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LÍVIA BATISTA CAMPOS

ISOLAMENTO, CULTIVO E CRIOPRESERVAÇÃO DE FOLÍCULOS OVARIANOS PRÉ-ANTRAIS DE CATETOS (*PECARI TAJACU* LINNAEUS, 1758)

MOSSORÓ-RN
2019

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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: Alexandre Rodrigues Silva, Prof. Dr.

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

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Defendida em: 18 / 01 / 2019.

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Dedicatória,

À Giselia Ferreira Batista Campos (mãe),
pessoa que mais amo nessa vida

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“Disse-lhe Jesus: Não te hei dito que, se creres, verás a glória de Deus*?”*

João 11:40

RESUMO

A manipulação de oócitos inclusos em folículos ovarianos pré-antrais permite aumentar o potencial reprodutivo e garantir a conservação da biodiversidade. Neste sentido, objetivou-se estabelecer protocolos eficientes para o isolamento, cultivo e criopreservação de folículos pré-antrais de catetos. Em um primeiro experimento, ovários de seis fêmeas adultas foram destinados a diferentes métodos de isolamento folicular: enzimático utilizando a colagenase tipo IV, mecânico, utilizando lâmina de bisturi, e associação de ambos. Dentre estes, a maior quantidade ($P < 0,05$) de folículos foi obtido pelo método enzimático (961,7 ± 132,9), o qual também resultou na maior percentagem de folículos viáveis (98,7 ± 0,6%). Em adição, a integridade dos folículos obtidos pelo método enzimático foi confirmada pela microscopia eletrônica de varredura e por fluorescência (86%) após cultivo de 24 h. No segundo experimento, identificou-se a expressão dos receptores ALK-5 (tipo I) e BMPRII (tipo II) em fragmentos ovarianos de catetos por meio de PCR. Em seguida, procedeu-se o cultivo in *vitro* de tecido ovariano de seis animais por 1 ou 7 dias com Fator de Crescimento e Diferenciação 9 GDF-9 (0, 50, 100 ou 200 ng/mL). Verificou-se que os folículos cultivados por 7 dias com 200 ng/mL de GDF-9 mantiveram o diâmetro folicular e oocitário similar àqueles observados no dia 1. Em comparação com o controle fresco, a porcentagem de folículos em crescimento foi significativamente em todos os tratamentos, especialmente na concentração 200 ng/mL de GDF-9 por 7 dias. Ainda, a presença de GDF-9 a 200 ng/mL melhorou a proliferação celular após o cultivo. No experimento 3, o tecido ovariano foi vitrificado tanto em uperfície sólida como no sistema cryosystem com 3M etilenoglicol (EG), 3M dimetilsulfóxido (DMSO) ou 1,5 M EG associado a 1,5 M DMSO. Após vitrificação, constatou-se que todos os tratamentos mantiveram, de modo similar, a proporção de folículos viáveis e a ocorrência de proliferação celular. No entanto, todos os tratamentos mantiveram a proporção de PFs normais como o grupo controle (75,6 ± 8,6%), exceto ($P <0,05$) os vitrificados por SSV com EG (52,8 ± 5,9%) ou a combinação de CPAs (54,5 ± 10,4%). Finalmente, a partir da análise da expressão de caspase 3-ativada, apenas as amostras processadas por SSV com EG (43,4%) ou CPAs (33,4%), bem como aquelas vitrificadas em OTC com EG (46,7%), forneceram valores semelhantes aos encontrados para grupo controle fresco (36,7%). Diante destes resultados, sugere-se que o método enzimático é um procedimento eficiente para o isolamento de folículos pré-antrais de catetos; existe receptores BMPR2 e ALK-5 para o GDF-9 no cortex ovariano de cateto e que o GDF-9 na concentração de 200 ng / mL é importante para o desenvolvimento *in vitro* de folículos ovarianos dessa espécie. Ainda, a vitrificação usando o método OTC com etilenoglicol (3M) como crioprotetores é eficiente para preservação do tecido ovariano de catetos.

Palavras chave: folículos pré-antrais, foliculogenese e biobancos

ABSTRACT

The manipulation of oocytes included in pre-antral ovarian follicles increases the reproductive potential and ensures the conservation of biodiversity. In this sense, the objective was to establish efficient protocols for the isolation, culture and cryopreservation of preantral follicles of peccary (*Pecari tajacu*). In a first experiment, ovaries of six adult females were assigned to different methods of follicular isolation: enzymatic using collagenase type IV, mechanical using a scalpel blade, and association of both. Among these, the highest amount ($P < 0.05$) of follicles was provided by the enzymatic method (961.7 ± 132.9), which also provided the highest proportion of viable follicles (98.7 ± 0.6%). In addition, the integrity of the follicles obtained by the enzymatic method was confirmed by scanning electron microscopy and by fluorescence (86%) after 24h culture. In the second experiment, the expression of ALK-5 (type I) and BMPRII (type II) receptors was identified in ovarian fragments of hips by means of PCR. Then, *in vitro* culture of ovarian tissue of six animals for 1 or 7 days with GDF-9 (0, 50, 100 or 200 ng / ml) was performed. The follicles cultured for 7 days with 200 ng / mL of GDF-9 maintained the follicular and oocyte diameter similar to those observed on day 1. Compared to fresh control, the percentage of growing follicles was significantly increased in all the treatments, especially in 200 ng / ml GDF-9 for 7 days. In addition, the presence of 200 ng / ml GDF-9 improved cell proliferation after cultivation. In experiment 3, ovarian tissue was vitrified on solid surface and cryosystem systems with 3 M ethylene glycol (EG), 3 M dimethylsulfoxide (DMSO) or with 1.5 M EG plus 1.5 M DMSO. After vitrification, all treatments were found to have similarly maintained the proportion of viable follicles and the occurrence of cell proliferation. However, all treatments maintained the proportion of normal PFs as the control group (75.6 ± 8.6%), except ($P < 0.05$) those vitrificated by SSV with EG (52.8 ± 5.9%) or the combination of CPAs (54.5 ± 10.4%). Finally, from the analysis of 3-activated caspase expression, only the samples processed by SSV with EG (43.4%) or CPAs (33.4%), as well as those glazed with OTC with EG (46.7%), provided values similar to those found for the fresh control group (36.7%). Considering these results, it is suggested that the enzymatic method is an efficient procedure for the isolation of preantral follicles of peccaries; there are BMPR2 and ALK-5 receptors for GDF-9 in the ovarian cortex and GDF-9 at a concentration of 200 ng / mL is important for the in vitro development of ovarian follicles of this species. Further, vitrification using the OTC method with ethylene glycol (3M) as cryoprotectants is efficient for preservation of ovarian tissue from peccaries.

Key words: preantral follicles, folliculogenesis and biobanks

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LISTA DE ABREVIATURAS E SIGLAS

ACP	Agente crioprotetor
ACP®	Água de coco em pó
ALK-5	Receptor de ativina tipo kinase-5
αMEM	Alfa meio essencial mínimo
ANOVA	Análise de Variância
BMPR II	Receptores tipo II do GDF-9
CAD	DNase ativada por caspase
cm	Centímetro
DMSO	Dimetilsulfoxido
$DMSO_2$	Dimetilsulfona
DNA	Ácido desoxirribonucleico
EG	Etilenoglicol
FOPAs	Folículos ovarianos pré-antrais
FSH	Hormônio folículo estimulante
GDF-9	Fator de crescimento e diferenciação-9
g/mol	Gramas por mol
h	Hora
HE	Hematoxilina eosina
M	Molar
mm^3	Milímetro cúbico
McCoy	Meio McCoy
MEM	Meio essencial mínimo
MOIFOPA	Manipulação de oócitos inclusos em folículos ovarianos pré-antrais
MSM	Metilsulfonilmetano
N_2	Nitrogênio líquido
ng/mL	Nanograma por mililitro
NORs	Regiões organizadoras do nucléolo
OTC	Ovarian tissue cryosystem
PAS	Ácido periódico de Schiff
PBS	Solução salina tamponada fosfatada
PCR	Reação em cadeia da polimerase
pg/mL	Picograma por mililitro
RNA	Ácido ribonucleico
R-Smad	Receptores-regulados SMADs (*receptor-regulated SMADs)*
Smad-Co	Smad comum
SSV	Superficie sólida
TCM199	Meio de cultivo tecidual 199
TGF-β	Fatores de crescimento transformante-β
2D	Bidimensional
3D	Tridimensional

µm	Micrômetro
mL	mililitro
mim	minuto
CIV	Cultivo *in vitro*
MIV	Maturação *in vitro*

LISTA DE SÍMBOLOS

±	Mais ou menos
<	Menor
≤	Menor igual
%	Porcentagem
®	Marca Registrada
<	Menor
≤	Menor igual
%	Porcentagem
®	Marca Registrada
°C	Grau Celsius

SUMÁRIO

1. INTRODUÇÃO

Os catetos, também conhecidos como caititus, porcos-do-mato ou pecaris, são mamíferos que pertencem à ordem Artiodáctila, subordem Suiforme e família Tayassuidae, a qual é representada por três espécies: o queixada (*Tayassu pecari*), o cateto (*Pecari tajacu*) e o taguá (*Catagonus wagneri*) (SOWLS, 1997). Em termos de risco de extinção, os catetos ocupam o status de pouco preocupante em praticamente todos os biomas, sendo classificados como espécie estável. Contudo, nas regiões leste e sul da Argentina a espécie já foi extinta (GANGORA et al., 2011).

Os catetos desempenham uma importante função ecológica no equilíbrio e na composição de cadeias alimentares, contribuindo substancialmente para a manutenção de seus predadores (CAVALCANTI; GEESE, 2010). Adicionalmente, esses animais são considerados bons dispersores de sementes (MORALES et al., 2015) e sua capacidade de modificar o ambiente através da abertura de clareiras e crescimento de plântulas confere aos catetos o título de engenheiro do ambiente (BECK et al., 2010). Além de sua importância ecológica, essa espécie pode ser empregada como modelo experimental (ARGÔLO-NETO et al., 2016) para espécies cujos níveis populacionais estão reduzidos, como o queixada e o taguá (IUCN, 2018).

Paralelamente, os catetos apresentam peculiaridades que os distinguem de outras espécies, como o sabor e a maciez da carne e a resistência do couro, as quais agregam valor econômico ao animal, isso estabelece um excelente potencial de exploração comercial. Esse potencial tem grande perspectiva de ser explorado, uma vez que os catetos possuem uma boa adaptabilidade em cativeiro, o que permite sua exploração comercial de maneira controlada, resultando ainda na diminuição e/ou eliminação da caça predatória e na possibilidade de manutenção da estabilidade da sua população (MIRANDA et al., 2010).

Diante disso, a aplicação de técnicas de reprodução assistida em cativeiro aparece como uma forma de melhorar o aproveitamento do potencial reprodutivo dos catetos e garantir a conservação da espécie (NOGUEIRA; NOGUEIRA FILHO, 2011). Dentre as biotécnicas, pode-se destacar a Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais (MOIFOPA), também conhecida como “Tecnologia do Ovário Artificial” (FIGUEIREDO; LIMA, 2017). Dentre as possíveis aplicações desta biotecnicas podem-se exemplificar: a pequisa fundamental, com o aumento dos conhecimentos acerca da foliculogênese; a criopreservação de material biológico para a produção de biobancos de células germinativas; fonte para testes toxicológicos para a indústria farmacêutica; incremento na produtividade de animais de alto valor genético, bem como, preservação de espécies ameaçadas de extinção

(HAAG et al., 2013). Tal biotécnica consiste nas etapas de isolamento, conservação (resfriamento e criopreservação) e/ou cultivo *in vitro* de folículos ovarianos pré-antrais, visando a estocagem, ativação, crescimento e maturação *in vitro* do folículo primordial até o folículo pré-ovulatório (FIGUEIREDO et al., 2008).

Assim, torna-se necessário inicialmente o desenvolvimento de métodos eficientes que possibilitem o isolamento de uma grande quantidade de folículos pré-antrais dos ovários de catetos. Adicionalmente, é essencial a realização de estudos voltados para a compreensão da fisiologia ovariana de catetos, no que se refere à atuação de substâncias que estão envolvidas no controle da foliculogênese. Esse estudo possibilitará o desenvolvimento de protocolos de cultivo *in vitro* que sejam capazes de manter a viabilidade dos folículos pré-antrais de catetos, e estimular seu crescimento, resultando no fornecimento de milhares de oócitos viáveis. Por fim, faz-se necessário o aprimoramento de protocolos de criopreservação, a fim de possibilitar o estoque de um grande número de folículos pré-antrais por longos períodos e também facilitar o transporte de material genético para diferentes centros reprodutivos. Desta forma, no futuro, a partir de um único ovário será possível a obtenção de centenas de folículos pré-antrais, que serão cultivados e submetidos a outras biotécnicas, como a fecundação *in vitro* e clonagem, viabilizando a produção *in vitro* de embriões e o aumento no número de crias nascidas. Para melhor compreensão do assunto será abordado uma revisão de literatura.

2. REVISÃO DE LITERATURA

2.1. Aspectos reprodutivos de fêmeas de catetos

Avanços significativos na compreensão da fisiologia reprodutiva dos catetos foram obtidos nas duas últimas décadas. Em geral, as fêmeas desta espécie atingem a maturidade sexual entre 8 e 14 meses de idade, sendo classificadas como poliéstricas não estacionais, com a capacidade de reproduzir durante todo o ano (BELLATONI, 1991; MAYOR et al., 2004; SILVA et al., 2002). As fêmeas apresentam ciclo estral de 21,0 ± 5,7 dias, sendo em média 6 dias de fase estrogênica e 15 dias de fase progesterônica (MAIA et al., 2014). A gestação dos catetos dura em torno de 138 ± 5 dias (MAYOR et al., 2005) e o tamanho da ninhada varia de 1,7 a 1,9 filhotes (GOTTDENKER; BODMER, 1998).

