarian preantral follicle population Spix's yellow-toothed cavies (*Galea spixii* Wagler, 1831). **Reproduction, Fertility and Development,** 2015. (doi.org/10.1071/RD15249).

PUKAZHENTHI, B.; COMIZZOLI, P.; TRAVIS, A.J.; WILDT, D.E. Applications of emerging technologies to the study and conservation of threatened and endangered species. **Reproduction, Fertility and Development,** *v.*18, p.77–190, 2006.

RICHINGS, N.M.; SHAW, G.; TEMPLE-SMITH, P.D.; RENFREE, M.B. Growth and histology of ovarian follicles after cold storage in the tammar wallaby. **Reproduction, Fertility and Development**, v.8, n.6, p.677–88, 2006.

SANTOS, C.S.; SILVA, A.M.; MAIA, K.M.; RODRIGUES, G.S.O.; FEIJÓ, F.M.C.; ALVES, N.D.; OLIVEIRA, M.F.; SILVA, A.R. Composition of semen and foreskin mucosa aerobic microbiota and its impact on sperm parameters of captive collared peccaries (*Pecari tajacu*). **J. Appl. Microbiol.** v.129, p.521–531, 2020.

SANTOS, E.A.A.; LIMA, G.L.; PRAXEDES, E.C.G.; SILVA AM, MAIA KM, OLIVEIRA MF, SILVA APR, SILVA AR. Estimation, morphometry and ultrastructure of ovarian preantral follicle population in agouti (*Dasyprocta leporina*). **Pesquisa Veterinária Brasileira**, v.38, p175–182, 2018.

SANTOS, E.A.A.; SOUSA, P.C.; MARTINS, J.A.M.; MOREIRA, R.A.; MONTEIROMOREIRA, A.C.O.; MORENO, F.B.M.B.; OLIVEIRA, M.F.; MOURA, A.A.; A.R. SILVA. Protein profile of the seminal plasma of collared peccaries (*Pecari tajacu* Linnaeus, 1758). **Reproduction,** v.147, p.753–764, 2014a.

SANTOS, R.R.; CELESTINO, J.J.H.; LOPES, C.A.P.; MELO, M.A.P.; RODRIGUES, A.P.R.; FIGUEIREDO, J.R. Criopreservação de folículos ovarianos pré-antrais de animais domésticos**. Revista Brasileira de Reprodução Animal**, v.32, n.1, p.9-15, 2008.

SANTOS, R.R.; THARASANIT, T.; VAN HAEFTEN, T.; FIGUEIREDO, J.R.; SILVA, J.R.V.; VAN DEN HURK, R. Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods. **Cell Tissue Res**, v.327, p.167–176, 2007.

SARAGUSTY, J.; ARAV, A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. **Reproduction,** v.141, p.1–19, 2011.

SARAIVA, M.V.A. Dynamic medium produces caprine embryo from preantral follicles grown in vitro. **Reproductive Sciences**, v. 12, p. 1135–1143, 2010.

SILVA, A.R.; CASTELO, T.S.; LIMA, G.L.; PEIXOTO, G.C.X. Conservation of germplasm from wild animalsof the Caatinga biome. **Acta Science Veterinary**, v.38, p.373–389, 2010.

SILVA, J.R.V.; VAN DEN HURK, R.; ANDRADE E.R.; NUNES A.P.A.; F.V.A FERREIRA, R.N.B LÔBO, J.R. FIGUEIREDO. Survival and growth of goat primordial follicles after in vitro culture of ovarian cortical slices in media containing coconut water. **Animal Reproduction Science**, v.81**,** p**.**273–286, 2004.

SILVA, A.M.; BEZERRA, J.A.B.; CAMPOS, L.B.; SOUSA, P.C.; SILVA, A.R. Comparação de diferentes métodos de coloração na avaliação morfológica de espermatozoides epididimários de preá *(Galea spixii).* In: VII Congresso Norte Nordeste de Reprodução Animal, Mossoró - RN. **Revista Acta Veterinaria Brasilica**, Sup.2, Mossoró-RN: UFERSA, v.8, p.37–38, 2014.

SILVA, A.R.; CASTELO, T.S.; LIMA, G.L.; PEIXOTO, G.C.X. Conservation of germplasm from wild animals of the Caatinga biome. **Acta Science Veterinary**, v.38, p.373–389, 2010.

SILVA, C.M.; SERAKIDES, R.; NASCIMENTO, E.F.; NUNES, V.A.; RIBEIRO, A.F.C. Quantification of the nucleolar organizer regions (NORs) as a parameter to measure proliferation of granulosa cells. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia** v.55, p.551–556, 2003d.

SILVA, M.A.; PEIXOTO, G.C.X.; CASTELO, T.S.; LIMA, G.L.; SILVA, A.M.; OLIVEIRA, M. F.; SILVA, A. R. Cryopreservation of collared peccary (*Peccary tajacu*) semen using different freezing curves, straw sizes, and thawing rates. **Cryobiology**, p.50–55, 2013.

SOLA, M.C. **Manutenção de micro-organismos: conservação e viabilidade.** Seminários aplicados, Programa de Pós-Graduação em Ciência Animal da Universidade Federal de Goiás, 2012.

SOMFAI, T.; DINNYÉS, A.; SAGE, D.; MAROSÁN, M.; CARNWATH, J.W.; OZAWA, M.; KIKUCHI, K.; NIEMANN, H. Development to the blastocyst stage of parthenogenetically activated *in vitro* matured porcine oocytes after solid surface vitrification (SSV). **Theriogenology,** v.66, n.2, p.415–422, 2006.

SOUZA, P.C.; RAMOS, R.S.L.; GUIMARÃES, D.A.A.; FERREIRA, A.C.S.; BASTOS, L.V.; BATISTA, C.R.; OHASHI O.M. Determinação da população folicular em cutias (*Dasyprocta* sp.) criadas em cativeiro. **Revista Brasileira de Reprodução Animal**, v.27, n.2, p286–287, 2003.

TAKAHASHI, K.; YAMANAKA, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. **Cell,** v.126, p.663–676, 2006.

TELFER, E.E.; WATSON, E.D. Method for isolating preantral follicles from mare ovaries. **Journal of Reproduction and Fertility Archive**, v.56, p.447–453, 2000.

TING, A.Y.; YEOMAN, R.R.; CAMPOS, J.R. Morphological and functional preservation of pre-antral follicles after vitrification of macaque ovarian tissue in a closed system. **Humam Reproduction**, v.28, n.5, p.1267–1279, 2013.

VAJTA, G.; HOLM, P.; KUWAYAMA, M.; BOOTH, P.J.; JACOBSEN, H.; GREVE, T.; CALLESEN, H. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Molecular, Reproduction and Development,** v.51, p.53– 8, 1998.

WANDERLEY, L.S.; LUZ, H.K.; FAUSTINO, L.R.; LIMA, I.M.; LOPES, C.A.; SILVA, A.R.; BÁO, S.N.; CAMPELLO, C.C.; RODRIGUES, A.P.; FIGUEIREDO, 52 J.R. Ultrastructural features of agouti (*Dasyprocta aguti*) preantral follicles cryopreserved using dimethyl sulfoxide, ethylene glycol and propanediol. **Theriogenology,** v.77, n.2, p.260–7, 2012.

WOWK, B.; LEITL, E.; RASCH, C. M.; MESBAH-KARIMI, N.; HARRIS, S. B.; FAHY, G.M. Vitrification enhancement by synthetic ice blocking agents. **Cryobiology**, v.40, p.228–236, 2000.

XING, W.; ZHOU, C.; BIAN, J.; MONTAG, M.; XU, Y.; LI Y. Solid-surface vitrification is an appropriate and convenient method for cryopreservation of isolated rat follicles. **Reproductive Biology and Endocrinology**, v.8, p.42, 2010.

ZHANG, AQ.; WANGB, S.; PING-BAO, Y.C.; ZHANG, D.L.; YA-JIE Z.D.; RU-XIN, C.D.; FU, Y.; ZHANG, J. Effects of L-carnitine on follicular survival and graft function following autotransplantation of cryopreserved-thawed ovarian tissues. **Cryobiology,** v.71, p.135–140, 2015.

CAPÍTULO I

Reproduction in agouti *(Dasyprocta spp.):* A review of reproductive physiology for developing assisted reproductive techniques

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Reproduction in agouti (*Dasyprocta spp.*): A review of reproductive physiology for developing assisted reproductive techniques

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Abstract

Dasyprocta spp. (agouti) include wild rodents with highlighted ecological and economic importance, and are considered experimental models for endangered hystricognath rodents. Of late, development of techniques to conserve their genetic material as well as the formation of biobanks is increasing. In this context, this review describes the main advances in the knowledge of the reproductive morphophysiological specificities of agouti as well as the development and improvement of assisted reproductive techniques aimed at conservation, multiplication, and exploitation of their reproductive potential under captivity.

Keywords: biobank, rodentia, wildlife.

Introduction

Rodents account for the largest order of mammals in the world and are found in the most varied types of habitats (Emmons and Feer, 1997). Of these, agouti (Fig. 1), a hystricognath rodent, *Dasyprocta spp.* or *Dasyprocta aguti*, is found throughout the area of Neotropical America and is distributed into 13 different species that constitute the genus *Dasyprocta* (IUCN, 2018).

Agouti is currently suggested as an experimental model for other species of hystricognath rodents that are vulnerable to extinction (Costa and Martins, 2008) because they can easily adapt to captivity and, thus, exhibit prolificity, precocity, and relatively short gestation period (Hosken and Silveira, 2001). These characteristics facilitate studying their physiology and exploring their zootechnical potential (Pachaly *et al*., 1999; Ribeiro *et al*., 2008).

Owing to their diverse and substantial distribution, these wild rodents represent an important protein source for human consumption (Lopes *et al*., 2004) as well as for obtaining skin, leather, and bristle (Silva *et al*., 2010). Moreover, they are important seed dispersers and maintain the ecological balance (Silva *et al*., 2013).

Still, with their economic and ecological relevance, agouti present a potential market that justifies the research focused on its conservation (Souza *et al*., 2003) to improve reproduction management of the commercial breed (Bonaudo *et al*., 2005). In this context, this review describes the main advances in research on the reproductive morphophysiological specificities of agouti as well as the development and improvement of assisted reproductive techniques (ART) aimed at conservation, multiplication, and exploitation of their reproductive potential under captivity.

General characteristics of male and female agouti

The term agouti, also called *Dasyprocta spp.*, refers to the third largest frugivorous rodent (Henry, 1999). The *Dasyprocta* genus comprises approximately 13 species of agouti, viz., *D. azarae*, *D. coibae*, *D. croconota*, *D. fuliginosa*, *D. guamara*, *D. iacki*, *D. kalinowskii*, *D. leporina*, *D. mexicana*, *D. prymnolopha*, *D. punctata*, *D. ruatanica*, and *D. variegata* (IUCN, 2018). The main differences found between these species are related to coat coloration and their conservation status (IUCN, 2018; Table 1). However, some species are not easily recognizable owing to taxonomic limitations and absence of any modern taxonomic revision (Emmons and Feer, 1997). Cytogenetic studies are essential because each species is characterized by a typical karyotype, which may differ from others with respect to form, size, and number of chromosomes (Lima, 2000). By cytogenetic analysis of 30 animals of the genus *Dasyprocta* (*D. prymnolopha*, *D. leporina*, and *D. fuliginosa*), Ramos *et al.* (2003) identified that the individuals presented two cellular lineages, with 2n = 64 and 2n = 65 chromosomes. The karyotypes showed similarity, and chromosomal polymorphism was not detected by Giemsa conventional staining and G banding. All analyzed specimens presented a diploid number of 64 or 65 chromosomes. This variation was observed with a frequency of approximately 70% in cells with 2n = 64 and 30% in cells with 2n = 65. There was no variation in the pattern of nucleolus organizer regions (NORs) in the species studied, which was used to verify chromosomal polymorphism (Ramos *et al*., 2003).

Agouti are distributed over a wide area of Neotropical America and are found in a great diversity of habitats from the south of Mexico, through Central America, to Argentina, Uruguay, and Paraguay (Deutsch and Puglia, 1988). They exhibit an extraordinary variety of ecological adaptations, supporting existence in the coldest and more torrid climates. They can thrive in regions with the highest floristic cover at high altitudes (Emmons and Feer, 1997).

Agouti are medium-sized animals, weighing between 2 and 5 kg, with an average height of 23 cm

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and length of 50 cm, measured from the muzzle to the base of the tail, with the tail being generally bristle. The head and thorax show fur colors ranging from gray to bright orange covered by long, harsh, and strong pelage, with predominating brown and light or dark yellowish or golden colors. The top of the head, neck, and middle of the back between the shoulders are sometimes dark with long bristles; the ears are small and bare, similar to the tail that is almost vestigial (Smythe, 1987; Emmons and Feer, 1997). In addition, they possess four long and curved incisor teeth, whose macroscopic characteristics reveal structure similar to that of other mammals (Oliveira *et al*., 2012). Between males and females there is no sexual dimorphism; therefore, to differentiate them, it is necessary to perform sexing through palpation, where the difference between the male's prominent penis and the female's two vaginal folds, forming the vulva, is easily observed (Guimarães *et al*., 2009).

These animals have adapted to terrestrial life by a reduction in functional fingers and a vestigial thumb, with four digits on the forelimbs and three on the hindlimbs. Furthermore, the claws of the forelimbs are slightly arched, indicating the ability to excavate, although they are not true diggers. The hindlimbs are much larger than forelimbs, enabling them to jump (Oliveira *et al*., 2012).

These animals have diurnal and crepuscular habits, and when threatened, they may present nocturnal habits. They are naturally herbivorous feeding on leaves, roots, flowers, fungi, seeds, and, particularly fruits found in the soil (Santos *et al*., 2005). In case of fruit shortage, agouti spend less time resting and more time foraging, looking for seeds that have been buried previously (Smythe, 1987). Such behavior of digging, hiding, and seeking, also observed in *Myoprocta spp*., is essential for the dispersion of plant species (Forget and Milleron, 1991). Thus, agouti act on aggregate distribution of tree seeds that is secondary to the action of long-distance dispersal animals, such as tapirs (*Tapirus terrestris*) (Lange and Schmidt, 2007). These species are also important links in the food chain because they are prey to birds, snakes, and wild carnivores, thereby maintaining the environmental balance (Hosken and Silveira, 2001). In addition, agouti are economically important because they enable the commercialization of skin, leather, and bristle (Silva *et al*., 2010). Yet, to their economic and ecological importance, because of their stable world population, they can be used as experimental models for endangered agouti, such as *D. ruatanica*, *D. coibae*, and *D. mexicana* (IUCN, 2018).

Because they are considered as important protein sources, agouti have been used for human consumption. Their meat is white, rich in nutrients, soft, tasty, and an excellent source of calcium and phosphorus, with low caloric value (each 100 g of meat has approximately 120 Kcal) (Barbosa, 2010). The commercialization of agouti meat provides low market value, requiring little manpower. The system of fattening and development of matrices are two activities that can be practiced at the same time, potentiating producer profit. According to some researchers, the meat of these animals is as appreciated as paca meat, the most sought-after in the market among the wild species (Barbosa, 2010). For example, in Trinidad and Tobago, and Central America, agouti meat is considered a spice; therefore, this animal has been intensively hunted (Roopchand, 2002).

Breeding agouti under captivity is an alternative for its preservation in the natural habitat and allows the exploration of their zootechnical potential, thereby allowing studies on their physiological characteristics (Pachaly *et al*., 1999; Lopes *et al*., 2004). However, studies with the appropriate methods of productive and reproductive management of wild animals are limited, which could reflect in higher productivity, enabling the preservation of some species by reduction in predatory hunting (Lopes *et al*., 2004).

Hence, it is necessary to apply captive breeding programs and ART as tools to ensure the increase in their reproductive potential and conservation of their genetic material (Comizzoli *et al*., 2001), allowing the increase in their zootechnical potential and genetic variability; thus, enabling the study of their physiological characteristics.

Table 1. Main differences among agouti's species (*Dasyprocta spp.*)

Species	Coats	Conservation status
Dasyprocta azarae	Black or white or pale orange	Listed as potentially vulnerable in Argentina
Dasyprocta coibae	Cream or black	Vulnerable
Dasyprocta leporina	Red-rumped	Estable
Dasyprocta iacki	-	Insufficient data
Dasyprocta guamara	Dark	Near threatened
Dasyprocta kalinowskii	-	Insufficient data
Dasyprocta croconota	-	Insufficient data
Dasyprocta fuliginosa	Black	Least Concern
Dasyprocta mexicana	Mexican Black	Critically Endangered
Dasyprocta prymnolopha	Black-rumped	Least Concern
Dasyprocta punctata	Uniform reddish brown	Least Concern
Dasyprocta ruatanica	Black or Cream	Endangered
Dasyprocta variegata	Brown	Insufficient data

Source: IUCN, 2018.

Figure1. A red-rumped agouti (*Dasyprocta leporina*) specimen.

Reproductive morphophysiological characteristics of male agouti

In agouti, the internal reproductive system is composed of accessory glands (vesicular glands, prostate, and bulbourethral glands), testis, and epididymis (delimited by adipose tissue, i.e., caput epididymal) that are paired and fully coated by cremaster muscle, allowing a greater range of movement and internalization of the testicle in the abdomen (Paula and Walker, 2012). In addition, the epididymis of *Dasyprocta spp*. is composed of principal, basal, halogen, apical, and clean cells with pseudostratified, columnar, and stereociliated epithelium (Arroyo *et al*., 2014).

According to Mollineau *et al.* (2006) in *D. leporina*, the testicular length, diameter, and weight are 3.67 ± 0.12 cm, 1.67 ± 0.04 cm, and 5.03 ± 0.52 g, respectively. In addition, the ductus deferens has a length of 10.98 ± 0.40 cm and mean diameter of 0.14 ± 0.01 cm. The absence of a scrotum, a characteristic observed in agouti, has been reported by Menezes *et al.* (2003).

