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ANDRÉIA MARIA DA SILVA

ESTEBELECIMENTO DE PROTOCOLOS PARA A CRIOPRESERVAÇÃO DE TECIDO TESTICULAR DE CATETOS (*Pecari tajacu* LINNAEUS, 1758)

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

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Orientador: Prof. Dr. Alexandre Rodrigues Silva

Co-orientadora: Profa. Dra. Alexsandra Fernandes Pereira

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Joanna de Ângelis

RESUMO

A criopreservação de tecido gonadal masculino pode ser usada na conservação de material genético, no intuito da formação de um banco de germoplasma permitindo a manutenção da variabilidade genética em animais pré-puberes e adultos. Diante disso, objetivou-se estabelecer um protocolo eficiente de criopreservação de tecido testicular de catetos. Para tanto, o estudo foi dividido em dois experimentos, sendo utilizados 10 animais adultos, 5 em cada experimento, oriundos do Centro de Multiplicação de Animais Silvestres da UFERSA. No experimento I, cinco pares de testículos foram fragmentados (9 mm^3) e alocados em grupos não-vitrificados (controle) e vitrificados em superfície sólida (VSS), após a exposição a diferentes crioprotetores (dimetilsulfóxido - DMSO 3,0 M, etileno glicol - EG 3,0 M e a combinação 1,5 M DMSO/1,5 M EG). As amostras não-vitrificadas e vitrificadas foram avaliadas quanto à histomorfologia, ultraestrutura, viabilidade e potencial de capacidade proliferativa. A conservação adequada da organização ultraestrutural do túbulo seminífero em termos de presença de lúmen e junções celulares foi observada somente com o uso da combinação DMSO/EG. Independentemente do crioprotetor, a vitrificação conservou efetivamente a visualização e a condensação nuclear das células de maneira semelhante à observada no grupo não vitrificado. Além disso, a combinação DMSO/EG proporcionou uma melhor conservação das membranas basais dos túbulos seminíferos que o DMSO ($P < 0,05$). Somente a combinação DMSO/EG manteve o potencial de capacidade proliferativa para espermatogônia (3,69 Regiões Organizadoras de Nucléolos - NORs/célula) e célula de Sertoli (3,19 NORs/célula) semelhantes aos controles (3,46 e 3,31 NORS/célula, respectivamente). Portanto, DMSO/EG é melhor que o DMSO ou o EG sozinho para VSS de tecido testicular de cateto adulto. No experimento II, cinco pares de testículos foram fragmentados (3 mm^3) e alocados a grupos não-criopreservados (controle) e criopreservados usando três métodos de criopreservação: congelação lenta (CL), vitrificação em criotubos (VC) e VSS, sendo então expostos a diferentes combinações de crioprotetores (1,5 M DMSO/1,5 M EG, 1,5 M DMSO/1,5 M glicerol – G, e 1,5 M G/1,5 M EG). As amostras, não-criopreservadas e criopreservadas foram avaliadas quanto à fragmentação de DNA, viabilidade, potencial de capacidade proliferativa e histomorfologia. Apenas no uso de DMSO/EG durante a CL e VC foi possível conservar a integridade do DNA de modo similar ao controle ($P>0,05$). Em adição, observou-se que, independentemente do método de criopreservação, as combinações DMSO/EG e DMSO/G foram capazes de conservar a viabilidade ($P>0,05$). Todos os tratamentos mantiveram o potencial de capacidade proliferativa para espermatogônia de modo similar; entretanto, apenas o G/EG, nos métodos de CL e VSS, foram inferiores ao controle para célula de Sertoli ($P>0,05$). Finalmente, o protocolo de CL usando as combinações DMSO/EG e DMSO/G foram melhores em evitar o aparecimento de edemas que G/EG – CL e DMSO/G – VSS ($P<0.05$). Assim, sugere-se a utilização da combinação DMSO/EG associada aos métodos de congelação lenta ou vitrificação para a criopreservação de tecido testicular de catetos adultos.

Palavras chave: biobanco; germoplasma; testículo; célula germinativa.

ABSTRACT

Cryopreservation of male gonadal tissue can be used in the conservation of genetic material, in order to form a germplasm bank allowing the maintenance of genetic variability in prepubertal and adult animals. Therefore, the aim was to establish an efficient protocol for cryopreservation of testicular tissue of collared peccaries. Therefore, the study was divided into two experiments, using 10 adult animals, 5 for each experiment, from the UFERSA Center for Wild Animals Multiplication. In experiment I, five pairs of testicles were fragmented (9 mm^3) and allocated to non-vitrified (control) and vitrified solid-surface (SSV) groups following exposure to different cryoprotectants (3.0 M dimethyl sulfoxide (DMSO), 3.0 M ethylene glycol (EG) or 1.5 M DMSO/1.5 M EG). After warming, samples were evaluated for histomorphology, ultrastructure, viability, and proliferative capacity potential. The appropriate conservation of the ultrastructural organization of the seminiferous tubule in terms of lumen presence and cell junctions was only observed at the use of DMSO/EG combination. Regardless of the cryoprotectant, the vitrification effectively preserved cell nuclear visualization and condensation similarly as observed at the non-vitrified group. Moreover, DMSO/EG combination provided a better preservation of basal membranes of seminiferous tubules than DMSO ($P < 0.05$). Only the DMSO/EG combination maintained the proliferative capacity potential for spermatogonia (3.69 Nucleolus Organizing Regions - NORs/cell) and Sertoli cell (3.19 NORs/cell) similar to controls (3.46 and 3.31 NORS/cell, respectively). In conclusion, DMSO/EG in combination is better than DMSO or EG alone for SSV of testicular tissue biopsies from adult collared peccaries. In experiment II, five pairs of testicles were also fragmented (3 mm^3) and allocated to non-cryopreserved (control) and cryopreserved groups using three cryopreservation methods: slow freezing (SF), cryotube vitrification (CV) and solid surface vitrification (SSV), and then exposed to different combinations of cryoprotectants (1.5 M DMSO/1.5 M EG, 1.5 M DMSO/1.5 M glycerol - G, and 1.5 M G/1.5 M EG). Non-cryopreserved and cryopreserved samples were evaluated for histomorphology, viability, proliferative potential and DNA fragmentation. Only in the use of DMSO/EG during SF and CV, it was possible to preserve DNA integrity in a similar way to control ($P > 0.05$). In addition, it was observed that, regardless the cryopreservation method, the DMSO/EG and DMSO/G combinations were able to preserve viability ($P > 0.05$). All treatments maintained the proliferative capacity potential for spermatogonia in a similar manner; however, G/EG in the SF and SSV methods impaired the proliferative potential of Sertoli cells ($P > 0.05$). Finally, the SF protocol using the DMSO/EG or DMSO/G combinations were better at preventing edema than G/EG for SF and DMSO/ G for SSV ($P < 0.05$). In conclusion, we suggest the use of a DMSO/EG combination associated with slow freezing or vitrification in cryotubes for cryopreservation of testicular tissue of adult peccaries.

Key words: biobank; germplasm; testicle; germ cell.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância
GFRα-1	Glial Cell-Derived Neurotrophic Factor Family Membrane Receptor Alpha-1
DNA	Ácido desoxirribonucleico
RNA	Ácido ribonucleico
mRNA	Ácido ribonucleico mensageiro
2D	Bidimensional
Cm	Centímetro
CL	Congelação lenta
DMSO	Dimetilsulfóxido
DC	Distrito de Columbia
DMEM/F12	Dulbecco's modified Eagle's medium/Ham's F12
EG	Etilenoglicol
FBS	fetal bovine serum
FCS	fetal calf serum
G ou GLY	Glicerol
g/mol	Gramas por mol
°C/min	Grau por minuto
H	Hora
HSA	human serum albumin
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
ICMBIO	Instituto Chico Mendes de Biologia
MEM	Meio essencial mínimo
µm	Micrômetro

mg/mL	Miligrama por mililitro
mm^3	Milímetro cúbico
mim	minutos
M	Molar
N_2	Nitrogênio líquido
NORs	Regiões organizadoras do nucléolo
RN	Rio Grande do Norte
SF	Slow Freezing
PBS	Solução salina tamponada fosfatada
cSSCs	Spermatogonial stem cells
SS	Sterile saline
SSV	Solide Surfice vitrification
3D	Tridimensional
USA	United States
V	Vitrification
VC	Vitrification in cryotubes

LISTA DE SÍMBOLOS

~	Aproximadamente
°C	Grau Celsius
>	Maior
±	Mais ou menos
®	Marca Registrada
<	Menor
%	Porcentagem
™	Símbolo trademarks

SUMÁRIO

1. INTRODUÇÃO

O *Pecari tajacu*, conhecido como cateto, caititu ou porco-do-mato, é uma espécie exclusiva das Américas, estando presente em praticamente todos os biomas (BODMER; SOWLS, 1996). Esta espécie desempenha importante função ecológica no equilíbrio e na composição de cadeias alimentares, contribuindo para a manutenção de seus predadores, assim como na dissipação de sementes (GARLA, 1998). Embora sua população internacional seja considerada estável (IUCN, 2019), um levantamento realizado pelo Instituto Chico Mendes de Biologia (ICMBIO) demonstrou que no Brasil, a espécie está ameaçada nos biomas Mata Atlântica e Caatinga, uma vez que enfrenta grandes problemas relacionados à caça predatória, à fragmentação e redução de seu habitat natural (DESBIEZ et al., 2012).

No intuito de diminuir o impacto da caça predatória e também fornecer uma fonte de proteína alternativa para a população, alguns criatórios têm sido implantados com a autorização do Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA). A criação de cateto em cativeiro poderia se tornar uma alternativa para o aproveitamento de áreas improdutivas de propriedades rurais, dada a sua fácil adaptação ao ambiente (NOGUEIRA-FILHO et al., 1999; SANTOS et al., 2004),). Para tanto, faz-se necessária a aplicação de programas de reprodução em cativeiro e técnicas de reprodução assistida para garantir a conservação do material genético dessa espécie (SANTOS et al., 2004), obter mais conhecimentos sobre protocolos de criopreservação adequado, e informações básicas da espécie em estudo.

Nesse contexto, a formação de criobancos surge como ferramenta importante para conservação e multiplicação de espécies de interesse zootécnico ou ecológico, permitindo o armazenamento de uma variedade de tecidos e células (AMSTISLAVSKY; TRUKSHIN, 2010). A criopreservação de tecido gonadal masculino tem representado uma relevante opção na conservação das espécies, sendo o objeto de inúmeras pesquisas (COMIZZOLI et al., 2012; POTHANA et al., 2015; LEE et al., 2016). A conservação de tecido testicular é uma técnica de reprodução assistida que permite o armazenamento de fragmentos contendo um grande número de espermatogônias, cujo principal papel é a produção ilimitada de espermatozoides (NING et al., 2012), e células germinativas em vários estágios de desenvolvimento (PICTON; KIM; GOSDEN, 2000). Além disso, a técnica promove o restabelecimento tanto da função reprodutiva como endócrina após o período de armazenamento (GOSDEN, 2002), e possibilita salvaguardar o material genético proveniente

de animais em qualquer estágio reprodutivo, ou que tenham morrido subitamente (THUWANUT et al., 2013).

Com o propósito de conservação de tecido gonadal masculino podem ser empregados diferentes protocolos de criopreservação como vitrificação e congelação lenta. A vitrificação consiste na solidificação de uma solução utilizando baixas temperaturas, mas sem a formação de cristais de gelo (LIEBERMANN et al., 2002). Esta técnica foi empregada na criopreservação de tecido testicular de espécies como suínos (ABRISHAMI; ABBASI; HONARAMOOZ, 2010), humanos (CURABA et al., 2011), primatas não humanos (POELS et al., 2012), caninos (LEE et al., 2016) e preás (SILVA et al., 2019). Na congelação lenta os fragmentos de tecido testicular são submetidos a uma redução gradual de temperatura, no qual é usada uma taxa de arrefecimentos de -1°C/min até atingir -80°C, seguindo -259°C/min entre -80 e -196°C, com baixas concentrações de crioprotetor para reduzir uma possível toxidade para as células (TRAVERS et al., 2011). O uso desta foi descrito para ovino (PUKAZHENTHI et al., 2015), felino (CHATDARONG; THUWANUT; MORRELL, 2016), e primata (FAYOMI et al., 2019).

Além disso, o sucesso de sistemas de criopreservação depende da escolha de crioprotetores, os quais atuam minimizando os efeitos deletérios causados durante a criopreservação (GURTOVENKO; ANWAR, 2007; COOPER et al., 2008). Nesse contexto, para tecido testicular tem disso usado dimetilsulfóxido (DMSO - DEVI et al., 2016), etilenoglicol (EG - KANEKO et al., 2013, 2019) e glicerol (G – LIMA et al., 2018)) como crioprotetores intracelulares, estes podem ser usados sozinhos ou em associações. Estudos realizados em animais domésticos, como equinos (GÓMEZ et al., 2019), suínos (ABRISHAMI; ABBASI; HONARAMOOZ, 2010) e ovinos (PUKAZHENTHI et al., 2015; SINGH et al., 2019) foram utilizados DMSO, em suínos também foi utilizado o EG (KANEKO, et al., 2013; 2019). Além disso, o DMSO em combinação com o EG foi usado para criopreservação testicular em camundongos (YAMINI et al., 2016), e a combinação DMSO com G, e EG com G foram usadas na vitrificação testicular em felinos (LIMA et al., 2018).

Nesse sentido, o estabelecimento de um protocolo para a criopreservação de tecido testicular em catetos é necessária visando a utilização deste valioso material genético na formação de bancos de germoplasma. Para tanto, faz-se faz necessário o estudo do crioprotetor ideal, assim como qual o protocolo de criopreservação mais eficiente na conservação do tecido testicular da espécie.

2. JUSTIFICATIVA

Apesar da importância estratégica do semiárido nordestino para o Brasil, pouco tem sido feito em termos de uma política que leve ao conhecimento do potencial da região visando o desenvolvimento científico e tecnológico e sua sustentabilidade. Assim o cateto apresenta-se como um animal de grande importância ecológica e grande potencial econômico.

Nesse contexto, o estudo da criopreservação do seu material genético é de grande valia seja para a conservação e multiplicação de genótipos zootecnicamente valiosos. Em especial, o tecido testicular apresenta uma grade diversidade celular, com presença de toda linhagem espermatogênica, contudo não há um protocolo de conservação considerado eficiente, levando à necessidade da condução de novos estudos relativos a esta área. Em utilizando o cateto como modelo experimental, tais estudos seriam importantes não apenas para a formação de bancos de germoplasma da espécie, mas também para espécies correlatas filogeneticamente como o queixada (*Tayassu pecari*) e o taguá (*Catagonus wagneri*).

Assim, para o estabelecimento de um protocolo de criopreservação eficiente, justifica-se a avaliação de diferentes fatores, como o tipo de crioprotetor mais adequados ao tecido testicular da espécie, e a identificação da taxa de arrefecimento da temperatura mais indicada, por meio do uso da vitrificação ou da congelação lenta.

3. HIPÓTESES

A combinação de crioprotetores é superior ao uso destes isolados para a manutenção da qualidade do tecido testicular submetido a criopreservação por vitrificação;

O método de vitrificação em superfície sólida proporciona eficiência similar à vitrificação em criotubo e à congelação lenta para a conservação de tecido testicular em catetos.

3. OBJETIVOS:

Objetivo Geral:

- Estabelecer um protocolo adequado para a criopreservação de tecido testicular de catetos.

Objetivos Específicos:

- Comparar o uso de diferentes crioprotetores e suas associações na vitrificação de tecido testicular de catetos;
- Comparar diferentes métodos de criopreservação (congelação lenta, vitrificação em criotubos e vitrificação em superfície sólida) sobre a conservação de tecido testicular de catetos;
- Verificar a existência de correlação entre crioprotetores intracelulares e técnicas de criopreservação.

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CAPÍTULO I

Criopreservação e cultivo de tecido testicular como ferramenta na conservação de mamíferos silvestres

Testicular tissue cryopreservation and culture as a tool for the conservation of wild mammals

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Resumo

A criopreservação do tecido testicular se apresenta como ferramenta promissora para a reprodução assistida, possibilitando o armazenamento de fragmentos contendo grande número de células germinativas em várias fases de desenvolvimento, incluindo espermatogônias indiferenciadas que podem ser cultivadas, garantindo a produção ilimitada de espermatozoides. Nesta revisão, são abordados aspectos técnicos relativos ao processamento e aplicabilidade da criopreservação e do cultivo de tecido testicular em mamíferos silvestres, ressaltando seus desafios e perspectivas.

Palavras-chave: biodiversidade, tecidos gonadais, vida selvagem, biobanco.

Abstract

Cryopreservation of the testicular tissue is a promising tool for assisted reproduction, allowing the storage of fragments containing a large number of germ cells in various stages of development, including undifferentiated spermatogonia that can be cultured, guaranteeing the unlimited production of spermatozoa. In this review, technical aspects related to the processing and applicability of testicular tissue cryopreservation and culture in wild mammals are discussed, highlighting their challenges and perspectives.

Keywords: *biodiversity, gonadal tissues, wildlife, biobank.*

Introdução

A cada dia, mais e mais espécies tem sua população reduzida e, muitas delas, atingem níveis críticos, denotando sua iminente extinção. Devido a isso, esforços multidisciplinares têm sido conduzidos na tentativa de frear esse ritmo, ou de desenvolver alternativas para garantir a sobrevivência das espécies (Comizzoli, 2015). Assim, o desenvolvimento de biotecnologias que garantam a conservação de espécies silvestres é ainda lento, porém promissor (Pukazhenthi et al., 2006).

A possibilidade de formação de bancos de germoplasma surge como uma grande aliada aos programas de conservação (Comizzoli, 2015). Principalmente no que se refere ao germoplasma masculino, inúmeras pesquisas vêm sendo conduzidas quanto ao desenvolvimento de protocolos para criopreservação de espermatozoides, sejam obtidos por eletroejaculação (Maia et al., 2018) ou a partir do epidídimo (Silva et al., 2017a). Paralelamente, a criopreservação do tecido testicular se apresenta como ferramenta promissora para a reprodução assistida, possibilitando o armazenamento de fragmentos contendo grande número de células germinativas em várias fases de desenvolvimento (Picton et al., 2000), incluindo as espermatogônias indiferenciadas que podem ser cultivadas, garantindo a produção ilimitada de espermatozoides (Ning et al., 2012; Lee et al., 2013; Sato et al., 2015) . Aliada a técnicas de xenoenxertos de células ou tecidos, esta tecnologia pode promover a restauração tanto da função gametogênica como endócrina após o período de criopreservação (Gosden, 2002).

A conservação do tecido testicular tem sua importância uma vez que, em mamíferos, a espermatogênese é continua a partir da puberdade, na qual as espermatogônias multiplicam-se e iniciam um processo de diferenciação em sucessivos estágios até a produção de espermatozoides (Russell et al., 1993). Esta regulação envolve todo o microambiente em torno destas células, chamado de nicho espermatogonial, que fornece um suporte físico e produz sinais que regulam a auto renovação e diferenciação espermatogonial (Ning et al., 2012). Com isso, protocolos de criopreservação do tecido testicular devem preservar, não só a espermatogônia-tronco, mas todo o nicho em torno delas, incluindo as células de Sertoli, células peritubulares mióides, células de Leydig e outros componentes intersticiais (Ning et al., 2012). Devido a essa grande variedade de tipos celulares, a preservação do tecido testicular apresenta desafios no ponto de vista criobiológico (Lima et al., 2017).

Nesse contexto, o objetivo dessa revisão é abordar aspectos técnicos relativos ao processamento e aplicabilidade da criopreservação e do cultivo de tecido testicular em mamíferos silvestres, ressaltando seus desafios e perspectivas.

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Coleta e preparação do tecido

O tecido testicular pode ser coletado de animais sexualmente maduros e imaturos, assim como de animais que acabaram de vir a óbito. Em animais imaturos, não existe a produção de espermatozoides, logo, quando estes morrem, todo o seu potencial genético é perdido. Dessa forma a criopreservação do tecido testicular associada às técnicas de transplantes gonadais, como o xenoenxerto (Honaramooz et al., 2002), pode ser uma solução para diminuir a perda genética (Pothana et al., 2015). Nos casos de animais mortos ou submetidos à castração, é sugestiva a coleta do testículo por completo, para posterior preservação (Thuwanut et al., 2013; Pothana et al., 2015).