Estudos evidenciaram aspectos importantes associado à regulação hormonal do ciclo estral das fêmeas. Assim, foi descrito um estro com duração de 2 a 4 dias, ocorrendo a cópula entre os dias 1 e 2 após o pico de estradiol (MAYOR et al., 2007). Anatomicamente, nesse período, a genitália externa apresenta-se hiperêmica, edemaciada e com a presença de muco (GUIMARÃES et al., 2011). Paralelamente, o epitélio vaginal durante o estro exibe predominância de células superficiais intermediárias, abrangendo cerca de 60% de todas as células. Na fase estrogênica, o pico médio de estrógeno observado foi de 55,6 ± 20,5 pg/mL, enquanto que durante a fase luteal o valor mais alto alcançado pela progesterona foi 35,3 ± 4,4 ng/mL, ambos analisado por ELISA (MAIA et al., 2014).

Em relação as gônadas, os catetos apresentam ovários (Figura 1) completamente envoltos pela bursa ovárica com estrutura mural semelhante à maioria das espécies de mamíferos: uma região parenquimatosa e funcional na porção externa do órgão, o córtex, no qual estão inseridos os folículos ovarianos em diferentes estágios de crescimento ou regressão e uma região de estroma localizada internamente, denominada de medula. Semelhante ao observados em outras espécies, a maioria dos oócitos das fêmeas de cateto é estocados no ovário inseridos em folículo pré-antrais ou não cavitários, que correspondem aos folículos primordiais, primários e secundários. Os folículos mais abundantes são os primordiais, que estão no ovário em estágio de quiescência sendo, portanto, a principal reserva de gametas femininos da espécie (LIMA et al., 2013). Estes folículos são caracterizados pela presença de uma camada única de células da pré-granulosa de morfologia pavimentoso, e após sua ativação entram em fase de desenvolvimento, aumentam de diâmetro e dão origem aos folículos primários, os quais exibem uma única camada simples de células da granulosa de morfologia cuboide (MAYOR et al.,

2006). Na sequência, são formados os folículos secundários, caracterizados pela presença de duas ou mais camadas de células da granulosa envoltas ou não por células da teca, e, em seguida, são originados os folículos terciários, também conhecidos como cavitários, caracterizados pela presença de uma cavidade repleta de líquido denominada de antro (MAYOR et al., 2006).

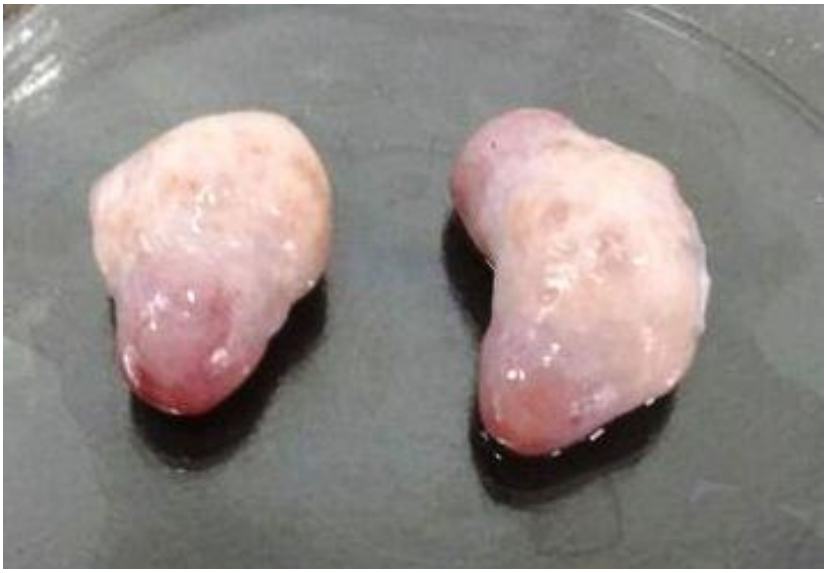

Figura 2- Ovários de catetos (~ 2 anos de idade). Fonte: Arquivo pessoal

Em estudos da estimativa da população folicular ovariana, Lima et al. (2013) encontraram uma média de 33273,45 ± 3019,30 folículos por ovário, dos quais a maioria pertence à categoria de folículo primordial (91,56 %), seguido de primário (6,29 %) e secundário (2,15 %). A maioria dos folículos pré-antrais são encontrados nos ovários com a morfologia normal (94,4 %), e um número reduzido estão em processo de atresia (5,6 %). Um aspecto morfológico importante relatado por esses autores foi a presença de grandes quantidades de gotas lipídicas no citoplasma dos oócitos de catetos, a qual foi confirmada por meio da microscopia eletrônica de transmissão (LIMA et al., 2013).

Por meio da ultrassonografia, durante o pico estrogênico foram encontrados folículos ovarianos antrais medindo 0,2 ± 0,1 cm (MAIA et al., 2014). O corpo lúteo é ovoide e sobressai na superfície dos ovários (MAYOR et al., 2006), podendo medir aproximadamente 0,4 ± 0,2 cm (MAIA et al., 2014), sendo que, em fêmeas não prenhes, pode atingir o volume de 397,1 ± 569,3 mm^3, já em fêmeas prenhes pode ter até 1041,8 ± 758,8 mm^3 (MAYOR et al., 2006).

Recentemente, Borges et al., (2018) investigaram a relação entre o potencial meiótico e o tempo de MIV de oócitos de catetos e sugeriram que 48 horas é o tempo adequado para a

maturação *in vitro* de oócitos derivados de catetos quando comparado ao tempo de 24 horas, de acordo com o potencial meiótico observado.

Ressalta-se que as informações a respeito do desenvolvimento e aplicações de biotécnicas relacionadas às fêmeas ainda são escassas (LIMA et al., 2013; 2014; 2017). Diante desse fato, são necessários mais estudos acerca da aplicação de biotécnicas que visem o aumento do potencial reprodutivo da espécie, que possa contribuir tanto para a conservação dos gametas femininos, como para a produção animal, tais como a manipulação de folículos ovarianos pré-antrais.

2.2. Manipulação de folículos ovarianos pré-antrais

Sabe-se que os folículos pré-antrais representam cerca de 90 a 95 % de toda população folicular e, desta forma, armazenam a grande maioria dos oócitos presentes em ovários mamíferos. Contudo, a grande maioria destes folículos (99,9%) não chega até à ovulação, sendo eliminados por meio de um processo conhecido por atresia folicular (FIGUEIREDO et al., 2007). Visando evitar a enorme perda folicular que ocorre naturalmente *in vivo*, vem sendo desenvolvida a biotécnica MOIFOPA, que consiste na manipulação de oócitos inclusos em folículos ovarianos pré-antrais, também conhecida como "Ovário Artificial" (FIGUEIREDO et al., 2017). Essa biotécnica possibilita o resgate dos folículos pré-antrais a partir do ambiente ovariano e, posteriormente, os folículos obtidos poderão ser conservados, e/ou cultivados *in vitro* até a maturação, possibilitando a recuperação de um grande número de oócitos que poderão ser empregados em outras biotécnicas reprodutivas como a produção de embriões *in vitro*, transgenia e clonagem. Assim, essa biotécnica pode ser dividida nas etapas de isolamento, conservação (resfriamento e/ou criopreservação) e cultivo *in vitro* (FIGUEIREDO et al., 2008).

Assim, a tecnologia do ovário artificial apresenta grande potencial, e no futuro, poderá contribuir para o aumento da eficiência reprodutiva de animais de alto valor zootécnico ou em risco de extinção, redução do intervalo entre gerações, uso de animais que não respondem a tratamentos de superovulação, obtenção de descendentes de um animal mesmo após sua morte, otimização e padronização de outras biotécnicas como a fecundação *in vitro*, clonagem e transgenia, além de contribuir para a elucidação de mecanismos implicados na foliculogênese na fase pré-antral (FIGUEIREDO et al., 2011).

Até o presente momento, os resultados mais satisfatórios desta biotécnica foram obtidos a partir de estudos *in vitro* com o cultivo de folículos pré-antrais de camundongos, os quais demonstraram que é possível a obtenção de crias vivas a partir de oócitos oriundos de folículos

cultivados *in vitro* (O'BRIEN et al., 2003). Em outras espécies como caprinos (SARAIVA et al., 2010), ovinos (ARUNAKUMARI et al., 2010), bubalinos (GUPTA et al., 2008), suínos (WU et al., 2001) e primatas não-humanos (XU et al., 2011), o cultivo *in vitro* de folículos secundários resultou na produção de oócitos maturados, os quais foram fecundados *in vitro*, gerando embriões, porém, apenas um número restrito e extremamente variável de embriões tem sido relatado. Contudo, em outros estudos em bovinos (GUTIERREZ et al., 2000) e caninos (SERAFIM et al., 2010), os folículos pré-antrais foram isolados e cultivados *in vitro,* mas se desenvolveram somente até o estádio antral.

Em catetos, estudos iniciais já foram realizados por LIMA et al., (2014) que realizaram a conservação dos folículos ovarianos pré-antrais (FOPAs) a curto prazo, verificando que a Água de coco em pó (ACP®) (66,7 %) foi mais eficiente que a solução salina tamponada fosfatada (PBS) (49,4 %) para preservar a integridade morfológica dos FOPAs após armazenamento por 36 h. Posteriormente, os autores realizaram a conservação do tecido ovariano a longo prazo, utilizando o método de vitrificação em superfície sólida (VSS) e observaram que 70 % dos folículos vitrificados mantiveram a integridade morfológica quando vitrificados com o dimetilsulfoxido (DMSO), etilenoglicol (EG), independentemente da concentração utilizada (3 M ou 6 M) (LIMA et al., 2012). Em adição, o cultivo *in vitro* também já foi descrito com sucesso para folículos ovarianos de catetos. Os autores obtiveram uma média de 53,0 ± 3,7 % de foliculos pré-antrais morfologicamente normais após o cultivo, os quais apresentaram proliferação nuclear, bem como, a manutenção da matriz extracelular após 7 dias quando cultivados com TCM199 suplementado com FSH (LIMA et al., 2017). Apesar de promissores os estudos são requerido afim de aprimorar esta técnica e obter resultados mais satisfatórios.

Nesse sentido, embora a tecnologia do Ovário Artificial venha apresentando resultados satisfatórios em espécies domésticas, em outras espécies silvestres como nos catetos, necessita ser aprimorada para que possa contribuir significativamente para aumentar a eficiência reprodutiva e também para a conservação da espécie. Para tanto, é necessário o aperfeiçoamento prévio das três etapas da técnica descritas anteriormente: isolamento, criopreservação e cultivo *in vitro* de folículos pré-antrais.

2.2.1. Isolamento folicular

A definição do protocolo de isolamento dos folículos pré-antrais é um importante passo para garantir a obtenção de um maior número de folículos viáveis, para se proceder as técnicas

de cultivo e/ou criopreservação, ou para que esses folículos possam ser utilizados em outras biotécnicas (TELFER, 1996).

O princípio do método de isolamento folicular consiste na dissociação ou separação dos folículos pré-antrais dos demais componentes do estroma ovariano (fibroblastos, fibras colágenas e elásticas, fibronectina), utilizando-se para isto, instrumentos mecânicos ou a digestão enzimática (FIGUEIREDO et al., 2008).

Os primeiros registros de estudos de FOPA isolados ocorreram nas décadas de 60 e 90 utilizando procedimentos enzimáticos em ovários de camundongos (GROB, 1964) e mecânicos em ovários bovinos (FIGUEIREDO et al., 1993). No isolamento enzimático, há uma exposição do tecido ovariano à ação de enzimas, como a colagenase, a pronase e a DNAse, permitindo o isolamento de muitos pequenos folículos (DEMEESTERE et al., 2005). Contudo, grande parte dos folículos pré-antrais isolados pode apresentar danos na membrana basal ou nas células da teca, principalmente, quando o tempo de incubação não é rigorosamente controlado (DEMEESTERE et al., 2005).

O isolamento enzimático tem sido realizado em várias espécies, como equinos (TELFER; WATSON, 2000), ovinos (MURUVI et al., 2005), bubalinos (GUPTA et al., 2007; SHARMA et al., 2009), camelídeos (SABER, 2009), suínos (AHN et al., 2012), macacos (HORNICK et al., 2012), equinos (HAAG et al., 2013), humanos (LIERMAN et al., 2015), bovinos (ANTONINO et al., 2017) e murinos (YOUNG et al., 2017), apresentando resultados bastante promissores.

Em suínos, a espécie doméstica mais próxima filogeneticamente dos catetos, vários métodos de isolamento para recuperação folículos pré-antrais foram aplicados (HIRAO et al., 1994; WU et al., 2001; MAO et al., 2002), e os resultados foram pouco promissores devido ao reduzido número de folículos pré-antrais recuperados. No entanto, Kerong et al. (2007) utilizando ovários de fêmeas pré-puberes, aplicaram o método enzimático por colagenase tipo I e obtiveram bons resultados, aumentando o rendimento da recuperação dos folículos ovarianos, conseguindo recuperar uma média de 82,43 ± 9,41 % de folículos primordiais. Já, Choi et al., (2008) sugeriram que a utilização da digestão enzimática com colagenase tipo IV é uma ferramenta útil para recuperar folículos pré-antrais de suínos, enfatizando que esta enzima é mais eficaz quando utilizada com agitação durante 30 minutos, sendo obtido uma média de 143,2 ± 66,7 folículos por ovário.

Uma alternativa para o isolamento de folículos pré-antrais é o método mecânico. A grande vantagem do isolamento mecânico é que, ao contrário do enzimático, este mantém a integridade da estrutura folicular, a membrana basal intacta, e ainda as interações entre oócito,

células da granulosa e células da teca (DEMEESTERE et al., 2005), sendo mais econômico e de maior facilidade de execução (DOMINGUES et al., 2003). Contudo, permite, em geral, a recuperação de um número inferior de folículos (TELFER et al., 2000). Neste método, os folículos podem ser isolados do córtex ovariano com auxílio de equipamentos como o *tissue chopper* (FIGUEIREDO et al., 1993), míxer (NUTTINCK et al., 1993), instrumentos simples como tesouras cirúrgicas (CARAMBULA et al., 1996), pequenos fórcepses (HULSHOF et al., 1994), filtros de dissociação celular (JEWGENOW, 1998) e agulhas dissecantes (JEWGENOW e PITRA, 1991). O isolamento mecânico tem sido testado com sucesso em folículos pré-antrais de bovino (ITOH et al., 2002), primata (DOMINGUES et al., 2003), humano (MARTINEZ-MADRID et al., 2004), ovino (TAMILMANI et al., 2005), bubalino (SHARMA et al., 2009), canino (ALVES, 2012), equino (HAAG et al., 2013) e murino (ZHANG et al., 2016).