The penis (Fig. 2) is doubled caudally with a U-bend lying down, with a mean length of 9.90 ± 0.43 cm, and contains paired ventral keratinous spicules. It is observed that the glans penis presents a rounded dilation, called urethral torus or penile flower, at the time of erection (Carvalho *et al*., 2008; Mollineau *et al*., 2012). Four steps are documented in the erection process: stage 1, protrusion of the penis from the preputial orifice; stage 2, opening of the lateral cartilages of the penis; stage 3, flowering of the head of the glans penile flower, eversion of the intromitting bag, and protrusion of the keratinous spicules; and stage 4, protrusion of the keratinous spurs and ejaculation (Mollineau *et al*., 2012).

Regarding reproductive development in males, Costa *et al.* (2010) observed that the spermatogenic cycle lasts for 9.5 ± 0.03 days, and the total duration of spermatogenesis is 42.8 ± 0.16 days. According to Assis Neto *et al*. (2003a), the period from birth to five months comprises the pre-pubertal period, in which the presence of gonocytes is observed with the absence of tubular lumen in the testicular cords as well as the presence of spermatogonia, spermatocytes, spermatids, Sertoli and Leydig cells (Arroyo *et al*., 2017); from six to eight months of age is the transition phase (pre-puberty to puberty), in which 40% to 90% of the testicular cords are in the process of tubular lumination, coinciding with the appearance of the first primary spermatocytes and rounded spermatids. Puberty in male agouti is established at around the age of nine months, where it is possible to observe the presence of all cell types and spermatozoa in the testicular lumen after eight stages of the cycle of the seminiferous epithelium (Assis Neto *et al*., 2003b). According to Arroyo *et al*., (2017), the main changes in the testis of agouti (*Dasyprocta spp.)* occur between the prepubescent and prepubertal periods, when the germinal epithelial organization occurs and the Sertoli cells undergo morphological and functional changes to form the spermatozoa.

Morphological analysis of agouti *(Dasyprocta spp.)* sperm demonstrated that these cells present an oval-shaped head, like a shovel with a rounded head apex, and a flat base (Fig. 3). Sperm head is tapered, without prominence of the acrosome or evidence of the perforatorium (Arroyo *et al*., 2017). Likewise, it has been reported that the base of the head is symmetric and the tail is extended and sharpened (Mollineau *et al*., 2006; Ferraz *et al*., 2009). Regarding the morphometry, the sperm presented distinct results among species, 48.0 ± 0.3 µm and 40.12 ± 2.4 µm for the species *D. leporina* and *D. prymnolopha*, respectively (Mollineau *et al*., 2008; Ferraz *et al*., 2011).

In this context, in wild animals, it is necessary to determine the anatomy and physiology of the species before implementing ART, to adapt to the characteristics of each species. Further, it is often necessary to perform tests of interaction between different factors that may affect the success of the technique.

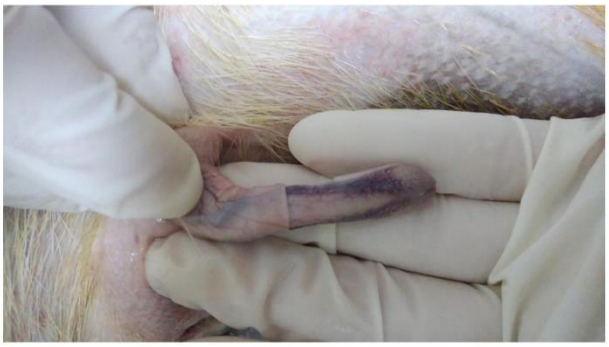

Figure 2. Agouti's (*Dasyprocta leporina*) penis with prepuce partly removed.

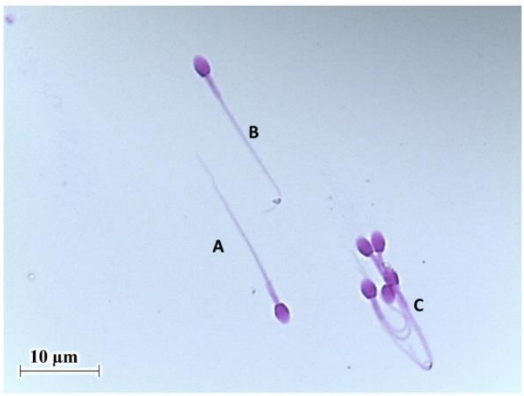

Figure 3. Morphology of agouti's (*Dasyprocta leporina*) epididymal sperm stained with Bengal rose. (A) Normal spermatozoa; (B) abnormal spermatozoa with tail folded; (C) abnormal spermatozoa with high tail folded.

ART for male agouti

Application of ART as an initial step for developing methods for sperm recovery is required. Of late, methods for obtaining spermatozoa by retrograde epididymal washing (Ferraz *et al*., 2011; Silva *et al*., 2011, 2012; Castelo *et al*., 2015) or electroejaculation (Mollineau *et al*., 2008; Martinez *et al*., 2013; Castelo *et al*., 2016) have been described for agouti.

Obtaining epididymal spermatozoa offers the possibility of acquiring and using the genetic material immediately after the animal's death (Goovaerts *et al*., 2006) because the genetic material is morphologically viable with a suitable degree of maturity and fertilizing capacity (Monteiro *et al*., 2011). There are two basic methods for obtaining spermatozoa from the epididymis, viz., retrograde washing and flotation. The most used method is retrograde washing (Martinez-Pastor *et al*., 2006). This method consists of injecting an aqueous medium followed by tail cutting, in the direction from the ductus deferens to the tail epididymis, for the recovery of sperm (Comizzoli *et al*., 2001). In agouti, Ferraz *et al.* (2011) were the first to demonstrate the possibility of obtaining spermatozoa by means of retrograde washing in animals previously submitted to orchiectomy, and they obtained a concentration of 748 ± 418.66 spermatozoa/mL. Subsequently, the technique was applied in the same species of agouti by Silva *et al.* (2011, 2012), in which a volume of 300 ± 2 µL was recovered with a concentration of 1.4 ± 0.3 × 10^9 spermatozoa/mL. Using the same method in *D. leporina*, Castelo *et al.* (2015) were able to recover a total volume of 1.65 ± 0.22 mL with a concentration of 1.04 ± 0.2 × 10^6 spermatozoa/mL.

Electroejaculation is another efficient way of obtaining sperm, primarily because the death of the animal is not necessary. This method of semen collection is based on the induction of ejaculatory reflex through the application of electrical stimuli on the nerve plexus located in the pelvic floor of the animal. A lubricated transrectal probe connected to an electrical stimulator is introduced in the rectum of the animal under anesthesia or without anesthesia to achieve stimuli (Silva *et al*., 2004). Mollineau *et al.* (2008) were the first to describe the use of electroejaculation in *D. leporina* using ketamine as an anesthetic; they applied a charge of 6 V for 5 s followed by increasing the charge by 1 V until 12 V with 5 s intervals of rest. Thus, the authors obtained an efficiency of 30% of ejaculates containing spermatozoa. In a subsequent study using the same electroejaculation protocol, but starting at 2 V, an efficiency of 40% of ejaculates containing spermatozoa was obtained (Mollineau *et al*., 2010a). Additionally, Castelo *et al.* (2016) demonstrated the interaction between different types of electrical stimuli, as sine or quadratic waves, and electroejaculation using ring or strip electrode apparatus with better results than those reported by Mollineau *et al.* (2008, 2010). The authors obtained 70% of ejaculates, with 57% of ejaculates containing spermatozoa, when using the protocol with the ring electrode associated with sinusoidal stimuli (Castelo *et al*., 2016). However, Martinez *et al*. (2013) obtained better results using four brown agouti (*D. azarae*) and obtained 100% success in semen collection. For this, the authors used an association of azaperone and meperidine as preanesthetic medication. Afterwards, the animals were anesthetized with the combination of ketamine and xylazine, followed by lumbosaccharide application of lidocaine. The protocol of stimulation comprised four sets of 20 electrostimulations for 3 s each with 2, 4, 6, and 8 V, with a 2-min interval between each series. The results of evaluation of sperm characteristics obtained from such experiments are shown in Table 2.

With respect to the development of preservative protocols, Silva *et al.* (2011) evaluated the performance of Tris and powdered coconut water (ACP-109c) diluents on the cryopreservation of epididymal sperm derived from *D. leporina*, wherein the samples were centrifuged, and extended in the same diluents in addition to egg yolk (20%) and glycerol (6%). After sperm cryopreservation and thawing, they observed that 26.5 ± 2.6% were motile sperm with 2.6 ± 0.2 vigor in the ACP-109c group, which was significantly better than 9.7 ± 2.6% motile sperm with 1.2 ± 0.3 vigor found in the Tris group. They verified that ACP-109c would be the most suitable diluent for processing and cryopreservation of these cells. Subsequently, the same group, studying the interactions between straw size (0.25 or 0.50 mL) and thawing rates (37°C for 60 s or 70°C for 8 s) for epididymal sperm, demonstrated that epididymal sperm of agouti could be efficiently cryopreserved in both 0.25 mL or 0.50 mL straws and thawing should be conducted at 37°C for 60 s. The use of 0.25 mL and 0.5 mL straws thawed at 37°C for 60 s provided a value of 26.6% and 18.4% for sperm motility, respectively (Silva *et al*., 2012).

Furthermore, for cryoprotectants (CPAs), Castelo *et al.* (2015), using glycerol, dimethylsufoxide (DMSO), and dimethylformamide (DMF) at 3% and 6% concentrations, demonstrated that 6% glycerol was the most appropriate for cryopreservation of spermatozoa of *D. leporina* compared to that by other CPAs, in which it was possible to recover spermatozoa with a mean motility of 39.5 ± 4.6% after thawing.

Through electroejaculation, Mollineau *et al.* (2010a) diluted the ejaculates of *D. leporina* in ultra-high-temperature (UHT) milk, unpasteurized coconut water, or pasteurized coconut water under refrigeration at 5°C. After 24 h of storage, best results were achieved in the samples diluted in UHT milk, with sperm motility values of 59.5 ± 7.75%. For cryopreservation, however, only 12.5% of sperms presenting progressive motility were obtained after thawing at 30°C for 20 s using the same milk diluent (Mollineau *et al*., 2010b). Recently, Castelo *et al.* (2016) cryopreserved samples derived via electroejaculation conducted on *D. leporina.* They demonstrated that the use of an extender containing ACP-109c with 20% egg yolk and 6% glycerol was able to yield 31.2% motile sperms after thawing.

Recovery of epididymal sperm appears as the most viable alternative for male gamete retrieval in this species to use viable sperms for the development of cryopreservation procedures. However, in agouti, obtaining sperm by electroejaculation is still a challenge, and based on these findings, we can infer that the main obstacle for the improvement of ARTs in male agouti is the low efficiency of electroejaculation protocols. Standardizing these protocols requires studying factors that may affect the success of cryopreservation that are appropriate to the inherent characteristics of each species. In addition to the type of CPAs, there is need for consideration of important factors such as their concentration and effects on sperm fertilizing ability.

Despite the advances already achieved (Tab. 3), the need for further studies is highlighted, with the objective of improving the protocols of electroejaculation with respect to time, interval between series, and anesthetic planes as demonstrated for other domestic and wild species. In the cryopreservation protocols, incorporation of new additives as detergents based on sodium dodecyl sulfate (SDS) and new CPAs, such as *Aloe vera* extract, may improve the quality and longevity of the sperm cell. To our knowledge, cryopreservation of testicular tissue has not been reported, which would represent a method for the conservation of the biodiversity of this species with future application in *in vitro* culture and optimization of diverse reproductive biotechniques.

Table 2. Values (Mean ± SEM) for the agoutis' (*D. Leporina*) sperm parameters obtained by electroejaculation and retrograde epididymal washing

Sperm parameters	Eletroejaculation	Retrograde epididymal washing
Volume (mL)	0.6 ± 0.1	1.65 ± 0.22
Sperm concentration (× 10^9 sperm/mL)	307.5 ± 69.6	822.5 ± 85.0
Sperm motility (%)	93.7 ± 4.7	96.2 ± 2.4
Vigor (0-5)	5.0 ± 0.0	5.0 ± 0.0
Membrane integrity (%)	74.0 ± 4.0	90.5 ± 2.1
Osmotic response (%)	66.2 ± 4.0	79.7 ± 2.6
Sperm morphology (%)	77.2 ± 4.1	80.7 ± 8.1

Source: Castelo (2015).

Table 3. State of the art of assisted reproductive techniques (ARTs) applied for agoutis' (*Dasyprocta spp.)* males.

Species	ART	Source
Dasyprocta azarae	Eletroejaculation	Martinez *et al*., (2013)
Dasyprocta leporina	Retrograde epididymal washing	Silva *et al*., (2011) Castelo *et al*., (2015)
	Eletroejaculation	Mollineau *et al*., (2008; 2010a) Castelo *et al*., (2016)
	Refrigeration of sperm	Mollineau *et al*., (2010a; 2010b) Silva *et al*., (2011; 2012)
	Crypreservation of epididymal spermatozoa	Castelo *et al*., (2015)

Reproductive morphophysiological aspects of female agouti

The ovaries (Fig. 4) of the agouti are located in the sublumbar region, caudally to the kidney, in the abdominal cavity presenting an ellipsoid or oval shape, flattened dorsoventrally (Almeida *et al*., 2003). According to the morphometric data, the right ovary weighs an average of 0.082 g, with a length of 0.83 cm, width of 0.49 cm, and thickness of 0.24 cm; whereas, the left ovary weighs 0.058 g, with a length of 0.74 cm, width of 0.45 cm, and thickness of 0.23 cm (Almeida *et al*., 2003). Histologically, the ovaries are covered by a simple cubic epithelium on a layer of connective tissue rich in fibers, and a high volume of accessory corpora lutea are described in this species (Weir, 1974). The ovaries are light yellow in color, with a smooth outer surface and small translucent areas, suggestive of the presence of follicles in different categories (Santos *et al*., 2018).

The follicle, a morphofunctional unit of the ovary, presents dimensions varying between the follicular classes (Fig. 5). The primordial follicle is 18.62 ± 3.40 µm in diameter, with oocyte of 12.28 ± 2.37 µm and nucleus of 6.10 ± 0.93 µm; the primary follicle is 23.75 ± 5.70 µm in length, oocyte of 14.22 ± 3.00 µm, and nucleus of 6.70 ± 1.24 µm; and the secondary follicle is 88.55 ± 17.61 µm in length, oocyte of 52.85 ± 17.56 µm, and nucleus of 22.33 ± 17.61 µm (Santos *et al*., 2018). In addition, the follicular population in *D. leporina* is estimated at 4419.8 ± 532.26 and 5397.52 ± 574.91 follicles in the right and left ovary, respectively. A high number of polyovular follicles, representing 7.51% of the follicles, are observed.

In general, for all females agouti, puberty is established at around the age of nine months; however, further information on the establishment of this event in different species of *Dasyprocta* is limited. The estrous cycle lasts for a mean period of 34.2 ± 2.1 days, with the existence of short cycles of only 12 days (Weir, 1971). However, in *D. prymnolopha*, Guimarães *et al.* (1997) conducted a study through vaginal cytology analyses and verified a mean duration of 30.69 ± 4.65 days for the estrous cycle, with variations between 19 and 40 days. Subsequently, they confirmed their results through hormonal measurements, performed twice a week, and determined the mean duration of the estrous cycle as 32.05 ± 4.17 days. There were no statistical differences in 17β-estradiol levels between the cycle phases. However, two peak periods of 17β-estradiol were observed, the first being in the metestrus (75.88 pg/mL) and the second during the proestrus (77.26 ± 20.71 pg/mL). In the estrus, the initial progesterone concentration is low (2.83 ± 2.34 ng/mL), but an increase in the progesterone level is observed at 24 h (9.02 ± 2.34 ng/mL) (Guimarães *et al*., 2011). For *D. leporina*, the estrous cycle was characterized as being polyestrous continuous with an average duration of 28 days, ranging from 24 to 31 days. Ultrasound analysis revealed no differences in ovarian morphology during the different phases of the estrous cycle. Follicles during the estrogenic phases (proestrus and estrus) were identified with an average diameter of 1 ± 0.5 mm. In only 12.5% of luteal phases, corpora lutea, measuring 1.4 ± 0.9 mm, were identified (Campos *et al*., 2015). The females of these species have a vaginal occlusion membrane, the perineum or operculum, the presence of which enables identification of the estrous phase, as observed in *D. prymnolopha* (Weir *et al*., 1974), and

which opens only during the estrous cycle and parturition.

Overall, for all the agouti species, there are few studies on the reproductive morphology and physiology. However, extensive knowledge on the female reproduction is important for the application of auxiliary reproductive biotechniques for its conservation and management.

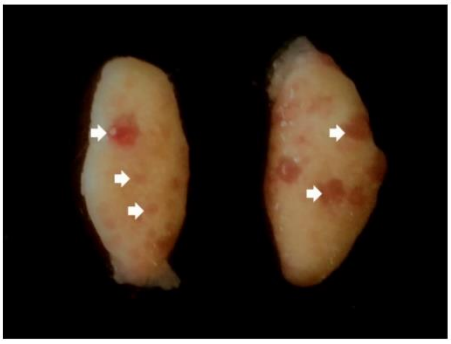

Figure 4. Ovary of the species *Dasyprocyta leporina*, with follicles at various stages of development (white arrows).

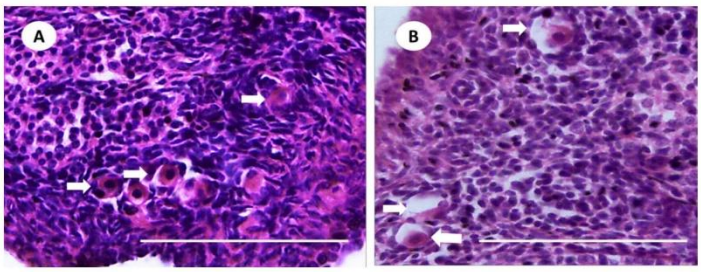

Figure 5. Photomicrographs of agouti (*Dasyprocta leporina*) ovarian sections. (A) Aggregates of primordial follicles displaying an oocyte surrounded by one layer of flattened cells (white arrows). (B) Degenerated follicles displaying oocyte cytoplasm retraction and disorganization of granulosa cells (white arrows).

Gestation physiology and monitoring

Kleiman et al. (1979) observed that for agouti, an average gestation period was 112 days, which varied among the 13 existing species. The occurrence of postpartum estrus has been reported in agouti and occurs between 18 and 20 days (Weir, 1971). The placenta is lobulate, monohemochorial, and is connected to the uterus through the mesoplacenta (Rodrigues et al., 2003).