Em indivíduos vivos, amostras testiculares poderiam ser obtidas por meio de biopsias, conforme descrito para humanos (Uijldert et al., 2017), nos quais a técnica tem sido aplicada, principalmente, para salvaguardar o potencial reprodutivo de homens que serão submetidos à quimioterapia para tratamento de câncer, que poderia afetar a viabilidade das células germinativas (Uijldert et al., 2017). Embora esta possibilidade não tenha, ao nosso conhecimento, sido ainda utilizada em espécies silvestres, ela é citada como importante alternativa.

Para coleta e transporte das amostras, diferentes meios são relatados. Em felinos selvagens, Thuwanut et al. (2013) reportaram o uso da solução salina esterilizada suplementada com penicilina-estreptomicina a 1% para a lavagem e transporte dos testículos. Em saguis (*Callithrix jacchus*), as amostras foram arrefecidas com gelo em meio de Eagle modificado por Dulbecco (Gibco) (Schlatt et al., 2002). Já no trágulo pintado (*Moschiola indica*), as amostras foram transportadas em solução salina fosfatada (PBS) (Pothana et al., 2015).

Após a chegada ao laboratório, o material pode ser fragmentado para então ser submetido a criopreservação. Neste caso, o tamanho do fragmento usualmente varia entre 0,3 mm³ (Thuwanut et al., 2013) a 3 mm³ (Silva et al., 2017b). Em sagüis (*Callithrix jacchus*), foram utilizados fragmentos de tecido testicular de 0,5-1,0 mm³ (Schlatt et al., 2002), bem como 0,3 mm³ para diferentes felídeos selvagens (Thuwanut et al., 2013), 1-2 mm³ em trágulo pintado (*Moschiola indica*) (Pothana et al., 2015), e 3 mm³ em catetos (*Tayassu tajacu*) (Silva et al., 2017b).

Métodos de criopreservação

Em geral, duas técnicas são reportadas para a preservação de tecido testicular, seja em animais domésticos ou silvestres: a criopreservação lenta e a vitrificação, cuja eficiência depende de diferentes fatores abordados a seguir.

Congelação lenta

Esta se baseia em uma redução gradual de temperatura, associada a baixas concentrações de agentes crioprotetores, o que apresenta a vantagem da baixa toxicidade de tais substâncias. Contudo, sua capacidade para prevenir a formação de cristais de gelo nestas concentrações nos tecidos torna-se limitada (Massip et al., 1995; Dobrinsky, 1996). Em animais silvestres, a congelação lenta do tecido testicular já foi reportada em saguis (*Callithrix jacchus*) (Schlatt et al., 2002), macaco Rhesus (*Macaca mulatta*) (Jahnukainen et al., 2007), lince ibérico *(Lynx pardinus*) (Leon-Quinto et al., 2009), gato-da-selva (*Felis chaus*), leão *(Panthera leo)*, leopardo *(Panthera pardus)*, cervo-do-Timor *(Rusa timorenses)*, cervídeo muntjac-de-tenasserim *(Muntiacus feae)*, e o goral (*Capricornis sumatraensis)* (Thuwanut et al., 2013).

Para a congelação lenta, é necessário o uso de um congelador programável (Travers et al., 2011), ou container específico, como o Mrfroster®, dentro de um freezer -80ºC (Pukazhenthi et al., 2015). Quando comparado a outros protocolos de criopreservação, a congelação lenta é considerada um processo demorado, podendo durar mais de 24 h (Pothana et al., 2015). Qualquer que seja o método, a congelação lenta segue, basicamente, a seguinte sequência: incubação dos fragmentos em meio de congelação; colocação dos fragmentos no equipamento de congelação; armazenamento das amostras em nitrogênio líquido, e, posteriormente, aquecimento das amostras e remoção dos crioprotetores (Schlatt et al., 2002).

Em tecido testicular imaturo do trágulo pintado *(Moschiola indica*), por exemplo, os fragmentos foram colocados por 30 min no meio HEPES DMEM / F12 suplementado com 10% de dimetil-sulfóxido (DMSO) e 80% de soro fetal bovino (SFB). Em seguida, foram acondicionados em criotubos e estes colocados em congelador programável a -80ºC por 24 h, quando foram então armazenados em nitrogênio líquido. Posteriormente, o aquecimento dos criotubos foi realizado em banho-maria a 37 °C, em meio contendo DMEM HEPES suplementado com SFB a 10%, 100 UI/mL de penicilina-estreptomicina, 40 mg/mL de gentamicina, onde foram realizadas três lavagens. Embora tenha sido observado aumento na degradação de DNA de 22,8 ± 2,0% para 46,3 ± 3,4%, constatou-se desenvolvimento até espermatócito primário no fragmento submetido ao xenotransplante (Pothana et al., 2015).

Vitrificação

Esta é uma alternativa à congelação lenta, devido à sua praticidade e baixo custo, e, consequentemente, maior rentabilidade (Vajta et al., 1998). Em geral, pode ser realizada em qualquer laboratório ou mesmo na sala de

operação do paciente/animal ou a campo, e imediatamente após a morte de um animal (Amorim et al., 2011).

Nesta técnica, são utilizadas rápidas taxas de congelação (20.000 a 40.000°C/min – Lin et al., 2008) associadas a altas concentrações de crioprotetores para promover o aumento da viscosidade, visando inibir a união de moléculas de água para formar cristais de gelo (Mukaida e Oka, 2012). Assim, o estado vitrificado consiste em um sistema amorfo, que carece de estrutura organizada, mas possui as propriedades mecânicas e físicas de um sólido (Taylor et al., 2004). Em resumo, a técnica consiste em equilibrar os fragmentos em um meio com alta concentração de crioprotetor por alguns minutos, seguindo-se da remoção deste meio, transferência do fragmento para uma superfície sólida em contato direto com o nitrogênio líquido e armazenamento dos fragmentos em criotubos, os quais são estocados em botijão criobiologico, para posterior aquecimento em banho-maria à 37°C, em solução contendo concentrações decrescentes de sacarose objetivando a remoção do crioprotetor (Thuwanut e Chatdarong, 2012; Baert et al., 2015).

A técnica já vem sendo desenvolvida com êxito em animais domésticos como os suínos, nos quais, inclusive, a vitrificação de tecido testicular imaturo associada ao xenotransplante possibilitou a produção de espermatozoides viáveis, os quais foram utilizados para injeção intra-citoplamástica (ICSI) e produção de embriões que foram transferidos, resultando no nascimento de leitões (Kaneko et al., 2013). Em animais silvestres, a aplicação da técnica é ainda recente, conforme reportado para os macacos Rhesus (*Macaca mulatta)* (Poels et al., 2012), catetos *(Pecari tajacu*) (Silva et al.,, 2017b), e preás (*Galea spixii)* (Lago et al., 2017).

Fatores que afetam a criopreservação de tecido testicular

Crioprotetores

Conforme utilizados para a criopreservação de outros germoplasmas, a adição de agentes crioprotetores (ACPs) é essencial para que as células resistam às lesões resultantes do processo (Jang et al., 2017). Neste sentido, vários estudos examinaram a criopreservação de suspensões de células testiculares ou fragmentos de tecido utilizando ACPs penetrantes de diferentes pesos moleculares, como glicerol (92,10 g/mol), etileno glicol (EG; 62,07 g/mol), dimetil-sulfóxido (DMSO; 78g/mol), ou propanodiol (76,9 g/mol) (Abrishami et al., 2010; Hu et al., 2015). Dentre estes, o uso isolado do DMSO tem sido destacado por apresentar resultados promissores na conservação de tecido testicular de sagui (*Callithrix jacchus*) (Schlatt et al., 2002), lince-ibérico *(Lynx pardinus*) (Leon-Quinto et al., 2009), e trágulo pintado (*Moschiola indica*) (Pothana et al., 2015); porém, a combinação do DMSO com o EG tem também sido efetivamente reportada para gato-das-selvas (*Felis chaus*), leão *(Panthera leo)*, leopardo *(Panthera pardus)*, (Thuwanut et al., 2013) e cateto *(Pecari tajacu)* (Silva et al., 2017b).

A associação dos ACPs penetrantes com os não penetrantes, em especial a sacarose, tem também se mostrado efetiva para a criopreservação de tecido testicular. Esse açúcar confere proteção às membranas celulares quanto a lesões oriundas do frio por meio de sua ligação com as cabeças dos grupos de fosfolípidos presentes na membrana (Anchordoguy et al., 1987). A incorporação deste açúcar foi reportada com êxito para congelação lenta do tecido testicular de trágulos (Pothana et al., 2015) e na vitrificação em macacos (Poels et al., 2012), catetos (Silva et al., 2017b) e preás (Lago et al., 2017).

Idade do animal

A princípio, espera-se que tecnologia para a conservação do tecido testicular seja aplicável a todo e qualquer indivíduo. Entretanto, a maioria das pesquisas tem mostrado uma maior eficiência para animais imaturos, principalmente se associando ao xenoenxerto (Pothana et al., 2015), suscitando um grande desafio para o desenvolvimento de protocolos aplicáveis a animais adultos (Arregui e Dobrinski 2014). Tais diferenças devem-se, particularmente, ao fato de que o tecido testicular de animais imaturos é caracterizado pela presença das espermatogônias indiferenciadas como únicas células germinativas presentes no epitélio seminífero. Neste sentido, o tecido apresentaria um metabolismo reduzido, o que favoreceria sua resistência à hipóxia derivada do processamento para a preservação (Assis-Neto et al., 2003; Aponte et al., 2005). Porém, no animal maduro, o desenvolvimento dos túbulos seminíferos é completo, com a presença das células germinativas, desde a espematogônia até o espermatozoide, assim como a maturação de células de Sertoli e formação da barreira hematotesticular (Assis-Neto et al., 2003; Aponte et al., 2005). Neste contexto, para os adultos, permanece o desafio inerente ao desenvolvimento de métodos eficientes que possibilitem a conservação de todos os tipos celulares presentes (Ning et al., 2012).

Cultivo de tecido testicular

A manutenção *in vitro* de gônadas masculinas de mamíferos tem sido relatada como uma ferramenta eficiente para avaliação e desenvolvimento do tecido criopreservado (Yokonishi et al., 2014; Lee et al., 2016), sendo também uma estratégia promissora para preservar a fertilidade do macho, em especial quando utilizado em associação com a criopreservação (Yokonishi et al., 2014).

Inicialmente, o método clássico de cultivo *in vitro* de órgãos utilizando-se o gel de agarose foi utilizado com sucesso para induzir a espermatogênese completa em ratos neonatos, cujo tecido testicular havia sido

previamente submetido à congelação lenta ou vitrificação. Neste trabalho, inclusive, os espermatozoides gerados foram utilizados para produção de prole (Sato et al., 2011a).

Diferentes meios de cultura têm sido reportados para o cultivo de tecido testicular. Em camundongos (Sato et al., 2015) e bovinos (Cai et al., 2016), tem-se utilizado αMEM; já em cão (Lee et al., 2016) e suíno (Lee et al., 2013), o meio StemPro-34 tem se mostrado melhor que o DMEM. Além disso, a adição de fatores como o fator de crescimento epidermal – mEGF, o fator básico de crescimento de fibroblastos – BFGF, e o fator neurotrófico derivado de células gliais – GDNF, tem sido reportados por incrementar a proliferação celular (Lee et al., 2013; 2016). Ainda, é necessária a adição de fonte de proteínas como o SFB ou albumina (Sato et al., 2015), de hormônios como rFSH e hCG (Reda et al., 2014) ou b-estradiol e progesterona (Lee et al., 2013), e de fontes de energia como a glicose e o piruvato (Sato et al., 2011b).

Alternativamente, ao invés de simplesmente realizar o crescimento celular em uma superfície plana, na placa de Petri, outras estratégias de cultura de células foram desenvolvidas. Assim, a disposição das células em um arranjo tridimensional (3D) pode simular um túbulo seminífero, onde a composição do meio de cultivo em contato basolateral pode ser manipulado através da adição de componentes que estimulam a proliferação e auto renovação espermatogonial, mimetizando as condições endócrinas de um indivíduo adulto (Gadella e Ferraz 2015).

Em adição, o xenoenxerto de fragmentos testiculares, que é considerado um método de cultivo in vivo, consiste no enxerto de pequenas peças (1-2 mm³) de parênquima testicular de uma espécie sob a pele ou cápsula renal de um camundongo imunodeficiente orquiectomizado (Abrishami et al., 2010). Ao contrário das tentativas para reproduzir a espermatogênese em condições *in vitro*, o xenoenxerto de fragmentos testiculares tem a vantagem de manter a arquitetura complexa do testículo (Pukazhenthi et al., 2006). Por meio deste, foi possível a obtenção de prole a partir de espermatozoides obtidos pelo xenoenxerto de tecido testicular criopreservado oriundo de suínos (Kaneko et al., 2013) e camundongos (Sato et al., 2011a) pré-púberes.

Ao contrário, quando xenotransplantado o tecido testicular de animais sexualmente maduros, os fragmentos tendem a degenerar. Possivelmente, o processo degenerativo é devido à demora na angiogênese, pois os animais adultos têm uma população celular diversa no epitélio seminífero de seus testículos, logo sem aporte sanguíneo para suprir as necessidades fisiológicas (Arregui e Dobrinski 2014). Tal fato suscita o desafio para o estabelecimento dessa tecnologia nesta categoria de indivíduos.

Considerações finais

Em geral, o potencial reprodutivo contido no testículo dos animais é descartado quando estes morrem ou são castrados, portanto, a capacidade de salvar este material pode representar um valioso recurso para o banco genético (Pukazhenthi et al., 2006). Esta tecnologia permitiria a conservação do material genético de animais em qualquer fase reprodutiva, e mesmo dos que vierem a óbito subitamente (Thuwanut et al., 2013), provendo uma fonte de gametas masculinos que poderia ser utilizada em um futuro próximo. Tal afirmação, inclusive, é evidenciada pelo recente relato amplamente divulgado pela mídia acerca da produção de um filhote de macaco a partir de espermatozoides produzidos por xenoenxerto de tecido testicular pré-púbere previamente criopreservado (Fayomi et al., 2019).

Esta biotécnica pode ser adaptada a partir de protocolos desenvolvidos para animais domésticos e otimizada para as espécies silvestres, podendo ser utilizada no armazenamento de tecido testicular de machos geneticamente valiosos (Leon-Quinto et al., 2009). Diante do exposto, no entanto, fica clara a necessidade do aprimoramento de protocolos adequados à criopreservação e cultivo, em especial, de animais adultos que venham subitamente a óbito. Assim, com todos os passos dominados, esta poderá ser uma biotecnica altamente utilizada na formação de bancos de germoplasma animal.

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CAPÍTULO II

Cryopreservation and culture of testicular tissues – an essential tool for fertility preservation in wild mammals.

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Running head: Testicular tissue preservation in mammals.

Abstract

Preservation of gonadal samples represents a huge potential to optimize reproductive management and ensure sustainability of rare mammalian genotypes. Specifically, testicular tissues contain a large number of germ cells, including early developmental stages (spermatogonia and spermatocytes) that can result in viable spermatozoa after grafting or culture. Resulting sperm cells then can be used for assisted reproductive techniques. The objectives of this review are to describe the current advances, limitations and perspectives related to the use of testicular tissue preservation as a strategy for the conservation of male fertility in wild mammal species. At this point, testicles can be obtained from mature or immature individuals, immediately after death or castration, but testicular biopsy would also be an alternative to obtain samples for living individuals. Testicular fragments can be then cryopreserved by using slow or fast freezing, or even vitrification methods, being this last one more adequate to be used under field conditions. The composition of the cryomedia can vary according to species-specific characteristics, especially regarding the cryoprotectant type and concentration. Finally, sperm have been usually obtained after xenografting of the testicular fragments into severely immunodeficient rats, while *in vitro* culture conditions lack to be improved.

Keywords: biodiversity, testis, tissue cryopreservation, 3D culture method, xenografting,

Introduction

Many wild species are currently endangered, which could result in a major loss of biological diversity in few decades. To prevent extinction, various efforts have been conducted from preservation of natural habitats to creations of germplasm banks (Comizzoli, 2015; Leon-Quinto et al., 2009). Most biobanking efforts have been focused on the development of effective protocols for preservation of sperm cells (Castelo et al., 2015; Maia et al., 2018), oocytes (Czarny and Rodger, 2010; Mrowiec et al., 2019) and embryos (Anav et al., 2019; Dresser et al., 1988). Preservation of other germplasms as the somatic (Borges et al., 2018; Leon-Quinto et al., 2009) and gonadal (Lima et al., 2019; Mouttham and Comizzoli, 2016; Praxedes et al., 2018) tissues has been recently emerging, including the cryopreservation of testicular tissue that recently led to the birth of live offspring in Rhesus monkey (Fayomi et al., 2019).

Testes contain a large number of germ cells, especially spermatogonia that can provide an unlimited source of male gametes if appropriately preserved and cultured (Sato et al., 2011). Specially in domestic species but also in some wild mammals, several studies have evidenced collection and preservation of testes from individuals under different conditions, such as of sexually immature (Lima et al., 2018b, 2018a) as mature animals (Macente et al., 2017), besides of living (Abrishami et al., 2010b) and *post-mortem* (Pothana et al., 2015) individuals. In general, a first strategy consists of conserving testicular tissue fragments appropriately using cryopreservation systems, as slow freezing, fast freezing, and vitrification (Curaba et al., 2011; Thuwanut et al., 2013; Yokonishi et al., 2014). Nevertheless, the use of these systems depends on some factors, as the choice of cryoprotectants (Lima et al., 2018b) and technical procedures (Travers et al., 2011). After tissue immediate recovery or cryopreservation, as second and third biotechnological strategies, fragments can be cultured *in*

vivo or *in vitro* to obtain viable spermatozoa destined to other reproductive techniques (Kaneko et al., 2013; Sato et al., 2011).

Despite the progress, adequate conditions for the direct application of that technology in wild animals remain a challenge, especially due to species-specific differences that lead to the needs for improvement and adaptations of current protocols. Therefore, this review aims to describe current advances, limitations and perspectives related to the use of gonadal tissue preservation as a strategy for the conservation of male fertility in wild mammal species.

Collecting and processing testicular tissues

Collection of testicular samples from wild species generally occur in places where it is possible to monitor the individuals (in zoos or animal parks), where tissues can be collected immediately after animals' death or castration. An initial study conducted by Schlatt et al., (2002) showed the possibility of recovering the testes from newborn marmoset monkeys (*Callitrix jacus*) derived from an institutional colony in Germany. Monkey testes were xenografted to castrated immunodeficient mice and were able to produce viable spermatocytes. After, the possibility of recovering and culturing fresh testicular tissue from wild species was demonstrated for various species as in Indian black bucks (*Antilope cervicapra L.* – Goel et al., 2011), bisons (*Bison bison* – Abbasi et al., 2011), and collared peccaries (*Pecari tajacu* – Campos-Junior et al., 2014).

One study reported the use of testicular tissues from a wild species for the biobank formation (Leon-Quinto et al., 2009). Testes and epididymis were recovered from seven Iberian lynx (*Lynx pardinus*) from five weeks to five years of age. Collections were conducted between 24 and 60 h post-mortem. However, only epidydimal and testicular sperm

cells were collected for cryopreservation but no testicular tissues were processed. This missed opportunity also has been observed in a recent rhinoceros report (Roth et al., 2016).

Only in 2012, Poels et al. reported a successful trial of recovery and cryopreservation of testicular tissues from an adult non-human primate, the Rhesus monkey (*Macaca mulata*), opening the possibility of using this germplasm for biobank formation. An impressive study in this area was conducted in Thailand (Thuwanut et al., 2013). Authors demonstrated the possibility of recovering and cryopreserving testes from adult felids like jungle cats (*Felis chaus*), lions (*Panthera leo*) and leopards (*Panthera pardus*). It also included adult ungulates like Rusa deer (*Rusa timorensis*), Fea's muntjacs (*Muntiacus feae*), and Sumatran serows (*Capricornis sumatraensis*). Samples were obtained during necropsy that was conducted within 56 h post-mortem and transported to the laboratory in sterile saline supplemented with 1% penicillin-streptomycin at 4 ºC. However, authors highlighted that timing of testis removal *post-mortem* was an important factor affecting testicular morphology (detachment of the epithelium of the seminiferous tubules for instance). Moreover, they evidenced that high ambient temperatures experienced by animals before death could also affect the quality of testicular sperm and testicular morphology (Thuwanut et al., 2013).