Em suínos, 599160 folículos foram recuperados por ovário no procedimento mecânico utilizando o *tissue chopper* e 76,44 % desses folículos estavam viáveis após o isolamento (ALVES, 2010). Posteriormente, Ahn et al. (2012) compararam o método mecânico, com auxílio de uma agulha dissecante, com o método enzimático, utilizando colagenase de tipo I sob agitação durante 30 minutos a 39º C. Os autores observaram que o método enzimático permitiu uma melhor taxa de recuperação de folículos pré-antrais (mecânico 68 vs. enzimático 128), mas a percentagem de folículos morfologicamente normais aumentou significativamente com a recuperação mecânica (mecânico 95 % vs enzimático 74 %), sugerindo que o isolamento mecânico é o melhor método de isolamento de folículos pré-antrais em suínos.

Outra alternativa para o isolamento de folículos pré-antrais é a associação dos métodos mecânico e enzimático, a qual possibilita o isolamento de grande número de folículos quando comparados com a aplicação dos métodos isolados (FIGUEIREDO et al., 1993), tendo sido aplicada com sucesso em bovinos (FIGUEIREDO et al., 1993, OKTAY et al., 1997), humanos (DONG et al., 2014) e ovinos (SADEGHNIA et al., 2016).

Em catetos, o isolamento mecânico dos FOPAs já foi realizado utilizando o *tissue chopper* ajustado para o intervalo de secções de 87,5 μm, seguindo um protocolo descrito em bovino por Figueiredo et al. (1993), contudo os autores realizaram tal técnica no intuito de proceder análises foliculares e não descreveram informação acerca da quantidade de folículos obtidos (LIMA et al., 2013).

No âmbito da pesquisa aplicada, o desenvolvimento de protocolos de isolamento de folículos pré-antrais seja mecânico ou enzimático, sozinho ou em associação, é de fundamental importância para obtenção de um grande número de folículos pré-antrais, os quais poderão ser utilizados em programas de criopreservação e/ou cultivo *in vitro*.

2.2.2. Cultivo *in vitro*

O cultivo *in vitro* de folículos pré-antrais é uma técnica que vem sendo largamente empregada com o intuito de avaliar o efeito de diferentes substâncias, em diferentes concentrações e em distintas fases do desenvolvimento folicular. A fim de estimular o desenvolvimento folicular *in vitro* mimetizando assim os eventos que ocorrem *in vivo* com os folículos que ovulam no ovário, permitindo a obtenção de oócitos aptos a serem maturados e utilizados em outras biotécnicas (MAO et al., 2002). Além disso, possibilita o estudo dos fatores que controlam a foliculogênese, e pode ser utilizado como importante ferramenta para avaliar a eficiência da criopreservação, uma vez que lesões foliculares não detectadas pós-descongelação podem evoluir e serem manifestadas durante o cultivo (FAUSTINO et al., 2011).

Vale ressaltar que o sistema de cultivo e a composição do meio são fatores fundamentais para promover o desenvolvimento durante o cultivo *in vitro* de folículos ovarianos pré-antrais a estágios mais avançados (PENG et al., 2010). Os sistemas de cultivo rotineiramente adotados são o cultivo de folículos pré-antrais inclusos no próprio tecido ovariano (cultivo *in situ*) que além da praticidade, tem a vantagem de manter o contato celular (ABIR et al., 2006) e a integridade tridimensional dos folículos, fornecendo um complexo sistema de suporte que se assemelha ao ovário *in vivo* (PICTON et al., 2008). No entanto, neste tipo de sistema, embora haja uma significativa ativação folicular, poucos folículos primários cultivados progridem até o estágio de folículo secundário (FORTUNE, 2003).

Outra forma de cultivo é na forma isolada (cultivo *in vitro* de folículos isolados). Este pode ser realizado de duas formas: 1) bidimensional (2D), quando o folículo se desenvolve sobre a própria placa de cultivo ou sobre uma matriz extracelular, como por exemplo, de colágeno **(**DEMEESTERE et al., 2005); e 2) tridimensional (3D), quando o folículo se desenvolve encapsulado em gota de alginato, fibrina ou outros biomateriais (XU et al., 2011). Ainda, esses dois tipos de cultivo também podem ser combinados, realizando primeiramente os procedimentos do cultivo *in situ*, para obtenção de um maior número de folículos pré-antrais em desenvolvimento, e posteriormente, o isolamento e cultivo dos folículos secundários isolados.

Outro fator que deve ser levado em consideração para a obtenção do sucesso do cultivo de FOPA *in vitro* é a composição do meio, visto que, é no meio de cultivo que os folículos encontrarão subsídios necessários para dar suporte ao seu crescimento (TELFER et al., 2000). Assim, os meios de cultivo de base utilizados para folículos pré-antrais devem ser ricos em vitaminas, aminoácidos e minerais, e dentre os mais comumente utilizados destacam-se o Meio

Essencial Mínimo (MEM) simples (MATOS et al., 2007) ou alfa-modificado (αMEM) (JIMENEZ et al., 2016), o Meio de Cultivo Tecidual 199 (TCM199) (LIMA et al., 2017) e o McCoy´s (MCLAUGHLIN et al., 2010). Embora sejam meios de cultivo ricos, os meios-base podem ser suplementados com outros compostos, como antioxidantes, aminoácidos, proteínas, micronutrientes, glicoproteínas, antibióticos e tampões (FIGUEIREDO et al., 2008).

Além de todos os suplementos supracitados, diversos autores têm investigado os efeitos de diversos fatores de crescimento para promoção do completo e eficiente desenvolvimento dos folículos pré-antrais *in vitro* (BERGAMO et al., 2014; ROCHA et al., 2016; COOK-ANDERSEN et al., 2016). Dentre estas substâncias, podemos destacar o fator de crescimento e diferenciação-9 (GDF-9), o qual atua no desenvolvimento de folículos pré-antrais (MARTINS et al., 2008; SASSEVILLE et al., 2010; READER et al., 2016). Entretanto, ainda não está completamente elucidada qual a sua atividade na foliculogênese inicial.

2.2.2.1. Importância do fator de crescimento e diferenciação-9 (GDF-9) no desenvolvimento folicular

O GDF-9 é um membro da superfamília de fatores de crescimento transformante-β (TGF-β) que tem sido descrita como muito importante no desenvolvimento folicular inicial (CHANG et al., 2002). O GDF-9 é majoritariamente expresso e secretado pelo oócito (CHANG et al., 2002) e atua no ovário de forma autócrina e parácrina após ligação à receptores específicos TGF-β de tipo I (ALK 5) (MOORE et al., 2003; MAZERBOURG et al., 2004; HUANG et al., 2009) e receptores tipo II (BMPRII) (VITT et al., 2002).

O GDF-9 liga-se ao receptor de BMP tipo II (BMPRII) que desempenha a função de ativar os receptores tipo I (ALK5) por fosforilação de uma região reguladora intercelular. O receptor de tipo I, uma vez ativado, fosforila proteínas citoplasmáticas da família de SMADs conhecidas como *receptor-regulated SMADs* (R-SMADs), incluem Smads 1, 2, 3, 5 e 8. Uma vez ativadas, as moléculas de R-Smad interagem com outra molécula de Smad, chamada Smad 4, que consiste em um parceiro comum para todo o R-Smad, conhecido como Smad comum (Smad-Co). Este complexo Smad / CoSmad (Smad4) é, finalmente, translocado para o núcleo celular para interagir com fatores de transcrição específicos que regulam a expressão de genes alvo (NISHIMURA et al., 1998; GILCHRIST et al., 2008; PAULINI, 2010; PENG et al., 2013) (Figura 2).

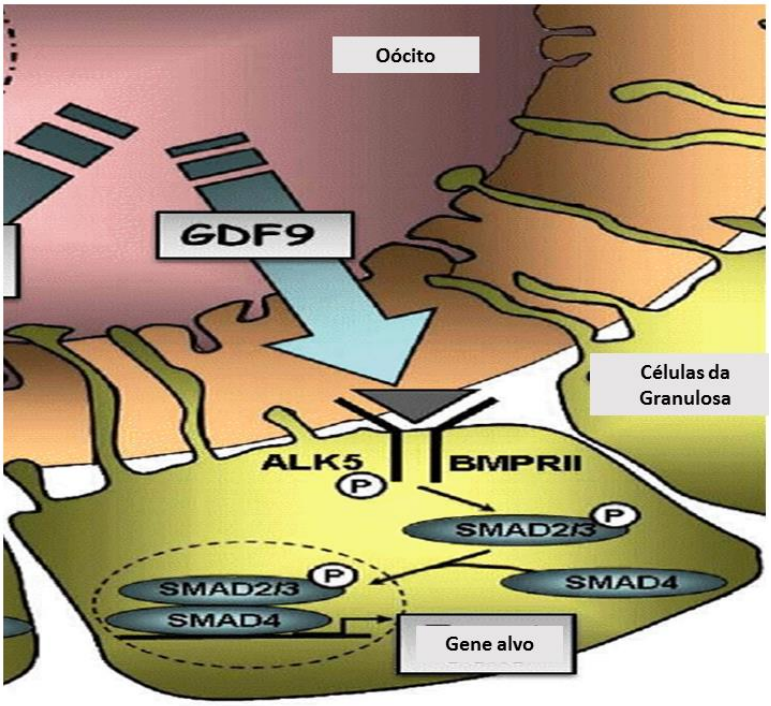

Figura 3 - Representação esquemática das vias de sinalização do fator de crescimento e diferenciação (GDF9) após a ligação a receptores específicos do Tipo I (ALK 5) e receptor do Tipo II (BMPRII) (adaptado CASTRO et al., 2016).

Receptores para o GDF-9 tem sido descritos em oócitos de folículos primordias de ovinos (BODENSTEINER et al., 1999), marsupiais (ECKERY et al., 2002), caprinos (SILVA et al., 2005), humanos (ORON et al., 2010), suínos (SUN et al., 2010), bovinos (HOSOE et al., 2011), cão (ABDEL-GHANI, 2012) e bubalinos (ABDEL-GHANI et al., 2016), enquanto que em camundongos (ELVIN et al., 1999) e ratos (HAYASHI et al., 1999), só são expressos a partir do estádio de folículo primário. Ainda, foi descrito a expressão do GDF-9 nas células da granulosa e da teca em suínos (QUINN et al., 2004; SUN et al., 2010; LIM et al., 2014), bovinos (HOSOE et al., 2011) e caninos (REYES et al., 2013; MAUPEU et al., 2015). Em catetos, até o presente momento não há dados sobre a expressão do GDF-9 e seus receptores no ovário, fazendo-se necessário a investigação do padrão de expressão desse fator nesta espécie.

Além dos estudos sobre expressão do GDF-9, é indispensável conhecer o efeito desse fator sobre a foliculogênese. O interesse pelo GDF-9 iniciou após a descoberta de que sua deleção causa bloqueio no desenvolvimento de folículos pré-antrais e, consequentemente, leva à infertilidade em camundongos (DONG et al., 1996). Com isso, vários estudos foram realizados no intuito de verificar o efeito do GDF-9 no desenvolvimento dos folículos pré-antrais, sugerindo que este fator de crescimento atua promovendo o crescimento de folículos primários e recrutamento de células da teca (hamster: WANG ROY, 2004; humanos: OTA et

al., 2006; caprinos: MARTINS et al., 2008), estimula manutenção da viabilidade folicular a proliferação de células da granulosa (ratas: VITT et al, 2000; bovinos: SPICER et al., 2006; camundongas: SASSEVILLE et al., 2010; humanos: READER et a., 2016), exerce efeito sinérgico com o FSH (murinos: HAYASHI et al., 1999; ratas: CHOI et al., 2014), inibe a expressão de receptores de LH (LH-R), estimula a síntese de progesterona e estrógeno (camungondos: ELVIN et al., 1999; ratas: VITT et al., 2000) e atua na expansão das células do cumulus (camundongas e humano: ELVIN et al, 1999, caprinos: MARTINS et al., 2008, ovinos: PENG et al., 2010).

Em suínos, a injeção *in vivo* de fragmentos de genes de GDF-9 na medula ovariana permitiu, após sete dias, a progressão do folículo primordial para o folículo primário ou dos primários à fase secundária, indicando a atividade do GDF-9 na ativação e crescimento folicular (SHIMIZU et al., 2006). Esta progressão demonstra que GDF-9 tem papel crítico na arquitetura ovariana da maioria das espécies e é um fator essencial para o desenvolvimento folicular adequado, interferindo consideravelmente na função, crescimento e formação de células granulosa e teca durante o crescimento do folículo (VITT; HSUEH, 2001; OTSUKA et al., 2011).

Entretanto, para obter tais efeitos, faz-se necessário a utilização de uma concentração ideal deste fator de crecimento. Martins et al., (2008), investigaram o efeito de diferentes concentrações de GDF-9 (1-200 ng/mL) sobre a sobrevivência e a ativação dos folículos pré-antrais cultivados *in situ*, bem como, a sua progressão subsequente para folículos secundários. Os autores concluíram que 200 ng/mL de GDF-9 foi suficiente para manter a sobrevivência de folículos pré-antrais e promover a ativação dos folículos primordiais caprinos. Esses resultados foram semelhantes ao encontrados em humanos (HREISSON et al., 2002), hamster (WANG e ROY 2004) e búfalos (ABDEL-GHANI et al., 2016), porém diferentes dos achados em ratos (100 ng/ml - ORISAKA et al., 2006), equinos (50 ng/mL – BERGAMO et al., 2014), ovinos (25 ng/mL – ROCHA et al., 2016) e murinos (camundongos C57/Bl6) (500 ng/mL - COOK-ANDERSEN et al., 2016).

Em catetos, até o momento, não se tem informação sobre o papel do GDF-9 no desenvolvimento folicular, sugerindo-se assim a investigação deste fator sobre a sobrevivência, ativação e crescimento dos folículos pré-antrais cultivados *in vitro*, melhorando assim a compreensão da atuação desse fator na foliculogênese. Diante disso, para validar a eficiência do protocolo de criopreservação e/ou cultivo *in vitro* é essencial submeter os folículos pré-antrais à avaliações como análise morfológica, integridade da membrana, proliferação celular e apoptose.