Sousa *et al.* (2012) performed multifrequency sonography (5-7.5 MHz) using a microconvex transducer to observe the characteristics related to the pregnancy age of the agouti *D. prymnolopha*. The first uterine morphological changes were observed on day 9 as an anechoic spherical structure, with slightly hyperechoic margins, and gestational sac was observed at only around 76 days after mating. In a recent study with the same species, B-mode ultrasonography associated with Doppler allowed the evaluation of the vascular network and determination of the reference values for blood flow necessary to maintain fetal viability at different gestational ages (Sousa *et al*., 2017). In addition, morphogenetic analysis of the fetuses of *D. leporina* from 30 to 100 days revealed stages of embryonic and fetal development (Oliveira *et al*., 2017), demonstrating the progress of species-oriented studies.

ART applied to female agouti

Understanding the estrous cycle of a species is essential for the development of ART (Durrant, 2009). High variability among wild species, duration of the estrous period, and difficulty in determining the exact time of ovulation led to the development of useful methods for monitoring the estrous cycle (Pimentel *et al*., 2014).

In a study conducted on animals bred in the Caatinga biome, it was possible to characterize the estrous cycle of *D. leporina* by means of exfoliative vaginal cytology (Fig. 6) and ultrasonography. Vaginal cytology revealed the predominance of superficial cells in the stages of proestrus and estrus, followed by intermediate cells in the metaestrus, thereby allowing the distinction of the follicular and luteal phases (Campos *et al*., 2015). Similar cytological findings have also been reported for *D. prymnolopha* (Guimarães *et al*., 1997). Additionally, the external genitalia of *D. leporina* presented changes related to the typical signs of the estrous phase, such as the opening of vulvar lips with the presence of mucous secretion. Such changes coincided with the presence of an ovarian follicle (with an average diameter of 1 ± 0.5 mm, varying from 0.4 to 1.6 mm) as observed by ultrasonography, with the predominance of superficial cells (Campos *et al*., 2015). Furthermore, ultrasonography revealed that there were no differences in ovarian morphology during the different phases of the estrous cycle, but it was possible to observe follicles in the follicular phases and corpora lutea, measuring 1.4 ± 0.9 mm, in the luteal phases (Campos *et al*., 2015). The results of ovarian morphometry detected by ultrasonography in the different phases of the estrous cycle in *D. leporina* were similar to those observed in black agouti (*D. fuliginosa*) (Mayor *et al*., 2011).

The cycles of female *D. leporina* were confirmed by blood hormonal measurements. Females were monitored throughout their estrous cycle, and estrogen (E_2) and progesterone (P_4) concentrations were determined at all stages by enzyme linked immunosorbent assay (ELISA). E_2 levels were 1212-3500 pg/mL, with the peak value coinciding with the observed estrus. However, two additional peak values for E_2, one in the metaestrus and one in proestrus were also recorded. The concentrations of P_4 reached a maximum value of 4.23 ng/mL, and the increase in P_4 occurred immediately after the second peak of E_2 in metaestrus, with the highest concentrations of P_4 recorded in diestrus (Singh *et al*., 2016). However, in agouti, there are still few reports on monitoring of the estrous cycle through hormonal dosages.

The induction of estrus was reported for *D. leporina*, in which the hormonal protocols consisting of peritoneal administration of cloprostenol (5 µg) alone or in combination with a gonadotropin-releasing hormone (GnRH) analog (30 µg) were compared. Using only cloprostenol, estrus induction was verified in only 40% of females that manifested estrus signs and presented estrogen peak at 3 and 6 days after drug administration. However, combination therapy with both the hormones yielded estrus induction in 60% of animals, but 40% of the animals manifested estrus at day 4 and 20% at day 10 day after drug administration (Peixoto *et al*., 2018). Although these protocols promoted estrus induction, the authors do not suggest this method as an effective means to achieve synchronization of estrus induction in *D. leporina*.

Parallel to these studies, cryopreservation of ovarian tissue has been developed with the aim of creating biobanks using the female germplasm. For *D. leporina*, preservation of up to 64% preantral follicles (PFs) was achieved using a conventional freezing method with different CPAs, such as DMSO, ethylene glycol (EG), and propanediol (PROH) at 1.5 M. However, transmission electron microscopy analyses revealed that PROH provided the most efficient preservation of the ovarian tissue ultrastructure and thus, is suggested for use in agouti (Wanderley *et al*., 2012).

Another study performed a cryopreservation protocol based on solid surface vitrification of *D. leporina* ovarian tissue. It was verified that regardless of the CPA used (DMSO 3.0 M or 6.0 M, EG 3.0 M or 6.0 M, and a combination of both agents), it was possible to preserve up to 70% of the follicular morphology. Moreover, DNA fragmentation was not observed in any of the groups exhibiting preserved follicular viability similar to that observed in the non-vitrified group (Praxedes, 2017).

In addition, the first ovarian tissue xenograft of non-vitrified and vitrified fragments of *D. leporina* was reported in 2017. Grafts are tools that can be used to measure the survival of tissue after cryopreservation and, in case of ovarian grafts, to obtain knowledge regarding follicular dynamics of various species. Praxedes *et al.* (2018) using a combination of DMSO and EG in immunosuppressed mice for both the non-vitrified (80%) and vitrified (16%) groups observed that ovarian activity was recovered after 20.6 ± 8.6 days of xenografting. The recovery of ovarian activity was characterized by the presence of typical signs of proestrus and estrus, associated with the increase in E_2 concentrations in recipient severe combined immunodeficiency (SCID) mice. Microscopically, primordial, primary, transitional, and secondary follicles, corpora lutea, and hemorrhagic body were observed in the grafts exhibiting normal morphology for the species studied (Praxedes *et al*., 2017).

Even with the advances in studies on reproductive biotechniques (Tab. 4) aimed at conserving the genetic material of female agouti, it is necessary to improve the existing protocols that allow better rates of preservation of morphological integrity and viability of follicles before they become atresic. These studies are needed not only to safeguard genetic material, but also for use in other biotechniques, such as *in vitro* fertilization and cloning.

However, in wild animals, cryopreservation of female gametes and conditions necessary for the complete development of *in vitro* PFs are still not well established. Thus, many studies are aimed at developing and adapting efficient *in vitro* culture systems to evaluate the effect of different substances (gonadotropins and intra-ovarian factors) on initial oocyte development for obtaining information about the mechanisms involved in folliculogenesis.

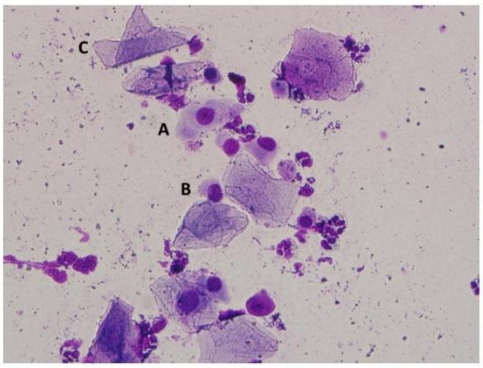

Figure 6. Exfoliative vaginal cytology of agouti (*Dasyprocta leporina*). (A) intermediate cell; (B) parabasal cells; (C) surface cell.

Table 4. State of the art of assisted reproductive techniques (ARTs) applied for agoutis' (*Dasyprocta spp.)* females.

Species	ARTs	Source
Dasyprocta leporina	Monitoring the estrous cycle	Campos *et al*., (2015)
		Singh *et al.,* (2016)
	Gestational monitoring	Sousa *et al.,* (2017)
		Oliveira *et al*., (2017)
	Induction of estrus	Peixoto (2016)
	Cryopreservation of ovarian tissue	Wanderley *et al.,* (2012)
		Praxedes (2017)
	Xenograft of fresh and vitrified ovarian tissue	Praxedes et al., (2017)
Dasyprocta prymnolopha	Monitoring the estrous cycle	Guimarães *et al.,* (1997)
	Gestational monitoring	Souza *et al.,* (2012)

Conservation and culture of agouti somatic tissue

The establishment of somatic cryobanks has been suggested as an important tool for conservation of endangered (Loi *et al*., 2001 or zootechnically valuable species (Loi *et al*., 2001, Pereira *et al*., 2013), as an alternative to the conservation of animal biodiversity. It allows the optimization of other reproductive biotechniques in association with nuclear somatic cell transfer (SCNT, also known as cloning). In agouti, studies focused on the formation of biobanks derived from somatic tissue are still nascent and little is known about the use of this genetic source.

Thus, aimed at the formation of germplasm banks, a study used peripheral ear tissue of *D. leporina* and analyzed different techniques of vitrification (solid surface and conventional vitrification) for the conservation of somatic samples. Vitrification consisted of exposing the fragments in DMEM medium supplemented with 20% DMSO, 20% EG, 0.25 M sucrose, and 10% fetal bovine serum for 5 min. Based on histological analyses, it was observed that solid surface vitrification better preserved the somatic tissue (Costa *et al*., 2015).

Thus, the interest in various tissue sources is primarily based on the possibility of using different cell types as a nucleus donor cell in SCNT (Arat *et al*., 2011). Therefore, samples derived from the skin are widely used for tissue preservation in addition to the formation of cryobanks, reproduction by SCNT, and pluripotency studies (Borges *et al*., 2015).

Final Considerations

The *Dasyprocta spp.*, besides being considered an alternative source of protein, has great potential as an experimental model for studying reproductive biotechniques and the formation of germplasm banks. However, it presents distinct limitations. The evolution and adaptation of different protocols to the characteristics of each species is not characterized. Moreover, it should be noted that very little is known about the morphophysiological characteristics of male and female agouti. This knowledge is essential for future application in sustainable production systems as well as for the development and improvement of protocols that guarantee the maintenance of cellular viability and allow the conservation, multiplication, and preservation of the biodiversity of these species and other related species.

References

Almeida MM, Carvalho MAM, Cavalcante Filho MF, Miglino MA, Menezes DJA. 2003. Estudo morfológico e morfométrico do ovário de cutias (*Dasyprocta aguti*, Linnaeus, 1766). *Braz J Vet Res Anim Sci*, 40:55-62.

Arat S, Caputcu AT, Akkoc T, Pabuccuoglu S, Sagirkaya H, Cirit U, Nak D. 2011. Using cell banks as a tool in conservation programmes of native domestic breeds: the production of the first cloned Anatolian Grey cattle. *Reprod Fertil Dev*, 23:1012-1023.

Arroyo MAM, Oliveira MF, Santos PRS, Assis Neto AC. 2014. Ultrastructure of the epididymis and vas deferens of agoutis at different stages of sexual development. *Anim Reprod Sci*, 149:273-280.

Arroyo MAM, Silva FFS, Santos PRS, Silva AR, Oliveira MF, Assis Neto AC. 2017. Ultrastructure of spermatogenesis and spermatozoa in agoutis during sexual development. *Reprod Fertil and Dev*, 29(2):383-393.

Assis-Neto AC, Carvalho MAM, Melo MIV, Miglino MA, Oliveira MF, Almeida MM. 2003a. Aspectos biométricos do desenvolvimento testicular e corporal de cutias (*Dasyprocta agouti*) criadas em cativeiros. *Braz J Vet Res Anim Sci*, 40:154-160.

Assis-Neto AC, Melo MIV, Carvalho MAM, Miglino MA, Oliveira MF, Menezes DJA, Papa PC, Kfoury Júnior JR. 2003b. Análise quantitativa do estabelecimento da espermatogênese em cutias (*Dasyprocta aguti*) criadas em cativeiro. *Braz J Vet Res Anim Sci*, 40:180-184.

Barbosa DFSF, Silva JBA, Oliveira MF, Araujo SAC. 2010. Avaliação qualitativa de carnes de catetos (*Tayassu tajacu*, LINNAEUS, 1758) e cutias (*Dasyprocta aguti*, Linnaeus, 1758) Criados em cativeiro. Dissertação Mestrado. - Universidade Federal Rural do Semi-Árido, Mossoró, RN.

Bonaudo T, Le Pendu Y, Faure JF, Quantz D. 2005. The effects of deforestation on wildlife along the transamazon highway. *Eur J Wildlife Res*, 51(3):199-206.

Borges AA, Queiroz Neta LQ, Santos MVO, Santos MLT, Lima GL, Oliveira MF, Silva AR, Pereira AF. 2015. Isolation of somatic cell derived from ear tissue of collared peccary (*Pecari tajacu* Linnaeus, 1758) submitted to different vitrification techniques. *Anim Reprod*, 12(1):842.

Campos LB, Peixoto GCX, Lima GL, Castelo TS, Souza ALP, Oliveira MF, Silva AR. 2015. Monitoramento do ciclo estral de cutias (*Dasyprocta leporina* Lichtenstein, 1823) através de citologia esfoliativa vaginal e ultrassonografia. *Pesq Vet Bras*, 35:188-192.

Carvalho MAM, Machado Júnior AAN, Silva RAB, Conde Júnior AM, Menezes DJA, Righi DA. 2008. Arterial supply for the penis in Agoutis *(Dasyprocta prymnolopha*, Wagler, 1831). *Anat Histol Embryol*, 37:60- 62.

Castelo TS. 2015. Obtenção e conservação de espermatozoides de cutia (dasyprocta leporina linneaus, 1753) do Semiárido brasileiro. Tese doutorado. Programa de Pós Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO), Natal, RN. 134f.

Castelo TS, Silva AM, Bezerra LG, Costa CY, Lago AE, Bezerra JA, Campos LB, Praxedes EC, Silva AR. 2015. Comparison among different cryoprotectants for cryopreservation of epididymal sperm from agouti (*Dasyprocta leporina*). *Cryobiology*, 71:442-447.

Castelo TS, Souza ALP, Lima GL, Peixoto GCX, Campos LB, Oliveira MF, Silva AR. 2016. Interactions among different devices and electrical stimulus on the electroejaculation of captive agoutis (*Dasyprocta leporina*). *Reprod Domest Anim*, 50:492-496.

Comizzoli P, Mermillod P, Cognie Y, Chai N, Legendre X, Mauge R. 2001. Succesful *in vitro* production of embryos in the red deer (*Cervus elaphus*) and the sika deer (*Cervus nippon*). *Theriogenology*, 55:649-659.

Costa CAS, Borges AA, Santos MVO, Queiroz Neta LB, Santos MLT, Franca PHF, Oliveira MF, Silva AR, Pereira AF. 2015. Aplicação da vitrificação para fins de conservação de tecido somático de cutias (*Dasyprocta leporina*). In: Seminário de Iniciação Científica da UFERSA, 21, 2015, Mossoró, RN. Anais... Mossoró: UFERSA.

Costa GMJ, Leal MC, Ferreira AC, Guimarães DA, França LR. 2010. Duration of spermatogenesis and spermatogenic efficiency in large neotropical rodent species: the Agouti (*Dasyprocta leporina*) and Paca (*Agouti paca*). *J Androl*, 31:489-499.

Costa PM, Martins CF. 2008. Conservação de recursos genéticos animais através de biotécnicas de reprodução. *Universitas Ciências da Saúde*, 6:39-55.

Deutsch LA, Puglia LRR. 1988. Paca, p.45-50. *In*: Deutsch LA and Puglia LRR (ed.), Os Animais Silvestres: Proteção, doenças e manejo. Editora Globo, Rio de Janeiro, 191p.

Durrant BS. 2009. The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). *Theriogenology*, 71:113-122.

Emmons LH, Feer F. 1997. Neotropical rainforest mammals: a Field Guide. (2 ed). Chicago: University of Chicago Press. 307p.

Ferraz MS, Conde Junior AM, Fortes EAM, Almeida HM, Rodriguez NM, Silva AR, Carvalho MAM. 2009. Caracterização morfométrica de espermatozóides epididimários de cutias (*Dasyprocta aguti*) criadas em cativeiro. *In:* Congresso Brasileiro de Reprodução Animal, 18, 2009, Belo Horizonte, MG. Anais... Belo Horizonte: CBRA. (CD-ROM), p.291. Resumo.

Ferraz MS, Menezes DJA, Pessoa GT, Cabral RM, Illera MJ, Silva AR. 2011. Collection and evaluation of epididymal sperm in captiveagoutis (*Dasyprocta aguti*), *Theriogenology*, 75:459.

Forget PM, Milleron T. 1991. Evidence for secondary seed dispersal by rodents in Panama. *Oecologia*, 87:596-599.

Goovaerts IGF, Hoflanck GG, Van Soom A, Dewulf J, Nichi M, Kruif A, Bols PE. 2006. Evaluation of epididymal semen quality using the Hamilton-Thorne analyser indicates variation between the two caudae epididymides of the same bull. *Theriogenology*, 66:323-330.

Guimarães DA, Moreira D, Vale WG. 1997. Determinação do ciclo reprodutivo da cutia (*Dasyprocta prymnolopha*) através do diagnóstico colpocitológico. *Acta Amaz*, 27(1):55-64.

Guimarães DAA, Ramos RSL, Garcia GW, Ohashi OM. 2009. The stimulatory effect of male agouti (*Dasyprocta prymnolopha*) on the onset of female puberty. *Acta Amaz*, 39(4):759-762.

Guimarães DA, Ramos RL, Ohashi OM, Garcia GW, Vale WG. 2011. Plasma concentration of progesterone and 17β-estradiol of blackrumped agouti (*Dasyprocta prymnolopha*) during the 505 estrous cycle. *Revta Bras Tróp*, 59(1):29-35.

Henry O. 1999. Frugivory and the importance of seeds in the diet of the orange-rumped agouti (*Dasyprocta leporina*). *J Trop Ecol*, 15:291-300.

Hosken FM, Silveira AC. 2001. Criação de cutias. Viçosa, MG: Aprenda fácil, 4:21-22.

IUCN Red List Categories and Criteria, Version 3.1. IUCN, Gland, Switzerland and Cambridge, UK. Available on: www.iucnredlist.org. Accessed on: January 26th 2018.

Kleiman DG, Eisenberg JF, Maliniak E. 1979. Reproductive parameters and productivity of caviomorph rodents. In: Vertebrate ecology in the northern Neotropics. Eisenberg JE (ed.). Smithsonian Institution Press, Washington, D.C. pp.173-183.