Environmental temperatures, however, seem to act in different ways on distinct species, since Pothana et al. (2015) reported the recovery of the testes from immature Indian spotted mouse deer (*Moschiola indica*) that were found approximately one hour post-mortem due to heat stress during summer in India. Authors reported that testes were cryopreserved, xenografted to immunodeficient mice, and resumed spermatogenesis (Pothana et al., 2015) .

Moreover, testicular tissue technology would also be an alternative for the germplasm preservation in wild species in which procedures for semen collection is low efficient as for the agoutis (Castelo et al., 2015) or when spermatogenesis has not started yet (Lima et al., 2018). However, to the best of our knowledge, no other procedures for testicular tissues

recovery in living individuals are reported, except for full castration. One possibility would be testicular biopsies, which has been currently used as an elective methodology for the collection of testicular samples destined to studies related to reproductive physiology and pathologies in wild animals (Bitencourt et al., 2007; Lopate et al., 1989). Although in humans, testicular biopsy is a widely used technique to recovery tissue before being submitted to cryopreservation, especially for men who will undergo chemotherapy (Faure et al., 2016).

Besides testicular tissue recovery, adequate media for washing and transport also are essential. Various media can be effectively used for this purpose as the sterile saline supplemented with 1% penicillin-streptomycin used in the Thai study (Thuwanut et al., 2013) or the phosphate buffer saline (PBS) used for Indian spotted mouse deer (Pothana et al., 2015) and chimpanzee (*Pan troglodytes* – Pothana et al., 2016). As an alternative, the Dulbecco's modified eagle (Gibco) medium was reported for transport of ice-cooled testes of domestic species as pigs (Abrishami et al., 2010a) and dogs (Lee et al., 2016).

Another important factor related to the cryopreservation is the size of the testicular tissue fragment used for the procedure. It can vary from 0.5 – 1 mm^3 as reported for Capuchin monkeys (*Callithrix jacchus*) (Schlatt et al., 2002), 1-2 mm^3 in Indian Spotted mouse deer (*Moschiola indica*) (Pothana et al., 2015) and 3 mm^3 for wild felids and cervids (Thuwanut et al., 2013). However, fragments as large as 9 to 20 mm^3 were also reported for prepubertal rhesus macaques (Fayomi et al., 2019).

Cryopreservation methods

After recovery, tissues are then destined to cryopreservation. Different methods have been reported for this purpose, considering different cooling rates, as well as cryoprotectant

types and concentrations. In general, methods used for wild species are adapted from previous experiments conducted in laboratory and domestic models (Table 1).

Slow and fast freezing methods

During slow freezing, tissue fragments are submitted to gradual reduction of temperature using a cooling rate of -1°C/min between 2 and -80°C, following -259°C/min between -80 and -196°C, at low cryoprotectant concentrations to reduce possible cellular toxicity (Travers et al., 2011). On the other hand, fast freezing or two-step freezing methods use three cooling rates of -10.8°C/min between 4 to -50°C, -18°C/min between -50 to -90°C/min, and -252°C/min between -90 to -196 °C (Thuwanut et al., 2013; Thuwanut and Chatdarong, 2012).

Among these methods, the slow freezing has been widely used for the conservation of testicular tissue from laboratory and domestic mammals (Table 1). In ovine, the slow freezing of neonatal lamb testes was far superior to vitrification in preserving cellular integrity and function after xenografting, including allowing ~10% of tubules to retain the capacity to resume spermatogenesis and yield mature spermatozoa (Pukazhenthi et al., 2015). By this moment, the most promising results obtained from the use of slow freezing of testicular tissue are related to the production of viable sperm by *in vitro* culture, resulting in the birth of viable offspring in immature mice (Yokonishi et al., 2014).

Table 1. Cryopreservation and culture of testicular tissues from laboratory species and domestic models.

Species	Used strategy	Aim	Main Results	Authors
Mouse	Cryopreservation and i*n vivo* culture	Evaluation of spermatogenesis in immature testicular pieces in xenogeneic recipients after cryopreservation	Birth of offspring following transplantation of cryopreserved immature testicular pieces	Shinohara et al., 2002
	Cryopreservation and i*n vivo* culture	Cryopreservation and transplantation of stem cells into the seminiferous tubules	The combination of cryopreservation and transplantation of stem cells produced viable sperm resulting in the birth of viable offspring	Kanatsu-Shinohara, 2003
	Cryopreservation and i*n vivo* culture	Comparison of slow freezing and vitrification techniques of immature mouse testes	Production of viable sperm by in vitro culture of testicular tissue preserved using both methods, resulting in the birth of viable offspring	Yokonishi et al., 2014
	Cryopreservation *and in vitro* culture	Comparation of slow freezing with vitrification techniques	The vitrification protocol resulted in success rates better than slow freezing in maintaining testicular tissue structure, tubular morphology, tissue functions, and production of flagellated spermatozoa	Dumont et al., 2015
	Cryopreservation and i*n vivo* culture	Comparison of slow freezing and vitrification techniques of neonatal mouse testes	The complete spermatogenesis was obtained after testicular tissue controlled-rate freezing and vitrification	Yildiz et al., 2018
Rat	Cryopreservation	Evaluation of several protocols for cryopreservation of rat immature testicular tissue	A testicular tissue piece of 7.5mg cryopreserved in cryovial using 1.5M DMSO, an equilibration time of 30 min at 4 °C showed fewer morphological alterations than the other protocols tested.	Travers et al., 2011
	Cryopreservation	Evaluation of the cryoprotectants toxicity in the vitrification of testicular tissues from immature and adult mice	DMSO would be preferable for the preservation of testes from immature individuals, while EG would be more indicated for adults.	Unni et al., 2012
Swine	Cryopreservation and *in vivo* culture	Evaluation of cryopreservation protocols, cryoprotectant and cryoprotectant exposure times in immature testis	Obtaining elongated spermatids after programmed slow-freezing using glycerol, as well as after vitrification using glycerol with 5- or 15-min exposures or using DMSO for a 5-min exposure.	Abrishami et al., 2010
		Evaluation of vitrification and culture on spermatogenesis in immature testis	Obtaining of spermatozoa fertile for production of live piglets	Kaneko et al. 2013
		Evaluation complete spermatogenic activity after cryopreservation and grafting into nude mice	Obtaining of spermatozoa fertile for production of embryo by intracytoplasmic injection	Kaneko et al., 2019

Bovine	Cryopreservation	Comparison among different cryoprotectants in adult testis	Cell viability rates were better for DMSO (30-80%), when compared to propanodiol (30-60%) and glycerol (20-40%)	Wu et al., 2011
		Investigation the effects of concentration various cryoprotectants on the cell viability as well as expression of spermatogenesis-related genes	10% DMSO exhibited the highest cell viability and mRNA expression level of the spermatogenesis-related genes, CREM, Stra8 and HSP70-2.	Zhang et al., 2017
		Evaluation at the molecular level in post-thaw bovine calf testicular tissue, the effects of different solution cryopreservation	30 mg/ml BSA combined with 5% DMSO may best protection against cryodamage for the spermatogenesis of bovine calf testicular tissue	Li et al., 2018
Sheep	Cryopreservation and *In vivo* culture	Comparison between to slow freezing and vitrification techniques	Slow freezing of neonatal lamb testes was superior to vitrification in preserving cellular integrity and function after xenografting, including allowing ~10% of tubules to retain the capacity to resume spermatogenesis and yield mature spermatozoa.	Pukazhenthi et al., 2015
	Cryopreservation and *In vivo* culture	Evaluation the influence of sphingosine-1-phosphate (S1P) on explants of frozen-thawed testis	S1P promotes germ cell proliferation during first week of culture and may exert an anti-apoptotic influence on the seminiferous cord in sheep testicular explants *in vitro*.	Singh et al., 2019
Horse	Cryopreservation	Comparison amount cryoprotectant solution and controlled cooling rate	PBS + 1.5 M dimethyl sulfoxide in a programmable freezer preserved structural integrity	Gómez et al., 2019
Feline	Cryopreservation	Comparison among different cryoprotectants in the vitrification of prepubertal testes	DMSO associated with glycerol resulted in a better preservation of proliferative potential of testicular cells when compared to EG plus glycerol and DMSO plus EG	Lima et al., 2017
		Comparison among different warming condition for the vitrification of prepubertal testes.	Warming at 50ºC for 5 seconds can be successfully used to ensure reanimantion for vitrified testicular tissue from immature cats	Lima et al. 2018
Canine	Cryopreservation and *in vivo* and *in vitro* culture	Evaluation of conditions of slow freezing and culture of spermatogonial stem cells	StemPro®−34 SFM as culture medium and DMSO as cryoprotectant were adequate for testis freezing, providing cells viable to develop during both in vivo or in vitro culture.	Lee et al., 2016

Among wild species (Table 2), promising results with slow freezing methods have been reached in non-human primates as adult white-headed marmosets (*Callithrix geoffroyi*), mandrills (*Mandrillus sphinx*) and chimpanzees, which tissues derived from these three species were able to express sperm- and spermatid-specific proteins (PRM2 and TNP1 respectively), proving maintenance of spermiogenesis after cryopreservation (Pothana et al., 2016). Authors also highlight differences between species, since mandrill and marmoset testicular tissues can be effectively cryopreserved in media containing 10% dimethyl sulfoxide – DMSO and 80% fetal bovine serum – FBS, whereas chimpanzee requires only 20% DMSO without FBS. The most promising result obtained in primates, however, was recently reported by Fayomi et al. (2019), who demonstrated the birth of a healthy female derived from intracytoplasmic injection of a sperm produced by the autologous graft of cryopreserved testicular tissue.

For wild ungulates (Table 2), Thuwanut et al. (2013) demonstrated that fast freezing tended to cause less damage to sperm cells recovered from testicular tissues of the Rusa deer, Fea's muntjac and Sumatran serows than slow freezing; however, protective effects of fast freezing on testicular intra-tubular cells was likely compromised, evidencing the incidence of apoptosis. In addition, Pothana et al. (2015) reported the slow freezing of testes from immature Indian spotted mouse deer and highlighted that increase on DMSO concentration impairs the spermatogenesis development during the xenograft of cryopreserved testicular fragments. Subsequently, Pothana et al., (2017) conducted the slow freezing of the testes from hog deer (*Hyelaphus porcinus*), barking deer (*Muntiacus muntjac*) and sambar deer (*Rusa unicolor*), showing that it was possible to detect the expression of TNP1 and PRM2 proteins, which prove the maintenance of spermiogenesis after cryopreservation. However, authors highlight that even among cervids, different species require different freezing protocols since

the presence of FBS in the cryomedia is necessary for cryopreservation of sambar and hog deer testes but not for the barking deer.

In carnivore species (Table 2), using both slow and fast freezing methods for the cryopreservation of testicular tissue from jungle cats, lions and leopards, viable sperm cells with intact DNA were only obtained from jungle cats. Moreover, fast freezing provoked more apoptotic changes in testicular tissue from all three felids than slow freezing (Thuwanut et al., 2013).

Finally, a preliminary study conducted in a wild hystricognath rodent (Table 1), the red-rumped agouti (*Dasyprocta leporina*), revealed that ethylene glycol – EG would be a cryoprotectant more appropriate for the preservation of the mitochondrial activity of testicular cells than the DMSO or the combination of these cryoprotectants (Silva et al., 2019b).

Vitrification Methods

Vitrification consists in a rapid decrease of temperature using a cooling rate of -20,000 to -40,000 °C/min and high cryoprotectant concentrations what can cause toxicity to cells and tissue (Lin et al., 2008). It is a practical system that is more cost-effective than slow freezing.

This technique has been applied for the testicular conservation in some laboratory and domestic mammals (Table 1). The most promising results were reported in swine, in which successfully generated porcine offspring were produced utilizing sperm from immature testicular tissues after vitrification and transplantation into nude mice (Kaneko et al. 2013). Also, an interesting study showed that both slow freezing and vitrification were equally efficient for the preservation of immature mice testicular tissue that resulted in the obtaining of viable sperm after *in vitro* culture, and then in the production of live offspring (Yokonishi et al. 2014).

Table 2. Cryopreservation of testicular tissues in wild mammals.

Species	Used strategy	Aim	Main outcomes	Authors
Antilope cervicapra L.	Cryopreservation and *In vivo* culture	Evaluation of vitrification slow freezing and *in vivo* culture of spermatogonial stem cells in mature testis	Recovery of spermatocytes and round spermatids post-grafting	Goel et al., 2011
Rhesus monkey	Cryopreservation and *in vivo* culture	Evaluation of vitrification and culture of spermatogonial stem cells in immature testis	Proliferative activity was maintained after vitrification and *in vivo* culture	Poels et al., 2012
	Cryopreservation and *in vivo* culture	Evaluation of slow freezing and autologously grafted in immature testis	Graft-derived sperm were competent to fertilize rhesus oocytes and the birth of a healthy female baby	Fayomi et al., 2019
Felis chaus *Panthera leo* *Panthera pardus* *Rusa timorenses* *Muntiacus feae* *Capricornis sumatraensis*	Cryopreservation	Comparison of the slow freezing and fast freezing rate	The two methods conserved the testicular tissue from wild animals	Thuwanut et al., 2013
Moschiola indica	Cryopreservation and *In vivo* culture	Cryopreservation of the testis tissue from immature and after evaluation the establishment spermatogenesis with xenografting.	The use of 10% DMSO and 80% FBS were the most advanced for pachytene spermatocytes in 15.4 ± 3.4%.	Pothana et al., 2015
Mandrillus sphinx *Pan troglodytes* *Callithrix geoffroyi*	Cryopreservation	Cryopreservation of testicular tissue of three adult primates	The results suggest that mandrill testis and marmoset testis can be cryopreserved in 10% DMSO with 80% FBS, whereas chimpanzee testis in 20% DMSO.	Pothana et al., 2016

Hyelaphus porcinus *Muntiacus muntjak* *Rusa unicolor*	Cryopreservation	Comparison of the effect of different cryoprotectants	20% DMSO in combination with 20% FBS is acceptable for cryopreservation of testis of three species of cervids	Pothana et al., 2017
Collared peccary	Cryopreservation	Comparison of different cryoprotectants for the vitrification of testes from mature individuals	The combination 1.5 M EG and 1.5 M DMSO provided preservation of the ultrastructure and the proliferative capacity of spermatogonia and Sertoli cells	Silva et al., 2019a
Dasyprocta leporine	Cryopreservation	Comparison of different cryoprotectants using slow freezing Comparison between slow freezing and vitrification methods	1.5 M EG was appropriate for the preservation of testicular cells mitochondrial activity Both methods were adequate for the conservation of the testicular histological architecture	Silva et al 2019b Silva et al 2019c
Galea spixii	Cryopreservation	Comparison of different cryoprotectants on seminiferous tubule morphology.	3 M EG was efficient to maintain histological architecture of testicular tissues	Silva et al 2019d

For wild species, however, the use of vitrification was applied to a few species (Table 2). Promising results were reported for non-human primates, in which the preservation of testicular tissue integrity, maintenance of proliferating spermatogonia and Leydig cell functionality was demonstrated for Rhesus monkey (Poels et al. 2012).

In ungulate species (Table 2), Silva et al., 2019a recently reported the use of solid surface vitrification for testicular tissues of adult collared peccary. Authors highlighted that 1.5 M EG and 1.5 M DMSO combination instead of isolate use of these cryoprotectants provided a better preservation of testicular structure (tubular lumen, cells junctions and cell membrane integrity) as well as of proliferative capacity of spermatogonia and Sertoli cells.

Preliminary studies conducted in wild hystricognath rodents also highlighted the use of a solid surface vitrification protocol (Table 2). For the red-rumped agouti (*Dasyprocta leporina*), Silva et al., 2019c verified that both slow freezing and vitrification methods were adequate for conservation of the testicular histological architecture. On the other hand, EG at a 3.0 M concentration was more adequate than DMSO at a 3.0 M for the vitrification of histological architecture of testicular tissues from the Spix' yellow-toothed cavy (*Galea spixii* – Silva et al., 2019d).

Grafting and in vitro culture of testicular tissues

After cryopreservation, the great challenge is to provide adequate conditions for tissues to resume spermatogenesis. Currently, most promising results have been achieved through an association between cryopreservative techniques and *in vivo* culture. This procedure has been conducted by the transplant of small fragments (1-9 mm^3) of testicular parenchyma both for the same donor individual (autografting) (Fayomi et al., 2019) or for

different interest species as the immunodeficient orchiectomized mouse (xenografting) (Kaneko et al., 2013).

As previously cited, an important novelty for the science related to the autologous grafting of cryopreserved testicular tissues from prepubertal Rhesus monkey, that resulted on the production of sperm and offspring, was recently reported (Fayomi et al., 2019). In spite of the importance of this work, its applicability for wildlife is limited, since there is no sense at recovering testicular tissues from an endangered individual and then devolve it to him.

On the other hand, current literature reports the successful use of xenografting to support complete spermatogenesis thus resulting in spermatozoa production after cryopreservation of testicular tissues from immature individuals in some domestic models as ovine (Pukazhenthi et al., 2015) or swine, which resulted in the birth of piglets (Kaneko et al., 2013).

Regarding wild species (Table 3), in spite that sperm was obtained after xenografting of fresh samples from immature ferrets (*Mustela putorius furo*) (Gourdon and Travis, 2011), white-tailed deers (*Odocoileus virginianus*) (Abbasi and Honaramooz, 2012) and collared peccaries (Campos-Junior et al., 2014), same was not achieved when frozen samples were used. At this point, xenografting of cryopreserved samples provided the maintenance of proliferating spermatogonial cells in Rhesus monkey (Poels et al., 2012), while the development of spermatogenesis until spermatocytes was observed in marmoset (Schlatt et al., 2002), and the establishment of spermatogenesis with initiation of meiosis was verified in Indian spotted mouse deer *(Moschiola indica)* (Pothana et al., 2015).

For mature individuals from both domestic (Arregui et al., 2008) or wild species, the complete spermatogenesis after testicular tissue cryopreservation and xenografting lacks to be reached, once cell degeneration was verified for Iberian lynx (*Lynx pardinus*) (Arregui et al., 2014). Possibly this is due to the delay in angiogenesis, since adult animals have a diverse cell

range in their testicles, without blood supply to physiological needs (Arregui et al., 2008). Then, to xenografting of fragments derived from mature mammals, some hypotheses have been generated (Arregui et al., 2008). The timing and the progression of tubular degeneration after grafting of adult testis tissue appear to be related to the intensity of spermatogenesis at the time of grafting, as observed in any animals tested until now (Arregui et al., 2008). At this point, the improvement of protocols for sperm production from the xenotransplantation of testicular tissue of mature individuals remains as scientific challenge.

To trespass the obstacle for reestablishing complete spermatogenesis in adult individuals, a recent study demonstrated the possibility of inducing infertility in mice and then injecting spermatogonia cells derived from adult dogs into the mice testicles, where it was possible to verify the repopulation capacity of germ cells (Pieri et al., 2019). This study shows an alternative for the use of samples from domestic and wild adult animals that have not yet succeeded in the xenotransplantation of testicular fragments.

In addition, the *in vitro* culture of male gonadal tissue has been reported as an alternative for preserving the mammals' fertility (Lee et al., 2013). Its success depends of the optimization of culture medium and supplements, as specific growth factors, hormones, vitamins and lipids (Valk et al., 2010), besides the development of adequate *in vitro* systems, as classical or 2D and three-dimensional or 3D method (Sato et al., 2011). Overall, *in vitro* culture has been used for the conservation of testicular tissue in various domestic species (Table 3), but in wild mammals, research remains limited to the primates (Huleihel et al., 2015).

Table 3. Culture of testicular tissues in wild mammals.