2.2.3. Criopreservação de tecido ovariano

A técnica de criopreservação de fragmentos ovarianos possibilita a conservação do material genético até que os protocolos empregados para o cultivo sejam desenvolvidos (FIGUEIREDO et al., 2007). Essa técnica consiste na conservação do material biológico, por tempo indefinido, em temperaturas negativas para que, quando aquecido, este material possa prosseguir seu desenvolvimento (BAO et al., 2010) por cultivo *in vivo* (transplante ou xenotransplante) ou *in vitro*. Essa biotécnica tem sido largamente investigada em caprinos (CARVALHO et al., 2011), primatas não humanos (XU et al, 2011; TING et al., 2013), murinos (YOUM et al., 2014), humanos (SUZUKI et al., 2015) e ovinos (BANDEIRA et al., 2015), com resultados bastante promissores.

A criopreservação pode ser realizada por dois métodos distintos: congelação lenta ou vitrificação. O primeiro método baseia-se na utilização de baixas concentrações de agentes crioprotetores (ACPs) e na redução lenta e gradual da temperatura. Embora no método de congelação lenta seja realizada uma redução gradativa de temperatura, ainda assim, este pode acarretar severos danos celulares ocasionados pela formação intracelular de gelo e danos osmóticos (STACHEKI e COHEN, 2004).

Já na vitrificação, os fluidos passam do estado líquido diretamente para um estado sólido amorfo denominado vítreo (MUKAIDA; OKA, 2012). Para que esta transição ocorra é necessária uma alta viscosidade do meio, a qual é obtida pela utilização de elevada concentração de ACPs, e uma rápida redução da temperatura (WOWK, 2010). Essa técnica vem se destacando como uma alternativa prática, eficiente e de baixo custo (BANDEIRA et al., 2015; SUZUKI et al., 2015; BRITO et al., 2018). Ainda, apresenta menores danos durante o resfriamento, visto que as amostras passam rapidamente pelos intervalos mais críticos de temperatura (15°C e -5°C) (WOWK et al., 2000). Diante de tais vantagens, diversos métodos de vitrificação foram desenvolvidos, dentre os quais merece destaque a vitrificação em superfície sólida (FATEHI et al., 2014; PRAXEDES et al., 2017) e *ovarian tissue cryosystem* (OTC) (BANDEIRA et al., 2015; BRITO et al., 2018).

2.2.3.1. Metódos de vitrificação

A vitrificação em superfície sólida consiste na sobreposição da amostra a ser congelada em um cubo de metal, que pode, inclusive, ser confeccionado artesanalmente com o uso de papel alumínio. O cubo de metal, quando posicionado acima do N_2 líquido, por ser um bom condutor de calor, proporciona um rápido resfriamento da amostra, condição necessária para

uma vitrificação eficiente. Posteriormente, a amostra deve ser armazenada em criotubos e mantida em N_2 líquido (AL-AGHBARI e MENINO, 2002). Essa técnica apresenta como principais vantagens o baixo custo, a facilidade no procedimento, bem como uma boa condução do calor (SANTOS et al., 2007).

Devido a tais características, a vitrificação em superfície sólida tem se tornado uma alternativa bastante promissora para a criopreservação de tecido ovariano, como observado em bovino (CELESTINO et al., 2010), ratos (XING et al., 2010), macacos (TING et al., 2011), caprino (CARVALHO et al., 2011), humanos (KAWAMURA et al., 2013), camundongos (FATEHI et al., 2014), preá (PRAXEDES et al., 2015), cutias (PRAXEDES et al., 2017). Em murinos, os resultados foram bastante satisfatórios, sendo possível o nascimento de crias viáveis após o aquecimento do tecido ovariano (MIGISHIMA et al., 2003; CHEN et al., 2006).

Em catetos, essa técnica já teve resultados promissores. Nesse estudo, os autores realizaram a vitrificação do tecido ovariano utilizando como agentes crioprotetores o etilenoglicol (EG) e dimetilsulfoxido (DMSO) isolados e em diferentes concentrações (3 ou 6 M). Os autores concluíram que tanto o EG (3M: 80,3 ± 9,1; 6M: 75,3 ± 3,4) como o DMSO (3M: 76,0 ± 2,5; 6M: 72,6 ± 3,5) promoveram a conservação da morfologia e viabilidade dos folículos independentemente da concentração do agente crioprotetor utilizada (LIMA et al., 2012).

Outro método promissor de vitrificação faz uso de um sistema específico denominado *ovarian tissue cryosystem* (OTC). Esse sistema segue o mesmo princípio da vitrificação em superfície sólida, contudo apresenta a vantagem de facilitar o processo de remoção de ACPs, não havendo a necessidade do uso de criotubos ou palhetas e ausência do contato do operador com a solução de vitrificação. Adicionalmente, pode ser aplicado para vitrificação de vários fragmentos de tecido do ovário simultaneamente, bem como, grandes fragmentos de tecido (hemiovário) ou mesmo o ovário inteiro (CARVALHO et al., 2013a).

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

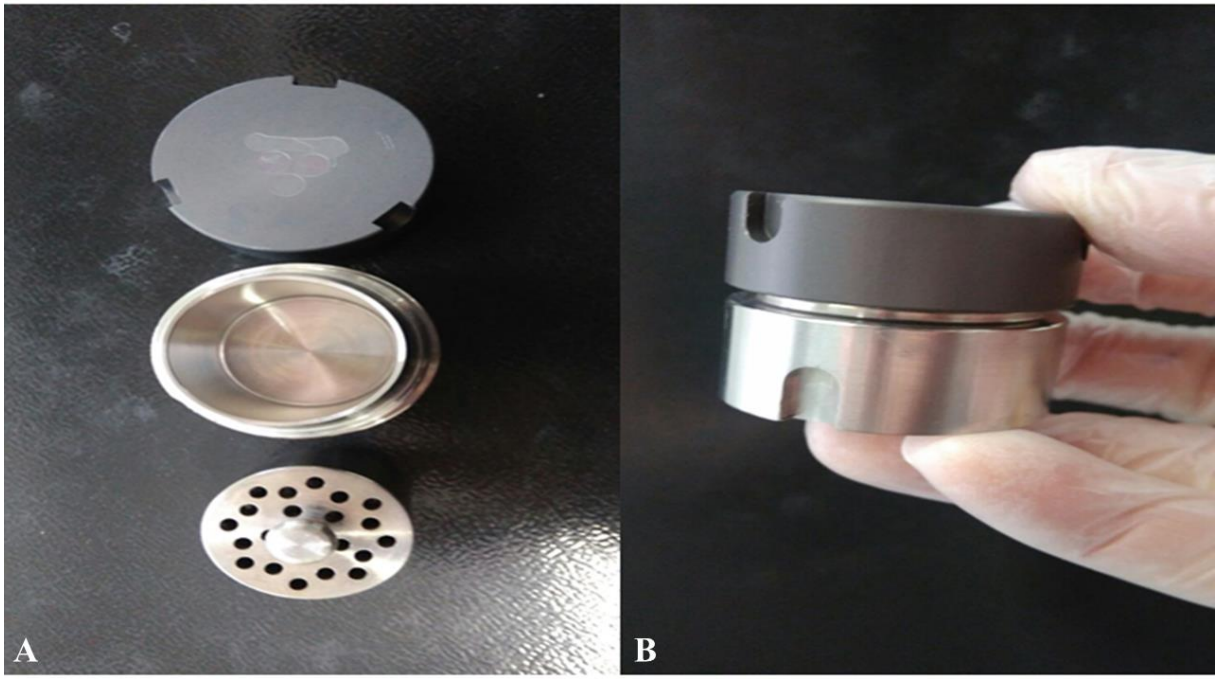

Figura 4 - Ovarian tissue cryosystem (OTC). (A) OTC aberto permitindo a visualização de suas partes constituintes. (B) OTC fechado. Fonte: Arquivo pessoal

O sistema já foi utilizado com sucesso na vitrificação de tecido ovariano de caprino, na qual os autores verificaram que o método permitiu a manutenção da morfologia (61,4 % de folículos normais) e a viabilidade (90 % de folículos viáveis), com conservação da ultraestrutura folicular (CARVALHO et al., 2013b) e menor taxa na fragmentação do DNA do folículo (FAUSTINO et al., 2014). Em ovinos, Bandeira et al., (2015) demonstraram que a vitrificação pela técnica de OTC permitiu a melhor conservação da morfologia e da viabilidade folicular, além de manter a integridade dos componentes da matriz extracelular em comparação com a técnica de superfície sólida.

Portanto, a técnica de OTC tem se mostrado bastante promissora para a criopreservação de tecido ovariano de pequenos ruminantes, podendo ser extrapolada para outras espécies, inclusive para os catetos. Ademais, considerando os resultados promissores nas outras espécies é possível que este método possa ser mais adequado ao tecido ovariano de cateto, possibilitando assim, a conservação dos gametas femininos da espécie.

Todavia, independente da técnica de criopreservação utilizada faz-se necessário a adição de agentes crioprotetores que garantem uma maior proteção celular durante a redução de temeratura, uma vez que eles protegem as células contra a desidratação, resfriamento e danos causados pela redução extrema de temperatura (GOSDEN et al., 2014).

2.2.3.2. Agentes Crioprotetores (ACP)

Em geral, os ACPs são divididos em dois grupos: os crioprotetores extracelulares e intracelulares. Entre os crioprotetores extracelulares encontram-se os sacarídeos, como trealose, glicose e macromoléculas como a polivinilpirrolidona e hialuronato de sódio. Estes permanecem fora da célula e interagem com moléculas de água livre da solução, influenciando indiretamente nos processos osmóticos de desidratação celular. Desse modo, evitam a formação de cristais de gelo ao retirar água do interior da célula, bem como, o protegem por meio da estabilização da membrana celular (PEREIRA e MARQUES, 2008).

Já os crioprotetores intracelulares são substâncias de baixo peso molecular que possuem a capacidade de penetrar na célula e formar pontes de hidrogênio com as moléculas de água presentes no citosol. Com isso, o ponto de congelação da água diminui, consequentemente, reduz a probabilidade de formação de cristais de gelo no interior da célula. Dentre os crioprotetores intracelulares, destacam-se glicerol, dimetilsulfóxido (DMSO), propanodiol e etilenoglicol (EG) (HERRAIZ et al., 2016). Destes, os mais utilizados na criopreservação de tecido ovariano são EG e DMSO por causarem menos danos para os FOPAs (LIMA et al., 2012; LUNARDI et al., 2012; FATEHI et al., 2014; PRAXEDES et al., 2015; GASTRAL et al., 2017).

O etilenoglicol (EG) é um álcool de fórmula molecular $C_2H_4(OH)_2$, obtido a partir da hidrólise do óxido de eteno (MARTINS et al., 2005). Em relação aos outros crioprotetores, este possibilita uma maior penetração nas células devido ao baixo peso molecular (62,07 g/mol) e ainda, apresenta um efeito tóxico baixo. Todavia, sua metabolização pode origina metabólitos tóxicos como o ácido glicólico e oxalato, sendo seus subprodutos potencialmente tóxicos (CORLEY et al., 2005). Esse crioprotetor tem sido amplamente empregado na criopreservação de tecido ovariano felino (LIMA et al., 2006), ovino (AMORIM et al., 2006), caprino (CELESTINO et al., 2008), macaco (HASHIMOTO et al., 2010), murino (AMORIM et al., 2011), bovino (LUNARDI et al., 2012), humano (SHEIKHI et al., 2013), equino (GASTRAL et al., 2017) e suíno (GABRIEL et al., 2017).

Já o dimetilsulfóxido (DMSO) é um composto orgânico de fórmula molecular C_2H_6SO com uma massa molar 78,13 g/mol (CARPENTER, 1994). Este composto possui uma elevada capacidade higroscópica que é decorrente de sua intensa afinidade pelo hidrogênio, formando pontes mais fortes que às formadas entre moléculas de água (ROSE; HODGSON, 1993; RAND-LUBY et al., 1996). Ainda, tem a capacidade de interagir com membranas de células, e atravessá-las rapidamente por difusão. Além disso, é uma substância considerada

relativamente atóxica, atua removendo radicais livres liberados durante o processo de descongelação. Todavia, ao sofrer metabolização, o DMSO é oxidado em dimetilsulfona ($DMSO_2$), também conhecida com metilsulfonilmetano (MSM), a qual pode acidificar o meio, podendo causar prejuízos (RODRIGUES et al., 2006).

Em caprinos, a utilização de DMSO foi capaz de manter a morfologia dos FOPAs após a criopreservação (CASTRO et al., 2011). Resultados similares foram relatados em hamster (KAGABU; UMEZU, 2000), bem como em bovino (LUCCI et al., 2004), suíno (GANDOLFI et al., 2006), cão (ISHIJIMA et al., 2006), ovino (PINTO et al., 2008), felinos silvestres (WIEDEMANN et al., 2013), rato (FATEHI et al., 2014) e preá (PRAXEDES et al., 2015).

Em catetos, o uso do EG e DMSO foi avaliado isoladamente na vitrificação de tecido ovariano em duas concentrações (3 ou 6 M). Os autores concluíram que tanto o EG (3M: 80,3 ± 9,1; 6M: 75,3 ± 3,4) como o DMSO (3M: 76,0 ± 2,5; 6M: 72,6 ± 3,5) promovem a conservação da morfologia dos FOPA independentemente da concentração utilizada (LIMA et al., 2012).

A escolha do crioprotetor mais adequado depende de vários fatores; um dos mais importantes é a toxicidade à célula criopreservada. Porém, vale ressaltar que a toxicidade biológica dos agentes crioprotetores está diretamente relacionada às suas respectivas concentrações (PALASZ et al., 2000). Diante disso, uma estratégia para diminuir a toxicidade das altas concentrações de crioprotetores necessárias para atingir o estado vítreo é a utilização dos agentes crioprotetores em associação, com a finalidade de reduzir a toxicidade específica de cada crioprotetor individualmente, pelo uso de concentrações relativamente baixas (HERRAIZ et al., 2016; SUGISHITA et al., 2016). Além disso, o uso de crioprotetores associados permite uma maior permeabilidade à célula do que o uso individual do crioprotetor (VICENTE e GARCÍA-XIMÉNEZ, 1994).