Lange RR, Schimidt EMS. 2007. Tratado de animais selvagens. Roca: São Paulo, Brasil, p.476.

Lima, JFS. 2000. Diversidade cariológica de roedores de pequeno porte do Estado do Tocantins, Brasil. Tese (Doutorado) - Universidade Estadual Paulista, Instituto de Biociências de Rio Claro, SP, Brasil. 183f.

Loi P, Ptak G, Barboni B, Fulka J, Cappai P, Clinton M. 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat Biotechnol*, 19:962-964.

Lopes JB, Cavalcante RR, Almeida MM, Carvalho MAM, Moura SG, Filho LAD, Conceição WLF. 2004. Desempenho de Cutias (*Dasyprocta prymnolopha*) Criadas em Cativeiro do Nascimento até o Desmame em Teresina, Piauí. *Rev Bras Zoo*, 33(6):2318-2322.

Martinez AC, Oliveira FS, Abreu CO, Martins LL, Pauloni AP, Moreira N. 2013. Colheita de semên por eletroejaculação em cutia-parda (*Dasyprocta azarae*). *Pesq Vet Bras*, 33(1):86-88.

Martinez-Pastor F, Garcia-Macias V, Alvarez M, Chamorro C, Herraez P, Paz P, Anel L. 2006. Comparison of two methods for obtaining spermatozoa from the caudaepidydimis of Iberian red deer. *Theriogenology*, 65:471-485.

Mayor P, Bodmer RE, And Lopez-Bejar M. 2011. Functional anatomy of the female genital organs of the wild black agouti (*Dasyprocta fuliginosa*) female in the Peruvian Amazon. *Anim Reprod Sci*, 123:249-257.

Menezes DJA, Carvalho MAM, Assis-Neto AC, Oliveira MF, Farias EC, Miglino MA, Medeiros GX. 2003. Morfologia dos órgãos genitais externos do macho de cutia (*Dasyprocta aguti*, Linnaeus, 1766). *Braz J Vet Res Anim Sci*, 40(2):148-153.

Mollineau WM, Adogwa A, Jasper N, Young K, Garcia G. 2006. The gross anatomy of the male reproductive system of a neotropical rodent: the agouti (*Dasyprocta leporina*). *Anat Histol Embryo*, 35:47-52.

Mollineau WM, Adogwa AO, Garcia GW. 2008. A preliminary tech-nique for electro-ejaculation of agouti (*Dasyprocta leporina*). *Anim Reprod Sci*, 7:92-108.

Mollineau WM, Adogwa AO, Garcia GW. 2010a. Improving the Efficiency of the Preliminary Electroejaculation Technique Developed for Semen Collection from the Agouti (*Dasyprocta leporina*). *J Zoo and Wildlife Medic*, 41:633-637.

Mollineau WM, Adogwa AO, Garcia GW. 2010b. Liquid and frozen storage of agouti (*Dasyprocta leporina*) semen extended with UHT Milk, unpasteurized coconut water, and pasteurized coco-nut water. *Vet Med Int*, 2011:1-6.

Mollineau WM, Sampson T, Adogwa AO, Garcia GW. 2012. Anatomical Stages of Penile Erection in the Agouti (*Dasyprocta leporina*) Induced by Electro-Ejaculation. *Anat Histol Embryol*, 41:392-394.

Monteiro GA, Papa FO, Zahn FS, Della JA Jr, Melo CM, Maziero RR, Avanzi BR, Alvarenga MA, Guasti PN. 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Anim Reprod Sci*, 127:197-201.

Oliveira FS, Martins LL, Canola JC, Oliveira PT, Pécora JD, Pauloni AP. 2012. Macroscopic description of teeth of Azarae agouti (*Dasyprocta azarae*). *Pesq Vet Bras*, 32(1):93-95.

Oliveira GB, Araújo Júnior HN, Silva Costa H, Silva AR, Moura CEB, Oliveira Rocha HA, Miglino MA, Oliveira MF. 2017. Post implantation development of red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758). *Anim Reprod Sci*, 182:35-47.

Pachaly JR, Lange RR, Margarido TCC. 1999. Punção venosa em cutias (*Dasyprocta sp.*). *Hora Vet*, 18A:62-64.

Paula TAR, Walker NJ. 2012. Reproductive morphology and physiology of the male capybara. *In*: Moreira JR; Ferraz, KMPMB, Herrera EA, MacDonald DW. Capybara: Biology, use and Conservation of Exceptional Neotropical Species. Springer, New York, 109-115.

Peixoto GCX, Maia KM, Almeida LM, Campos LB, Oliveira GB, Oliveira MF, Brito AB, Domingues SFS, Silva AR. 2018. Indução do estro em cutias (*Dasyprocta Leporina*) Utilizando protocolos a base de prostaglandina isolada ou em associação com análogo de GnRH. *Arq Bras Med Vet Zoo*, 70:806-814.

Pereira AF, Feltrin C, Almeida KC, Carneiro IS, Avelara SRG, Alcântara Neto AS, Sousa FC, Melo CHS, Moura RR, Teixeiraa DIA, Bertolini LR, Freitas VJF, Bertolini M. 2013. Analysis of factors contributing to the efficiency of the in vitro production of transgenic goat embryos (*Capra hircus*) by handmade cloning (HMC). *Small Rumin Res*, 109(2):163-172.

Praxedes ECG. 2017. Conservação de tecido ovariano de cutias (*Dasyprocta leporina* Lichtenstein, 1823) criadas em cativeiro no semi-árido nordestino. Dissertação Mestrado. Universidade Federal Rural do Semi-Árido, Mossoró, RN. 105f.

Praxedes ECG, Lima GL, Bezerra LGP, Santos FA, Bezerra MB, Guerreiro DD, Rodrigues APR, Domingues SFS, Silva AR. 2018. Development of fresh and vitrified agouti ovarian tissue after xenografting to ovariectomised severe combined immunodeficiency (SCID) mice. *Reprod Fertil Dev*, 30 (3):459-468.

Ramos RSL, Vale WG, Assis FL. 2003. Karyotypic analysis in species of the genus Dasyprocta (Rodentia: Dasyproctidae) found in Brazilian Amazon. *An Acad Bras Cienc*, 75(1):55-69.

Ribeiro EEA, Batista MCS, Carvalho MAM, Silva JAL. 2008. Níveis iônicos e enzimáticos de cutias (*Dasyprocta sp.)* hígidas, criadas em cativeiro, influência do sexo e da idade. *Arq Bras Med Vet Zootec*, 70:651-655.

Rodrigues RF, Miglino MA, Ferraz RHS, Morais-Pinto L. 2003. Placentação em cutias (*Dasyprocta aguti*, Carleton MD): aspectos morfológicos. *Braz J Vet Res Anim Sci*, 40:133-137.

Roopchand A. 2002 Wildlife and the Oil Industry: Catshill Reserve a Case Study. M.Sc. Student project, The Department of Food Production, Faculty of Science and Agriculture, University of the West Indies, St Augustine, Trinidad and Tobago.

Santos CR, Filho HO, Barbosa OR, Cardozo RM, Araujo DN, Fornari DC, Guaragni M, Fregadolli RM. 2005. Etologia de capivaras (*Hydrochaerism hydrochaeris* L. 1766) jovens semiconfinadas no Norte do Estado do Paraná. *Acta Sci Anim Sci*, 1(27):163-169.

Santos EAA, Lima GL, Praxedes ECG, Silva AM, Maia KM, Oliveira MF, Rodrigues APR, Silva AR. 2018. Estimation, Morphometry and Ultrastructure of Ovarian Preantral Follicle Population in Agouti (*Dasyprocta leporina*). *Pesq Vet Bras*, 38(1):175-182.

Silva DC, Fagundes NC, Teixeira FB, Penha NE, Santana LN, Mendes-Oliveira AC, Lima RR. 2013. Anatomical and histological characteristics of teeth in agouti (*Dasyprocta prymnolopha* Wagler, 1831). *Pesq Vet Brasil*, 33:51-57.

Silva MA, Peixoto GCX, Santos EAA, Castelo TS, Oliveira MF, Silva AR. 2011. Recovery and cryopreservation of epididymal sperm from agouti (*Dasiprocta aguti*) using powdered coconut water (ACP-109c) and Tris extenders. *Theriogenology*, 76:108-1089.

Silva MA, Peixoto GCX, Sousa PC, Bezerra FS, Bezerra AC, Silva AR. 2012. Interactions between straw size and thawing rates on the cryopreservation of agouti (*Dasyprocta aguti*) epididymal sperm. *Reprod Domest Anim*, 47:4-6.

Silva AR, Castelo TS, Lima GL, Peixoto GCX. 2010. Conservation of germplasm from wild animalsof the Caatinga biome. *Acta Sci Anim Sci*, 38:373-389.

Silva AR, Morato RG, Silva LDM. 2004. The potential for gamete recovery from nondomestic canids and felids. *Anim Reprod Sci*, 81:159-175.

Singh MD, Morris MJ, Guimaraes DA, Bourne G, Garcia, GW. 2016. Serological evaluation of ovarian steroids of red-rumped agouti (*Dasyprocta leporina*) during the estrous cycle phases. *Anim Reprod Sci*, 175:27-32.

Smythe, N. 1987. The paca (*Cuniculus paca*) as a dosmetic source of protein the Neotropical, Humid Lowlands. *Appl Anim Behav Sci*, 17:155-170.

Sousa F, Pessoa GT, Moura LS, Araújo JR, Rodrigues R, Barbosa M, Diniz AN, Souza AB, Silva EG, Lucena LU, Sanches MP, Silva-Filho OF, Guerra PC, Sousa JM, Neves WC, Alves FR. 2017. Organogenesis and foetal haemodynamics during the normal gestation of healthy black-rumped agoutis (*Dasyprocta prymnolopha*, Wagler, 1831) bred in captivity. *Reprod Domest Anim*, 52(1):60-66.

Sousa FCA, Alves FR, Fortes EAM, Ferraz MS, Machado Júnior AAN, Menezes DJA, Carvalho MAM. 2012. Pregnancy in Hystricomorpha: gestational age and embryonic fetal development of agouti (*Dasyprocta prymnolopha* Wagler, 1831) estimated by ultrasonography. *Theriogenology*, 78(6):1278-1285.

Souza PC, Ramos RSL, Guimarães DAA, Ferreira ACS, Bastos LV, Batista CR, Ohashi OM. 2003. Determinação da população folicular em cutias (*Dasyprocta sp.)* criadas em cativeiro. *Rev Bras Reprod Anim*, 27(2):286-287.

Wanderley LS, Luz HK, Faustino LR, Lima IM, Lopes CA, Silva AR, Báo SN, Campello CC, Rodrigues AP, Figueiredo JR. 2012. Ultrastructural features of agouti (*Dasyprocta aguti*) preantral follicles cryopreserved using dimethyl sulfoxide, ethylene glycol and propanediol. *Theriogenology*, 77(2):260-7.

Weir B. 1971. Some observations on reproduction in the female agouti (*Dasyprocta aguti*). *J Reprod Fertil*, 24:203-211.

Weir BJ, Rowlands JW. 1974. Functional anatomy of the hystricimorph ovary. *Symp Zool Soc Lond*, 34:303-332.

Capítulo II

In vitro culture of fresh and vitrified ovarian preantral follicles enclosed in *Dasyprocta leporina* ovarian tissues using TCM-199 supplemented with FSH.

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Cultivo *in vitro* de folículos ovarianos pré antrais de *Dasyprocta leporina* incluoso em tecido ovariano, frescos e vitrificados usando TCM199 suplementado com FSH.

In vitro culture of fresh and vitrified ovarian preantral follicles enclosed in Dasyprocta leporina ovarian tissues using TCM-199 supplemented with FSH**.**

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Abstract

Considering the relevance of establishing biodiversity conservation tools, the present study aimed to investigate the use of TCM-199 supplemented with different follicle-stimulating hormone (FSH) concentrations on *in vitro* culture of fresh and vitrified preantral follicles enclosed in agouti ovarian tissues. In a first experiment, six pairs of ovaries were fragmented and

cultured *in vitro* for 6 days according to the groups: 10 ng/mL pFSH (FSH10 group) and 50 ng/mL (FSH50 group). Non-cultured tissues were considered as a control group. In the second experiment, vitrified/warmed fragments derived from four pairs of ovaries were cultured in the presence of the best concentration established in the previous experiment (cryopreserved and cultured group). Non-cryopreserved (fresh control group) and cryopreserved but non-cultured (non-cultured group) tissues were used as controls. For both experiments, preantral follicles were evaluated for morphology, development, and viability. After culturing fresh samples, the FSH50 group showed a higher percentage of morphologically normal follicles when compared to FSH10 group ($P < 0.05$). Moreover, this same response was observed for primordial follicles. Regardless of the concentrations of FSH used during *in vitro* culture, no difference was observed regarding the percentage of viable follicles ($P > 0.05$). Thus, the FSH50 group was used for further experiment, in which a total of 76.2 ± 7.2% normal PAFs previously vitrified was found after 6-day culture, also presenting the highest values ($P < 0.05$) for primordial follicles morphology (95.2 ± 4.7%). Nevertheless, *in vitro* culture did not improve the viability of preantral follicles derived from cryopreserved tissues ($P > 0.05$). In summary, we described for the first time the *in vitro* culture of preantral follicles of red-rumped agouti and observed that 50 ng/mL pFSH favors the morphology, development and viability of these follicles.

Keywords: Wildlife, female germplasm, follicular development, vitrification, biobank.

1. Introduction

Given the emergence of the sixth phase of mass extinction (Lueders and Allen, 2020), efforts directed towards the development of biodiversity conservation strategies become a growing demand that involves different sectors of society. Into an ecosystem, the loss of a unique component would represent a great damage for all the species that

inhabit there. As a pray for carnivores and a seed disperser, the agouti largely contributes for the equilibrium of their habitats (Hosken, Silveira, 2001). From the 13 catalogued agouti species, only three remain presenting a stable population, including the *Dasyprocta leporina*, the red-rumped agouti that inhabits Brazilian Caatinga (Emmons and Reid, 2016). At this sense, various efforts related to ex situ conservation strategies, as the biobank formation (Castelo et al. 2015; Praxedes et al., 2020), have been conducted to help on the efforts to maintain the stability of free-living *D. leporina* populations and provide technologies for its captive sustainable breeding (Lall et al., 2020).

Recently, the establishment of a solid-surface vitrification (SSV) protocol for red-rumped agouti ovarian tissues preservation was demonstrated, denoting the possibility of conserving valuable female germplasm in biobanks (Praxedes et al., 2020). Despite the goal reached by this study, the posterior use of the samples after rewarming remains a great challenge. At this sense, the development of *in vitro* culture (IVC) systems that provide appropriate conditions for the ovarian preantral follicles (PAFs) to grow (Cecconi et al., 2004) is a step extremely important for the wildlife conservation puzzle.

Due to its rich composition, the Tissue Culture Media 199 (TCM-199) has been indicated as an adequate media for ovarian tissue IVC even for wild species (Madboly et al., 2017; Lima et al., 2018). Among the supplements incorporated to the media, the follicle-stimulating hormone (FSH) stands out, since it is highlighted for presenting indirect action on follicles initial development through the stimuli of paracrine factors from the ovarian stroma and from the follicles (Van Den Hurk & Zhao, 2005), thus promoting initial development through the stimulation of cell proliferation and steroid synthesis (Martins et al., 2008). Because the heterogeneity of the wild species physiology, however, the determination of adequate concentrations of media supplements that support the development of ovarian follicles is a key point for the establishment of effective IVC

systems. In fact, the FSH concentration in the medium varies even for domestic species, which can present distinct responses. While goat PAFs grow at the use of 50 ng/mL FSH in culture media (Matos et al., 2007), bovine PAFs do not respond to any FSH concentrations varying from 1 to 100 ng/mL, even during a 14-days culture (Fortune et al., 1998).

Therefore, the aim was to evaluate the effect of an IVC system based on the use of TCM-199 supplemented with different concentrations of pFSH (10 and 50 ng/mL) on the morphology, development, and viability of red-rumped agouti PAFs enclosed in ovarian tissue. Then, the effect of this IVC system was checked on the same parameters of red-rumped agouti PAFs subjected to vitrification.

Materials and methods

The Ethics Committee of Federal Rural University of Semi-Arid (UFERSA, no. 23091.005916/2015-74) and the Chico Mendes Institute for Biodiversity Conservation (no. 66618-1) approved the experimental protocols. Unless stated otherwise, chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and collection of ovaries

Six mature agouti females aging 2–3 years and weighing 2.2–2.7 kg, from the Center of Multiplication of Wild Animals, UFERSA (Mossoró, Brazil; 5º10'S, 3º10 ' W), were used for the study. These individuals were distributed as six females for the first experiment, and four for the second one. For ovarian collection, animals were fasted for 12 h, restrained using a hand net, and premedicated with intramuscular administration of 15 mg/kg ketamine hydrochloride (Ketalar; Pfizer, São Paulo, Brazil) and 1 mg/kg xylazine hydrochloride (Rompun; Bayer, São Paulo, Brazil). After 15 min, anesthesia

was induced with intravenous administration of sodium thiopental (Thiopentax; Cristalia, São Paulo, SP, Brazil), and the animals were subsequently euthanized with intravenous 1 mL/kg potassium chloride (Castelo et al., 2015). Immediately thereafter, there was opening of the abdomen and recovery of the ovaries that were washed in 70% ethanol, followed by two lavages in Minimum Essential Medium (MEM). The ovaries were transported within 1 h to the laboratory in MEM at 4 °C.

Experimental design

Two experiments were designed (Fig. 1). In the first experiment, pairs of ovaries were fragmented and cultured *in vitro* for 6 days according to the groups: 10 ng/mL pFSH (FSH10 group) and 50 ng/mL (FSH50 group). Non-cultured tissues were considered as a control group. In the second experiment, vitrified/warmed fragments were cultured in the presence of the best concentration established in the previous experiment (cryopreserved and cultured group). Non-cryopreserved (fresh control group) and cryopreserved but non-cultured (non-cultured group) tissues were used as controls. For both experiments, preantral follicles were evaluated for morphology, development, and viability.