Species	Used strategy	Aim	Main outcomes	Authors
Bison bison	*In vivo* culture	Evaluation of the xenografting in immature testis	Resulted in testicular maturation and complete development of spermatogenesis in the grafts	Abbasi et al., 2011
Marmoset monkeys *(Callitrix jacus)*	*In vivo* culture	Evaluation of the xenografting in immature testis	Recovery of spermatozoa post-grafting	Schlatt et al, 2002
Rhesus monkey	*In vivo* culture	Evaluation of germ cell differentiation in mature testis	All cells degenerated	Arregui et al., 2008
	In vitro culture	Evaluation of kisspeptin on hormonal secretion in adult testis	The kisspeptin has no role in testicular regulation related to testosterone and inhibin release	Tariq and Shabab, 2017
Mustela putorius furo	*In vivo* culture	Evaluation of the xenografting in immature testis	Recovery of spermatozoa post-grafting	Gourdon and Travis, 2011
Odocoileus virginianus	*In vivo* culture	Evaluation of the xenografting in immature testis	Recovery of spermatozoa post-grafting	Abbasi and Honaramooz, 2012
Collared peccary	*In vivo* culture	Evaluation of spermatogenesis in immature testis	The sperm recovery from the xenografts resulted in diploid embryos after intracytoplasmic injection	Campos-Júnior et al.. 2014
Lynx pardinus *Gazella cuvieri* *G. dama mhorr*	*In vivo* culture	Evaluation of the effect of donor adult and freezing on testicular survival after grafting was also assessed	Degenerated	Arregui et al., 2014

Final considerations

Considering the large potential for testicular tissues be used for the formation of biobanks, it is necessary that zoos and reserves have staff people able to recover the samples immediately after the animals' death and conduct adequate procedures for lavage and transport of the samples.

Additionally, it is necessary to consider that although conventional freezing is the method chosen for a couple of researchers, it has a disadvantage of needing a long time-consuming process, besides needing programmable freezers. At this point, vitrification raises as an alternative more appropriate to be conducted under field conditions. Moreover, there is a lot to learn from domestic models, once statements currently applied for the vitrification of testes from domestic cats (Lima et al. 2018), for instance, could be adapted to the wild felids, in which vitrification is not yet reported. Anyway, we may consider that there is probably no ideal cryopreservation protocol for all species, but each species has its own characteristics and sensitivity to cell preservation. The study of different cryopreservation techniques and cryoprotectants concentrations should be tested for each species to determine what it is the most adequate protocol.

Despite the possibilities of using xenografts for sperm development, *in vitro* culture has become an interesting tool since it may help in understanding the complete communication of spermatogenesis in adult individuals. Nevertheless, more studies are needed regarding the culture and cryopreservation conditions, culture media and supplementation, to ensure the success in obtaining spermatozoa for the species of interest.

Although the success of testicular tissue cryopreservation and culture has been evidenced in some domestic and wild mammals, limitations especially regarding species-specific peculiarities and the sexual age of the animal have been important factors for the

success of these techniques. Nevertheless, the age of the animal is still the most representative factor for the conservation of testicular fragments, since that the testis presents morphological differences between individuals of different sexual maturity. Thus, adult-derived tissues, regardless of organ source, are generally vulnerable to *ex vivo* conditions and difficult to maintain under these conditions due to the cell diversity they exhibit.

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CAPÍTULO III

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Combination of intracellular cryoprotectants preserves the structure and the cells proliferative capacity potential of adult collared peccary testicular tissue subjected to solid surface vitrification

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ABSTRACT

The objective was to evaluate different permeating cryoprotectants to vitrify testicular tissue biopsies from adult collared peccaries. Five pairs of testicles were dissected into fragments (9 mm^3) that were allocated to non-vitrified (control) and vitrified groups using a solid-surface method following exposure to different cryoprotectants (3.0 M dimethyl sulfoxide (DMSO), 3.0 M ethylene glycol (EG) or 1.5 M DMSO + 1.5 M EG). After warming, samples were evaluated for histomorphology, ultrastructure, viability, and proliferative capacity potential. The appropriate conservation of the ultrastructural organization of the seminiferous tubule in terms of lumen presence and cell junctions was only observed at the use of DMSO/EG combination. Regardless of the cryoprotectant, the vitrification effectively preserved cell nuclear visualization and condensation similarly as observed at the non-vitrified group. Moreover, DMSO/EG combination provided a better preservation of basal membranes of seminiferous tubules than DMSO ($P < 0.05$). The occurrence of cell swelling was more evident in the use of DMSO than EG ($P < 0.05$), but both isolate cryoprotectants were similar to the DMSO/EG combination. Only the DMSO/EG combination maintained the proliferative capacity potential for spermatogonia (3.69 NORs/cell) and Sertoli cell (3.19 NORs/cell) similar to controls (3.46 and 3.31 NORS/cell, respectively). Moreover, ~40% cell viability was found after vitrification independent of cryoprotectant. In conclusion, DMSO/EG in combination is better than DMSO or EG alone for SSV of testicular tissue biopsies from adult collared peccaries.

Keywords: Biobank, Male germplasm, Wildlife, Tissue cryopreservation.

1. Introduction

Collared peccaries (*Pecari tajacu*) are wild ungulates that inhabit the Americas and perform an important ecological function as a seed disperser and as a main component of the food chain, thus contributing to the maintenance of their predators, the big felids [15]. Although they are globally considered as a species of least concern [20], they are vulnerable or even extinct in some Latin American regions, such as the Atlantic Forest [15] and the North Argentina [24]. Hence, the need for development of strategies for their conservation is imminent, including the formation of germplasm banks.

In this regard, the conservation of male gonadal tissue, a relatively recent technology for both domestic [28, 30] and wild mammals [36, 39], has increased over the years. This technology allows the use of the germplasm derived from male gonadal tissues in other assisted reproductive techniques, such as intracytoplasmic sperm injection [44] and *in vitro* fertilization [23]. Moreover, it allows for safeguard of genetic material from animals at any reproductive stage or that have suddenly died [39]. However, it is a great challenge in preserving the testicular tissue derived from adult individuals who have initiated the spermatogenesis, thus containing different cell types in the seminiferous tubules with a complex metabolism, which is more sensitive to manipulation as compared to prepubertal animals [2, 38].

The establishment of the vitrification method and the cryoprotectants employed are essential stages that need to be optimized in the species of interest. The vitrification of tissue samples consists of the solidification of a solution using low temperatures, but with the reduction of the formation of ice crystals The solid-surface vitrification (SSV) is a simple and economical method that can avoid potential risk of cellular injury caused by the formation of intracellular ice [25]. In collared peccaries, a preliminary study using the SSV method with ethylene glycol (EG) as intracellular cryoprotectant provided promising results related to an

adequate morphological preservation of the germinative epithelia of testicular tissue [7]; however, further evaluation of other morphological and functional parameters is necessary to define an adequate protocol.

Besides EG, dimethyl sulfoxide (DMSO) has also been proposed as a cryoprotectant for the preservation of mammalian testicular tissues [26, 43]. It is necessary to highlight that there are marked differences between DMSO and EG with regards to their molar mass (78.13 g/mol vs 62,07 g/mol, respectively), which is directly related to their capacity to penetrate the cell [13, 18]. In fact, DMSO interacts with the lipid membrane and induces pore formation for water passage causing cell dehydration and consequently reduces the ice crystal formation inside the cells during cryopreservation [13, 22]. On the contrary, EG is able to permeate the membrane cell faster [11, 34], resulting in reduced osmotic stress and preventing the formation of ice crystals during cryopreservation [21, 40]. Although EG was efficient for the cryopreservation of swine testis [26], DMSO presented better results for mouse testis [16]. Alternatively, combination of substances allows the reduction of the intracellular concentration of cryoprotectants, which minimize their toxic effects, thus providing efficient tissue preservation for some species, as reported for the testes derived from wild felids, deer [39], and sheep [37]. Collectively, these different results highlight the need for determining adequate protocols for different species.

Addressing the need for the germplasm conservation of species, the aim of this study was to evaluate the influence of different intracellular cryoprotectants followed by solid surface vitrification on testicular tissue derived from collared peccaries.

2. Materials and methods

2.1. Chemicals and media

Unless otherwise noted, all chemicals and media were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-vimentin antibody and goat Anti-mouse IgG (H&L HRP) were purchased from Abcam (Cambridge, CA, USA).

2.2. Bioethics and animals

All the experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.004271/2014-71) and Chico Mendes Institute for Biodiversity Conservation (no. 37329). All males belong to the Centre of Multiplication of Wild Animals (Mossoró, RN, Brazil; 5°10'S, 37°10'W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912).

2.3. Testis collection and experimental design

Every year, a programmed slaughter is conducted for population control and the biological material is destined for several experiments. For the present study, five mature males of 3–5 years of age, weighing 15–20 kg, were used, with each individual considered as a replicate. After slaughtering, the pairs of testicles were washed in saline solution (NaCl 0.9%) and transported to the laboratory in same solution at 22 °C for 30 min.

In the laboratory, the testicles were separated from surrounding tissues and washed with three successive baths of saline solution. Then, all testis were fragmented into 9.0 mm^3 slices (3 × 3 × 1 mm) and 32 fragments derived from each individual were distributed randomly into non-vitrified [fresh control] and vitrified fragments, according to the

cryoprotectant to be tested [3.0 M dimethyl sulfoxide (DMSO), 3.0 M ethylene glycol (EG), and combination of 1.5 M DMSO and 1.5 M EG]. For each treatment, a total of eight fragments were used. These fragments were equally distributed (two fragments per assay) for histo-morphological assessment, cell viability after different dissociation procedures, cell proliferative capacity potential through quantification of nucleolar organizer regions (NOR) and scanning electron microscopy.

2.4. Cryopreservation of testicular tissues

A vitrification solution (VS) constituted of Minimum Essential Medium (MEM) supplemented with 0.25 M sucrose, and 10% (v/v) fetal bovine serum (FBS) was used. To this VS, isolate cryoprotectants (DMSO or EG) at a 3.0 M concentration or its combination (DMSO/EG) containing a 1.5 M concentration of each cryoprotectant was added.

For the solid-surface vitrification (SSV) procedures, we adopted a methodology previously described for swine testicular tissues [26] with a few modifications. Briefly, a total of 24 fragments were vitrified; for each treatment group, eight fragments were exposed to the corresponding VS (4.0 mL) for 5 min; tissues were then dried on sterile gauze. Next, the fragments were individually placed on a metal cubic surface partially immersed in liquid nitrogen and transferred to cryovials for storage in liquid nitrogen (-196 ºC).

After one week, the cryovials were maintained for 1 min at 25 °C and immersed in a water bath at 37 °C for 30 sec. For removal of cryoprotectants, all fragments were washed three times for 5 min in MEM, 10% FBS, and with decreasing concentrations of sucrose (0.50, 0.25 M, and without sucrose).

2.5. Effect of intracellular cryoprotectants on testicular three-dimensional surface morphology

For evaluation of testicular three-dimensional surface, fragments were fixed in 2.5% glutaraldehyde solution for 24 h. Then, samples were fixed in osmium for 30 min, followed by washing with PBS and then subsequently dehydrated in 15%, 20%, 25%, 30%, 50%, 70%, 90% ethanol for 5 min each, and three times in 100% ethanol for 10 min. After dehydration, the critical point drying equipment used was Baltec CPD 030 and the samples were dried following the manufacturer's instructions. After drying, the samples were kept in a desiccator until their set-up on stubs and metallization. The samples were fastened with an adhesive tape on brass stubs and then gold coated in the metallizer Sputter Coater SCD050/LEICA and were observed under a scanning electron microscope JSM-6510/JEOL [9]. After processing, we detected possible damages on the surface of testicular tissue caused by vitrification and observed the cellular organization of the seminiferous tubule through the presence or absence of lumen [9].

2.6. Testicular cell morphology analysis

For evaluation of morphology by conventional histology, fragments derived from both control and vitrified groups were fixed in Bouin solution for 24 h, sectioned at 5.0 µm, stained with hematoxylin-eosin and evaluated by using a light microscope (Olympus CX 31 RBSFA, Tokyo, Japan). The morphological parameters for tubular structure, ruptures of the basement membrane, swelling, tubular cell loss, separation of the basal membrane and vacuolation were assessed [17], according to a score scale established for human testicular tissue and adapted for this study (Table 1). Then, testicular tissues that were classified as

score 3, were considered morphologically normal and fragments classified with a score of 0 were considered unfit in their testicular morphology. For each parameter, five seminiferous tubules of six different fields were assessed, resulting in a total of 30 seminiferous tubules for each treatment.

To identify the Sertoli cells and evaluate their morphology, we conducted an immunohistochemistry assay based on the identification of the vimentin. First, we validated the primary antibody (anti-vimentin monoclonal antibody – ab8979) in collared peccaries using dogs as a reference species (Fig. 1). Briefly, the tissues were fixed using 4% paraformaldehyde for 12 h at 22 °C, and they were processed via routine histology, embedded in paraffin and sectioned in series (5.0-μm thickness). Subsequently, samples were dewaxed in xylene and rehydrated in decreasing alcohol baths. The antigen retrieval was performed by submerging the slides in citrate buffer, pH 6.0, for 15 min. After cooling down, slides were washed twice in PBST (PBS and 0.1% Triton X-100) for 5 min each. Tissue sections were saturated with blocking solution (UltraCruz® blocking reagent) for 2 h at 22 °C. Samples then were incubated overnight with primary antibody (1:300) at 4 °C in a humidified chamber. A negative control in which the primary antibody was absent was included in each trial, while the fresh canine testicular tissue was considered as a positive control. After extensive washings in PBST, the samples were incubated in 0.3% hydrogen peroxide in PBS for 15 min at 22 °C. Then, tissue sections were incubated with secondary antibody (Goat Anti-Mouse IgG H&L – ab205719; 1:100) for 1 h at 22 °C in darkened container. After more washes in PBS and PBST, one drop of diaminobenzidine agent (DAB, 1:5) was added to each slide and immersed in distilled water after 10 min in a dark environment. After the suspension, all reactions were colored in Harris hematoxylin for 10 sec, then washed with absolute alcohol, xylene, and mounted with Permount® (Fisher Chemical™ Permount™ Mounting Medium, SP15-500). Lastly, 15 seminiferous tubules were analyzed and Sertoli

cell morphology was classified with a score scale, based on parameters of shape, detachment from the basement membrane or cell loss [17]. Sertoli cells were evaluated in scores from 0 to 4 as showed in Table 1 [17].

2.7. Testicular cells proliferative capacity potential

Proliferative capacity potential was evaluated by quantification of nucleolar organizer regions (NORs) in the spermatogonia, spermatocytes, spermatids, and Leydig and Sertoli cells using silver staining technique [5].

Table 1. Morphological parameters of tubules from testis of collared peccaries.

Parameter	**Scores**				
	#4	**#3**	**#2**	**#1**	**#0**
Distinction spermatogonia/Sertoli	--	Easy distinction	Difficult distinction	Impossible to distinguish	--
Nuclear view	--	Easy view	Dificult view	Impossible to view	--
Nuclear condensation	--	No condensation	Some (< 50%) cells with nuclear condensation	Most cells (< 50%) nuclear condensation	--
Tubular cell swelling	--	No swelling	> 50% cells without swelling	> 50% cells with swelling	--
Tubular cell loss	--	No cell loss	< 75% cell types lost	> 75% cell types lost	--
Rupture from basal membrane	--	No rupture	Partly ruptured (< 50%)	Mostly ruptured (> 50%)	--
Shrinkange from basal membrane	--	No shrinkange	Partly shrinkange (< 50%)	Mostly shrinkange (> 50%)	--
Tubular structure	--	Structure intact	All cell types present although slightly disordered structure	Random distribution of remaining cells	--
Sertoli cells	Normal Sertoli cells	Some Sertoli cells are round or detached from basal membrane	Most Sertoli cells are round or detached from basal membrane	All Sertoli cells are round or detached from basal membrane	No Sertoli cells left

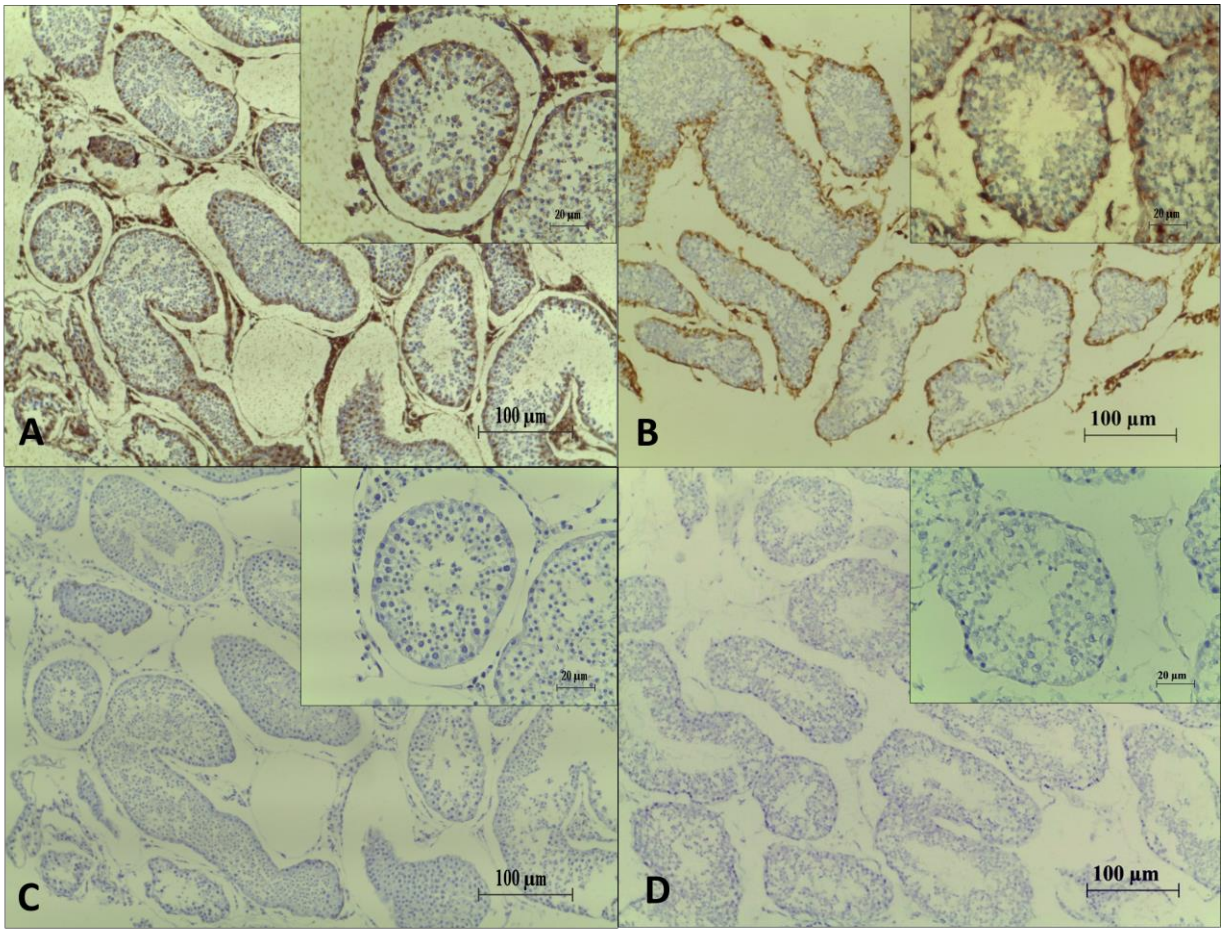

Figure 1. Validation the primary antibody to Sertoli cell morphology in testicular tissues from collared peccaries using dogs as a reference species evaluated by immunohistochemistry (vimentin staining). **(A)** Positive dog's testicular tissue, **(B)** Positive collared peccarie's, **(C)** and **(D)** Negative control testicular tissue from dogs and collared peccaries respectly.

The slides were soaked in silver solution prepared in 1 part of 2% gelatin in 1% aqueous formic acid and 2 parts of 50% aqueous silver nitrate solution and were exposed in a dark room for 30 min. Subsequently, the slides were washed in 5% thiosulfate solution for 10 min [6]. For analysis of each group, NOR dots were counted within the nucleoli of spermatogonia, spermatocytes, spermatids, and Leydig and Sertoli cells of 10 randomly selected nuclei in 10 fields at 1000× magnification, resulting in 500 cells for each treatment [8].

2.8. Testicular cells viability

To obtain cells isolated from vitrified and non-vitrified tissues, fragments were dissociated using an enzymatic dissociation [1]. Briefly, we used 0.2% collagenase type IV, 0.1% hyaluronidase, 0.01% DNase type I in DMEM at 37 °C for 20 min under slow stirring and the reaction was stopped by the addition of an equal volume of FBS [1].