A veracidade de tais informações já foi demonstrada em estudos realizados em suínos (MONIRUZZAMAN et al., 2009), camundongos (HASEGAWA et al., 2006; NASRABADI et al., 2015), babuínos (NYACHIEO et al., 2013), ratos (YOUM et al., 2014), gatos domésticos (MOUTTAM e COMIZZOLI, 2016; BRITO et al., 2018) e em cutias (PRAXEDES et al., 2017).

Diante de tais informações, maiores estudos no intuito de verificar o efeito da associação de EG e DMSO na vitrificação de tecido ovariano de catetos faz-se necessário, visando assim aperfeiçoar os protocolos de vitrificação existentes.

3. JUSTIFICATIVA

Os catetos (*Pecari tajacu*) desempenham uma importante função ecológica no equilíbrio e na composição de cadeias alimentares, contribuindo substancialmente para a manutenção de seus predadores. Em relação ao risco de extinção, esses animais apresentam uma situação pouco preocupante na maioria dos biomas, porém, nas regiões leste e sul da Argentina a espécie já é extinta.

Adicionalmente, um crescente interesse pela criação em cativeiro tem sido observado, haja vista a possibilidade da comercialização de sua carne e pele no mercado internacional, bem como a venda de animais machos e fêmeas para servirem como reprodutores em criatórios comerciais. Por esses motivos, o incentivo à reprodução destes animais em cativeiro se torna um procedimento de importância econômica, como também ecológica, com o intuito de prevenir a possível degradação da espécie.

Diante do exposto, as técnicas de reprodução assistida precisam ser desenvolvidas e aplicadas, as quais são essenciais tanto para a conservação da espécie como para a produção animal. Desse modo, a Manipulação de oócitos inclusos em folículos ovarianos pré-antrais (MOIFOPA) ou a tecnologia do Ovário Artificial permite tanto a obtenção de conhecimentos básicos da foliculogênese da espécie, como também o resgate de centenas de folículos a partir de um ovário, e posteriormente seu crescimento *in vitro* até a maturação, permitindo sua aplicação em outras biotécnicas reprodutivas.

Inicialmente, a definição do protocolo de isolamento dos folículos pré-antrais de catetos é necessária para a obtenção de um maior número de folículos viáveis, os quais poderão ser utilizados em programas de criopreservação e/ou no cultivo *in vitro.* Adicionalmente, na busca por um eficiente sistema de cultivo de folículos pré-antrais, diversos pesquisadores têm aprofundado as investigações sobre o efeito e a concentração ideal de diferentes fatores de crescimento, de conhecida eficiência sobre a regulação da foliculogênese. Já é bem estabelecido que o GDF-9 estimula o desenvolvimento de folículos pré-antrais *in vitro* em diferentes espécies, mas em catetos, o papel desta substância sobre o desenvolvimento folicular é desconhecido. Diante disso, constatou-se a necessidade de se estudar os efeitos de diferentes concentrações de GDF-9 sobre o cultivo *in vitro* de folículos pré-antrais dessa espécie, permitindo assim o estabelecimento de estratégias para melhorar a ativação e o crescimento folicular *in vitro.*

Concomitantemente, protocolos de vitrificação fazem-se necessários para a manutenção da morfologia e viabilidade de folículos pré-antrais em baixas temperaturas até o momento da

sua utilização, quando um sistema de cultivo *in vitro* estiver adequadamente desenvolvido. Diante disso, sugere-se o aperfeiçoamento dos protocolos atualmente utilizados, particularmente, pela proposta de utilização do *ovarian tissue cryosystem* (OTC), juntamente com a associação de crioprotetores etilenoglicol (EG) e dimetilsulfóxido (DMSO) visando equilibrar os efeitos tóxicos da solução de vitrificação para que no final, possibilite um aumento na taxa de conservação de tais folículos ovarianos de catetos.

Dessa forma, este trabalho contribuirá para a conservação em longo prazo de células germinativas femininas, assim como, tornará os catetos uma espécie mais prolífica e produtiva, devido à possibilidade do aproveitamento máximo de folículos pré-antrais antes que os mesmos sofram atresia.

4. HIPÓTESES CIENTÍFICAS

-O isolamento mecânico promove uma maior recuperação de folículos pré-antrais de catetos que o método enzimático;

-O GDF-9 é importante durante o desenvolvimento *in vitro* dos folículos pré-antrais de catetos;

-O sistema de OTC pode ser utilizado com sucesso na vitrificação de tecido ovariano de catetos.

-A associação do dimetilsufoxido (DMSO) e etilenoglicol (EG) é eficiente para a conservação do tecido ovariano de catetos submetido à vitrificação em OTC;

5. OBJETIVOS

5.1. Objetivo Geral:

Otimizar protocolos eficientes para o isolamenteo, cultivo e criopreservação de folículos ovarianos pré-antrais derivados de catetos.

5.2. Objetivos Específicos:

Comparar a eficiência dos procedimentos mecânico e enzimático sozinhos ou em associação no isolamento de folículos ovarianos pré-antrais de catetos;

Verificar o efeito do GDF-9 sobre a morfologia, a viabilidade, o desenvolvimento, a ativação e a proliferação das células da granulosa dos folículos pré-antrais de catetos cultivados *in vitro* por 7 dias;

Identificar os receptores para o GDF-9 no córtex do ovário de catetos por meio da técnica de PCR convencional;

Comparar os métodos de vitrificação em superfície sólida ou utilizando o sistema OTC quanto à manutenção da morfologia, viabilidade e apoptose de folículos pré-antrais de catetos;

Comparar os efeitos do uso do dimetilsufoxido (DMSO) e etilenoglicol (EG) isolados ou em associação na vitrificação de tecido ovariano de fêmeas de cateto sobre a morfologia, a viabilidade e a apoptose de folículos pré-antrais criopreservados.

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Capítulo I - Advances and challenges of using ovarian preantral follicles to develop wildlife biobanks

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Abstract

Extinction rate of mammalian species has been accelerated in recent decades. It therefore is important to preserve and store genetic materials in cryo-banks for immediate or future studies and assisted reproductive techniques. Along with the creation of biobanks, research efforts focusing on vitro culture of germplasms to produce mature gametes are critical. Specifically, in vitro culture of oocytes from preantral follicles has been developed, since these follicles represent 90-95% of follicular population on ovarian cortex biopsy. This review presents main advances and discusses limitations and perspectives for the application of isolation, cryopreservation and *in vitro* culture of wild animals PFs and its future applications for germplasm biobanking.

Keywords: conservation, genetic material, cryopreservation, *in vitro* culture.

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Introduction

Conservation of endangered animals is necessary to balance and maintain functioning ecosystems. To mitigate the issues, *in situ* and *ex situ* conservation programs have been applied and their success depends directly on developing reproductive biotechnologies,[1] especially those geared to females.

In vitro culture of oocytes recovered from preantral follicles (PFs), besides serving as a model to investigation of initial folliculogenesis, has an impact on assisted reproduction of endangered species.[1] Considering that ovary contains thousands of primordial follicles that can remain viable even several hours after the animal's death, utilization of these follicles is an alternative for preservation of genetic material.[2]

PFs development has become an increasingly recognized area of study in the last two decades.[2] For its use, follicles must be rescued from ovarian environment, cryopreserved and stored indefinitely. Subsequently, these PFs can be cultured *in vivo*[3] or *in vitro*[4] to obtain mature oocytes able to be used for others reproductive techniques.[1] Despite promising results, ideal conditions for cryopreservation and especially for complete *in vitro* development of PFs in wild animals is a great challenge for researchers, which is characterized by low repeatability of its results. Main limiting factors are lack of knowledge on basic reproductive biology of wild species, absence of standardization for protocols used for PFs' obtaining, preservation and culture, as well as species-specific differences.[2] In this context, this review presents main advances and discusses limitations and perspectives for the application of isolation, cryopreservation and *in vitro* culture of wild animals PFs and its future applications for germplasm biobanking.

Ovarian Follicle Isolation

PFs can be *used* to formation of animal germplasm bank *in situ*, *i.e.* inserted into entire ovary (for example in small rodents) or ovarian fragments, or even individually isolated from ovarian cortex. Mechanical and / or enzymatic methods are used to dissociate follicles from other components of ovarian stroma (fibroblasts, collagen and elastic fibers, fibronectin, etc).[5]

There are three methods for PFs' isolation: mechanical, enzymatic or the combination of both (Table 1). For mechanical isolation, some devices like tissue chopper, mixer, surgical scissors, small forceps or dissecting needles are commonly used. This method maintains integrity of follicular structure, basal membrane, and interactions between oocytes/granulose and teak cells. However, it is relatively slow, laborious and, in general, provides recovery of a low number of follicles.[6]

For wildlife, *f*ollicular isolation by mechanical methods using cell dissociation sieve was initially described for wild felines with varying age. In this study, authors obtained an average of 980 follicles per ovary in cheetah (*Acinonyx jubatus*), 800 follicles in jaguar (*Panthera onca*), 100 follicles in bengal tiger (*Panthera tigris tigres*), 1080 follicles in siberian tiger (P*anthera tigris altaica*), 1440 in sumatran tiger (*Panthera tigris sumatrae*), 600 follicles in indian lion (*Panthera leo persica) and* 12.500 follicles in puma (*Puma concolor*). Still, puma isolated follicles were cultured *in vitro* for 14 days and a mean of 62.5 % viable follicles were obtained. Moreover, authors demonstrated possibility of recovering considerable amounts of viable PFs from really old females, including those with ovarian pathologies (polycystic ovaries).[7] Another important point is that authors studied, at one time, follicular population of seven wild felines, which consisted a great advance in knowledge of reproductive physiology of these species.

Table 1. Application of different methods for ovarian follicular isolation in wild species.

Authors and year	*Species*	*Isolation method*	*Number of follicles per ovary*	*Viability (%)*	*In vitro culture*	*Viable follicles after cultivation (%)*
JEWGENOW AND STOLTE, 1996	Cheetah (*Acinonyx jubatus*)	mechanical methods using cell dissociation sieve	980	32.5	-	-
	Jaguar (*Panthera onca*)	mechanical methods using cell dissociation sieve	800	18.7	-	-
	Puma (*Puma concolor*)	mechanical methods using cell dissociation sieve	12.500	49.6	14 days	62.5%
	Bengal tiger (*Panthera tigris tigres*)	mechanical methods using cell dissociation sieve	100	26.3	-	-
	Siberian tiger (P*anthera tigris altaica*)	mechanical methods using cell dissociation sieve	1080	18.7	-	-
	Sumatran tiger (*Panthera tigris sumatrae*)	cell dissociation sieve	1440	28.6	-	-
	Indian lion (*Panthera leo persica)*	cell dissociation sieve	600	37.7	-	-
DOMINGUES et al., 2003	Capuchin monkey (*Cebus appeals*)	*tissue chopper*	-1,000µm: 168,330 ± 17,590; -500µm: 300,830 ± 111,460; -750 µm: 95,800 ± 31,660; -250µm: 197,500 ± 50,278	-	-	-
BUTCHER AND ULLMANN 1996	Gray opossum (*Monodelphis domestica*)	-	-	-	-	-
NATION AND SELWOOD 2006	Dunnart (*Sminthopsis macroura*)	-	-	-	-	-

RICHINGS et al., 2006	Tammar wallaby (*Macropus eugenii*)	27-gauge needles	-	-	4 days	16-22%
CZARNY et al., 2009	Dasyuridae (Fat-tailed dunnart (*Sminthopsis crassicaudata*) Eastern quoll (*Dasyurus viverrinus*) Northern quoll (*Dasyurus hallucatus*) Tasmanian devil (*Sarcophilus harrisii*))	0.1% collagenase and 0.02% DNAse, incubated at 35 ° C for 30 min	-	-	up to 2 days	-
XU et al., 2011	Rhesus macaques (*Macaca mulata*)	1% de HSA, 0.08 mg/ml de Liberase Blendzyme 3 and 0.2 mg/ml de DNase for 1 h	-	-	-	-
HORNICK et al., 2012	Rhesus macaques (*Macaca mulata*)	0.08 mg/ml Liberase Blendzyme 3 with 0.2 mg/ml DNase for 30 min	-	-	-	-

Regarding Australian marsupials, mechanical methods provided the isolation of large PFs (120-230 µm) from gray opossum (*Monodelphis domestica*).[8] Large secondary follicles from Tammar wallaby (*Macropus eugenii*),[9] and Dunnart (*Sminthopsis macroura*)[10] were also obtained, but information regarding follicle size and number was not indicated.

Due to the importance of non-human primates for biomedical research, techniques for PFs manipulation are better established for them than in any other wild species. Mechanical procedure for isolating PFs using tissue choppers allowed the recovery of 68.330 ± 17.590 PFs from 1/4 ovary in capuchin monkey (*Cebus apella*).[11] In Rhesus monkey (*Macaca mulata*), approximately 50-200 "healthy" secondary follicles (125-225 µm in diameter) can be mechanically isolated from the ovarian pair.[12] Primate ovarian tissues, however, present dense connective tissue, which renders isolation of individual follicles somewhat difficult without enzymatic treatments.[5] As a disadvantage, many PFs isolated by this procedure can present basement membrane or teak cells damage, especially when the incubation time is not strictly controlled.[13] Initial studies on Rhesus macaque[14] and baboon[15] indicate that PFs can survive and grow after collagenase treatment. Due to variation among animals, it was difficult to uniformly control the level of stromal digestion that many times led to secondary follicle damage during isolation.[13] Posteriorly, efficiency of enzymatic methods using association of other enzymes (1% de HSA, 0.08 mg/ml de Liberase Blendzyme 3 and 0.2 mg/ml de DNase for 1 h - 28; 0.08 mg/ml Liberase Blendzyme 3 with 0.2 mg/ml DNase for 30 min)[16] was described to obtain PFs from Rhesus macaques. In the latter case, authors defined that association of enzymes can be efficient to isolate their PFs without compromising the integrity of follicles, allowing availability of more than 500 follicles, ranging in size from 25.0 to 69.6 µm in diameter.[16]

Regarding others wild animals, PFs were recovered by enzymatic isolation from ovaries of Fat-tailed dunnart (*Sminthopsis crassicaudata*), Eastern quoll (*Dasyurus viverrinus*),

Northern quoll (*Dasyurus hallucatus*) and Tasmanian devil (*Sarcophilus harrisii*)) using 0.1 % collagenase and 0.02 % DNAse, incubated at 35° C for 30 min. Follicles obtained were capable of producing immature oocytes that survived after vitrification process, presenting a mean of 69.42 ± 2.44 % viable oocytes. Given these results, authors developed a follicle isolation protocol that will play a fundamental role in biobank formation aiming at conserving Dasyurid marsupials.[17]

Given the above, comparison of results between species should be analyzed with caution considering important aspects such as differences in methodology, age of animals used and species-specific peculiarities. Table 1 summarizes types of isolation and number of follicles obtained in different species of wild animals. Still, a challenge faced by authors is isolation of primordial follicles. This follicular category is important for fertility preservation purposes, because, although these follicles are most abundant and are present in females of all ages, they are difficult to study due to their small size and susceptibility to dissociation upon isolation.[16] Thus, developing an efficient mechanism to isolate large numbers of primordial and small primary follicles, or resting follicles it's a promising breakthrough that will contribute to the utilization of reproductive potential of rare species.