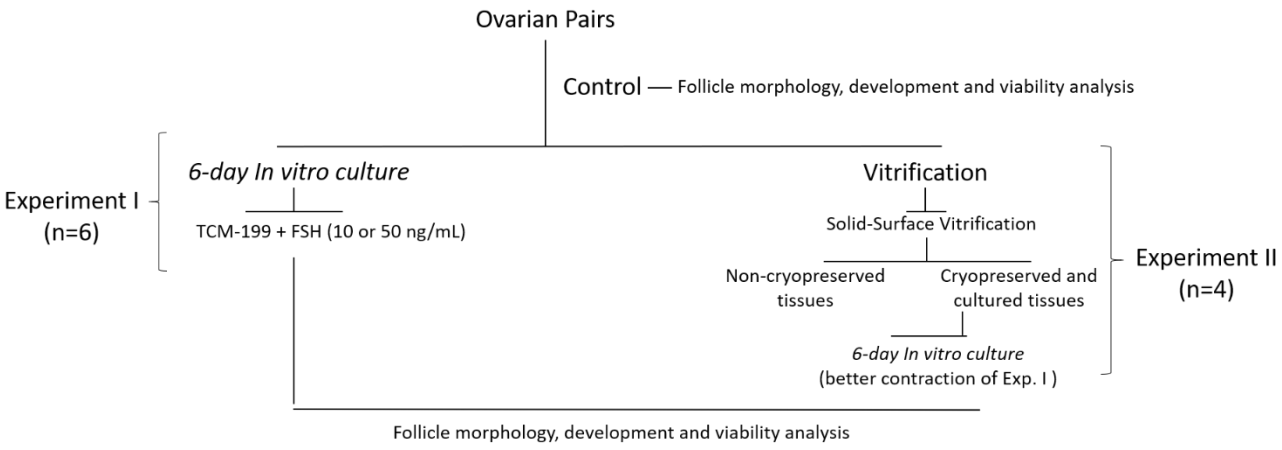

Figure. 1. Experimental design to assess the effect of different pFSH concentrations and solid surface vitrification (SSV) on agouti (*Dasyprocta leporina*) preantral follicles morphology, development and viability following in vitro culture for six days.

In vitro culture of ovarian fragments

Initially, ovarian tissues were divided in 12 fragments (9.0 mm^3 = 3 × 3 × 1 mm). Four fragments constituted the fresh control group that was immediately analyzed, and the others were distributed for cultured groups (4 fragments per group). For the IVC, fragments were allocated in plates placed in 24-well culture dishes containing 1.0 mL of culture medium consisted of TCM-199 supplemented with ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, and 5.0 ng/mL selenium), 0.23 mM sodium pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin, and different pituitary FSH (Folltropin®, Veterpharm, Canada) concentrations as 10 ng/mL (as reported for another rodent, the mouse; Hardy et al., 2017) or 50 ng/mL (as reported for goats; Magalhães et al., 2008). Samples were then cultured at 38.5 °C and 5% CO_2 in a humidified incubator. After IVC for 6 days (Cecconi et al., 2004), ovarian fragments were fixed in 4% paraformaldehyde solution for 12 h and subjected to histological processing for analysis.

Ovarian tissue vitrification

Ovaries were divided in 12 fragments, being four immediately analyzed. For SSV (Praxedes et. al., 2020), the other eight fragments were individually placed in 1.8 mL plastic tubes containing vitrification solution consisted of MEM supplemented with 10 mg/mL fetal calf serum (FCS), 0.25 M sucrose and 3.0 M ethylene glycol (EG). After exposure to the vitrification solution for 5 min, the samples were dried using sterile gauze and placed on aluminum foil on a LN_2 surface. Once vitrified, the samples were transferred (with nitrogen-cooled forceps) to cryovials for storage in LN_2 at −196 °C. After two weeks, samples were rewarmed at 25 °C for 1 min and immersed in a water bath at 37 °C for 5 sec. The cryoprotectants were removed by three consecutive washes

of 5 min in MEM supplemented with 10% FCS and decreasing sucrose concentrations (0.5, 0.25, and 0.0 M). After rewarming, four fragments were immediately analyzed and the others were cultured for 6 days, and then evaluated.

Histological evaluation

For morphological examination, after fixation in 4% paraformaldehyde solution for 12 h, the ovarian cortex fragments were dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Then, the ovarian tissue samples were serially sectioned at 7 µm, and each 5th section was assembled on slides and stained with hematoxylin and eosin for evaluation under a light microscope at a magnification of ×100.

When oocytes presented a regular shape with a homogeneous cytoplasm and well-organized granulosa cells, the PAFs were classified as morphologically normal; if they presented a pyknotic nucleus or ooplasma shrinkage with unorganized granulosa cells, PAFs were categorized as degenerated. Moreover, depending on their developmental stage, PAFs were also classified as primordial, primary, or secondary (Santos et al., 2018). Additionally, the proportions of healthy primordial and growing follicles were calculated before (fresh control) and after culturing to evaluate the follicular development at each treatment (Campos et al., 2021).

Viability analysis

The ovarian fragments were sliced using a scalpel blade, and placed on a stirrer with MEM for 10 min. After agitation, the solution was filtered in a 500 µm filtration screen and the suspension was centrifuged at 280 × g for 10 min. The suspension (90 µL) containing individual PAFs was added to 10 µL of 0.4% trypan blue solution and

subsequently incubated at 25 °C for 5 min. A total of 30 PAFs were evaluated per group under inverted microscopy (BX51TF, Olympus Co., Tokyo, Japan). The PAFs were classified as viable when the oocyte and <10 % of granulosa cells were not stained or were deemed non-viable when the oocyte and/or > 10 % of granulosa cells were stained (Lucci et al., 1999).

Statistical analysis

All statistical analyses were carried out using StatView 5.0 software (SAS Inc., Cary, NC, USA). Data were expressed as means and standard error of means (SEM). Results were analyzed by Smirnov–Kolmogorov and Bartlett tests to confirm normal distribution and homogeneity of variance, respectively. Comparisons among treatments regarding PAFs morphology, development and viability were evaluated by ANOVA followed by PLSD Fisher. Values were considered statistically significant when $P < 0.05$.

Results

Effect of TCM-199 plus different FSH concentrations on fresh PAFs

Regarding PAFs morphology found during the initial culture of fresh ovarian tissues from agoutis, a total of 558 follicles was evaluated (Table 1). The positive effect of TCM-199 supplemented with 50 ng/mL FSH was evident, since it provided 82.07 ± 7.43 % morphologically normal follicles, a valuer significantly higher than those obtained by using 10 ng/mL FSH ($P < 0.05$). The effective effect of the highest FSH concentration was also verified for primordial follicles morphology ($P < 0.05$).

Table 1. Values (means ± SEM) for normal morphology of *Dasyprocta leporina* (n=6) preantral follicles in non-cultured group (fresh control) and in ovarian tissues cultured in TCM-199 with different FSH concentrations (10 and 50 ng/mL) for six days.

Treatments	Morphologically Normal Preantral Follicles (%)		
	Primordial	Primary	Total
Fresh control group	61.5 ± 9.6[ab] (104/129)	85.8 ± 5.50[a] (39/50)	72.6 ± 6.7[ab] (143/179)
FSH 10 ng/ mL	47.3 ± 17.0[b] (86/117)	75.1 ± 4.7[a] (58/66)	61.2 ± 9.5[b] (144/183)
FSH 50 ng/ mL	80.1 ± 12.1[a] (109/131)	84.1 ± 8.7[a] (49/65)	82.1 ± 7.4[a] (158/196)

[a,b] Different superscript letters indicate significant differences in the same column ($P < 0.05$).

With regards to PAFs development, there were no significant differences among experimental groups. At observing the graph (Fig. 2), however, the tendency for an increase in the proportion of growing follicles after 6-day culture is evident at the use of TCM-199 plus 50 ng/mL FSH (54.33 ± 10.6%) when compared to fresh control (31.5 ± 9.5 %) and that cultured with 10 ng/mL FSH (41.3 ± 9.9 %).

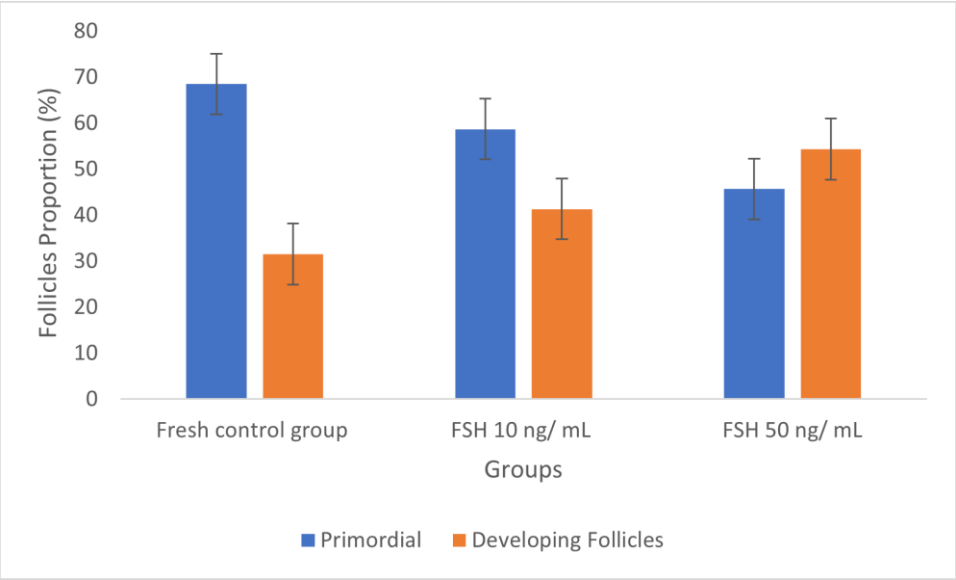

Figure 2. Proportions* (means ± SEM) of *Dasyprocta leporina* morphologically normal primordial and developing follicles in non-cultured group (fresh control) and in ovarian tissues cultured in TCM-199 with different FSH concentrations (10 and 50 ng/mL) for six days. *No statistically significant difference was observed ($P > 0.05$).

Treatments	Viability*	
	%	Viable/Total
Fresh control	90.56 ± 3.3	163/180
FSH 10 ng/ mL	83.89 ± 4.8	151/180
FSH 50 ng/ mL	81.67 ± 9.9	147/163

*No statistically significant difference was observed (P > 0.05).

Effect of culture system on morphology, development and viability of vitrified PAFs

Considering the fresh and vitrified samples cultured for 6 days, a total of 299 PAFs were analyzed for morphology (Table 3). When compared to the control group (71.8 ± 2.1%), *D. leporina* PAFs were efficiently preserved by SSV that presented 67.5 ± 13.9% normal PAFs immediately after rewarming. Moreover, a total of 76.2 ± 7.2% normal PAFs was found after 6-day IVC in TCM-199 supplemented with 50 ng/mL FSH, which also presented the highest values (P < 0.05) for primordial follicles morphology (95.2 ± 4.7%).

Table 3. Values (means ± SEM) for normal morphology of *Dasyprocta leporina* (n = 4) preantral follicles in fresh control group and in the samples subjected to solid surface vitrification (SSV) and then cultured in TCM-199 plus 50 ng/mL for 6 days.

	Morphologically Normal Preantral Follicles		
Treatments	Primordial	Primary	%MNPF*
Fresh control	68.4 ± 6.6[b] (44/63)	73.9 ± 18.7[a] (27/35)	71.8 ± 2.1[a] (71/98)
SSV non-cultured	74.2 ± 12.4[b] (54/79)	69.7 ± 13.5[a] (22/40)	67.5 ± 13.9[a] (76/119)
SSV cultured	95.2 ± 4.7[a] (24/26)	68.1 ± 9.4[a] (37/56)	76.2 ± 7.2[a] (61/82)

[a,b] Different superscript letters indicate significant differences in the same column (P<0.05).

Regarding PAFs development (Fig. 3), we found the proportions of 73.9 ± 18.7 %, 52.2 ± 13.5 % and 68.0 ± 9.4 % developing PAFs in the fresh, vitrified and vitrified-cultured samples, respectively. No differences were found among treatments (P < 0.05).

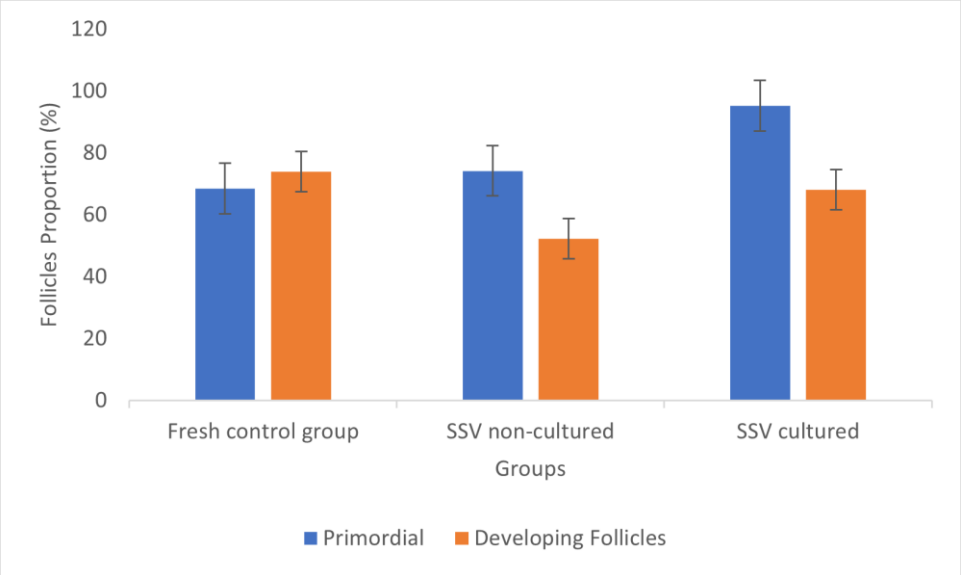

Figure 3. Proportions* (means ± SEM) of *Dasyprocta leporina* morphologically normal primordial and developing follicles in fresh control group and in the samples subjected to solid surface vitrification (SSV) and then cultured in TCM-199 plus 50 ng/mL for 6 days. *No statistically significant difference was observed (P < 0.05).

Immediately after vitrification (65.8 ± 11.4 %), PAFs viability was similar to that found for fresh control group (88.3 ± 4.81%). After 6-day culture in TCM-199 plus 50 ng/mL FSH, vitrified samples yet presented an amount of 60 ± 9.2%, similarly as those values observed immediately after rewarming (P > 0.05).

Table 4. Values (means ± SEM) for viability of *Dasyprocta leporina* (n=4) preantral follicles in fresh control group and in the samples subjected to solid surface vitrification (SSV) and then cultured in TCM-199 plus 50 ng/mL for 6 days.

Treatments	Viability	
	%	Viable/Total
Control group	88.3 ± 4.8[a]	106/120
SSV non-cultured	65.8 ± 11.4[ab]	79/120
SSV cultured	60.00 ± 9.16[b]	73/120

[a,b] Different superscript letters indicate significant differences in the same column ($P<0.05$).

DISCUSSION

To demonstrate the possibility of exploitation the female genetic material stored in biobanks, we present an initial attempt for the development of *in vitro* culture systems able to provide adequate conditions for the restoration of vitrified PAFs of *D. leporina*. Since the ovary contains thousands of follicles, collecting and preserving these follicles represent a huge opportunity for germplasm biobanking (Comizzoli et al., 2010). Therefore, the *in vitro culture* of oocytes recovered from preantral follicles, along with the efforts for the systematic collection and storage of germplasm, could enhance the management of endangered species populations (Campos et al., 2019).

As a media previously demonstrated for being efficient for laboratory rodents (Abedelahi et al., 2008), TCM-199 also supported the fresh and vitrified PAFs culturing in agoutis, a representative wild rodent species. This culture media is highlighted due its rich composition presenting amino acids, vitamins, ribonucleosides and deoxyribonucleosides, inorganic salts and energy sources (Mao et al.,2002). Despite its valuable compounds, however, supplements as growth factors and hormones are usually incorporated to the media to improve its effectiveness (Martins et al., 2008).

Little is known about folliculogenesis in agoutis (Santos et al., 2018), as well as which substances are involved in the initial follicular development. Based on the positive results demonstrated for other species (Matos et al., 2007a; Saraiva et al., 2010), we choose to verify the effects of different FSH concentrations supplemented to the culture media for agouti PAFs. In fresh samples, the most encouraging results were provided by

a 50 ng/mL FSH supplementation that allowed the follicle morphology and viability preservation, besides the follicle development. These results differ from those previously found for other rodent, the mice, in which a 10 ng/mL FSH concentration was efficient for culturing isolate PAFs (Hardy et al., 2017), which could indicate a variate response among different species.

FSH has been evidenced for promoting follicle initial growth through the stimulation of cell proliferation, steroid synthesis, and expression of receptors for epidermal growth factor (EGF) and luteinizing hormone (LH) (Martins et al., 2008). In laboratory rodents, a recombinant FSH has been commonly incorporated to the culture media for preantral follicles, mainly due to its high level of purity (Choi et al., 2008; Hardy et al., 2017). At the present research, however, we demonstrate the effective use of a commercial pituitary FSH for agouti ovarian tissues IVC. If replacing pituitary with recombinant FSH could improve the results obtained for agoutis PAFs, it remains a factor to be investigated.

As observed in previous studies (Praxedes et al., 2020), SSV provided the preservation of ~70% morphologically normal PAFs. This is an effective cryopreservation method that consists in an open system, being an excellent heat conductor that allows the sample to cool quickly and preserve a large percentage of morphologically normal follicles when using a lower volume of cryopreservation solution (Santos et al., 2007). Despite its efficiency, this is an open system that allows the contact of liquid nitrogen (LN2) with the ovarian fragments, which can expose tissues to cryogenic resistant pathogens (Grout et al., 2009). Therefore, other vitrification systems, as the ovarian tissue cryosystem (OTC) – a closed system (Carvalho et al., 2013), should be evaluated.