The cellular viability was assessed using trypan blue staining, and the test was conducted in duplicates. Briefly, the cells were suspended in MEM and a cell aliquot was stained with 0.4% trypan blue (in PBS, ratio 1:1) and counted in a Neubauer chamber. The cells were considered viable cells when they are not stained; the non-viable cells became blue due to diffusion of the dye into the dead cell [1].

2.9. Statistical analyses

Data were tested for normality and homoscedasticity using Shapiro-Wilk and Levene's tests, respectively (Stat View 5.0, SAS Institute, Inc. Cary, NC, USA) and values were arc sine-transformed before analysis when necessary. The effects of cryoprotectants on the testicular parameters were assessed by ANOVA followed by Tukey test. The scores obtained in classical histology were subjected to the Mann-Whitney's test for comparison among groups. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Morphological analysis

Using scanning electron microscopy we observed whether the seminiferous tubule lumen was preserved, whether visible spermatogonia had membrane damage and whether their junctions were preserved. We discovered that no damage, derived from vitrification, was evident on external structure of the samples that were vitrified using DMSO (Fig. 2B) or DMSO/EG combination (Fig. 2D) in comparison to fresh samples (Fig. 2A). However, damages on the external membrane of spermatogonia were noted in the use of EG (Fig. 2C). In contrast with other cryoprotectants, DMSO/EG combination was the only group where the seminiferous tubule lumen could be observed (Fig. 2D'), also conserving the tubule organization (Fig. 2D") where the cells were appropriately bound to membrane by evident junctions.

The morphological features in non-vitrified testicular tissue (fresh control) and after vitrification using different intracellular cryoprotectants are detailed in Figure 3. In all groups, a clear distinction between spermatogonia and Sertoli cells as well as a normal nuclear structure was observed (Fig. 3A–D). However, testicular tissues cryopreserved with DMSO resulted in the swelling (Fig. 3B). Moreover, space between basal membrane and cells in seminiferous tubules was noted for all experimental groups (Fig. 3A–D). DMSO resulted in the swelling (Fig. 3B). Moreover, space between basal membrane and cells in seminiferous tubules was noted for all experimental groups (Fig. 3A–D).

By a quantitative point of view, the vitrification did not affect the nuclear visualization or condensation regardless of the intracellular cryoprotectants used (Table 2). The other histo-morphological parameters were altered by vitrification ($P < 0.05$). DMSO/EG combination provided a better preservation of basal membrane than DMSO ($P < 0.05$), which provoked increased swelling when compared to EG ($P < 0.05$). Although vitrification

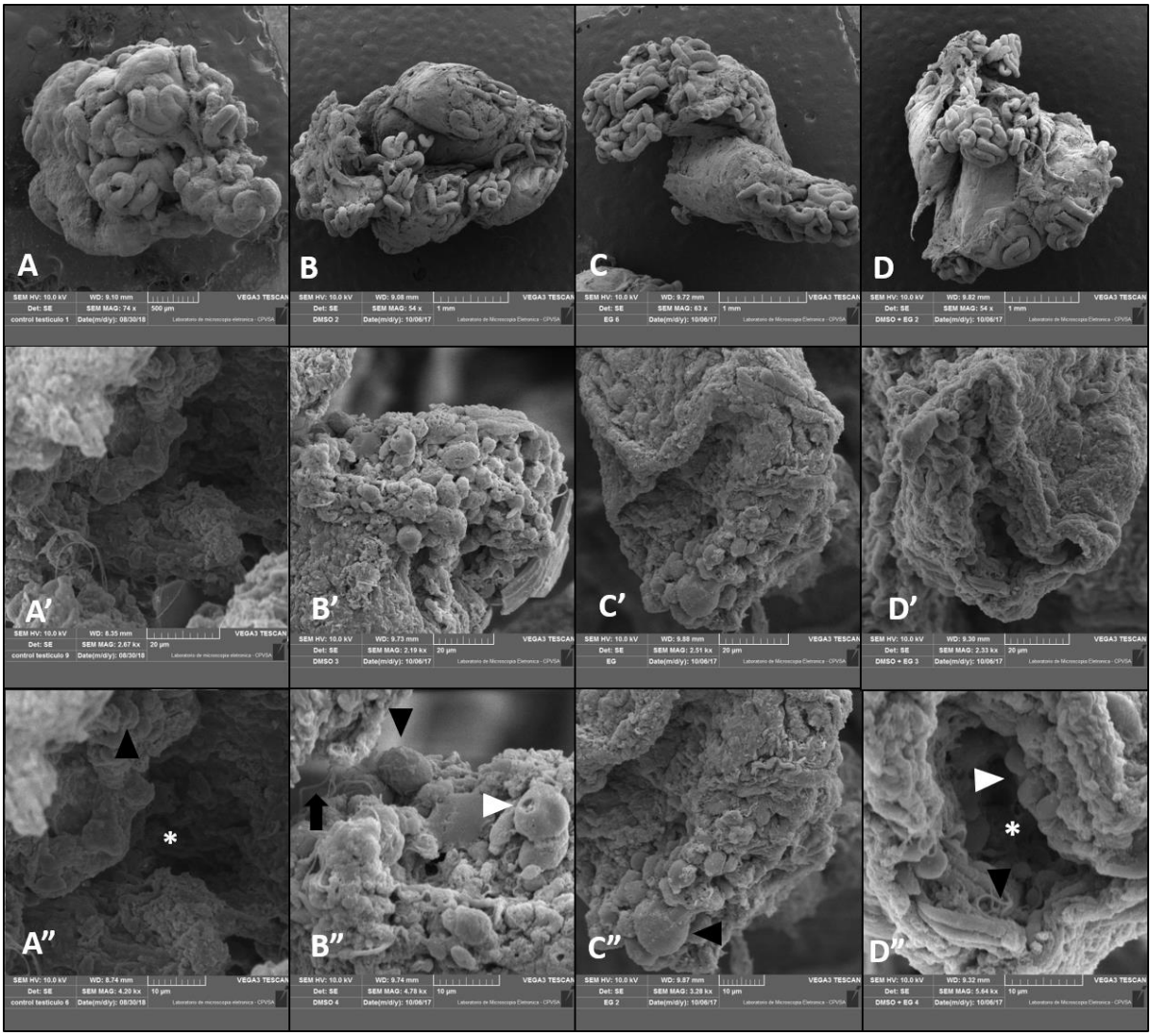

Figure 2. Scanning electron micrographics of the peccary non-vitrified and vitrified testicular tissues. **(A)** Non-vitrified group, **(B)** group vitrified with dimethyl sulfoxide (DMSO), **(C)** group vitrified with ethylene glycol (EG), **(D)** group vitrified with DMSO/EG combination. Letters A', B', C,' and D' are a magnification of A, B, C, and D, respectively. (A") Magnification of figure A': asterisk shows seminiferous tubule lumen and black arrowhead shows a spermatid; (B") Magnification of figure B': black arrow shows sperm tail, black arrowhead shows a normal spermatogonia and black arrowhead shows a spermatogonia with membrane damage; (C") Magnification of figure C' white arrowhead show a spermatogonia; (D") Magnification of figure D': asterisk shows seminiferous tubule lumen, black arrowhead shows a sperm tail and white arrowhead shows a spermatid with its junctions preserved.

affected the morphology of Sertoli cells in comparison to fresh control group, no difference was observed among vitrified groups for this indicator (Fig. 4A–E). Additionally, all groups containing DMSO resulted in a clear labeling of the cells, which facilitated the visualization of Sertoli cells (Fig. 4B and 4D). Moreover, EG group presented many Sertoli cells that were round and detached from the basal membrane (Fig. 4C).

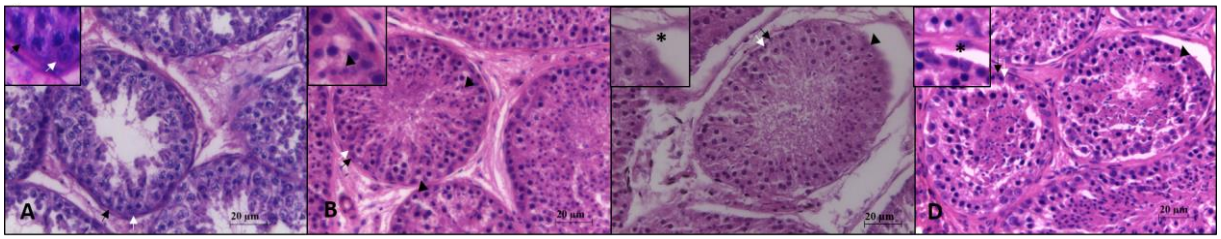

Figure 3. Histo-morphological evaluations of non-vitrified and vitrified testicular tissues in the collared peccary. **(A)** Non-vitrified group, **(B)** group vitrified with dimethyl sulfoxide (DMSO), **(C)** group vitrified with ethylene glycol (EG), **(D)** group vitrified in DMSO/EG combination. For Fig. A–D, black arrow shows Sertoli cell, and the white arrow shows spermatogonia. For Fig. B, arrowhead shows swelling, asterisk show higher magnification. For Fig. C and D, black arrowhead shows space between basal membrane and cells in seminiferous tubules, asterisk show higher magnification.

Table 2. Histomorphological evaluation of testicular non-vitrified and vitrified tissues with different intracellular cryoprotectants derived from collared peccaries.

	Non-vitrified	**DMSO**	**EG**	**DMSO/EG**
Distinction spermatogonia/Sertoli	2.98 ± 0.03^{a}	2.29 ± 0.04^{b}	2.34 ± 0.02^{b}	2.22 ± 0.05^{b}
Nuclear view	2.95 ± 0.02^{a}	2.05 ± 0.06^{a}	2.12 ± 0.06^{a}	2.13 ± 0.06^{a}
Nuclear condensation	2.74 ± 0.03^{a}	2.15 ± 0.06^{a}	2.25 ± 0.06^{a}	2.17 ± 0.06^{a}
Tubular cell swelling	2.63 ± 0.04^{a}	2.21 ± 0.05^{c}	2.39 ± 0.04^{b}	2.33 ± 0.04^{bc}
Tubular cell loss	2.66 ± 0.04^{a}	2.19 ± 0.04^{b}	2.13 ± 0.04^{b}	2.18 ± 0.04^{b}
Rupture from basal membrane	2.73 ± 0.04^{a}	2.17 ± 0.05^{c}	2.26 ± 0.04^{bc}	2.27 ± 0.04^{b}
Shrinkange from basal membrane	2.83 ± 0.04^{a}	2.08 ± 0.06^{b}	2.11 ± 0.05^{b}	2.13 ± 0.05^{b}
Tubular structure	2.64 ± 0.04^{a}	2.12 ± 0.04^{b}	2.06 ± 0.03^{b}	2.13 ± 0.04^{b}

Lowercase superscript letters indicate difference among columns ($P < 0.05$).

3.2. Testicular cell proliferative capacity potential

Regarding the quantification of NORs (Fig. 5A–G), we verified that the DMSO/EG combination was the unique treatment that was able to maintain the proliferative capacity

potential of the spermatogonia and Sertoli cells after vitrification, owing to the values that were similar to those calculated for fresh samples ($P > 0.05$).

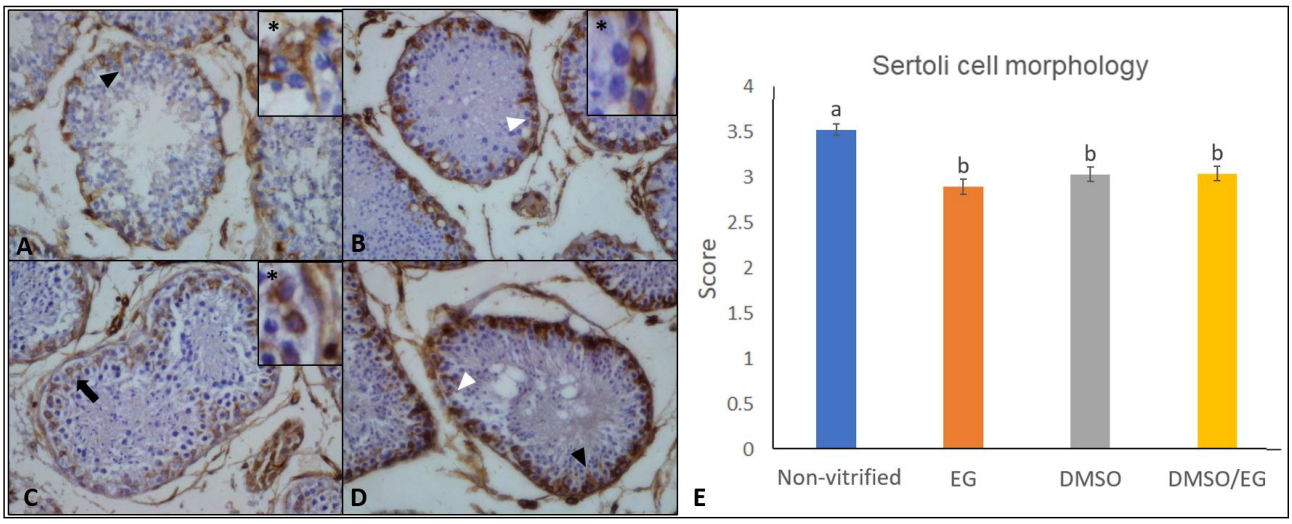

Figure 4. Sertoli cell morphology in testicular tissues from collared peccaries evaluated by immunohistochemistry (vimentin staining). **(A)** Non-vitrified group, **(B)** group vitrified with dimethyl sulfoxide (DMSO), **(C)** group vitrified with ethylene glycol (EG), **(D)** group vitrifies with DMSO/EG combination, **(E)** values (Means ± SEM) for all groups. For Fig. A, black arrowhead shows the morphologically normal Sertoli cell. For Fig. B, white arrowhead shows round Sertoli cells. For Fig. C, arrow shows Sertoli cell round and detached from basal membrane. For Fig. A–C, asterisk show higher magnification. For Fig. D, black arrowhead shows the morphologically normal Sertoli cell and the white arrowhead shows round Sertoli cells. Lowercase superscript letters indicate difference among treatments ($P < 0.05$).

3.3. Testicular cell viability

The vitrification affected the cell viability after warming ($P < 0.05$), regardless of the cryoprotectants and dissociation procedures (Fig. 6). Following Trypan blue assay, viability of the germinal cells was about 80% for fresh tissue and 30–40% in vitrified tissues.

4. Discussion

This study demonstrates the use of an SSV method and characterizes the benefit of a cryoprotectant combination (DMSO/EG) for testicular tissues derived from a wild species. This method was previously proven as an effective alternative for the cryopreservation of peccary somatic [6] and ovarian [31] tissues but had not been tested for testicular tissue.

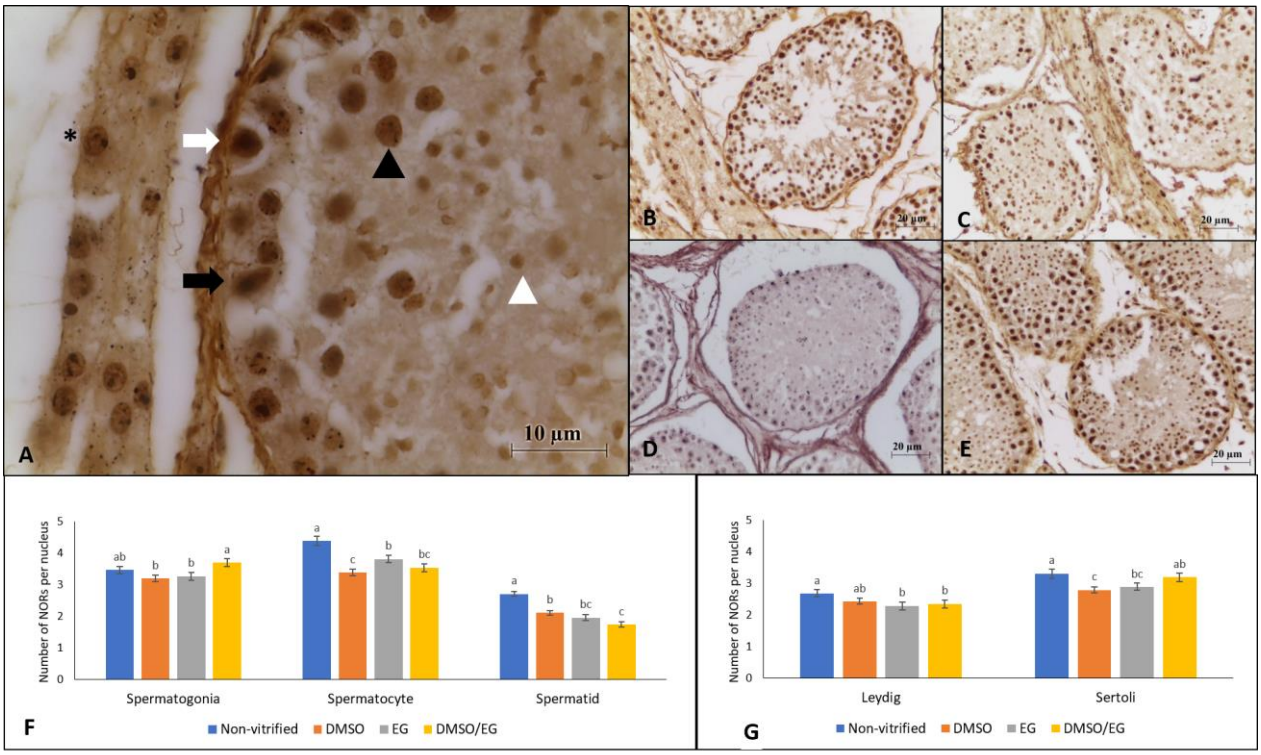

Figure 5. Proliferative capacity evaluated by quantification of nucleolar organizer regions (NORs) in peccary testicular germinative cells from non-vitrified (control) and vitrified. **(A and B)** Non-vitrified group. For Fig. A, black arrow shows Sertoli cell, white arrow shows spermatogonia, black arrowhead indicates spermatocyte, white arrowhead indicates spermatids, and asterisk indicates Leydig cell. **(C)** group vitrified with dimethyl sulfoxide (DMSO), **(D)** group vitrified with ethylene glycol (EG), **(E)** group vitrifies with DMSO/EG combination. Figures F and G show values (Means ± SEM) and different letters in the columns indicate differences among treatments ($P < 0.05$)

Through scanning electron microscopy, which allowed us to conduct an ultrastructural analysis at a nanometric scale [10], we provide novel data related to the possibility of adequately conserving the germinative cells and the lumen of the seminiferous tubule using DMSO/EG combination. The maintenance of the tubule lumen after vitrification is essential for the process of spermatogenesis because it is the natural path for the sperm to follow [2]. In addition, we verified the preservation of the cell junctions along the peccary seminiferous tubules, thus evidencing the maintenance of the tissue cyto-architecture [9] even after vitrification process.

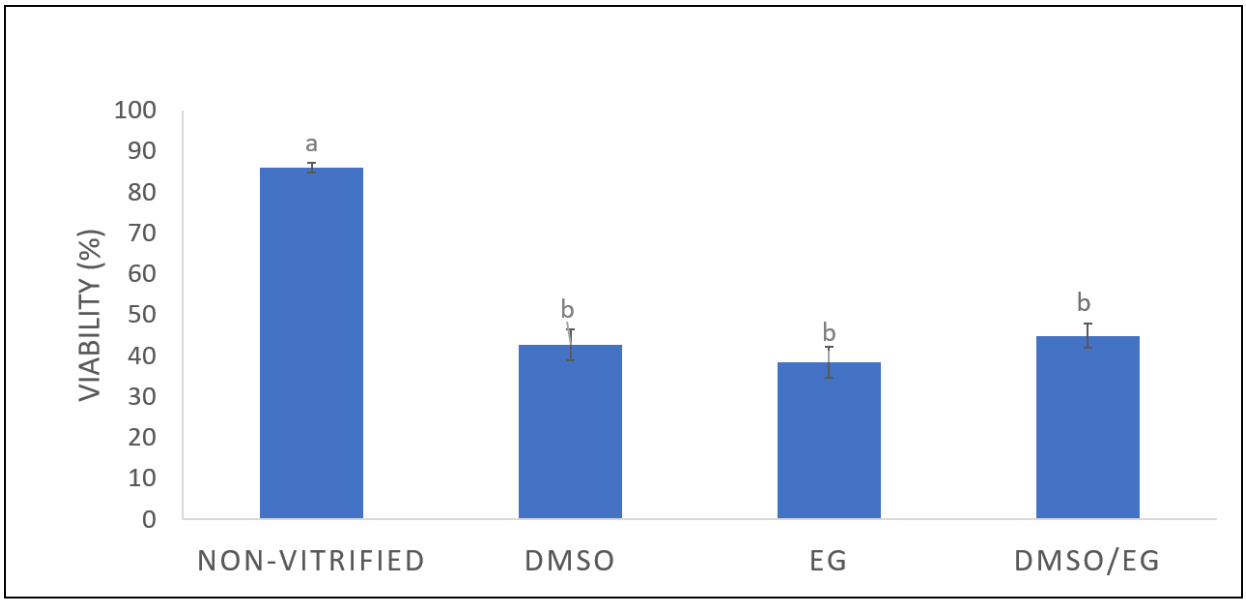

Figure 6. Values (Means ± SEM) of germinal cell viability evaluated by trypan blue assay from peccary non-vitrified and vitrified testicular tissue, using different cryoprotectants. Lowercase superscript letters indicate difference among cryoprotectants ($P < 0.05$).