Cryopreservation

Many studies have been conducted for cryopreservation of PFs from different wild animals with the objective of establishing efficient protocols that allow the maintenance of female gamete viability and maintenance of germplasm banks.[18] Cryopreservation consists of preserving biological material at low temperatures, in most cases cryobiological temperature of liquid nitrogen (-196ºC), allowing cell or tissue remain viable for indefinite time, and can be recovered viable after thawing process.[2]

Because its numerous advantages, cryopreservation using ovarian tissue ensures preservation of female germ cells, firstly by ovaries containing many oocytes included in PFs and second, because these follicles are more resistant to cryoinjury. This resistance is due to the small size of the oocyte, low metabolic rate, stage of cell cycle stopped in prophase I, small amounts of lipids, cortical granules and support cells and absence or small development of zona pellucida.[19]

For wildlife, however, cryopreservation of ovarian tissue is still a challenge and protocols lack to be optimized, especially due to biological diversity between cell types that compound tissue.[18] To succeed in this biotechnology some key factors for cell survival must be considered, such as type and concentration of cryoprotective agents; rate of reduction of freezing temperature; choice of cryopreservation method (slow freezing or vitrification) and techniques used to ensure removal of cryoprotectant agent.[2]

Cryopreservation is performed by two main methods, slow freezing and vitrification (Table 2). Slow freezing, conventional method, is characterized by exposing cells or tissues to low concentrations of cryoprotectants (~ 1.5 mol / l)[2] for a period ranging from 20[20] to 60 min.[21] This method was successfully applied for cryopreservation of agoutis' (*Dasyprocta leporine*) ovarian tissue,[22] providing 64% of morphologically normal follicles after rewarming. Moreover, in deers (*Cervus elaphus hispanicu*),[23] kangaroos (*Macropus giganteus)*,[9] and monkeys,[24] ovarian tissue slow freezing was also able to maintain PFs viability. In wild felids from Asia, slow freezing using EG followed by *in vitro* culture allowed obtaining high rates of viable PFs from Amur leopard (*Panthera pardus orientalis*), black-footed cat (*Felis nigripes*), oncilla (*Leopardus tigrinus*), Geoffroy's cat (*Leopardus geoffroyi*), Northern Chinese leopard (*Panthera pardus japonensis*), rusty-spotted cat (*Prionailurus rubiginosus*), serval (*Leptailurus serval*) and Sumatran tiger (*Panthera tigris sumatrae*).[4] These results confirm that freezing ovarian tissue to form germplasm banks of these species is viable.

The vitrification technique consists of ultrafast reduction of temperature with an average speed of 20000 a 40000 ºC/min, using high concentrations of cryoprotectants, providing viscosity to vitrification solution, allowing cell or tissue to behave as an amorphous, uncrystallized solid, known as vitreous state in which parts of molecular chains are disorganized, promoting some molecular mobility.[2] Among its main advantages are limited formation of ice crystals due to rapid passage through critical states of temperature reduction (-15 to 5º C) and reduction of processing time. In general, it can be performed in any laboratory or even in the operating room, simultaneously patient/animal surgery, and even field, even immediately after death of animal.[25]

Regarding wild animals, it was recently demonstrated that is possible to preserve more than 70% morphologically normal PFs after ovarian tissue using a solid surface vitrification method in agoutis[3], peccaries (*Pecari tajacu*)[26], and *Spix's yellow*-toothed cavies (*Galea spixii*).[27] In non-human primates, association between ovarian tissue vitrification using combinations of glycerol, ethylene glycol, dimethylsulfoxide and synthetic polymers (polyvinylpyrrolidone – PVP) with *in vitro* culture of isolate secondary follicles preserved PFs morphology (52 ± 2 %) and function.[28] Moreover, vitrification of *Papio anub*is ovarian tissue followed by autotransplantation, resulted in high rates of survival (36 ± 46 % – primordial; 68 ± 86 % – primary; 24 ± 20 % – secondary; 9 ± 8 % antral), follicular growth and ovulation (as indicated by presence of corpus luteum),[29] similarly as reported for cynomolgus monkeys *(Macaca fascicularis).*[30]

Although vitrification of ovarian tissue cannot be considered as a routine procedure in reproductive medicine, some important advances have been attained in domestic (sheep, goats, cows, pigs and dogs) and exotic (dasyurids and macaque monkeys) animals (Table 2). Despite advances, cryopreservation of ovarian tissue is still a challenge, but its potential for biobanking genetic female material is incontestable.

In vitro culture

In vitro culture of PFs presents a unique challenge of mimicking dynamics of the ovarian environment, a methodical sequence of cell communication, and interaction with secretory, hormonal, and growth factors.[31] In recent years, attention has been devoted to the possibility of obtaining mature oocytes from the culture of frozen–thawed PFs. The development of a system that allows *in vitro* growth of PF follicles, resulting in oocytes capable of being matured and fertilized *in vitro* would be of great importance, it could contribute to long-term preservation of female germ cells and multiplication of endangered animals in the future.[2]

Culture systems

PFs can be cultured *in situ*, inserted into the entire ovary or into cortical fragments. This system is commonly utilized to culture primordial and primary follicles and it presents as advantages the practicality and speed, besides ensuring the maintenance of the three-dimensional architecture of follicle and preservation of interactions between follicular cells and between follicle and cells of surrounding stroma. However, cortical tissue may act as a barrier to medium perfusion to follicles.[31] The *in situ* culture system was described for baboon, in which primordial follicles within pieces of ovarian cortex can survive and develop to secondary stages in serum-free culture.[32] This type of *in vitro* culture has already been described for Amur leopard (*Panthera pardus orientalis*)[4] and Peccary (*Pecari tajacu*).[33]

Table 2. Cryopreservation of ovarian follicles derived from wild species using different methods.

Authors and year	*Species*	*Biotechiniques*	*CPAs*	*Results*
GUNASENA et al., 1998	African elephants and *(Loxodonta Africana*	Xenograft and cryopreservation of tissue ovarian	1.4 M of DMSO	Presence of primordial and antral follicles after xenograft
LIMA et al., 2012	Collared peccaries *(Pecari tajacu)*	Vitrification of tissue ovarian	3 M of DMSO, EG and dimethylformamide (DMF)	72% of morphologically normal follicles
WANDERLEY et al., 2012	Red-rumped agoutis *(Dasyprocta leporina)*	Slow freezing of tissue ovarian	1.5 M of DMSO, EG and PROH	DMSO: 60.64±3.64 EG: 64.00±11.88 PROH: 62.00±6.91
WIEDEMANN et al., 2013	African lions (*Panthera leo)*	Cryopreservation of tissue ovarian and *In vitro* culture	1.5 M of EG	Primordial: 24.46% Primary: 8.23% Secondary: 0.59%
TING et al., 2013	Nonhuman primates	Vitrification and *in vitro* culture vitrified secondary follicles isolated	Glycerol, EG, DMSO and synthetic polymers	52% of morphologically normal follicles after thawing and IVC
AMORIM et al., 2013	Baboon *(Papio anubis*)	Vitrification of tissue ovarian and autotransplantation	10% of DMSO and 26% of EG	Primordial: 36% Primary: 68% Secondary: 24% Antral: 9%
PRAXEDES et al., 2015	Spix's yellow-toothed cavy (*Galea spixii)*	Vitrification of tissue ovarian	3 M of DMSO	70% of morphologically normal follicles
PRAXEDES et al, 2018	Red-rumped agouti *(Dasyprocta leporina)*	Xenograft of tissue ovarian vitrified	3 M of DMSO and EG	Fresh control: 61,1% primordial; 14,4% transition; 15,6% primary Vitrified control: 41,7% primordial; 26,7% primary Xeno-fresh 63,6% primordial; 9,1% transition; 9,1% primary Xeno-vitrified: 40% primordial; 7,5% transition; 12,5% primary

On the other hand, isolated follicle culture system has been especially used for secondary follicles. This system allows a better perfusion of medium and individual monitoring of follicles during growth period, which favors study of substances involved in oocyte development, differentiation of granulosa cells and regulation of autocrine/paracrine factors that control folliculogenesis.[34] However, this culture system requires a longer time interval, a more sophisticated technique and can be affected by isolation procedures (Table 3). Moreover, smaller resting primordial and early growing primary follicles represent a larger follicle population than secondary follicles and efforts to grow and mature individual primordial and primary follicles *in vitro* were poorly reported. In macaque, primordial or primary follicles can be isolated and maintain their viability when cultured in groups.[16] Moreover, preliminary experiments conducted in rhesus macaques indicate that it is possible to grow individual primary follicles (80-120 μm in diameter) *in vitro* to small antral stages, which function in steroidogenesis, local factor production, and oocyte maturation.[35]

In 2D system, secondary follicles are cultured directly on plastic surface or even on extracellular matrix. In *monodelphis domestica*, this culture system was able to support follicle growth; however, no advancement to antral phase was verified.[8] Similar results were reported for *Tamar wallaby*, in which follicle diameter increased 16% during culture, but there were no signs of antrum formation.[9] Already, in marmosets it was possible to develop mature oocytes (metaphase II (MII)) from secondary follicles (> 85 μm) submitted to 2D system.[36]

In three-dimensional (3D) system, follicles are cultivated completely included in extracellular matrix, consequently follicles have no contact with the plate and do not adhere to it.[15,35] Biomaterials have been applied to 3D follicle culture, which maintain cell-cell and cell-matrix connections important in regulating follicle development *in vivo.* This system allows maintenance of tridimensional follicular structure and prevents occurrence of deformations during *in vitro* culture.[37]

Currently, knowledge on development of PFs in wild animals is more developed in non-human primates, and most of this knowledge has been obtained from studies with isolated secondary follicles cultivated in a 3D system. These animals receive attention due to their phylogenetic similarities with humans, which make them important experimental models for biomedical research.[38] Encapsulated 3D culture system was first implanted in rhesus monkeys, in which secondary follicles supported survival and increases in diameter under two hydrogel conditions (0.5% and 0.25% alginate) after 30 days of culture.[12] From this work, alginate application to non-human primates resulted in growth of small preantral follicles through antral stage with production of ovarian steroids and local factors, as well as oocyte maturation,[12,15,35] being posteriorly adapted for capuchin monkeys (*Sapajus apella*).[39]

To improve 3D-culture, secondary follicles from baboon were cultured in a fibrin-alginate matrix, grew to antral stage and their oocyte achieved meiotic maturation.[15] Subsequently, same results were obtained, in rhesus macaque (*Macaca mulatta*).[40,41] However, Xu et al.[40] demonstrated that while not altering follicle survival, fibrin-alginate improves macaque primary, but not secondary follicle development in terms of growth, steroidogenesis, anti-Müllerian hormone (AMH)/vascular endothelial growth factor (VEGF) production and oocyte maturation.

Table 3. *In vitro* culture system, duration, media and results achieved in wild animals.

Authors and year	*Species*	*In vitro culture system*	*Duration of in vitro culture*	*Used medium*	*Results achieved*
JEWGENOW et al., 1996	Puma (*Puma concolor*)	*In situ*	14 days	Dulbecco's MEM	62.5% viable follicles
WIEDEMANN et al., 2013	Amur leopard (*Panthera pardus orientalis*)	*In situ*	14 days	Leibovitz	The overall amount of follicles found in FCS cultured pieces (120 follicles)
XU et al., 2009	Rhesus monkeys	encapsulated 3-D culture system	≤ 30 days.	Alpha minimum essential	55 ±15% of follicles
XU et al., 2011	Baboon	encapsulated 3-D culture system	14 days	Alpha minimum essential	Thirteen oocytes exhibited GVBD within 48 h
XU et al., 2011	Rhesus macaques	encapsulated 3-D culture system	40 days.	Alpha minimum essential	Healthy (n=11) and mature (n=1) oocytes
RODRIGUES et al., 2015	Rhesus macaques	encapsulated 3-D culture system	40 days.	Alpha minimum essential	androgens promote pre-antral follicle development but inhibit antral follicle growth and function in primates.
BABA et al., 2017	Rhesus macaques	encapsulated 3-D culture system	5 weeks	Alpha minimum essential	antrum formation

Culture media

Regardless of system used, *in situ* or isolated, *in vitro* culture success of PFs is directly related to medium composition used. The basic medium is a source of nutrients responsible for maintaining normal metabolic activities of different cell types present in follicular culture. In wild animals, studies comparing the efficiency of basic culture media are scarce.

In collared peccaries, it was recently demonstrated that TCM-199 is more efficient than α-MEM to *in vitro* development of PFs during *in vitro* culture for 7 days.[33] Another study that supplementation with fetal bovine serum (10%) instead of bovine serum albumin (0.1%) during a 14-days culture improves the PFs development in the Amur leopard (*Panthera pardus orientalis*).[4] These results point for the need to establish ideal media according to specific requirements of each species.