By this moment, the most effective way to provide conditions for the development of vitrified agouti PAFs is by the xenografting to immunodeficient mice (Praxedes et al., 2018). In parallel to xenografting, the development of IVC systems is highlighted for provide essential knowledge on the folliculogenesis by evidencing the effect of individual substances on the PAFs development. Besides it, it allows to mimic the dynamics of the ovarian environment, cell communications, and interaction with secretory, hormonal, and growth factors (Figueiredo et al., 2011). At this point, the present results represent only a small step on the journey to produce an effective culture system that allow the complete development of agouti PAFs up to ovulation and *in vitro* production of viable oocytes able to be used for other assisted reproductive technologies.

Of course, there is a perspective to improve the IVC system by trying other media as the MEM+ (Lima et al., 2018), other additives like the growth factors as the bone morphogenic protein 15 (BMP-15; Gomes et al., 2020) or the growth differentiating factor-9 (GDF-9; Campos et al., 2021), long-term culturing procedures (Choi et al., 2008), culturing isolate PAFs (Jamalzaei et al., 2016), and the use of three-dimensional systems (Asgari et al., 2015). Moreover, present data were only evaluated for morphology, development, and viability. Therefore, other morphological and functional approaches as the cell proliferation (Bandeira et al., 2015; Brito et al., 2018), DNA fragmentation (Brito et al., 2018), transmission electron microscopy (Carvalho et al., 2013), and quantitative RT-PCR (Brito et al., 2018) should be attempted to prove the efficiency of IVC systems for agouti PAFs.

In summary, we describe, for the first time, an in vitro culture system for red-rumped agouti preantral follicles enclosed in ovarian tissues. For this purpose, we suggest the use of a TCM-199 media supplemented with 50 ng/mL pituitary FSH. This is a

344 valuable information that contributes for biobanking the female germplasm from wild
345 hystricognath rodents.
346
347 **Declaration of interest**
348 None of the authors have conflict of interest.
349
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355 **REFERENCES**
356

357 Abedelahi A, Salehnia M, Allameh AA. The effects of different concentrations of sodium
358 selenite on the in vitro maturation of preantral follicles in serum-free and serum
359 supplemented media. J Assist Reprod Genet. 2008;25:483–488.
360 Asgari F, Valojerdi RM, Ebrahimi B, Fatehi R. Three-dimensional *in vitro* culture of
361 preantral follicles following slow-freezing and vitrification of mouse ovarian tissue.
362 Criobyology, 2015;71:529–536.
363 Bandeira FT, Carvalho AA, Castro SV, Lima LF, Viana DA, Evangelista J, Pereira MJS,
364 Campello CC, Figueiredo JR, Rodrigues APR. Two methods of vitrification followed by
365 in vitro culture of the ovine ovary: evaluation of the follicular development and ovarian
366 extracellular matrix. Reprod Domest Anim. 2015;50:177–185.

Brito DCC, Domingues SFS, Rodrigues APR, Maside C, Lunardi FO, Wu X, Figueiredo JR, Pieczarka JC, Santos RR. Cryopreservation of domestic cat (*Felis catus*) ovarian tissue: comparison of two vitrification methods. Theriogenology, 2018;111:69–77.

Campos LB, Silva AM, Praxedes ECG, Bezerra LGP, Lins TLB, Menezes VG, Matos MHT, Lima GL, Rodrigues APR, Silva AR. Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants. Cryobiology, 2019;91:77–83.

Campos LB, Silva AM, Praxedes ECG, Bezerra LGP, Lins TLB, Menezes VG, Matos MHT, Pereira AF, Figueiredo JR, Silva AR. Effect of growth differentiation factor 9 (GDF-9) on *in vitro* development of collared peccary preantral follicles in ovarian tissues. *Anim Reprod Sci.* 2021;226:106–717.

Carvalho AA, Faustino LR, Silva CM, Castro SV, Lopes CA, Santos RR, Bao SN, Figueiredo JR, Rodrigues APR. Novel wide-capacity method for vitrification of caprine ovaries: ovarian tissue cryosystem (OTC). Anim Reprod Sci. 2013;138:220–227.

Castelo TS, Silva AM, Bezerra LGP, Costa CYM, Lago AEA, Bezerra JAB, Campos LB, Praxedes ECG, Silva AR. Comparison among different cryoprotectants for cryopreservation of epididymal sperm from agouti (*Dasyprocta leporina*). Cryobiology, 2015;71:442–447.

Ceconi S, Cappachietti G, Russo V, Berardinelli P, Mattioli M, Barboni B. *In Vitro* Growth of Preantral Follicles Isolated from Cryopreserved Ovine Ovarian Tissue. Biol Reprod. 2004;70:12–17.

Comizzoli P, Songsasen N, Wildt DE. Protecting and extending fertility for females of wild and endangered mammals. Cancer Treat Res. 2010;156:87–100.

Choi JY, Lee BE, Lee EY, Yoon BK, Choi DS. Effect of activin A and insulin-like growth factor-I on in vitro development of preantral follicles isolated from cryopreserved ovarian tissues in the mouse. Cryobiology. 2008;57:209–215.

Emmons L, Reid F. 2016. *Dasyprocta leporina*. The IUCN Red List of Threatened Species e.T89497102A22197762. Downloaded on 17 February 2021.

Fauque P, Amor AB, Joanne C, Agnani G, Bresson JL, Roux C. 2007. Use of trypan blue staining to assess the quality of ovarian cryopreservation. *Fertil Steril,* 87:1200–1207.

Figueiredo JR, Celestino JJH, Faustino LR, Rodrigues APR. *In vitro* culture of caprine preantral follicles: Advances, limitations and prospects. Small Rumin Res. 2011;98:192–195.

Fortune JE, Kito S, Wandji SA, Srsen V. Activation of Bovine and Baboon Primordial Follicles in vitro. Theriogenology. 1998;49:441–449.

Gomes HAN, Campos LB, Praxedes ÉCG, Oliveira MF, Pereira AF, Silva AR, Saraiva MVA. BMP-15 activity on in vitro development of collared peccary (*Pecari tajacu Linnaeus*, 1758) preantral follicles. Rep Dom Anim. 2020;55:958–964.

Grout WW, Morris GJ. Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. Theriogenology. 2009;71:079–1082.

Hardy K, Fenwick M, Mora J, Laird M, Franks S. Onset and Heterogeneity of Responsiveness to FSH in Mouse Preantral Follicles in Culture. Endocrinology. 2017;158:134–147.

Hosken FM, Silveira AC. 2001. Criação de cutias. Viçosa MG: Aprenda fácil; 4:21–22.

Jamalzaei P, Valojerd RM, Ebrahimi B, Ali Farrokhi. Oocyte maturation and expression pattern of follicular genes during *in vitro* culture of vitrified mouse pre-antral follicles. Gene Expr Patterns. 2016;20:63–60.

Lall KR, Jones KR, Garcia GW. 2020. Natural Habitat, Housing, and Restraint of Six Selected Neotropical Animals in Trinidad and Tobago with the Potential for Domestication. *Scientifica* (Cairo). doi: 10.1155/2020/9741762.

Lima GL, Luz VB, Lima LF, Rocha R, Castro SV, Castelo TS, Rodrigues APR, Figueiredo JR, Silva AR. Interactions between different media and FSH supplementation on in vitro culture of preantral follicles enclosed in ovarian tissue derived from collared peccaries (*Pecari tajacu* Linneaus, 1758). Reprod. Domest. Anim. 2018;53:880–888.

Lucci CM, Amorim CA, Báo SN, Figueiredo JR, Rodrigues APR, Silva JRV, Gonçalves PB. 1999. Effect in the interval sections of ovarian tissue in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci. 1999;56:39–49.

Lueders E, Allen WRT. Managed wildlife breeding-an undervalued conservation tool? Theriogenology, 2020;150:48–54.

Madboly MM, Abdel-Aal SS, Elsayed EH. Impact of cryopreservation method on dromedary camel ovary structure, viability, and development of antral follicular oocytes. Impact of cryopreservation method on dromedary camel ovary structure, viability, and development of antral follicular oocytes. Anim Reprod Sci. 2017;184:120–131.

Magalhaes DM, Araujo VR, Lima-Verde IB, Matos MH, Silva RC, Lucci CM, Bao SN, Campello CC, Figueiredo, J. R. Impact of pituitary FSH purification on in vitro early folliculogenesis in goats. Biocell. 2009;33:91–97

Mao J, Wu G, Smith MF, McCauley TC, Cantley TC, Prather RS, Day BN. Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral follicular development and antrum formation in vitro. Biol Reprod. 2002;67:1197–1203.

Martins FS, Celestino JJH, Saraiva MVA, Matos MHT, Bruno JB, Rocha-Junior CMC, Lima-Verde IB, Lucci CM, Báo SN, Figueiredo JR. Growth and differentiation factor-9

stimulates activation of goat primordial follicles in vitro and their progression to secondary follicles. Reprod. Fertil. Dev. 2008;20:916–924.

Matos MHT, Lima-Verde IB, Luque MCA, Maia Júnior JE, Silva JRV, Celestino JJH, Figueiredo JR. Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability in vitro. Zygot. 2007;15:173–182.

Praxedes ECG, Lima GL, Bezerra LGP, Santos FA, Bezerra MB, Guerreiro DD, Rodrigues APR, Domingues SFS, Silva AR. Development of fresh and vitrified agouti ovarian tissue after xenografting to ovariectomised severe combined immunodeficiency (SCID) mice. Reprod Fertil Dev. 2018;30:459–468.

Praxedes ÉCG, Lima GL, Silva AM, Campos LB, Souza CMP, Moreira SSJ, Oliveira MF, Silva AR. Comparison of different intracellular cryoprotectants on the solid surface vitrification of red-rumped agouti (*Dasyprocta Leporina* Lichtenstein, 1823) ovarian tissue, Reprod Dom Anim. 2020;00:1–8.

Santos EAA, Lima GL, Praxedes ECG, Silva AM, Maia KM, Oliveira MF, Silva APR, Silva AR. Estimation, morphometry and ultrastructure of ovarian preantral follicle population in agouti (*Dasyprocta leporina*). Pesq Vet Brasil. 2018;38:175–182.

Santos RR, Tharasanit T, Van Haeften T, Figueiredo JR, Silva JRY, Van Den Hurk R. Vitrification of goat preantral follicles enclosed in ovarian tissue by using conventional and solid-surface vitrification methods. Cell Tissue Res. 2007;327:167–176.

Saraiva MVA, Celestino JJH, Chaves RN, Martins FS, Bruno JB, Lima-Verde IB, Matos MHT, Silva GM, Porfirio EP, Báo SN, Campello CC, Silva JRV, Figueiredo JR. Influence of different concentrations of LH and FSH on caprine primordial ovarian follicle development in vitro. Small Rumin Res. 2008;78:87-95.

462 Van Den Hurk R, Zhao J. Formation of mammalian oocyte and their growth,
463 differentiation and maturation within ovarian follicles. Theriogenology. 2005;63:1717–
464 1751.

CAPÍTULO III

Microbiological control and preantral follicle preservation using different systems for ovarian tissue vitrification in the red-rumped agouti

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Microbiological control and preantral follicle preservation using different systems for ovarian tissue vitrification in the red-rumped agouti

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Abstract

We evaluated the effect of open and closed systems used for ovarian tissue vitrification on the microbiological contamination and preservation of preantral follicles (PAFs) in the red-rumped agoutis. The ovaries from eight females were recovered and fragmented, with four cortexes fragments immediately fixed and evaluated (fresh group). The other fragments were processed for the solid-surface vitrification method (SSV) or an ovarian tissue cryosystem (OTC) using fetal calf serum, ethylene glycol, and sucrose as cryoprotectants, stored for two weeks, and rewarmed. Subsequently, fragments were subjected to a 24-h *in vitro* culture and assessed for microbiological load, PAF morphology, and DNA integrity. Cell proliferation was evaluated for fresh and vitrified samples. There was no fungal contamination; however, the vitrified samples from two individuals showed bacterial contamination of 79 200 colony forming units per milliliter (CFU)/mL for SSV and 3 120 CFU/mL for OTC. Regarding PAF morphology, both systems provided adequate preservation, with values higher than 70% normal follicles observed before and after culture. The TUNEL assay revealed that both SSV (52.39%) and OTC (41.67%) could preserve DNA integrity after vitrification and after 24 h of culture. For cell proliferation, low rates were detected for fresh (11.53%) and SSV (29.87%) samples; however, no evidence of proliferation was found when OTC was used. In summary, both open and closed systems were equally efficient in preserving agouti ovarian tissues, especially concerning the preantral follicle morphology and DNA

integrity; however, the OTC seems to be more effective for the control of the bacterial load.

Keywords: Rodent; Wildlife; Biobank; In Vitro Culture.

1. INTRODUCTION

The red-rumped agoutis (*Dasyprocta leporina* Linnaeus, 1758) acts as seed dispersers and important compounds of food chains in various ecosystems [20]. Because they can easily adapt to captivity, these species represent an important protein source for Latin American communities [19]. They are classified as having a 'least concern' population [13]; therefore, red-rumped agoutis can be used as experimental models for endangered species such as *D. mexicana* and *D. ruatanica*.

The development of assisted reproductive techniques has emerged as an important tool for agouti management and conservation. The most recent studies on the preservation of agouti female germplasm [26,27] suggest the use of ovarian tissue vitrification, which is a rapid cooling method that involves simple execution, economic profitability, and high speed of the preservation procedure [1]. Vitrification is known for its low formation of ice crystals, allowing greater cell morphology preservation [1]. In a study by Praxedes et al. [26], a solid-surface vitrification (SSV) method was adopted and demonstrated that agouti ovarian tissue could be preserved and xenografted in severe immunodeficient mice after which preantral follicles (PAFs) continued to develop until ovulation. However, the procedure could be improved as only 15% efficiency was achieved after transplantation. Some factors, such as the vitrification method, might interfere with the preservation efficiency of mammalian ovarian tissue [7]. Therefore, the evaluation of other vitrification methods for agouti ovarian tissues is justified.

Despite preserving a high percentage of ovarian follicles, the SSV method consists of an open system [31]. Thus, it allows the contact of liquid nitrogen (LN_2) with the ovarian fragments, which can expose tissues to cryogenic resistant pathogens [16]. To overcome this obstacle, the use of a closed device called an ovarian tissue cryosystem® (OTC) has been proposed [10] and proven to be efficient in preserving ovine [2] and collared peccary [9] ovarian tissues. The OTC consists of a closed stainless steel system that can withstand temperatures below −196 °C and above 200 °C, even under high pressure conditions, which allows for complete asepsis and reuse [10]. According to

various authors, the OTC efficiency is highlighted for providing bacterial control during ovarian tissue preservation procedures [2,9,10]; however, as far as we know, a microbiological approach to confirm this hypothesis remains to be investigated.

The aim of this study was to investigate the effectiveness of open (SSV) and closed (OTC) systems in controlling bacterial and fungal contamination and preserving the PAF morphology, DNA integrity, and cell proliferation during agouti ovarian tissue vitrification.

2. MATERIALS AND METHODS

The Ethics Committee of Universidade Federal Rural do Semi-Árido (UFERSA) (no. 23091.005916/2015-74) and the Chico Mendes Institute for Biodiversity Conservation (no. 66618-1) approved the experimental procedures. Unless stated otherwise, the reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Animals

The agouti ovaries were obtained from the programmed slaughtered conducted at the Center of Multiplication of Wild Animals, UFERSA (Mossoró, Brazil; 5°10′ S, 3°10′ W). For the present study, eight sexually mature (2–3 years of age) agoutis females were used, weighing 2.42 ± 0.19 kg (range 2.21–2.70 kg).

2.2. Source and processing of ovaries

After slaughtering, the ovaries were recovered and immediately immersed in 70% ethanol, followed by two lavages in minimum essential medium (MEM), and conducted to the laboratory within 1 h in MEM at 4 °C.

Each pair of ovaries was divided into 24 fragments (9 mm^3). These were randomly allocated into six groups: (1) a fresh control group, in which one ovary fragment was immediately fixed in 4% paraformaldehyde solution for 12 h for subsequent analysis; (2) a cultured control group, in which ovary fragments were cultured as described below prior to analysis, (3) an SSV control group, (4) an OTC control group, (5) a cultured SSV group, and (6) a cultured OTC group. For each group, we used four ovarian cortex

fragments, with each fragment subjected to microbiological analysis, as well as histological, DNA integrity, and cell proliferation evaluations.

2.3. Ovarian tissue vitrification

The fragments were individually stored in 1.8 mL plastic tubes containing vitrification solution, which consisted of MEM plus 10 $mg.mL^{-1}$ fetal calf serum and 0.25 M sucrose, with 3.0 M ethylene glycol as cryoprotective agents (CPAs). After 5 min, the fragments were dried using sterile gauze and putted on aluminum foil on a LN_2 surface for the SSV method. The samples were then stored in cryovials at −196 °C in LN_2 [27].

At the use of OTC, vitrification procedure was similar as that reported for the SSV method, but the exposure to the CPAs was proceeded inside the device, which was closed after solution remotion, and stored in LN_2 [9].

After two weeks, samples derived from both systems were placed at room temperature (25 °C) for 1 min and rewarmed in a water-bath at 37 °C for 5 s. For CPAs remotion, samples were consecutively washed in MEM supplemented with 10% fetal calf serum and decreasing sucrose concentrations (0.50, 0.25, and 0.0 M). Finally, the samples were evaluated.

2.4. In vitro culture (IVC) of ovarian fragments

For *in situ* IVC, each fragment was allocated into 24-well culture dishes filled of 1 mL of media and cultured for 24 h. Samples were cultured at 38.5 °C in 5% CO_2 in a humidified incubator. The culture medium was composed of TCM199 (pH 7.2–7.4) plus ITS (insulin 10 µg/mL, transferrin 5.5 µg/mL, and selenium 5.0 ng/mL), 0.23 mM sodium pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/mL bovine serum albumin. After IVC, samples were evaluated [32].

2.5. Microbiological analysis

Fragments of the fresh control and vitrified culture groups were evaluated for the presence or absence of bacteria and fungi in the samples [28]. A fragment of each sample was washed in 1 mL of sterile 0.85% saline, and 100 µL of this washing solution (0.85% saline) was serially diluted to 10-5. For quantification, 100 µL of each dilution tube was

distributed in triplicate in Petri dishes containing plate count agar and Sabouraud agar plus chloramphenicol. The first culture medium was incubated in a bacteriological oven at 37 °C for 24 to 48 h for the quantification of aerobic mesophilic bacteria, and the last medium was incubated in a greenhouse at 29 °C for 48 to 72 h for the quantification of molds and yeasts. After the incubation period, bacterial and fungal colonies were counted, and the results of the triplicate averages were described in colony forming units per milliliter (CFU/mL) multiplied by the dilution [28].