As verified through histological analysis, the vitrification did not affect the nuclear view and condensation of testicular cells regardless of intracellular cryoprotectants. Probably, the reduced formation of ice crystals derived from the rapid cooling rate used on the SSV method [4] would have contributed to the conservation of the cell's nucleus.

On the contrary, significant morphological changes were observed among non-vitrified and vitrified treatments for spermatogonia/Sertoli distinction, swelling, tubular cell loss, membrane rupture and distance between basal membrane and cells in seminiferous tubules, along with its structure and morphology. Such results could be related to the presence of the lumen and different cell population at the seminiferous tubules in the testicles of adult individuals [2], which increases the tissue sensitivity to manipulation and cryopreservation. It was evident that isolated use of DMSO increases the incidence of swelling and membrane rupture. It is well known that the pore formation in the phospholipid membrane is dependent on the DMSO concentration [22]. Thus, a high concentration of

DMSO can completely destroy the structure of the membrane lipid bilayer [22]. Probably, the concentration of 3.0 M was enough to produce the formation of several membrane pores that lead to the water inflow, increasing the occurrence of swelling and membrane rupture in peccary testicular tissue.

With the emphasis on the identification of the Sertoli cell using an immunohistochemistry assay for vimentin detection, we did not verify morphological differences on these cells regarding cryoprotectants. However, we observed that the DMSO/EG combination provided an effective conservation of proliferative capacity potential of Sertoli cell as well as of the spermatogonia using the Ag-NOR technique. This is an interesting result, highlighted by the fact that NORs are associated with argyrophilic proteins present in the chromosomes during the interphase stage [14, 27]. These proteins can contribute to a successful spermatogenesis when tissues are subjected to in vivo or in vitro culture. In fact, the spermatogonia form a pool of testicular tissue responsible for the formation of the germinative cell lineage with the capacity to produce unlimited spermatozoa, and Sertoli cells can support these cells during development [33].

Related to cell viability, we obtained a recovery of 40% cell viability from the vitrified tissue. The recovery values were higher than 23.4% cell viability as described for adult bovines [42]. It is evident, however, that adult testicular tissues are more sensitive to freezing techniques than immature ones, especially because 80% cell viability was reported for prepubertal individuals whose testes were vitrified [37].

As previously demonstrated for neonatal sheep [37], DMSO/EG combination was efficient for the conservation of testicular tissues derived from adult collared peccaries in terms of preservation of the nucleus condensation, cell proliferation capacity potential in spermatogonia and Sertoli cells, and the ultrastructure of the seminiferous tubules lumen. In fact, cryoprotectant combination can provide enough penetration into cells, thus avoiding the

intracellular ice formation, and reduce the osmotic stress [19], while the isolate use of cryoprotectants at high concentrations could have toxic effects on cellular organelles, thus impairing cellular functions [12, 41]. Nevertheless, the use of a low concentration of each cryoprotectant may have nullified its toxic effects on the cell.

Finally, we highlight that we used testicular tissues derived from adult individuals in the present study, which generally are difficult to conserve due to the cellular diversity and vulnerability to ex vivo conditions [38]. Although tissues from adult animals have already been cryopreserved in different species (feline – [32]; cervids – [35]; bovine – [42], no protocol effective enough to obtain sperm from cryopreserved testicular tissues, was yet described. This fact evidences the need for continuing the studies to produce viable sperm, and consequently, live offspring.

5. Conclusion

Our results allow us to suggest the use of a solid surface vitrification method associated to a DMSO/EG combination for the conservation of testicular tissue derived from adult collared peccaries. These novel data can be used as a basis for the formation of germplasm banks for the conservation of this wild ungulate.

Conflict of interest

The authors declare no conflicts of interest.

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CAPÍTULO IV

Conservation of testicular tissue derived from collared peccaries (*Pecari tajacu*) using slow freezing, vitrification in cryotube and solid surface vitrification.

Diferentes protocolos de criopreservação de tecido testicular de cateto.

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ABSTRACT

As an effort for the optimization of germplasm banks, the aim was to evaluable the effects of the cryopreservation and correlations with intracellular cryoprotectants in conservation of testicular tissue derived from adult collared peccaries. Five pairs of testicles were fragmented (3 mm^3) and allocated in non-cryopreserved (control) and cryopreserved using three sistems: slow freezing (SF), vitrification in cryotubes (VC), and solid surface vitrification (SSV), using different combination of cryoprotectants [1.5 M dimethyl sulfoxide (DMSO) and 1.5 M ethylene glycol (EG), 1.5 M DMSO and 1.5 M glycerol (G) and 1.5 M G and 1.5 M EG]. All samples were evaluated for viability, histomorphology, proliferative capacity potential, and DNA fragmentation. Regardless of the cryopreservation method, the combinations DMSO/EG and DMSO/G were able to preserve the viability ($P > 0.05$). Moreover, the SF protocol using the DMSO/EG and DMSO/G combinations were better for swelling preservation than G/ EG - SF and DMSO/G - SSV ($P < 0.05$). All treatments maintained the potential for proliferative capacity for spermatogonia ($P > 0.05$), but G/EG in SF and SSV protocols impaired the proliferative ability of Sertoli cells ($P < 0.05$). DNA fragmentation in SF and VC protocols were similar to control when using the DMSO/EG combination ($P > 0.05$). In conclusion, slow freezing and vitrification in cryotubes using DMSO/EG appear to be the most suitable methods for the cryopreservation of peccary testicular tissue.

Key words: *Pecari tajacu*, testicle, germplasm, germ cell.

INTRODUCTION

The collared peccaries (*Pecari tajacu* Linnaeus, 1758) are wild ungulates that inhabit the Americas, although they are globally considered as a species of least concern (Gongora *et al*., 2011). Therefore, this specie can be used as an experimental model for the development of conservative strategies to other closely related species as the white-lipped peccary *(Tayassu pecari)* (Keuroghlian *et al*., 2013) and the Chacoan peccary *(Catagonus wagneri)* (Altrichter *et al*., 2015), currently classified as vulnerable and endangered by IUCN, respectively. The peccaries perform an important ecological function as seed dispersers and as main components of the food chain, thus contributing to the maintenance of their predators, the big felids (Desbiez *et al*., 2012). To contribute for the peccaries conservation, our team have been studying several strategies for the storage of their germplasm as the cryopreservation of ejaculates (Campos *et al*., 2017), epididymal sperm (Bezerra *et al*., 2014), somatic tissue (Borges *et al*., 2018), ovarian tissue (Lima *et al*., 2019), and, recently, the testicular tissue (Silva *et al*., 2019).

In fact, cryopreservation of testicular tissue raised as a promising tool for assisted reproduction, allowing the storage of large numbers of germ cell fragments in various developmental stages (Lima *et al*., 2018a; Silva *et al*., 2019; Singh *et al*., 2019) including undifferentiated spermatogonia that can be cultured, thus providing an unlimited sperm production (Fayomi *et al*., 2019; Kaneko *et al*., 2019, 2013). Combined with cell or tissue xenograft techniques, this technology can promote the restoration of both gametogenic and endocrine function after the cryopreservation period (Gosden, 2002), and result in live offspring as demonstrated for swine (Kaneko *et al*., 2019, 2013), the domestic species most closely related to the peccaries (Benirshchke, 1974).

With the purpose of cryopreserving testicular tissue several cryopreservation protocol such as vitrification (Lima *et al*., 2019) and slow freezing (Pukazhenthi *et al*., 2015) have been developed. The vitrification involves converting the biomaterial into a glass-like, amorphous solid without triggering ice crystal formation, where the tissue fragments are exposure to high concentrations of usually multiple cryoprotectants and then plunging/storing directly in liquid nitrogen (Pereira *et al*., 2016). On the other hand, the slow freezing use low concentration cryoprotectant and then frozen gradually at ~1°C/min in a -80°C freezer overnight before plunging and storing in liquid nitrogen (Pukazhenthi *et al*., 2015).

In addition, the cryoprotectants also is variable among cryopreservation protocols. The most used have been DMSO and EG, but glycerol showed positives results too. Studies conducted with combination the cryoprotectant had tried less effect negative them. In domestic animals, DMSO in combination with EG was used for testicular cryopreservation in mouse (Yamini *et al*., 2016), while the combination DMSO plus glycerol was used for testicular vitrification in cat (Lima *et al*., 2018a). In wild animals, DMSO in combination with EG jungle cat, lion, leopard, rusa deer, fea's muntjac, sumatran (Thuwanut *et al*., 2013) as well as to peccaries (Silva *et al*., 2019). However, it is important verify the combination of main cryoprotective that has been used in other animals to peccary.

The goal of this study was evaluable the influence the different cryopreservation protocol, slow freezing, vitrification in cryotube and Solid Sufice Vitrification, using several combinations of cryoprotective (dimetilsufoxide - DMSO/ ethylene glycol - EG, DMSO/ glycerol- G, G/EG).

MATERIAL AND METHODS

Bioethics and animals

All the experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.004271/2014-71) and Chico Mendes Institute for Biodiversity Conservation (no. 37329). All males belong to the Centre of Multiplication of Wild Animals (Mossoró, RN, Brazil; 5°10'S, 37°10'W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912).

Testis collection and experimental design

Every year, a programmed slaughter is conducted for population control and the biological material is destined for several experiments. For the present study, five mature males, where each male was considered one replicate, aging from 3 to 5 years of age and weighing 15–20 kg, were used. After slaughtering, pairs of testicles were washed in saline solution (NaCl 0.9%) and transported to the laboratory in same solution at 22 °C for 30 min.

In the laboratory, the testicles were separated from surrounding tissues and washed with three successive baths of saline solution. Then, all testis were fragmented in 3.0 mm^3 (3

x 1 x 1 mm) and 120 fragments derived from each individual were distributed randomly into non-cryopreserved [fresh control] and cryopreserved fragments, according to the cryoprotectant to be tested [combination of DMSO and EG, DMSO and M G, and EG and M G,] and the cryopreservation methods [Slow freezing- SF, Vitrification in cryotube -VC, and Surface-Solid Vitrification - SSV]. In each treatment, 12 fragments were equally distributed, where 2 fragments were submitted to each assay, for histological assessment, cell viability, cell proliferative capacity potential through quantification of nucleolar organizer regions (NOR) and DNA fragmentation through TUNEL.

Cryopreservation

Slow freezing of testicular tissues

The slow freezing solution (FBS) constituted of Minimum Essential Medium (MEM) supplemented with 0.25 M sucrose, 10% (v/v) fetal bovine serum (FBS) and 1.5 M intracellular cryoprotectant (0.75 M DMSO/ 0.75 M EG, 0.75 M DMSO/ 0.75 M G and 0.75 M EG/0.75 M G) was used. We employed a final concentration of intracellular cryoprotectants of 1.5 M for all groups.

Each 2.0 mL cryovial (Fisher Scientific, Pittsburgh, PA) contained 12 tissue pieces immersed in 2.0 mL of the FBS at 21°C. Each cryovial then was placed into a Nalgene (Mr. Frosty, Fisher Scientific) freezing container with isopropyl alcohol at room temperature that then was transferred into a -80°C freezer overnight (Singh *et al*., 2019). This sistem is designed to provide a cooling rate of ~-1°C min-1.

Vitrification of testicular tissues

The vitrification solution (VS) constituted of Minimum Essential Medium (MEM) supplemented with 0.25 M sucrose, 10% (v/v) FBS and 3.0 M intracellular cryoprotectant (1.5 M DMSO/1.5 M EG, 1.5 M DMSO/1.5 M G and 1.5 M EG/1.5 M G) was used. We employed a final concentration of intracellular cryoprotectants of 3.0 M for all groups (Silva *et al*., 2019).

After placing threaded tissues, for five minutes, on an aseptic absorbent filter to remove the remaining vitrification solution, they were vitrified to VC and SSV: In VC the fragments were placed in cryotubes, plunged directly into liquid nitrogen, and stored (Lima *et*

al.,2018b). In the SSV the fragments are placed for 30 seconds on aluminum sheet as a solid surface of high conductivity in contact with nitrogen and then they are placed in cryotubes and stored for one week (Silva *et al*., 2019).

Warming of cryopreserved tissues

After one week, the cryovials were maintained for 1 min at 25 °C and immersed in a water bath at 37 °C for completely warming of testicular tissue fragments. For removal of cryoprotectants, all fragments were washed three times for 5 min in MEM, 10% FBS, and with decreasing concentrations of sucrose (0.50, 0.25 M, and without sucrose) (Silva *et al*., 2019).

Testicular cell morphology analysis

For evaluation of morphology by conventional histology, fragments derived from both control and cryopreserved groups were fixed in Bouin solution for 12 h, sectioned at 5.0 µm, stained with hematoxylin-eosin and evaluated by using a light microscope (Olympus CX 31 RBSFA, Tokyo, Japan). The morphological parameters for tubular structure, ruptures of the basement membrane, swelling, tubular cell loss, separation of the basal membrane and vacuolation were assessed such as table 1. Then, testicular tissues classified as score 3 were considered morphologically normal and fragments classified with a score of 0 were considered unfit for their testicular morphology. For each parameter, five seminiferous tubules of six different fields were assessed, resulting in a total of 30 seminiferous tubules for each treatment (Silva *et al*., 2019).

Testicular cells viability

To obtain cells isolated from cryopreserved and non- cryopreserved tissues, fragments were dissociated using an enzymatic dissociation (Silva et al., 2019). Briefly, we used 0.2% collagenase type IV at 37 °C for 20 min under slow stirring and the reaction was stopped by the addition of an equal volume of FBS. To cellular viability was incubated in a solution constituted by a mixture of propidium iodide (0.5 mg/mL in salt solution) and Hoechst 33342 (25 mg\mL in dimethyl sulfoxide) for 10 min at 37°C to detect germinative cell viability and

to enable the counting of nuclei, respectively. Then, 100 cells were counted as either dead red to fluorescence (propidium iodide) and live to blue fluorescence (Hochest 33342).

Table 1. Morphological parameters of tubules from testis of collared peccaries.

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

Testicular cells proliferative capacity potential

Proliferative capacity potential was evaluated by quantification of nucleolar organizer regions (NORs) in the spermatogonia and Sertoli cells using silver staining technique (Bandeira *et al*., 2015). The slides were soaked in silver solution prepared in 1 part of 2% gelatin in 1% aqueous formic acid and 2 parts of 50% aqueous silver nitrate solution and were exposed in a dark room for 30 min. Subsequently, the slides were washed in 5% thiosulfate solution for 10 min (Borges *et al*., 2018). For analysis of each group, NOR dots were counted within the nucleoli of spermatogonia and Sertoli cells of 10 randomly selected nuclei in 10 fields at 1000× magnification, resulting in 200 cells for each treatment (Chacur *et al*., 2015).

DNA fragmentation

Testicular tissues were fixed overnight in 4% paraformaldehyde solution, embedded in paraffin, sectioned in series (5 μm thickness), mounted on slides and evaluable using the

In-Situ Cell Death Detection kit (Roche) following the manufacturer's instructions. Sections were hydrated from ethanol 100% to 70%, rinsed twice 0.05% Triton X-100 in PBS for 5 min each, permeabilized with 0.5% Triton X-100 in PBS for 30 minutes, and rinsed once 0.05% Triton X-100 in PBS for 5 min. The TUNEL reaction mixture was prepared using the enzyme solution composed by terminal deoxynucleotidyl transferase (TdT) and label solution composed by nucleotide polymers. The sections incubated with TUNEL reaction mixture for 1 h at 37°C within a humidified darkened container. A negative control in which the TdT was omitted was included in each trial. The positive control was performed incubating the cells with DNase I recombinant (Sigma-Aldrich) for 10 min before to labeling procedures to induce DNA stands breaks. The nucleus of all cells was stained with Hoechst 33342 (1:100, Sigma-Aldrich) in a humidified chamber for 10 minutes at room temperature and then, the slides were mounted with Vectashield mounting medium (Vector laboratories). We evaluated 25 images per experimental group, which were captured using an Olympus BX41 epifluorescence microscope (Olympus Corporation) with SPOT advanced software 5.0. The pictures were evaluable for ImageJ software, which they were counted 25 images/treatment. Green cells were classified as fragmented DNA and blue cells as normal DNA.

Statistical analysis

Data were tested for normality and homoscedasticity using Shapiro-Wilk and Levene's tests, respectively (Stat View 5.0, SAS Institute, Inc. Cary, NC, USA) and values were arc sine-transformed before analysis when necessary. The effects of cryoprotectants on the testicular parameters were assessed by ANOVA followed by Tukey test. The scores obtained in classical histology were submitted to the Mann-Whitney's test for comparison among groups. Differences were considered significant when $P < 0.05$.

RESULTS

Morphological analysis

The morphological features in non-cryopreserved testicular tissue and after cryopreservation using different methods and cryoprotectant combinations are showed in Table 2. After warming, all the treatments were similar at preserving the tubular structure and preventing the rupture of basal membrane. However, occurrence of swelling was most

evident ($P < 0.05$) in the use of slow freezing with G/EG, while tubular cell loss was most predominant in SSV using DMSO/G ($P < 0.05$) – figure 1.

Viability

Using all the cryopreservation methods, groups containing DMSO/EG or DMSO/G combination provide results for testicular cell viability (Fig. 1) similar to those reported for non-cryopreserved samples. However, use of G/EG combination associated to all cryopreservation methods tested significantly impaired the cell viability in comparison to fresh samples ($P < 0.05$).

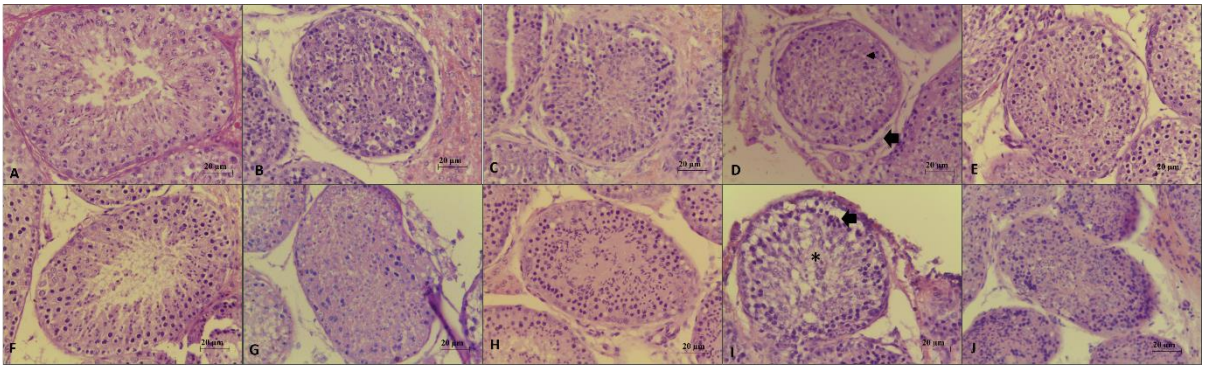

Figure 1. Histo-morphological evaluations of non-cryopreserved and cryopreserved testicular tissues in the collared peccary. **(A)** Non- cryopreserved group, **(B)** group cryopreserved for slow freezing (SF) with dimethyl sulfoxide (DMSO) and ethylene glycol (EG) **(C)** group cryopreserved by SF with DMSO and glycerol (G), **(D)** group cryopreserved by SF with EG/G, **(E)** group cryopreserved for vitrification in cryotubes (VC) with DMSO/EG, **(F)** group cryopreserved for V with DMSO/G, **(G)** group cryopreserved for V with EG/G. **(H)** group cryopreserved for surface solid vitrification (SSV) with DMSO/EG, **(I)** group cryopreserved for SSV with DMSO/G, **(J)** group cryopreserved for SSV with EG/G. For Fig. G, black arrowhead shows swelling, and the white arrow shows space between basal membrane and cells in seminiferous tubules. For Fig. I, black arrow shows swelling, asterisk shows tubular cell loss.