Among the most commonly substances added to culture medium, FSH stands out due to its importance in folliculogenesis regulation in mammals. Positive effects in follicle growth in the marsupial *Monodelphis domestica* (1.0-1.5 IU of FSH mL-1),[8] in the follicle survival of rhesus monkeys (500 mIU/ml of rhFSH)[42] and in follicle development of peccaries (*Pecari tajacu*) (50 ng/mL of FSH)[33] were reported. In contrast, a negative effect on follicle health by disrupting the integrity of oocyte and cumulus cells contact was verified in Baboon when 10 or 100 mIU/ml of FSH was used.[15,35]

The effects of growth factors as bone morphogenetic protein 4 (BMP4) at improving *in vitro* PFs development was demonstrated in capuchin monkeys (*Sapajus apella*). Moreover, the addition of mare serum gonadotrophin to culture media promoted positive effects on growth and survival of primate PFs.[39]

In Rhesus macaque, the presence of bovine fetuin in culture media associated to 5% of O_2 promoted high PFs survival rate, high percentage of healthy oocytes and later a higher maturation rate.[15,35] Moreover, association of FSH concentration and O_2 tension positively

influenced antral formation and production of vascular endothelial growth factor-A (VEGF-A) in primate PFs.[43]

Addiction of androgens (testosterone and dihydrotestosterone) to culture medium altered follicle survival, growth, steroid and anti-Müllerian hormone (AMH) production, and oocyte quality *in vitro*, in a dose- and stage-dependent manner in non-human primates. While androgens can act positively, excess levels of androgens may have negative impacts on primate folliculogenesis.[44] According to Ting and Stouffer[45], androgens appear to be a survival factor but hinder antral follicle differentiation, estrogen appears to be a survival and growth factor at pre-antral and early antral stage, whereas progesterone may not be essential during early folliculogenesis in primates. However, their growth-promoting effects are limited to preantral to early antral stages.[46] These authors reported that primate follicles pool is heterogeneous and differs between animals, therefore, even though only secondary follicles were selected, follicle growth and developmental outcomes might differ from one animal to another.

Addition of 25 pg/mL of 1,25-dihydroxy vitamin D3 (VD3) increased PFs survival and maintained anti-Mullerian production by antral follicles, while 100 pg/mL of VD3 improved antral follicle growth. Soon, VD3 supplement promoted oocyte growth in *in vitro*–developed follicles, suggesting that direct actions of VD3 on primate follicles appear to be both dose and stage dependent.[41]

The goal is to increase efficiency and reproducibility of producing mature, competent oocytes by PFs in vitro culture. For this it is necessary to continue research to better clarify factors involved in initial phase of folliculogenesis to define a culture system that provides oocytes suitable for application in other biotechnology, enhancing reproductive performance of rare genotypes and contributing to preservation of species.

In vivo culture

Ovarian tissue transplantation has been developed as a promising alternative.[47] Results of xenotransplantation to immunosuppressed rodents varied from activation of primordial follicles, followed by growth until the early antral stage in several species as marmosets,[21,48] elephants,[49] wallabies,[50] wombats,[51] lioness,[52] and baboons.[29] However, maturation of oocytes obtained from transplanted ovarian tissue has not yet been reported. Thus, fertility preservation using ovarian tissue banks and transplantation is still in the experimental phase and is being slowly adapted to several groups of wild mammals such as felines,[52] and non-human primates[29]. In wild rodents as agoutis, efficiency of ovarian tissue after vitrification was recently demonstrated through the confirmation of ovarian activity return after 40 days of xenotransplantation of fresh and vitrified ovaries fragments to ovariectomized SCID mice.[3] In fact, the main challenge is to establish the best technique to be used (allotransplantation or xenotransplantation), the size of the fragment to be used, the site of transplantation and especially the maturation of transplanted oocytes according to species studied.[2]

Final considerations

Initial studies related to the recovery of primordial and primary follicles have been conducted on few wild species and results are promising to improve culture systems that promote the development of these follicles. In relation to cryopreservation, protocols developed to date are efficient for storing many PFs in some species of wild animals, and although it is necessary to improve protocols, in practice they can already be applied to the formation of germplasm banks. In this sense, use of techniques associated with transplants, as well as a study on reproductive physiology of each species, can assume a primordial role in the advancement and improvement of protocols already developed, with positive effects on embryo development rates.

In addition to the lack of knowledge about reproductive physiology in wild species, there are still few studies on the development of PFs. Improvement of protocols developed until then to allow the recovery PFs and their cryopreservation and *in vitro* development can be very useful for preservation of rare species, as well as the creation of female germplasm banks.

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Capítulo II - Vitrification of collared peccaries' ovarian tissue using open or closed systems associated to different cryoprotectants

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Artigo a ser submetido Cryobiology

Qualis B1

Abstract

It was evaluate the effect of different vitrification methods using distinct cryoprotectants on the preservation of peccary preantral follicles. Ovarian pairs (n = 6) were divided into fragments destined to fresh analysis or vitrification through solid surface (SSV) or using the ovarian tissue cryosystem (OTC) with 3 M ethylene glycol (EG), 3 M dimethyl lsulfoxide (DMSO) or the combination 1.5 M EG plus 1.5 M DMSO. The preantral follicles (PFs) were evaluated for morphology, viability, cell proliferation and apoptosis through the analysis of caspase-3. After vitrification, all treatments maintained the proportion of normal PFs similar to control group (75.6 ± 8.6%), except ($P < 0.05$) those vitrified by SSV with EG (52.8 ± 5.9%) or the combination of CPAs (54.5 ± 10.4%). Regarding viability or cell proliferation, all the tested groups were similar. Regarding apoptosis, only the samples processed through SSV with EG (43.4%) or CPAs combination (33.4%), as well as those vitrified in OTC with EG (46.7%), provided similar values as to for fresh control group (36.7%). In conclusion, we suggest the use of a closed system, the OTC, associated with 3 M EG as the cryoprotectant for the vitrification of collared peccary ovarian tissue.

Keywords: follicles, vitrification, etigenoglicol

1. Introduction

It is known that the formation of germplasm biobanks for mammalian species such as peccary is of great importance for the conservation of the species. Even if globally considered as a stable population, poaching and deforestation are reducing the population of collared peccaries (*Pecari tajacu*) and they are already extinct in some biomes as the Atlantic Forest and Caatinga (Gongora et al. 2011). Due to its ecological importance as seed dispersers (Morales et al. 2015) and as a part of food chains for great carnivores (Cavalcanti and Geese, 2010), the need for the development of conservative strategies for the species is eminent. Besides the

conservation of peccaries in their natural habitats or even under captivity, the formation of biobanks arises as an alternative to safeguard their valuable biological material (Silva et al. 2017).

For this purpose, there is only one study demonstrating the possibility of preserving peccaries' ovarian tissue fragments through a solid surface vitrification (SSV) method using ethylene glycol (EG), dimethyl sulfoxide (DMSO) or dimethyl formamide as cryoprotective agents (CPAs) (Lima et al. 2012). In such study, even if SSV was proven to provide a large amount (~70%) of morphologically intact preantral follicles (PFs) after tissue rewarming of peccaries (Lima et al. 2012). Despite being used for the preservation of female germplasm of domestic (Carvalho et al. 2011) and wild (Praxedes et al. 2015, 2017) species, SSV is considered an open system that allows the contact between the sample and the nitrogen vapors, which could expose tissue to cryogenic resistant pathogens (Grout and Morris, 2009). Still, protocols need to be improved and more practical techniques are required.

In this context, the ovarian tissue cryosystem (OTC) raised as an alternative closed system efficiently proven for the preservation of ovarian tissue derived from goats (Faustino et al., 2014), sheep (Bandeira et al. 2015) and cats (Brito et al. 2018). Using the OTC, the cryoprotectant exposure and removal is easily performed, without any direct contact of the operator with the vitrification solution, and no need of cryovials or straws (Carvalho et al., 2013). Regardless the vitrification method used, high concentrations of CPAs and cooling rates are required, which can cause fatal cell damage and osmotic stress (Zhang et al. 2009). To reverse this problem, the combination of two types of CPAS was proposed as being less toxic and more effective than the isolated use, as demonstrated for the swine (Moniruzzaman et al. 2009), the domestic species more closely related to the peccaries.

Yet, PFs detailed analysis at this point would be largely requested, mainly because there are controversial findings indicating that vitrification may result in the emergence of apoptosis (Jafarabadi et al. 2015; Sheikhi et al. 2011) or not (Abdollahi et al. 2013).

Based on the above mentioned, the aim of this study was to compare different vitrification methods (VSS and OTC) associated to the use of different CPAs (EG and DMSO), alone or in combination, on the morphology, viability, cell proliferation and expression of markers for apoptosis in peccary ovarian PFs.

2. Material and Methods

The ethics committee of the UFERSA approved the experimental protocols as well as the animal care procedures used (Process no. 23091.006525/2016-82). The study was authorized by the Chico Mendes Institute for Biodiversity (SISBio Process no. 37329). All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1 Animals and collection of ovaries

The ovaries were obtained from six mature females, aging 2 years and weighting 22 kg, from the Centre for Multiplication of Wild Animals from UFERSA (Mossoró, RN, Brazil; 5°10'S, 37°10'W). Such females were subjected to the programmed slaughter that is conducted every year for population control at the Centre, being the carcasses destined to various experiments. After euthanasia of animals, ovaries (n=12) were washed twice in 70% alcohol and TCM-Hepes, and then transported to laboratory in the same medium.

2.2 Experimental design

The pairs of ovaries (n=6) were divided into 21 fragments (3 mm x 3mm x 1mm). For the fresh control group, two fragments were immediately fixed in Carnoy and destined to histological procedures for morphological evaluation; other fragment was fixed in paraformaldehyde for later immunohistochemical analysis to identify activated caspase-3 expression and assessment of cell proliferation; another one was subject to the isolation protocol to evaluate follicle viability. The other fragments were vitrified and further analyzed.

2.3 Ovarian tissue vitrification

For SSV (Lima et al. 2012), a vitrification solution composed of MEM supplemented with sucrose, 10% fetal bovine serum (FBS) and CPAs was used. As experimental groups, DMSO and EG were tested individually at 3M concentration, or in combination (DMSO 1,5 M plus EG 1,5 M). The ovarian fragments were exposed to vitrification solution for 5 min and placed on a metal cube surface partially immersed in liquid nitrogen (LN_2) for vitrification. Once vitrified, the sample was transferred to cryovials for storage in LN_2 (at -196°C).

The vitrification using the OTC followed the same procedure described for the SSV, however the entire process of exposure to the CPAs was conducted inside the OTC and then the solution was removed, the device closed and transferred to the LN_2 (Carvalho et al. 2013).

For both methods, the fragments remained stored for two weeks before being rewarmed at room temperature (25 °C) for 1 min, and immersed in a water bath at 37 °C for 5 s. The CPAs were removed by 3 consecutive washes of 5 min in MEM supplemented with 10% FCS and decreasing concentrations of sucrose (0.50 M, 0.25 M, and 0.0 M sucrose, respectively) (Lima et al. 2012). After the procedure, the fragments were analyzed.

2.4 PFs morphological analysis

After fixation in Carnoy for 12 h, the ovaries from the control and vitrified groups were dehydrated in a graded series ethanol (70, 80, 95 and 100%), clarified with xylene, embedded in paraffin wax and serially sectioned at 7 µm. Every 5th section was mounted on glass slides that were stained with hematoxylin-eosin and evaluated by light microscopy at a 400x magnification. The PFs developmental stages were classified as primordial, primary, or secondary (Silva et al. 2004).

The PFs were also identified and classified by light microscopy (400x) according to their integrity. Follicular morphology was evaluated based on the integrity of the oocyte, granulosa cells and basement membrane. PFs were classified and counted as morphologically normal, when containing an oocyte with regular shape and uniform cytoplasm, and organized layers of granulosa cells; or degenerated, when the oocyte exhibited pycnotic nucleus and/or ooplasma shrinkage, and occasionally granulosa cell layers became disorganized, detached from the basement membrane and/or included enlarged cells. The PFs were analyzed only in the sections where oocyte nucleus was and 30 follicles were counted per treatment (Silva et al. 2004).

2.5 PFs viability analysis

Fresh and vitrified fragments were subjected to the mechanical procedure for follicular isolation. To this purpose, the fragments were placed in Petri dish and submitted to dissociation with a scalpel blade. After agitation, the suspension was filtered through 500 µm nylon mesh filter and posteriorly, it was centrifuged at 40g for 10 minutes and evaluated with trypan blue (0.4 %) (Sigma Chemical Co., St. Louis, MO, USA). After 5 min, samples were evaluated under inverted microscopy and PFs were classified as viable when the oocyte and <10% of granulosa cells remained unstained or as non- viable when the oocyte and/or >10% of granulosa cells were stained (Amorim et al. 2003) and 30 follicles were counted per treatment.

2.6 Assessment of cell proliferation

The proliferation rate of follicular cells in all treatments was evaluated by quantifying the number of argyrophilic (Ag) nucleolus organizer regions (NORs) as previously reported for the species (Lima et al. 2018). The ovarian tissue fragments were fixed in 4% paraformaldehyde and subject to conventional histological processing. The blocks were then, the blocks were sectioned at 5μm and the sections were immersed in 1% potassium metabisulfite (3 min). The slides were impregnated with silver nitrate (2:1 colloidal solution) in a darkroom (30 min) and then placed in a solution of sodium metabisulfite (3 min) and sodium thiosulphate (3 min) to allow impregnation of the silver nitrate. To quantify the NORs, 30 developing follicles of each treatment were visualized under a light microscope (1000x) and counted NORs of all nuclei of visible granulosa cells.

2.7 Immunolocalization of Active Caspase-3

Immunohistochemistry was performed according to Barberino et al. (2017), with some modifications. Ovarian fragments from fresh and vitrified groups were fixed in paraformaldehyde. After 12 h of fixation, the ovarian tissue was dehydrated with increasing concentrations of ethanol, clarified in xylene, and embedded in paraffin. Sections (3 μm thick) from each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted in Starfrost glass slides (Knittel, Braunschweig, Germany). The slides were incubated in citrate buffer (Dinamica) at 95°C in a deckloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with H_2O_2 (EasyPath) for 10 min. Nonspecific binding sites were blocked using 10% normal goat serum (EasyPath) Subsequently, the sections were incubated in a humidified chamber for 55 min at room temperature with polyclonal rabbit anti- activated caspase-3 (1:150; Santa Cruz

Biotechnology, Santa Cruz, CA, USA). Thereafter, the sections were incubated for 20 min with EasyLink One-polymer (EasyPath). Protein localization was demonstrated with diaminobenzidine (DAB; EasyPath), and the sections were counterstained with hematoxylin (Vetec, Sao Paulo, Brazil) for 1 min. Negative controls (reaction control) were performed in the absence of the rabbit polyclonal anti- activated caspase-3. Sections were examined using the optical microscope and the follicles containing positively stained cells (granulosa cells/oocyte) for activated caspase-3 were counted manually (Desmeules and Devine, 2006).