2.6. Histological evaluation

After 12-h fixation in 4% paraformaldehyde solution, ovarian cortex fragments derived from all groups were dehydrated in ethanol at increasing concentrations, cleared in xylene, and embedded in paraffin wax. Then, the samples were sectioned at 7 µm, and each 5^{th} section was mounted on slides, processed for hematoxylin-eosin staining, and evaluated under a light microscope (×100).

To avoid double counting, PAFs were evaluated in each section in which they appeared and matched with the same follicles on adjacent sections. Then, they were categorized as primordial, primary, or secondary [29]. The PAFs were also counted and classified as morphologically normal (when presenting an oocyte with a regular form and homogeneous cytoplasm, and well-arranged layers of granulosa cells) or degenerated (if the oocyte presented a pyknotic nucleus or ooplasma shrinkage, and the granulosa cell layers were poorly arranged and separated from the basement membrane). To ensure that the follicles were counted only once, PAFs were exclusively evaluated when the oocyte nucleus was verified in the sections [29].

2.7. DNA integrity evaluation

Fresh, vitrified, and cultured ovarian tissues were processed for DNA integrity evaluation through the In-Situ Cell Death Detection kit (Roche) as recommended by the manufacturer's instructions [17]. Tissue samples from the non-cultured (control) and cultured groups were fixed with 4% paraformaldehyde in PBS (pH 7.2) and subsequently dehydrated and embedded in paraffin wax. Tissue sections (5 mm) mounted on Superfrost Plus slides (Knittel Glass, Bielefeld, NW, Germany) were deparaffinized with Citrisolve

(Fisher Scientific, Ottawa, Ontario, Canada) and rehydrated in a graded ethanol series. Antigen retrieval was performed by incubating the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, in a pressure cooker. To block endogenous peroxidase the slides were incubated with 3% H2O2 in methanol and then blocked for 1 h at room temperature (RT) using PBS containing 1% BSA. After washing, the slides were incubated with the TUNEL reaction mixture (50 lL) for 1 h at 378C. Converter POD was added and the location of the protein expression was demonstrated by incubation with DAB (0.05% DAB in Tris/HCl, pH 7.6, 0.03% H2O2). Finally, the sections were counterstained with hematoxylin. The follicles were considered with fragmented DNA when the oocytes were detected having dark brown stained nuclei (Yucebilgin et al., 2004). As an internal positive control, the sections were treated with 10 U/mL DNase I (InvitrogenTM, Carlsbad, CA) in 1 mg/mL BSA, for 10 min at RT, before incubation with the TUNEL reaction mixture to induce the nonspecific breaks in the DNA. The negative control sections omitted the terminal deoxynucleotidyl transferase enzyme [17].

2.8. Assessment of granulosa cell proliferative capacity

An immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was conducted to evaluate the proliferation rate of follicular cells [2] in fresh and vitrified samples. From the samples processed for histology, sections (5 µm) were mounted on slides coated with positively charged poly-L-lysine (Starfrost, Knittel, Germany). Samples were incubated in 0.01 M citric acid buffered (pH 6.0) plus Tween-20 and hydrogen peroxide in a pressure cooker for 5 min for epitopes activation. Then, samples were incubated in 5% normal goat serum and 3% Triton × 1000 diluted in PBS to block the endogenous peroxidase activity, and endogenous biotin. Then, the slides were stored overnight at 4 °C with anti-PCNA (1:3000; ab2426, Abcam, Inc., USA), rinsed in PBS, and incubated with the biotinylated anti-rabbit immunoglobulin G secondary antibody (1:500; AB97049, ABCAN, Inc., USA). After rinsing, the protein location was developed the diaminobenzidine (20 µL for 1 mL of substratum; K346811-2, Dako, Inc., Carpinteria, CA, USA) was developed for protein location. Then, the samples were counterstained with hematoxylin and eosin. For the analysis, negative controls were processed by substituting the primary antibody. After mounting, the slides were analyzed under light microscopy. The positive immunostaining presented more than 50% labeled granulosa cells, while negative showed less than 50% labeled granulosa cells [2].

2.9. Statistical analysis

Data were presented as means (± standard error of the means), except for bacterial load that was expressed as medians, and analyzed by StatView 5.0 (SAS Institute Inc., Cary, NY, USA) software. To evaluate normality and homogeneity of variance, the results were analyzed by Smirnov–Kolmogorov and Bartlett tests, respectively. Regarding PAF morphology, groups were compared using the analysis of variance followed by the Fisher's Least Significance Difference test. DNA integrity and PCNA results were combined as pools and analyzed by dispersion of frequency using the chi-square test. Statistical significances were set at $P < 0.05$.

3. RESULTS

3.1.Microbiological analysis of ovarian tissues

In the majority of procedures, the experiment was conducted under aseptic conditions with no contamination found in six individuals (Table 1). Fungal contamination was not found in any sample. However, bacterial contamination was identified in two individuals (animals 7 and 8). Considering these two animals, the median values were 106 375 CFU/mL for the fresh control group, 79 200 CFU/mL for SSV, and 3 120 CFU/mL for OTC.

Table 1. Total bacterial load (CFU/mL) at the agouti (*Dasyprocta leporina*) ovarian tissues in the fresh control group and in the vitrified groups using solid-surface vitrification (SSV) or ovarian tissue cryosystem (OTC), after 24 h *in vitro* culture.

Animal	**Treatment (CFU/mL)***		
	Fresh Control	SSV cultured	OTC cultured
Animals 1 – 6	0	0	0
Animal 7	3 150	1 400	440
Animal 8	209 500	157 000	5 800
Median Values for Animals 7 and 8	106 375	79 200	3 120

*Data expressed by descriptive statistics.

3.2. Percentage of morphologically normal PAFs

A total of 1 124 PAFs were analyzed (~30 per group): 279 from the control group and 569 from the vitrified group (Table 2). Most PAFs from the control group (74.8 ± 6.8%) and those found after IVC of fresh tissues (73.2 ± 7.1%) were categorized as morphologically normal. After vitrification and IVC, both systems (SSV and OTC) provided similar rates of morphologically normal PAFs ($P > 0.05$).

When the results were evaluated per follicular category (Table 2), both systems preserved the primordial and primary follicle morphology, even after culture ($P > 0.05$). Regarding secondary PAFs, only a few follicles from this category were found in a unique individual. Therefore, this follicle category was not included in the results.

Table 2. Percentage (normal/total) of morphologically normal ovarian preantral follicles of red-rumped agouti (*Dasyprocta leporina*), in the fresh control group and after solid surface vitrification (SSV) or using the *ovarian tissue cryosystem* (OTC) before and after *in vitro* culture (IVC) for 24 h. %MNPF: percentage of morphologically normal follicles.

Treatments	**Primordial***	**Primary**	**%MNPF***
Control	77.4 ± 6.8[a] (104/129)	72.2 ± 12.8[ab] (39/50)	74.8 ± 7.0[a] (143/179)
Control IVC	85.7 ± 3.7[a] (87/105)	62.1 ± 11.63[b] (49/77)	73.1 ± 6.9[a] (136/182)
SSV	71.8 ± 7.1[a] (86/117)	89.9 ± 4.60[a] (58/66)	80.8 ± 4.7[a] (144/183)
SSV IVC	79.8 ± 8.3[a] (109/131)	70.7 ± 13.18[ab] (49/65)	75.2 ± 7.6[a] (158/196)
OTC	76.9 ± 6.7[a] (77/134)	86.5 ± 5.49[ab] (55/66)	81.7 ± 4.3[a] (132/200)
OTC IVC	76.4 ± 4.8[a] (90/118)	73. ± 10.34[ab] (45/66)	74.7 ± 5.5[a] (135/184)

[a,b] Different lower case letters indicate significant differences among treatments within the same follicular group ($P < .05$); * No statistical difference was observed ($P > 0,05$).

The morphologically normal PAFs showed spherical oocytes, presenting a central or eccentrical nuclei that occupied most of the cytoplasm. Granulosa cells were arranged as concentric layers around the oocyte. These features were found in normal PAFs in the

control (Fig. 1A), vitrified (Fig. 1B), and cultured (Fig. 1C, white arrow) groups. After IVC, the most common alterations found in all the groups included oocyte retraction, separation of granulosa cells from the basement membrane, and pyknotic oocyte nuclei (Fig. 1C, black arrow).

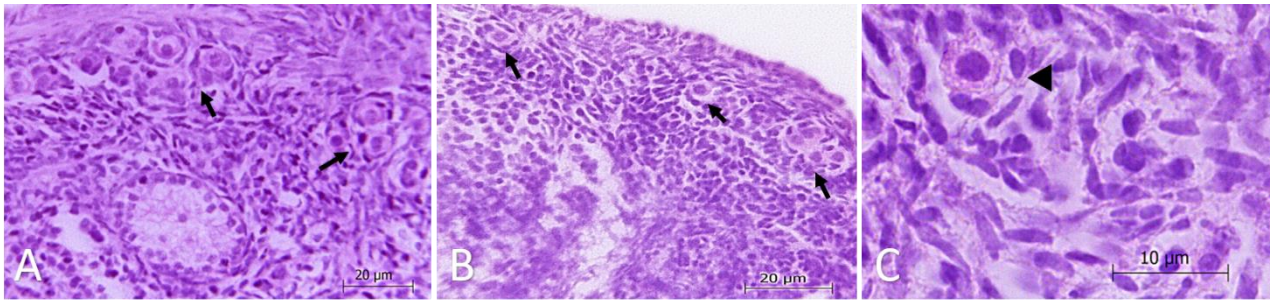

Figure 1

Histological features of agouti (*Dasyprocta leporina*) preantral follicles from fresh (A), vitrified (B) and rewarmed/in vitro cultured (C) groups. (A and B) Aggregates of intact primordial follicles (arrows); (C) degenerated follicle (white arrow head) and intact primary follicle (black arrow head).

3.2.PAFs DNA integrity

The TUNEL assay (Fig. 2) revealed a large number of PAFs with intact DNA in all experimental groups (Table 3). However, due to an unexpected technical problem, it was not possible to test fresh samples subjected to 24 h culture. As observed, the SSV method (52.39%) and OTC (41.67%) preserved DNA integrity after vitrification, and after 24 h of culture ($P > 0.05$).

Table 3 Average proportions of preantral follicles with intact DNA in ovarian tissues from agoutis (*Dasyprocta leporina*) at the fresh control group and after cryopreservation using solid surface vitrification (SSV) or ovarian tissue cryosystem (OTC), before and after a 24-h in vitro culture.

	Control		SSV		OTC	
	Fresh	Cultured	Non cultured	cultured	Non cultured	cultured
DNA integrity (%)*	70	-	52.3	100	41.6	45.4
(TUNEL negative/ total evaluated)	(14/20)		(11/21)	(1/1)	(5/12)	(5/11)

(*) No statistical difference was observed (P > 0.05); (-) no follicles were observed.

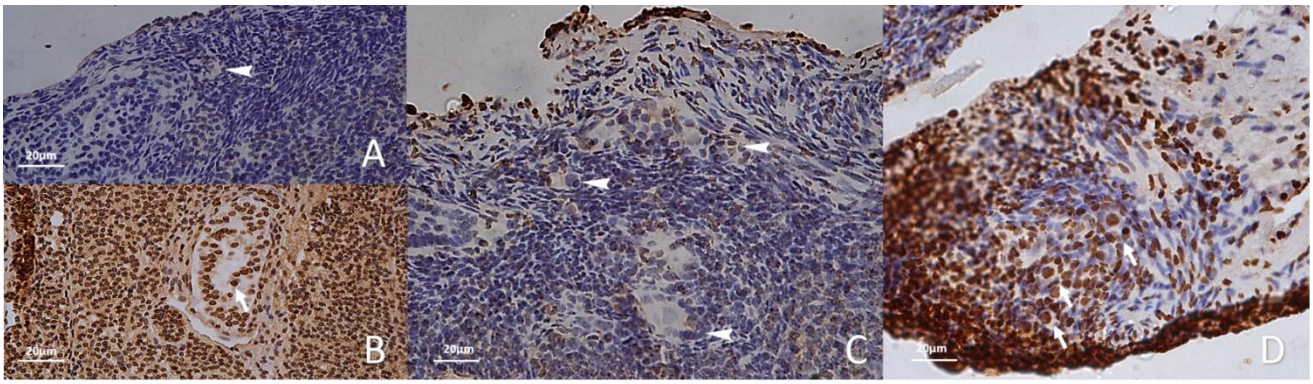

Figure 2

DNA integrity evaluated by TUNEL assay in ovary cells from agoutis. (A) Negative control, ovary cell incubated in label solution without TdT-catalyzed. (B) Positive control, ovary cell treated with DNase I, incubaded with TdT-catalyzed, stained with DAB and counterstained by hematoxylin. (C and D) Representative picture of DNA integrity evaluated by TUNEL assay in ovary cells from adult agouti, TdT-catalyzed, stained DAB and counterstained by hematoxylin, Arrowhead shows intact cell (hematoxylin), and the arrow shows the damaged cell (brown). Scale bar: 20 µm.

3.3. Granulosa cell proliferative ability

In general, the immunohistochemistry assay for PCNA detected low proliferation rates in the granulosa cells of agouti PAFs (Table 4) even in fresh samples. Higher rates (P < 0.05) were detected for the samples cryopreserved using the SSV method (29.87%), followed by those of the fresh control (11.53%). No evidence of granulosa cell proliferation was detected with the use of OTC (Table 4).

Table 4 Granulosa cells (GC) proliferation rate (%) in preantral follicles enclosed in ovarian tissues from agoutis (*Dasyprocta leporina*) at the fresh control group and after cryopreservation using solid surface vitrification (SSV) or ovarian tissue cryosystem (OTC).

	Control	SSV	OTC
PCNA	11.5[b]	29.8[a]	0[c]
(Positive GC/Total GC)	(24/208)	(43/144)	(0/211)
Nº of follicles evaluated	30	23	29

[a,b,c] Different letters indicate significant differences among treatments (P < 0.05).

4. DISCUSSION

To improve biotechnological strategies for female agouti, we investigated the use of different ovarian vitrification systems using morphological, functional, and, for the first time, microbiological approaches. We attempted to conduct the procedures under the most possible aseptic condition; however, bacterial contamination was still observed in the samples from two individuals. When procedures relating to tissue collection and processing are not conducted under complete aseptic conditions, the closed system provides a more effective control of bacterial load, which is reflected in an absolute lower number of bacterial CFU than that observed for the open system. The OTC is a fast and practical method in which the entire vitrification procedure is conducted inside the device and does not allow direct contact with LN_2 [10], thus avoiding the risk of contamination by microorganisms resistant to low temperatures [24]. In contrast, this characteristic was not observed for the SSV method, where the samples passed through LN_2 vapor before reaching the metallic surface [30].

Cryopreservation of samples via open systems that have direct contact with LN_2 can be contaminated with environmental microorganisms or opportunistic pathogens [14] and the bacterial load increases as LN_2 evaporates and is replenished [25]. Using open systems for cryopreservation of bovine embryos and semen, similar observations were reported because the authors found that cell contamination might be associated with a series of microbial agents that can contaminate LN_2 and consequently the samples [5]. Therefore, the use of OTC under field conditions should be adequate, especially because obtaining gonadal tissues from wild animals that suddenly die in zoos, reserves, or even in their natural habitats [8] is more common than that obtained from programmed slaughter under relative controlled conditions.

In the embryo and oocyte, the pellucid zone acts as a physical barrier of great molecular weight to the entry of pathogens, preventing infection by many types of microorganisms [35]. However, microorganisms can contaminate culture systems and thus affect the development of ovarian follicles [25]. Therefore, future studies should not only focus on quantifying the microorganisms present in the contaminated samples, but also identify them, to verify the effect of the microorganism activity and its toxins on follicular development.

In the present study, both systems acted in similarly concerning microbiological control for the majority of the procedures. In parallel, the SSV and OTC methods were both able to preserve the agoutis PAF morphology, before or after 24 h of culture, at rates up to 81%, values similar to those previously found with the SSV method (73.3%) for agouti ovarian tissue vitrification [27]. Moreover, the effectiveness of OTC in preserving PAF morphology in agoutis was more evident than that verified for goats (58.1%) [10], sheep (30.7%) [2], cats (56%) [7], and collared peccaries (58%) [9].

Both vitrification systems provided a similar preservation of DNA integrity in agouti PAFs, as evaluated by the TUNEL assay. In a previous study, we identified low DNA fragmentation in vitrified ovarian tissues of the same species, suggesting that the SSV method could provoke injuries mainly in the cell membranes and cytoplasmic organelles, thus providing preservation of the nuclear compartment [27], as also reported for humans [34]. The same appeared to occur with the use of OTC because this device follows the statements of other vitrification processes [2]. DNA preservation by both systems can also be attributed to the use of ethylene glycol as an intracellular cryoprotective agent [9]. Ethylene glycol has a low molecular weight (62.07 g/mol) that contributes for its penetration into tissues. It is also lower toxic than other intracellular cryoprotective agents, contributing to the preservation of DNA after cryopreservation [3]. In the present study, the sucrose acts as an extracellular cryoprotectant, also contributing to the maintenance of cellular DNA integrity, similar to that reported for goats [31]. Sucrose is a macromolecule that reduces the appearing of ice crystals during cryopreservation and facilitates the dehydration of cells before freezing [4].

There were no significant positive effects of 24 h culture on the morphology and DNA integrity of agouti vitrified PAFs. These findings contradict the recently postulated statements that the action of cryoprotectants on metabolism and function may not be highlighted when analysis occurs immediately after tissues rewarming, as the short-term IVC suggested before analysis [22,23]. After the cryopreservation process, cells need a time to adapt and resume their metabolism, allowing the identification of alterations or even cell death [11]. It is possible that IVC for only 24 h was not sufficient for promoting significant developmental alterations in agouti vitrified PAFs, being the longest culture more appropriate for this purpose, as observed for collared peccaries during a 6-days culture [15].