Testicular cells proliferative capacity potential

With regards to the quantification of NORs (Fig. 3A-B), our results showed that all treatments conserved the proliferative capacity potential to spermatogonia. Moreover, the use of G/EG combination in the SF and SSV significatively impaired the proliferative potential o e Sertoli cells ($P < 0.05$).

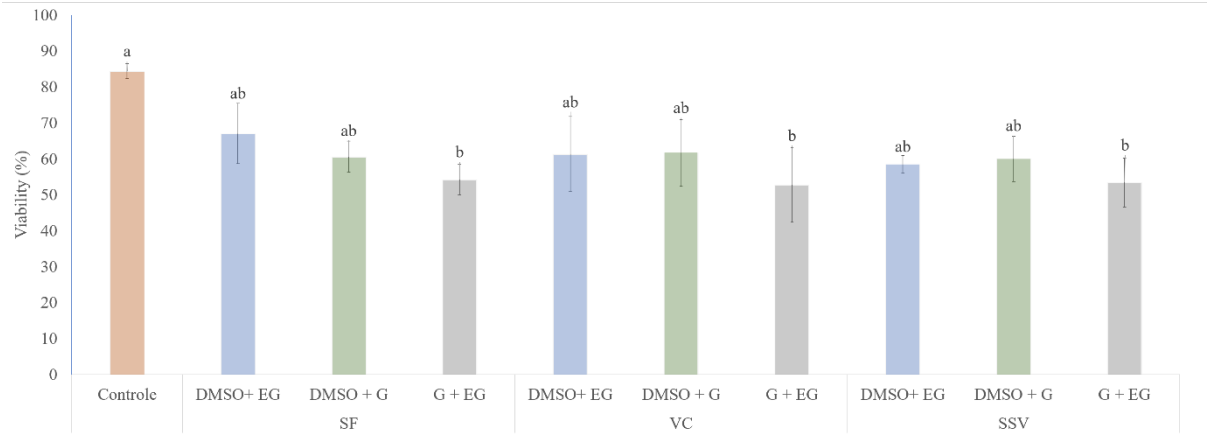

Figure 2. Values (Means ± SEM) of germinal cell viability evaluated by fluorescent probes assay from collared peccary non-cryopreserved and cryopreserved testicular tissue, using different protocol of cryopreservation, slow freezing (SF), vitrification in cryotubes (VC) and solid-surface vitrification (SSV) and different combination of cryoprotectants. Letters indicate difference among treatments ($P < 0.05$).

DNA fragmentation

After warming, only the groups cryopreserved by SF or VC using DMSO/EG ($P < 0.05$) were able to prevent DNA fragmentation, presenting values similar to those observed for non-cryopreserved samples (Fig. 4). The most evident occurrence of DNA damage ($P < 0.05$) was observed in the use of G/EG combination in any method of cryopreservation.

DISCUSSION

As an effort to contribute to the formation of biobank with germplasm derived from collared peccaries, we present novel information regarding the establishment of a protocol for the cryopreservation of testicular tissue from mature individuals. Our present results confirm the use of DMSO/EG combination for this purpose; however, we highlight the use of SF or VC, instead of SSV as previously demonstrated for the same species (Silva *et al.*, 2019).

Because the toxicity of high DMSO concentrations is well-known (Baert *et al.*, 2013; Unni *et al.*, 2012), a combination of EG and DMSO has been suggested to obtain a less toxic vitrification solution (Poels *et al.*, 2012). At the present study, the use of DMSO/EG combination not only provided an efficient preservation cell viability, proliferative potential of peccary testicular tissue, but it also prevented the DNA fragmentation, providing values similar as observed for non-cryopreserved samples. In fact, Noda *et al.*, (2017) recently demonstrated that DMSO is able to avoid physical-chemical mechanisms that lead to the

DNA double-strand breakaways (DSBs), which are related to the DNA fragmentation, occurring as a consequence of the ice crystals formation during cryopreservation.

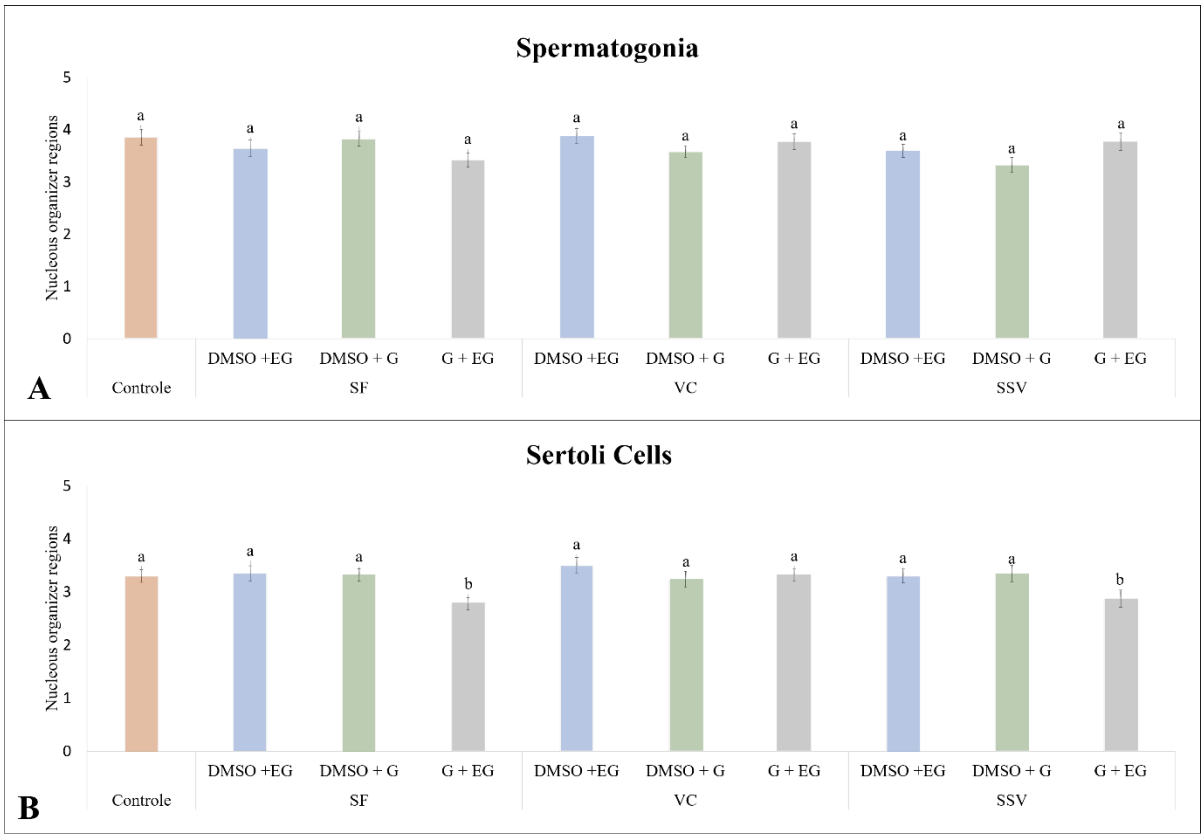

Figure 1. Proliferative capacity evaluated by quantification of nucleolar organizer regions (NORs) in peccary testicular germinative cells from non-cryopreserved (control) and cryopreserved, using different protocol of cryopreservation, slow freezing (SF), vitrification in cryotubes (VC) and solid-surface vitrification (SSV) and different combination of cryoprotectants. Lowercase superscript letters indicate difference among treatments ($P < 0.05$).

As largely known, DMSO (molar mass 78.13 g/mol) presents the capacity to interact with the lipid membrane and induces pore formation for water passage (Cooper *et al*., 2008; Gurtovenko and Anwar, 2007), thus causing cell dehydration and consequently reducing the possibility of ice crystal formation into the cells during cryopreservation (Chaytor *et al*., 2012; Gurtovenko and Anwar, 2007). Probably, the combination of DMSO and EG improves the protective mechanisms, since the EG, which presents a low molecular weight (molar mass 62,07 g/mol), is able to rapidly permeate the cell membrane (Chi *et al*., 2002; Phelps *et al*., 1999), resulting in a low water conductivity with reduced osmotic stress (Gilmore *et al*., 1995), as well as presenting the ability to bind to the water molecules thus preventing the formation of ice crystals (Weng *et al*., 2011).

Table 2. Morphological evaluation of testicular non-cryopreserved and cryopreserved tissues from collared peccaries.

	Controle	**SL**			**VC**			**SSV**		
		DMSO +EG	DMSO + G	G + EG	DMSO +EG	DMSO + G	G + EG	DMSO +EG	DMSO + G	G + EG
Swelling	2.63±0.04^{a}	2.02±0.05^{b}	2.09±0.05^{b}	1.85±0.05^{c}	2.00±0.06^{bc}	1.82±0.05^{bc}	1.87±0.05^{bc}	1.89±0.05^{bc}	1.84±0.06^{c}	1.94±0.05^{bc}
Tubular cell loss	2.78±0.03^{a}	2.35±0.05^{bd}	1.98±0.06^{cd}	2.15±0.05^{bd}	2.20±0.05^{b}	2.12±0.05^{bd}	2.18±0.07^{bd}	2.27±0.05^{b}	1.95±0.06^{d}	2.16±0.05^{bd}
Space between basal membrane and cells in seminiferous tubules	2.78±0.03^{a}	2.04±0.06^{bcd}	2.07±0.06^{bc}	1.78±0.07^{d}	2.02±0.06^{cd}	2.02±0.09^{cd}	2.02±0.06^{cd}	1.95±0.06^{cd}	2.27±0.06^{b}	2.23±0.05^{b}
Rupture from basal membrane	2.79±0.03^{a}	2.18±0.05^{b}	2.13±0.05^{b}	2.00±0.05^{b}	2.20±0.06^{b}	2.10±0.05^{b}	2.22±0.06^{b}	2.21±0.05^{b}	2.05±0.05^{b}	2.15±0.05^{b}
Tubular structure	2.84±0.03^{a}	2.06±0.04^{b}	2.05±0.05^{b}	1.93±0.04^{b}	2.07±0.05^{b}	1.91±0.05^{b}	1.84±0.05^{b}	1.95±0.05^{b}	1.91±0.05^{b}	2.09±0.14^{b}

Letters indicate difference among columns ($P < 0.05$).

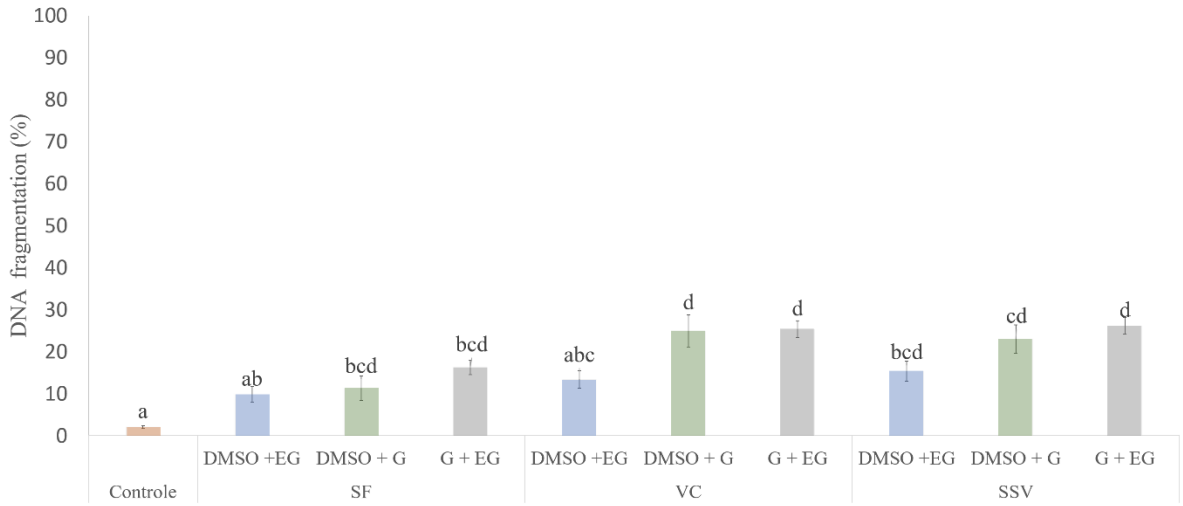

Figure 2. DNA fragmentation evaluations of non-cryopreserved and cryopreserved testicular tissues in the collared peccary, using different protocol of cryopreservation, slow freezing (SF), vitrification in cryotubes (VC) and solid-surface vitrification (SSV) and different combination of cryoprotectants. Different lowercase letters mean the statistical difference among treatments (P<0.05).

Regarding proliferative ability of testicular cells, groups containing DMSO were also efficient on the preservation nucleolar organizing regions (NORs) of spermatogonia and Sertoli cells, similarly as demonstrated on our previous study (Silva *et al*., 2019). The NORs are related to the argyrophilic proteins present in the chromosomes at the interphase stage, therefore indicating cell proliferation (Crocker *et al*., 1989; Lindner, 1993). It is a really interesting result since the proliferative capacity of both spermatogonia and Sertoli cell is a crucial step for the occurrence of spermatogenesis under culture conditions (Hunter *et al*., 2012).

We also highlight that groups containing DMSO in combination with EG or G were the most efficient on the preservation of testicular cells viability in the use of all cryopreservation protocols. Probably, the mechanism of pore formation for water passage induced by DMSO (Cooper *et al*., 2008; Gurtovenko and Anwar, 2007) would contribute to minimize the cryoinjury on cell membrane. In addition, values obtained for cell viability (58.5%) at the use of SSV were similar to those previously reported for peccaries using the same protocol (~50%) (Silva *et al*., 2019); however, best results were obtained when other cryopreservation methods as SF (67 %) or VC (61,3 %) were applied. According to Pukazhenthi *et al*. (2015), the success derived from SF protocol would be related to an imposed gradual exchange of water and cryoprotectant at a low temperature over a protracted interval. On the other hand, vitrification is a cryopreservation strategy that differs from SF due a vitreous state formation that provides a sufficient high cooling, which prevent ice

crystal damage, which sometimes happens in slow cryopreservation (Benvenutti *et al*., 2018). However, even among vitrification methods, there are marked differences related to the temperature drop. This is due to the differences related to the thermal conductivity of the surface in which tissues are placed since SSV uses an aluminum sheet while VC uses a polypropylene cryotube (Boudenne *et al*., 2004; Patti and Acierno, 2019). Despite the good performance of SF and VC, we highlight that VC presents the advantage of being a method of simple execution to be conducted under field conditions, where there is no freezer to be used for SF. Moreover, various studies have been indicating vitrification as the procedure of choice for the preservation of testicular tissue (Baert *et al*., 2013; Curaba *et al*., 2011; Poels *et al*., 2012).

In conclusion, our results suggest the use of a DMSO/EG combination for the slow freezing or vitrification in cryotubes of testicular tissue derived from collared peccaries. This information would be valuable for the establishment of protocols for the storage of genetic material from genetic valuable individuals that suddenly die, providing germplasm to be used in association to other reproductive assisted techniques.

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CONCLUSÕES

Nossos resultados mostram que a combinação DMSO/EG foi mais adequada que o uso isolado destes crioprotetores ou que as combinações DMSO/G e G/EG na conservação da organização ultraestrutural do túbulo seminífero em termos de presença de lúmen e junções celulares, viabilidade das células germinativas, capacidade proliferativa da espermatogônia e das células de Sertoli, e integridade do DNA. Adicionalmente, observou-se que todas as técnicas de criopreservação foram adequadas para a conservação de tecido testicular, sendo a VC mais eficiente para essa finalidade.

PERSPECTIVAS

As informações aqui presentes são passos para o estabelecimento de um protocolo de criopreservação de tecido testicular de catetos adultos, possibilitando o aproveitamento de tal tecido em animais abatidos ou mortos acidentalmente. Ressalta-se que essas informações são de grande valia para o estabelecimento de protocolos para estocagem de material genético de animais adultos, providenciando germoplasma para ser utilizado em associações com outras técnicas de reprodução assistida.

Nesse contexto, para a continuidade da pesquisa, os próximos passos consistem no desenvolvimento de técnicas de cultivo *in vitro* e *in vivo* que possibilitem a produção de espermatozoides viáveis, passíveis de serem utilizados na produção de embriões e obtenção de crias.

ANEXOS

Avaliação da viabilidade de tecido testicular fresco ou vitrificado de catetos (*Pecari tajacu*) utilizando diferentes métodos de dissociação celular

Evaluation of the viability of fresh and frozen testicular tissue from collared peccaries (Pecari tajacu) using different methods for cell dissociation

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A viabilidade é um importante parâmetro avaliado em células provenientes de tecidos biológicos frescos ou submetidos a criopreservação. Para tecido testicular, a dissociação celular através de digestão enzimática tem sido corriqueiramente reportada, contudo as enzimas apresentam custo elevado. No tecido ovariano, a viabilidade é avaliada a partir de um isolamento mecânico folicular, um método de baixo custo que poderia ser também adaptado ao tecido testicular. Assim, o objetivo desse estudo foi comparar os métodos de digestão enzimática e dissociação mecânica para análise de viabilidade celular em tecido testicular de catetos (*Pecari tajacu*), a fresco ou vitrificado utilizando diferentes crioprotetores. Fragmentos (3 mm^3) testiculares provenientes de indivíduos adultos foram avaliados a fresco e após vitrificação em superfície sólida com os crioprotetores dimetilsulfóxido (DMSO) ou etilenoglicol (EG) na concentração de 3M. Para a digestão enzimática, os fragmentos foram imersos em associação das enzimas colagenase tipo IV (0,2% wt/vol), hialuronidase (0,1% wt/vol) e DNase tipo I (0,01% wt/vol) por 20 minutos sob agitação lenta, seguindo-se adição de soro fetal bovino a 10% para inativação das enzimas, e centrifugação a 500 xg por 5 minutos. Na dissociação mecânica, os fragmentos foram fatiados com uma lâmina de bisturi esterilizada n° 24 por 5 minutos, suspensos em 2 mL de meio essencial mínimo, colocados sob agitação por 10 minutos, filtrados em malha de 500 mm e centrifugados a 500 xg por 5 minutos. Para avaliação da viabilidade, as amostras foram acondicionadas em tubo plástico contendo a proporção de 50 µL de azul de Tripan (0,4% solução) para 50 µL da suspensão celular, dispostas em câmara de Neubauer e avaliadas por microscopia óptica (x40), sendo contadas 200 células da linhagem germinativa. Aquelas células coradas em azul foram consideradas inviáveis; as células que permaneciam sem marcação foram consideradas viáveis. Os dados foram expressos em média ± erro padrão, sendo os métodos comparados pela ANOVA seguida do teste t de Student ($P < 0,05$), e as relações entre os métodos estabelecidas pelo teste de correlação de Spearman ($P < 0,05$). No tecido fresco, foram obtidas 85,9 ± 1,18% e 80,9 ± 1,1% de células viáveis a partir dos métodos enzimático e mecânico, respectivamente. No tecido vitrificado, foram obtidas 29,3 ± 3,0% e 33,9 ± 6,8% no uso do DMSO, bem como 29,5 ± 4,3% e 39,1 ± 6,6% no uso do EG, respectivamente para os referidos métodos. Comparando-se os métodos de dissociação celular dentro de cada tratamento (fresco ou DMSO ou EG), não foram evidenciadas diferenças significativas ($P > 0,05$). Ressalta-se que, embora a vitrificação tenha promovido uma redução de viabilidade celular ($P < 0,05$), os dois crioprotetores não diferiram entre si ($P > 0,05$). Destaca-se ter sido verificada uma correlação moderada e positiva entre os dois métodos de dissociação celular, com $R = 63,2\%$ ($P < 0,05$). Diante do exposto, conclui-se que tanto o DMSO quanto o EG podem ser utilizados para a vitrificação de tecido testicular de catetos. Além disso, sugere-se que o método de dissociação mecânica possa ser utilizado em substituição à digestão enzimática para obtenção de células de tecido testicular fresco ou criopreservado destinadas à avaliação de viabilidade com azul de Trypan. O referido método mecânico foi aqui inicialmente descrito para os catetos, mas acredita-se que possa ser adaptado a qualquer espécie mamífera, haja vista sua praticidade e baixo custo.