The proliferative activity in agouti ovarian tissues was evaluated by detecting the PCNA. In general, only a few proliferation rates were detected for the agouti granulosa

cells in fresh and SSV samples, and no activity was detected when OTC was used. PCNA is an auxiliary protein of DNA polymerases δ and ε that are enzymes involved in DNA synthesis and, therefore, proliferation markers [18,6,36]. On the other hand, detectable levels of PCNA can vary significantly between different cell types, cell cycle phases, normal cells, tissue fasteners, and the antigenic recovery used [12,33]. Therefore, the absence of PCNA marking in granulosa cells does not necessarily mean that they were not proliferating, but that it could be in phase M when PCNA expression was reduced [23].

Additionally, there is a theory that PCNA is not only a marker for the cell cycle, but also for DNA repair, leading to non-proliferative cells, such as follicles at an early stage of development, to express this marker [11], which could explain the low rates observed in some agouti primordial and primary PAFs. Only a few secondary PAFs were found in the present study, as evidenced by morphological analysis. In fact, the ovarian follicle population of agoutis was estimated to be approximately 5 000 PAFs per ovary, but only 0.35% were classified as secondary [29]. This could justify the difficulties of finding this follicle category in agouti ovarian tissue samples in the present study.

5. Conclusion

In summary, both open and closed systems are similarly efficient at preserving the morphological and functional features of agouti PAFs enclosed in ovarian tissues. Under aseptic conditions, however, we suggest the use of OTC for more efficient control of the bacterial load. The present study represents an important step for the formation of germplasm banks derived from agouti female germplasm, which will serve as a basis for applying this biotechnology to other endangered hystricognath rodents.

Declaration of interest

None of the authors have conflict of interest.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

References

[1] C.A. Amorim, M. Curaba, A. Van Langendonckt, M.M. Dolmans, J. Donnez, Vitrification as an alternative means of cryopreserving ovarian tissue, Reprod. Biomed. Online 23 (2010) 160–186, doi:10.1016/j.rbmo.2011.04.005

[2] F.T. Bandeira, A.A. Carvalho, S.V. Castro, L.F. Lima, D.A. Viana, J. Evangelista, M. Pereira, C.C. Campello, J.R. Figueiredo, A. Rodrigues, Two methods of vitrification followed by in vitro culture of the ovine ovary: evaluation of the follicular development and ovarian extracellular matrix, Reprod. Domest. Anim. 50 (2015) 177–185, doi:10.1111/rda.12463

[3] J.A.N. Bautista, H. Kanagawa, Current status of vitrification of embryos and oocytes in domestic animals: ethylene glycol as an emerging cryoprotectant of choice, Jpn. J. Vet. Res. 45 (1998) 183–191.

[4] I. Begin, B. Bhatia, H. Baldassarre, A. Dinnyes, C.L. Keefer, Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods, Theriogenology 59 (2003) 1839–1850, doi:10.1016/s0093-691x(02)01257-8

[5] A. Bielanski, H. Bergeron, P.C.K. Lau, J. Devenish, Microbial contamination of embryos and semen during long term banking in liquid nitrogen, Cryobiology 46 (2003) 146–152, doi:10.1016/s0011-2240(03)00020-8

[6] R. Bravo, R. Frank, P.A. Blundell, H. MacDonald-Bravo, Cyclin/PCNA is the auxiliary protein of DNA polymerase δ, Nature 326 (1987) 515–7, doi:10.1038/326515a0

[7] D.C.C. Brito, S.F.S. Domingues, A.P.R. Rodrigues, C. Maside, F.O. Lunardi, X. Wu, J.R. Figueiredo, J.C. Pieczarka, R.R. Santos, Cryopreservation of domestic cat (*Felis catus*) ovarian tissue: comparison of two vitrification methods, Theriogenology 111 (2018) 69–77, doi:10.1016/j.theriogenology.2018.01.015

[8] L.B. Campos, E.C. Praxedes, M.V.A. Saraiva, P. Comizzoli, A.R. Silva, Advances and challenges of using ovarian preantral follicles to develop biobanks of wild animals, Biopreserv. Biobank. 17 (2019) 334–341, doi:10.1089/bio.2018.0130

[9] L. B. Campos, A. M. Silva, E.C.G. Praxedes, L.G.P. Bezerra, T.L.B. Lins, V.G. Menezes, M.H.T. Matos, G.L. Lima, A.P.R. Rodrigues, A.R. Silva, Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants, Cryobiology 91 (2019) 77–83, doi:10.1016/j.cryobiol.2019.10.193

[10] A.A. Carvalho, L.R. Faustino, C.M. Silva, S.V. Castro, C.A. Lopes, R.R. Santos, S.N. Bao, J.R. Figueiredo, A.P.R. Rodrigues, Novel wide-capacity method for vitrification of caprine ovaries: ovarian tissue cryosystem (OTC), Anim. Reprod. Sci. 138 (2013) 220–227, doi:10.1016/j.anireprosci.2013.02.015

[11] W.J. Choi, H.J. Yeo, J.K. Shin, S.A. Lee, J.H. Lee, W.Y Paik. Effect of vitrification method on survivability, follicular growth and ovulation of preantral follicles in mice, J Obstet. Gynaecol. Res. 33 (2007) 128–33, doi:10.1111/j.1447-0756.2007.00498.x

[12] M.D. Coltrera, A.M Gown, PCNA/cyclin expression and BrdU uptake define different subpopulations in different cell lines, J. Histochem. Cytochem. 39 (1991) 23–30, doi:10.1177/39.1.1670579

[13] L. Emmons, F. Reid, 2016. *Dasyprocta leporina*. The IUCN Red List of Threatened Species (2016) e.T89497102A22197762, doi:10.2305/IUCN.UK.2016-2.RLTS.T89497102A22197762.en. Downloaded on 17 February 2021.

[14] M. Fountain, N. Ralston, J.B. Higgins, L. Gorlin, C. Uhl, Wheeler, J.H. Antin, W.H. Churchill, R.J. Benjamin, Liquid nitrogen freezers: a potential source of microbial contamination of hematopoietic stem cell components, Transfusion 37 (1997) 585–591, doi:j.1537-2995.1997.37697335152.x

[15] H.A.N. Gomes, L.B. Campos, E.C.G. Praxedes, M.F. Oliveira, A.F. Pereira, A.R. Silva, M.V.A. Saraiva, BMP-15 activity on in vitro development of collared peccary (*Pecari tajacu Linnaeus*, 1758) preantral follicles, Reprod. Domestic. Anim. 55 (2020) 958–964, doi:10.1111/rda.13735

[16] W.W. Grout, G.J. Morris, Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer, Theriogenology 71 (2009) 1079–1082, doi:10.1016/j.theriogenology.2008.12.011

[17] D.D Guerreiro, L.F. Lima, N.A.R. Sá, G.T. Mbemya, A.C.A. Ferreira, B.G. Alves, S.S. Maranhão, C. Pessoa, Pinheiro, A.C. Nogueira, T.C.M. Souza, M.V. Nora, J.R. Figueiredo, A.P.R. Rodrigues, Response of preantral follicles exposed to quinoxaline: A new compound with anticancer potential. Res. Vet. Sci. 128 (2020) 261–268, doi:10.1016/j.rvsc.2019.12.010B.

[18] P. Kurki, M. Vanderlaan, F. Dolbeare, J. Gray, E.M. Tan, Expression of proliferating cell nuclear antigen (PCNA/cyclin) during the cell cycle, Exp. Cell. Res. 166 (1986) 209–219, doi:10.1016/0014-4827(86)90520-3

[19] J.B. Lopes, R.R. Cavalcante, M.M. Almeida, M.A.M Carvalho, S.G. Moura, L.A.D. Filho, W.L.F Conceição, Performance of agouti (Dasyprocta prymnolopha) bred in captivity according to sex and parturition in Teresina, Piauí, Rev. Bras. Zootec. 33 (2004) 2318–2322, doi:10.1590/S1516-35982004000900018

[20] P. Mittelman, C. Kreischer, A.S. Pires, F.A.S. Fernandez, Agouti reintroduction recovers seed dispersal of a large-seeded tropical tree, Biotropica 54 (2020) 766–774, doi:10.1111/btp.12788

[21] L. Mouttham, J.E. Fortune, P. Comizzoli, Damage to fetal bovine ovarian tissue caused by cryoprotectants exposure and vitrification is mitigated during tissue culture, J. Assist. Reprod. Genet. 32 (2015) 1239–1250, doi:10.1007/s10815-015-0543-x

[22] L. Mouttham, P, Comizzoli, The preservation of vital functions in cat ovarian tissues during vitrification depends more on the temperature of the cryoprotectant exposure than on the sucrose supplementation, Cryobiology 73 (2016) 187–195, doi:10.1016/j.cryobiol.2016.07.013

[23] K. Oktay, R.S. Schenken, J.F. Nelson, Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat, Biol. Reprod. 53 (1995) 295–301, doi:10.1095/biolreprod53.2.295

[24] M. Piasecka-Serafin, The effect of the sediment accumulated in containers under experimental conditions on the infection of semen stored directly in liquid nitrogen (-196 degrees C), Bull Acad. Pol. Sci. Biol. 20 (1972) 263–267.

[25] K.O. Pomeroy, Stanley Harris, B.S. Joe Conaghan, Margaret Papadakis, Grace Centola, Rita Basuray, David Battaglia. Storage of cryopreserved reproductive tissues: evidence that cross-contamination of infectious agents is a negligible risk, Fert. Steril. 94 2010, doi:10.1016/j.fertnstert.2009.04.031

[26] E.C.G. Praxedes, G.L. Lima, L.G.P. Bezerra, F.A. Santos, M.B. Bezerra, D. D. Guerreiro, A.P.R. Rodrigues, S.F.S. Domingues, A.R. Silva, Development of fresh and vitrified agouti ovarian tissue after xenografting to ovariectomised severe combined immunodeficiency (SCID) mice, Reprod. Fertil. Dev. 30 (2018) 459–468, doi:10.1071/rd17051

[27] E.C.G. Praxedes, G.L. Lima, A.M. Silva, L.B. Campos, C.M.P. Souza, S.S.J. Moreira, M.F. Oliveira, A.R. Silva, Comparison of different intracellular cryoprotectants on the solid surface vitrification of red-rumped agouti (*Dasyprocta Leporina* Lichtenstein, 1823) ovarian tissue, Reprod. Domest. Anim. 55 (2020) 154–161, doi: https://doi.org/10.1111/rda.13600

564 [28] C.S. Santos, A.M. Silva, K.M. Maia, G.S.O. Rodrigues, F.M.C. Feijó, N.D. Alves,
565 M.F. Oliveira, A.R. Silva, Composition of semen and foreskin mucosa aerobic
566 microbiota and its impact on sperm parameters of captive collared peccaries (*Pecari*
567 *tajacu*), J. Appl. Microbiol. 129 (2020) 521–531, doi:
568 https://doi.org/10.1111/jam.14663

569 [29] E.A.A. Santos, G.L. Lima, E.C.G. Praxedes, A.M. Silva, K.M. Maia, M.F. Oliveira,
570 A.P.R. Silva, A.R. Silva, Estimation, morphometry and ultrastructure of ovarian
571 preantral follicle population in agouti (*Dasyprocta leporina*), Pesq. Vet. Bras. 38
572 (2018) 175–182, doi:10.1590/1678-5150-pvb-4946

573 [30] R.R. Santos, C. Amorim, S. Cecconi, M. Fassbender, M. Imhof, J. Lornage, M. Paris,
574 V. Schoenfeldt, B. Martinez-Madrid, Cryopreservation of ovarian tissue: an
575 emerging technology for female germline preservation of endangered species and
576 breeds, Ani. Reprod. Sci. 122 (2010) 151–163,
577 doi:10.1016/j.anireprosci.2010.08.010

578 [31] R.R. Santos, T. Tharasanit, T. Van Haeften, J.R Figueiredo, J.R.V. Silva, R. Van
579 Den Hurk, Vitrification of goat preantral follicles enclosed in ovarian tissue by using
580 conventional and solid-surface vitrification methods, Cell. Tissue Res. 327 (2007)
581 167–176, doi: https://doi.org/10.1007/s00441-006-0240-2

582 [32] S.S. Santos, F.C. Biondi, M.S. Cordeiro, M.S. Miranda, J.K. Dantas, J.R. Figueiredo,
583 O.M. Ohashi, Isolation, follicular density, and culture of preantral follicles of buffalo
584 fetuses of different ages. Anim. Reprod. Sci. 95 (2006) 1–15,
585 doi:10.1016/j.anireprosci.2005.08.012

586 [33] T. Scholzen, J. Gerdes, The Ki-67 protein: from the known and the unknown, J. Cell.
587 Physiol. 18 (2000) 311–22, doi:10.1002/(sici)1097-
588 4652(200003)182:3%3C311::aid-jcp1%3E3.0.co;2-9

589 [34] M. Sheikhi, K. Hultenby, B. Niklasson, M. Lundqvist, O. Hovatta, Preservation of
590 human ovarian follicles within tissue frozen by vitrification in a xeno-free closed
591 system using only ethylene glycol as a permeating cryoprotectant, Fertil. Steril. 100
592 (2013) 170–177, doi:10.1016/j.fertnstert.2013.03.018

593 [35] G. Vanroose, H. Naumyaek, A. Vansoom, M.T. Ysebaert, G. Charlier, P. Van
594 Oostueldt, A. Kruif, Structural aspects of the zona pellucida of in vitro-produced
595 bovine embryos: a scanning electron and confocal laser scanning microscopic study,
596 Biol. Reprod. 62 (2000) 463–9, doi:10.1095/biolreprod62.2.463

597 [36] R.D. Wood, M.K.K. Shivji, Which DNA polymerases are used for DNA-repair in
598 eukariotes? Carcinogenesis 18 (1997) 605–10, doi:10.1093/carcin/18.4.605

CONCLUSÕES GERAIS

- O sistema de cultivo *in vitro* foi eficiente para a preservação da morfologia, desenvolvimento e viabilidade de folículos pré antrais a fresco e vitrificados de cutias;

- O pFSH na concentração de 50 ng/mL auxilia no desenvolvimento *in vitro* de folículos ovarianos pré-antrais de cutias;

- A vitrificação do tecido ovariano de cutais pode ser eficientemente realizada utilizando-se tanto o método SSV quanto o OTC;

- Em condições assépticas, sugere-se o uso de OTC para um controle mais eficiente da carga bacteriana durante a vitrificação de tecido ovariano de cutias.

PERSPECTIVAS

Esse é o primeiro trabalho a descrever o processo de vitrificação de gametas femininos de cutias em condições assépticas seguido do cultivo *in vitro*, o que demonstrou ser uma técnica simples e eficaz para tais gametas, abrindo vertentes para a formação de bancos de germoplasma para a espécie, bem como desenvolvimento de protocolos para espécies silvestres e domésticas ameaçadas de extinção.

Nesse sentido, o desenvolvimento de novos protocolos de cultivo *in vitro*, ou até mesmo o aperfeiçoamento dos já existentes com a utilização de outras combinações com fatores de crescimento, ideais para cada tipo de célula são requeridos para o sucesso do desenvolvimento folicular *in vitro*, no intuito de se obter maiores aplicações da técnica do cultivo *in vitro* em roedores silvestres. Ainda, visando-se desenvolver novos estudos moleculares que podem auxiliar na identificação da interação de todos os constituintes celulares.

- **RESUMOS PUBLICADOS EM CONGRESSOS REGIONAIS**

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CULTIVO *IN VITRO* DE FOLÍCULOS OVARIANOS PRÉ-ANTRAIS DE CUTIAS (*Dasyprocta leporina*) PREVIAMENTE SUBMETIDOS A DIFERENTES MÉTODOS DE VITRIFICAÇÃO

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As cutias são animais silvestres que apresentam importantes funções ecológicas por serem dispersadoras de sementes, além de contribuírem para o equilíbrio da cadeia alimentar. Ademaiss, esses animais são utilizados como modelo experimental, tendo em vista seu potencial reprodutivo, tanto para o aperfeiçoamento de técnicas reprodutivas, quanto para a formação de bancos de germoplasma. Dessa forma, este trabalho teve como objetivo comparar diferentes métodos de vitrificação como a superfície sólida (SSV) e o *ovarian tissue cryosystem* (OTC), submetidos ao cultivo *in vitro* do tecido ovariano de cutias (*Dasyprocta leporina*) para avaliar a viabilidade de seus folículos ovarianos pré-antrais (FOPAS). Para tanto, foram coletados oito pares de ovários de cutias adultas, sexualmente maduras e eutanasiados, de acordo as recomendações do comitê de ética da UFERSA (Parecer nº 21/2018). Os ovários foram lavados em álcool 70% e Meio Essencial Mínimo (MEM), fragmentados e vitrificados utilizando-se a SSV e o OTC. Os fragmentos foram expostos à solução de vitrificação a 37ºC, constituída de MEM suplementado com 10% de soro fetal bovino (SFB); 0,25 M de sacarose e 3 M de etilenoglicol (EG). Após uma semana, os fragmentos foram aquecidos e submetidos ao cultivo *in vitro* por 24 horas. Em seguida, as amostras foram submetidas ao processamento histológico, com a desidratação em concentrações crescentes de etanol, a diafanização com xilol, inclusão em parafina histológica e montagem em lâminas, e coradas com Hematoxilina e Eosina. Os dados foram expressos em média e erro padrão (±EP) e o teste de Tukey foi usado para comparar os métodos de vitrificação, considerando-se uma significância de $P<0,05$. Foi observado que 53,33 ± 7,9 % de folículos apresentaram-se morfologicamente normais para o grupo controle fresco. Após a vitrificação, ambos os métodos, SSV (50,00 ± 5,8 %) e OTC (50,00 ± 5,5 %) não diferiram entre si, bem como, do grupo controle fresco. E ainda, foi verificado que houve a manutenção da viabilidade folicular quando foram submetidos ao cultivo *in vitro* por 24 horas (SSV: 53,84 ± 6,5 % e OTC: 50,22 ± 5,7 %). Assim, o cultivo *in vitro* evidenciou que ambos os métodos de vitrificação (SSV e OTC) foram eficientes para a manutenção da viabilidade folicular *in situ* da *Dasyprocta leporina*.

Palavras-chave: Biobanco, Vida Selvagem, Germoplasma, Ovário, Roedor.
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