Palavras-chave: digestão enzimática, isolamento mecânico, células viáveis, espermatogênese.
Keywords: *enzymatic digestion, mechanical isolation, viable cells, spermatogenesis.*

Financiamento: CAPES, CNPq

CRIOPRESERVAÇÃO DE TECIDO TESTICULAR DE CATETOS (*Pecari Tajacu*) POR DIFERENTES TÉCNICAS: RESULTADOS PRELIMINARES

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A criopreservação de tecido gonadal é uma inovação a ser usada na conservação de material genético de animais ecologicamente vulneráveis. Comparou-se a eficiência das técnicas de vitrificação em superfície sólida e congelação lenta na criopreservação de tecido testicular de catetos. Fragmentos (3mm³) de testículo foram coletados de machos adultos eutanasiados. Um fragmento (controle) foi avaliado quanto a viabilidade por sondas florescentes e morfologia por histologia clássica, utilizando-se escores de 1 a 3, levando-se em conta separação da membrana, vacuolização, integridade estrutural, perda de células e ruptura. Quatro fragmentos foram imersos por 5 minutos em MEM com 10% de soro fetal bovino (SFB), 0,25 M sacarose, 1,5 M dimetilsulfóxido (DMSO) e 1,5M etilenoglicol (EG), sendo vitrificados em superfície sólida (VSS) e armazenados em nitrogênio líquido. Outros quatro fragmentos foram estocados em criotubos contendo MEM com 10% SFB, 0,25 M sacarose. 0,75 M DMSO e 0,75M EG, sendo submetidos a congelação lenta (CL) em MrFroster® mantido em freezer -80 °C por 12 h, e armazenados em nitrogênio. Após duas semanas, as amostras foram aquecidas (37 °C) e reavaliadas. Os dados foram expressos em média (±EP), a viabilidade foi comparada pelo teste Tukey e a morfologia por Mann Whitney ($P < 0,05$). As células germinativas nas amostras frescas apresentaram 86 ± 6% de viabilidade, semelhante ($P > 0,05$) aos tratamentos VSS (56.5 ± 5%) e CL (60 ± 8%). Para morfologia, as amostras do controle receberam escore 1.2 ± 0, sendo observado aumento ($P < 0,05$) de danos nos tratamentos VSS (1.79 ± 0.03) e CL (1.83 ± 0.03). Estes resultados preliminares indicam que o tecido testicular de catetos poderia ser criopreservado por ambas as técnicas.

Palavras-chave: *Tayassu tajacu*; vitrificação; congelação lenta

Agradecimentos: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

EFEITO DA ASSOCIAÇÃO DE CRIOPROTETORES NA VIABILIDADE DO TECIDO TESTICULAR VITRIFICADO EM CATETOS

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A vitrificação em superfície sólida (VSS) é uma biotécnica inovadora para conservação de material genético de animais, quer estejam vivos ou que recentemente tenham vindo a óbito. O objetivo foi comparar diferentes associações de crioprotetores na VSS de tecidos testiculares de catetos (*Pecari tajacu*). Para tanto, fragmentos testiculares (3mm³) foram obtidos de dois machos adultos eutanasiados. Um fragmento (controle) foi avaliado a fresco quanto a viabilidade por sondas florescentes e a morfologia por histologia clássica, utilizando-se escores de 1 a 3, levando-se em conta separação da membrana, vacuolização, integridade estrutural, perda de células e ruptura. Para criopreservação, quatro fragmentos foram imersos em MEM com 10% de soro fetal bovino e 0.25 M de sacarose, adicionados de 1.5 M dimetilsulfóxido e 1.5 M glicerol (DMSO + G), ou 1.5 M etilenoglicol e 1.5 M glicerol (EG + G). Após 5 minutos, os fragmentos foram submetidos à VSS e armazenados em nitrogênio. Após duas semanas, as amostras foram aquecidas a 37 °C, removendo-se os crioprotetores, sendo procedidas avaliações. Os dados foram expressos em média (±EP), a viabilidade comparada pelo teste de Fisher's PLSD e a morfologia por Mann Whitney ($P < 0.05$). As amostras frescas apresentaram 86 ± 6% de viabilidade de células germinativas, semelhante ao tratamento DMSO + G (71 ± 5%), porém, observou-se redução ($P < 0.05$) da viabilidade no uso de EG + G (57.0 ± 4.0%). Quanto à morfologia, os tratamentos a preservaram de modo similar com escores de 1,92 ± 0,05 para DMSO + G e 1,71 ± 0,06 para EG + G; porém, ambos diferentes ($P < 0,05$) do controle a fresco (1.21 ± 0.02). Estes resultados preliminares sugerem o uso da combinação DMSO + G para conservação da viabilidade durante o procedimento de vitrificação testicular em catetos.

Palavras-chave: *Tayassu tajacu*, vitrificação, biobanco.

Agradecimentos: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

Combinations of different cryoprotectants for the vitrification of testicular tissues in collared peccaries (*Pecari tajacu*) – Preliminary results

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Development of biobanking strategies are critical for the management and conservation of collared peccary's populations. In addition to the cryopreservation of spermatozoa, oocytes and ovarian tissues, freezing testicular tissues is another potential source of germplasms from genetically valuable individuals. The objective of our study was to develop a vitrification protocol for peccary's testicular tissue using combinations of different cryoprotectants. Testes from two mature individuals were excised after euthanasia, washed in alcohol 70% and dissected into small fragments (3 mm^3). Three fragments constituting the fresh control group were immediately evaluated for: 1) viability by vital fluorescent probes (Hoechst 33358 and propidium iodide); 2) presence of Sertoli cells by immunohistochemistry using vimentin labelling and scored from 0 (all Sertoli cells lost from the tubule) to 4 (all Sertoli cells present); and 3) histo-morphology after fixation and staining (eosin/hematoxylin) before observations of 30 seminiferous tubules evaluated according to separation of the basal membrane, vacuolization, integrity, swelling, cell loss and rupture, and then scored from 1 (good preservation) to 3 (bad preservation). Other tissue biopsies were immersed in a MEM-based vitrification solution with 10% fetal bovine serum supplemented with 0.25M sucrose. Samples were exposed to different cryoprotectant combinations (three fragments per combination): 1.5 M dimethyl-sulfoxide (DMSO) + 1.5 M ethylene glycol (EG); 1.5 M DMSO + 1.5 M glycerol (G); or 1.5 EG + 1.5 G. After 5 min equilibration, tissues were plunged and stored in liquid nitrogen. After two weeks, samples were warmed at 37°C, washed step-wise in decreasing concentrations of sucrose for cryoprotectants removal and evaluated as described for fresh controls. Data were expressed as means ± standard error. Viability was compared using Fisher's PLSD and morphology by Mann Whitney test. Fresh samples contained 86 ± 6% viable cells and similar values ($P > 0.05$) were found for samples vitrified in DMSO + G (64 ± 3%) and EG + G (69 ± 6%). However, viability decreased ($P < 0.05$) with DMSO + EG (48 ± 16%). Regarding morphology, control samples had a score of 1.21 ± 0.02 for seminiferous tubules and 3.30 ± 0.10 for Sertoli cells. After warming, scores for seminiferous tubules (DMSO + EG: 1.97 ± 0.04; DMSO + G: 2.02 ± 0.03; EG + G: 1.84 ± 0.03) and Sertoli cells (DMSO + EG: 2.10 ± 0.20; DMSO + G: 2.00 ± 0.10; EG + G: 2.30 ± 0.10) were lower ($P < 0.05$) than fresh controls but no significant difference was found among treatments. Preliminary results suggest that combinations of DMSO + G or EG + G preserve testicular tissue viability after vitrification, but that morphology is not affected by the combination of cryoprotectants.

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VII International Symposium on Animal Biology of Reproduction

Reproductive: Biotechnology and Future

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CERTIFICATE

We certify that **Alexandre Rodrigues Silva** has attended the VII International Symposium on Animal Biology of Reproduction (ISABR 2018), with focus on: Reproductive: Biotechnology and Future, held in Aracaju, SE, Brazil from November 6h to 9h, 2018, and received a distinction in the competition for **TOP 5 BEST POSTER PRESENTATION**.

Title: Combinations of different cryoprotectants for the vitrification of testicular tissues in collared peccaries (Pecari tajacu) – Preliminary results

Authors: Silva AM, Moreira SSJ, Santos CS, Praxedes ECG, Brasil AV, Lima PLC, Campos LB, Dantas MRT, Oliveira MF, Comizzoli P, Silva AR

Carlos Eduardo Ambrósio
Chair of the VII ISABR 2018
Scientific Committee
President of the CBRA

Influência dos crioprotetores intracelulares sobre a conservação de tecido testicular de cutias (*Dasyprocta leporina*)

Influence of intracelular cryprotectants on the conservation of testicular tissue derived from agouti (Dasyprocta leporina) using different cryoprotectants

Andréia Maria Silva*, Luana Grasiele Pereira Bezerra, Erica Camila Gurgel Praxedes, Samara Sandy Jerônimo Moreira, Caio Sergio Santos, Moacir Oliveira Franco, Alexsandra Fernandes Pereira, Alexandre Rodrigues Silva

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A criopreservação de tecido testicular representa uma ferramenta promissora para a conservação de mamíferos silvestres, como as cutias. Essa tecnologia permite a maximização de material biológico masculino derivado de indivíduos *post-mortem*, possibilitando o armazenamento de fragmentos contendo um grande número de espermatogônias, as quais servirão como fonte de espermatozoides para uso em tecnologias de reprodução assistida. Neste sentido, o objetivo foi avaliar a influência de diferentes crioprotetores intracelulares [dimetilsulfóxido (DMSO), etilenoglicol (EG) e a sua associação] sobre a criopreservação de tecido testicular de cutias. Para tanto, testículos derivados de três cutias adultas *post-mortem* foram recuperados e transportados ao laboratório em solução fisiológica a 27°C dentro de 30 min. No laboratório, fragmentos testiculares (~9,0 mm^3) foram criopreservados por congelação lenta usando o sistema Mr. Frosty Freezing Container® a 1°C/min, em meio essencial mínimo acrescido de 10% de soro fetal bovino e 0,2 M de sacarose, contendo os seguintes crioprotetores intracelulares: DMSO 1,5 M, EG 1,5 M ou EG 0,75 M + DMSO 0,75 M. Fragmentos não criopreservados foram considerados grupo controle. Após duas semanas, fragmentos não criopreservados e criopreservados/aquecidos foram submetidos a digestão enzimática (DNAse, Hialuronidase e Colagenase IV) e avaliados para viabilidade usando Hoechst e iodeto de propídeo, e atividade mitocondrial usando a MitoTracker Red. Os resultados foram expressos como média ± erro padrão e analisados por ANOVA seguida do teste de Tukey ($P < 0,05$). Assim, foi observada uma viabilidade de 81,0% ± 0,6 no grupo controle superior em relação aos tratamentos ($P < 0,05$), os quais não diferiram entre si ($P > 0,05$), observando-se os valores de 38,7% ± 14,0 para DMSO, 33,3% ± 0,9 para EG, e 33,0% ± 5,2 para a associação. No tocante à atividade mitocondrial, houve diferença entre o controle fresco (1,0 ± 0) e os grupos tratados ($P < 0,05$), sendo que entre estes, o EG (0,6 ± 0,1) foi superior ($P < 0,05$) ao DMSO (0,5 ± 0,05) e à associação (0,5 ± 0,05). Com base na atividade mitocondrial, sugere-se que o etilenoglicol seja o crioprotetor intracelular mais adequado a ser utilizado na criopreservação de tecido testicular de cutias.

Palavras-chave: biobanco, vida selvagem, roedor, tecido gonadal.
Keywords: *biobank, wildlife, rodent, gonadal tissue.*

Comparação de diferentes técnicas de vitrificação para a conservação do tecido gonadal masculino usando a Cutia como modelo

***C**omparison of different vitrification techniques for the conservation of male gonadal tissue using the Agouti as a model*

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Nos últimos anos, tem-se enaltecido a conservação de tecido gonadal masculino como uma possível fonte para obtenção de espermatozoides visando a formação de bancos de germoplasma. Neste sentido, o sucesso de tais bancos depende, de sobremaneira, da escolha de técnicas de criopreservação adequadas para a manutenção da viabilidade do tecido pós-aquecimento. Portanto, o objetivo do presente estudo foi comparar a eficiência de duas técnicas de vitrificação [superfície sólida (VSS) ou convencional (VC)] para a conservação do tecido gonadal de cutias (*Dasyprocta leporina*), as quais representam um modelo interessante para extrapolação para outras espécies. Para tanto, fragmentos (9 mm^3) de tecido testicular foram coletados de cutias adultas post mortem e criopreservados utilizando as diferentes técnicas de vitrificação. Para ambas as técnicas, a solução de vitrificação consistiu de Meio Essencial Mínimo (MEM) com 10% de soro fetal bovino, 0,25 M de sacarose, e 1.5 M dimetilsulfóxido (DMSO). Fragmentos não criopreservados foram considerados como controle. Após duas semanas, fragmentos criopreservados/aquecidos e não criopreservados foram fixados em solução de Bouin por 12 h e processados histologicamente visando a avaliação morfológica, levando-se em conta os parâmetros de separação da membrana, vacuolização, integridade estrutural, perda de células, ruptura e separação de membrana. Tais parâmetros foram categorizados em escores variando de 1 (degenerado) a 3 (conservado). Os dados foram expressos em média e erro padrão, e os tratamentos foram comparados por ANOVA seguida do teste Tukey ($P < 0,05$). No tecido fresco, observaram-se os seguintes escores: 3,0 ± 0,02 para vacuolização, 2,3 ± 0,1 para integridade estrutural, 3,0 ± 0 para perda celular, 3,0 ± 0 para ruptura e 2,9 ± 0,1 para separação da membrana. Após o aquecimento das amostras, verificou-se que ambas as técnicas foram eficientes em conservar todos os parâmetros de modo similar ao controle, obtendo-se os seguintes escores: 2,8 ± 0,5 e 2,8 ± 0,5 para a vacuolização, 2,0 ± 0,3 e 2,1 ± 0,4 para integridade estrutural, 2,9 ± 0,3 e 2,9 ± 0,4 para perda celular, 2,9 ±0,4 e 2,9 ± 0,4 para ruptura, e 2,7 ± 0,6 e 2,9 ± 0,4 para separação de membrana, utilizando-se a VSS e VC, respectivamente. De acordo com os resultados apresentados, ambas as técnicas poderiam ser utilizadas para a conservação da arquitetura histológica do tecido testicular no modelo experimental Cutia.

Palavras-chave: bancos de recursos biológicos, vida selvagem, criopreservação, testículo.
***Keywords**: biological resource banks, wildlife, cryopreservation, testis.*

P277 - Mitochondria-Eating Protein Is Essential For Sperm Function, But Not Oocyte Quality, In In Vitro Fertilization.
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P279 - Mitofusin2 Is Required For Male Fertility.
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P282 - Soy-Isoflavones Regulates Steroidogenic Capacity In Testes Of Male Rats At All Ages.
Bamidele O. Jeminiwa, Rachel C. Knight, Erica M. Molina, John F. Fischer, Samantha M. Bradley, Benson T. Akingbemi

P284 - Mrnip Is Critical For Male Meiosis And Fertility.
Renata Prunskaite-Hyyrylainen, Julio Castañeda, Samina Kazi, Kaori Nozawa, Zhifeng Yu, Ramiro Ramirez-Solis, Martin M. Matzuk

P287 - NANOS2 Knockout Pigs As A Model To Devise Strategies For Treating Male Infertility.
Mariana I. Giassetti, Michela Ciccarelli, Ki-Eun Park, Bhanu P. Telugu, Jon M. Oatley

P290 - Mining and mRNA Expression Profiling Of WD-40 Family Genes Including DDB1- And CUL4-Associated Factor Genes In The Mouse And Human Testes.
Bhavesh Mistry, Maha Alanazi, Hana Fitwi, Olfat Alharazi, Mohamed Rajab, Abdullah Altorbag, Dilek Colak, Falah Almohanna, Abdullah Assiri

P292 - Short-Term Treatment With mTORC1 Inhibitors Rapamycin And Everolimus Negatively Impact Male Germ Cell Differentiation.
Oleksandr Kirsanov, Randall H. Renegar, Nicholas D. Serra, Christopher B. Geyer

P295 - Vitrification Of Testicular Tissue From Adult Spix's Yellow-Toothed Cavies' (Galea Spixii) Using Different Cryoprotectants.
Andreia Maria Da Silva, Ana Gloria Pereira, Erika Camila Gurgel Praxedes, Samara Sandy Jerônimo Moreira, Moacir Franco De Oliveira, Pierre Comizzoli, Alexandre Rodrigues Silva

P298 - Patterns Of The PRAMEY Expression In The Bovine Testis And Epididymis.
Weber B. Feitosa, Chandlar H. Kern, Wan-Sheng Liu

P302 - Steroidogenesis During Prenatal Testicular Development In Spix Cavies (Galea Spixii).
Amilton Cesa Santos, Alan James Conley, Moacir Franco Oliveira, Antonio Chaves De Assis Neto

P304 - Examination of centriole marker in spermatozoa separated by density gradient.
Mariam Asadullah, Emily L. Fishman, Ahmed Hussain, Andrew Gerts, Tariq Shah, Puneet Sindhwani, Tomer Avidor-Reiss

P307 - Mitochondrial Dynamic and Acrosomal Reaction are Disturbed In Spermatozoa from Stressed Adult Rats.
Silvana A. Andric, Isidora M. Starovlah, Sava M. Radovic, Tatjana S. Kostic

P295 - Vitrification of testicular tissue from adult Spix's yellow-toothed cavies' (*Galea spixii*) using different cryoprotectants

Saturday, 20 July 2019

08:00–08:00

Part of: Poster Session B

Speakers- Pierre Comizzoli- Samara Sandy Moreira- Moacir Oliveira- Ana Pereira- Erika Camila Praxedes- Alexandre Silva- Andreia Silva

The objective was to assess different cryoprotectants for the vitrification of testicular tissue from the Spix's yellow-toothed cavy, a wild rodent from the Caatinga biome. Testes from six adults were recovered and dissected in small fragments (3 mm3). Fresh tissues were evaluated for morphology according to scores (3 – adequate; 2 regular; 1 – poor) using the following criteria: separation of the basal membrane, structure integrity, cell swelling, cell loss, and rupture. Moreover, fresh controls were evaluated for proliferative activity by the Ag-NOR technique. Other fragments were immersed in a MEM-based solution with 10% of fetal bovine serum plus 0.25M sucrose for 5 min before exposure to either 3 or 6 M of dimethyl-sulfoxide (DMSO) or ethylene glycol (EG) for 5 min. Tissues then were cryopreserved using a solid-surface vitrification technique. After two weeks of storage in liquid nitrogen, samples were warmed at 37°C, washed in decreasing sucrose concentrations, and evaluated as described previously. Scores of fresh samples were: 2.97 ± 0.02 membrane separation, 2.49 ± 0.04 structure integrity, 2.81 ± 0.04 swelling, 2.97 ± 0.01 cell loss, and 2.97 ± 0.01 rupture. After warming, scores of 3M EG groups were similar to controls ($P > 0.05$) for cell swelling (2.71 ± 0.04), cell loss (2.98 ± 0.01) and membrane rupture (2.98 ± 0.01). The 3M DMSO group was only efficient to avoid cell swelling (2.72 ± 0.03). Regarding proliferative activity, fresh control presented 3.73 ± 0.09 nucleolar organizing regions for spermatogonia, 3.72 ± 0.11 for spermatocytes, 1.68 ± 0.08 for spermatids, 2.55 ± 0.11 for Leydig and 3.89 ± 0.11 for Sertoli cells, which was efficiently preserved after thawing using all cryoprotectants. Collective results suggest that 3M EG is optimal for testicular tissue vitrification in adult Spix's cavies. Research supported by CAPES (Financial Code 01), Brazil.