2.8 Statistical analysis

Data were expressed as mean and standard error of means (SEM) and subjected to Smirnov–Kolmogorov and Bartlett tests to confirm normal distribution and homogeneity of variance, respectively. For the morphology and cell proliferation assessments, comparisons between groups (fresh control versus vitrified by SSV or OTC using different CPAs) were conducted by one-way variance analysis (ANOVA) followed by the Fisher's PLSD test. For the analysis of viability and caspase-3 expression, data were taken as a pool and analyzed by dispersion of frequency with Chi-square test. Values were considered statistically significant at $P < 0.05$.

3. Results

A total of 1260 PFs were analyzed (~ 180 per treatment). Overall, 75.6% morphologically normal PFs were found in the fresh group. After vitrification, all treatments maintained the proportion of normal PFs similar to the control group, except those vitrified by SSV with EG or the combination of CPAs, in which a reduction on the percentage of morphologically normal PFs was evident ($P < 0.05$) (Table 1).

Through histological analysis, peccaries' PFs showed spherical oocyte, with the nuclei occupying most of the cytoplasm and positioned centrally or eccentrically in the oocyte. The granulosa cells were well organized around the oocyte, forming concentric layers. These features of morphologically normal PFs were found both in fresh control (Fig. 1A) and vitrified (Fig. 1B and 1C) groups, regardless of the method and cryoprotectant used. After the vitrification process, the main changes observed were the oocyte retraction, detachment of granulosa cells from the basement membrane and pyknotic oocyte nuclei (Fig. 1D) in all treated groups.

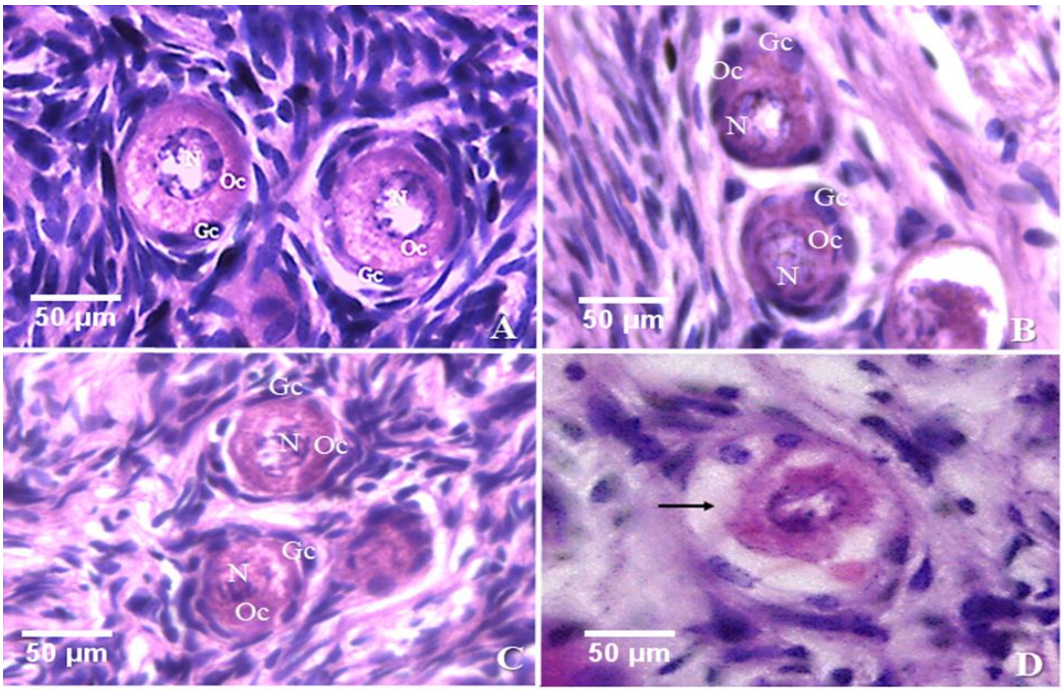

Fig. 1. Histological features of collared peccaries ovarian preantral follicles. Normal follicles from control group (A), after vitrification in SSV (B) and OTC (C) exhibited oocyte with homogenous cytoplasm (Oc), central nucleus (N) and intact and organized granulosa cells (GC). Atretic follicle (D) showed cytoplasmic retraction (arrow) or disorganization of granulosa cells.

Table 1 Percentage (mean ± S.E.M.) for normal morphology, viability, and cell proliferation in collared peccary preantral follicles subjected or not (fresh control) to vitrification by the solid surface method (SSV) or using the ovarian tissue cryosystem (OTC) with the cryoprotectants as ethylene glycol (EG) or dimethyl sulfoxide (DMSO), alone or in combination (EG+DMSO), n= 30 follicles/ treatments.

Treatments		Morphology (%)	Viability (%)	Cell proliferation (Number of NORs*)
Fresh control		75.6 ± 8.6[a]	84,0 ± 2,9	2.0 ± 0.2
SSV	EG	52.8 ± 5.9[b]	79,0 ± 5,3	2.1 ± 0.2
	DMSO	63.9 ± 7.6[ab]	82,0 ± 2,9	1.9 ± 0.1
	EG+DMSO	54.5 ± 10.4[b]	79,3 ± 3,2	2.0 ± 0.2
OTC	EG	67.8 ± 6.8[ab]	79,0 ± 4,3	2.0 ± 0.2
	DMSO	58.3 ± 8.7[ab]	78,0 ± 2,1	2.0 ± 0.1
	EG+DMSO	64.5 ± 7.7[ab]	77,0 ± 1,0	2.0 ± 0.1

[a,b] Superscript lowercase letters indicate significant differences among treatments ($P < 0.05$).

Regarding viability (Table 1), the percentage of viable PFs in the control group was 84%. After vitrification, all the tested groups were able to maintain the peccaries' PFs viability, with values varying from 77 to 82%. Furthermore, in the vitreous samples regardless of the treatment, the samples maintained cell proliferation ($P < 0.05$) (Table 1).

None of the negative controls (Fig. 2A) showed any immunoreactivity for active caspase-3, while the signal-positive active caspase-3 marked was observed in the positive controls (Fig. 2 C, D, E, F, G and H). Also, the healthy (primordial, primary and secondary) follicles were determined as active caspase-3 negative for both the control group (Fig. 2 B) and vitrified groups.

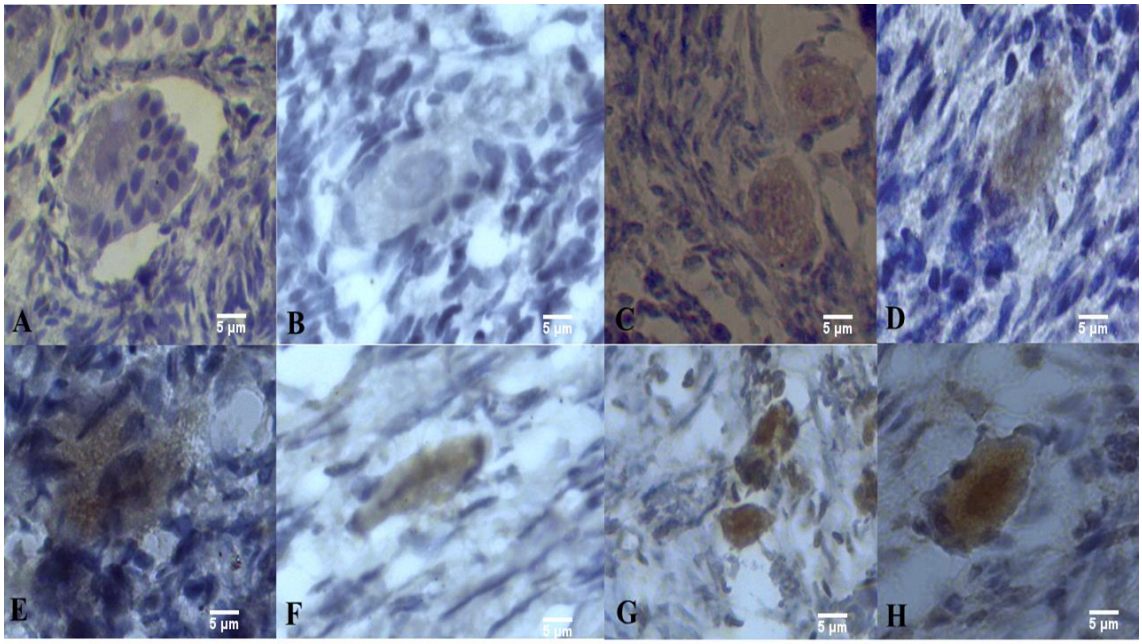

Fig. 2. Immunohistochemical analysis for the expression of activated caspase-3 in collared peccary ovarian preantral follicles. Folicles not marked with caspase-3 activated in the negative control (A) and fresh control group (B). Folicules marked with caspase-3 activated in the groups vitrified through solid surface method using ethylene glycol (C), dimethyl sulfoxide (D) or its combination (E); Groups vitrified through the ovarian tissue cryosystem with ethylene glycol (F), dimethyl sulfoxide (G) or its combination (H).

Only follicles with visible oocyte nuclei were analyzed. Moreover, a total of 36.7% PFs were marked for activated caspase-3 in the fresh control group. After vitrification, only the samples processed through SSV with EG (43.4%) or CPAs combination (33.4%), as well as those vitrified in OTC with EG (46.7%), provided similar values as those found for fresh control group (Fig. 3).

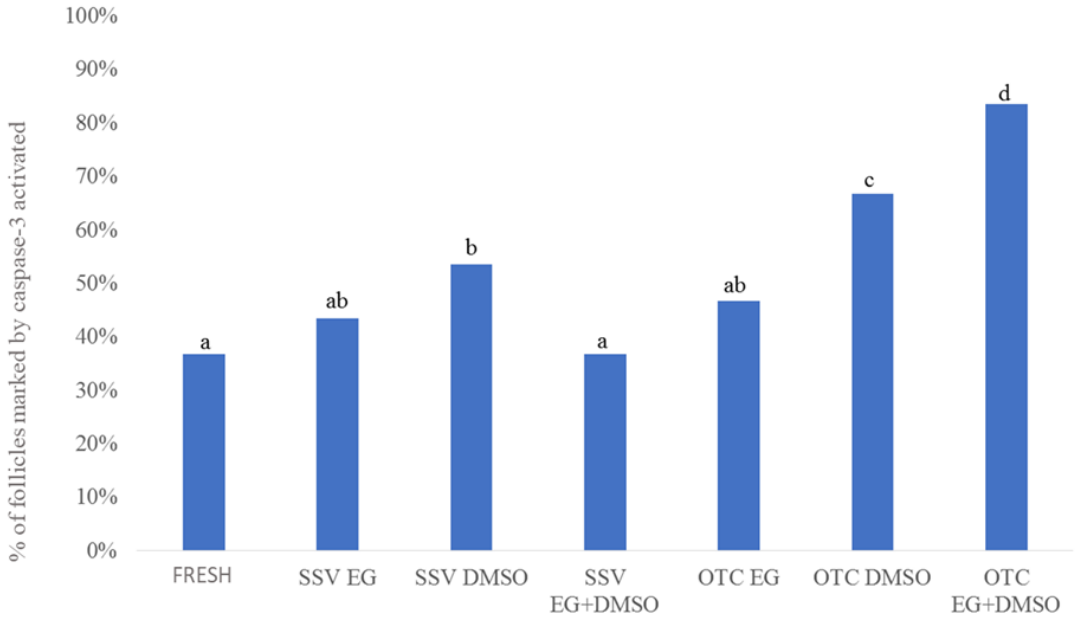

Fig. 3. Percentage apoptotic of collared peccary preantral follicles in the fresh control group and after vitrification through solid surface method or ovarian tissue cryosystem using different cryoprotective agents (EG, DMSO and EG + DMSO).

4. Discussion

The biobank formation based in female germplasm allows the storage of many PFs for long periods and facilitates the transport of genetic material among different reproductive centers (Bao et al. 2010). In this context, this study represents an important step on the

preservation of collared peccaries and related endangered species, as the Chacoan peccary (*Catagonus wagneri*, Altrichter et al. 2015), since it highlights the possibility of safeguard its female genetic material under different vitrification methods for an undetermined time.

The morphological examination of the peccary ovarian tissue showed the possibility of preserving more than 50% of the total PFs population when the samples were vitrified by both opened or closed systems. In fact, results reached with OTC were similar to those reported for goats (58.1% - Carvalho et al. 2013), but even higher than those verified for sheep (30.66% - Bandeira et al. 2015) and cats (37% - Brito et al. 2018). It is noteworthy that in the vitrification with OTC, the whole procedure of exposure and removal of the cryoprotective agent is carried out inside the device making the technique more practical and faster than other vitrification methods. In addition, the fragments do not come into contact with liquid nitrogen, thus avoiding contamination of the samples (Carvalho et al. 2013). In parallel, the SSV method can provide extremely fast rates of cooling due to heat exchange through direct contact with liquid nitrogen cooled surface (Xing et al. 2010), being also considered as a low-cost method (Santos et al. 2007).

Necessary to highlight that, in comparison to the fresh control group, OTC provided an efficient preservation of PFs morphology at using any of the tested cryoprotectants. Similarly, both EG or DMSO are the cryoprotectants usually suggested for the use of OTC in goats (Carvalho et al. 2013a), sheep (Bandeira et al. 2015) and cats (Brito et al. al. 2018). On the other hand, SSV was only efficient at preserving PFs morphology with values similar to the fresh control group in the use of DMSO. This cryoprotectant was efficient in sheep (Pinto et al. 2008), mice (Kim et al. 2010), goat (Castro et al. 2011), it is worth noting that DMSO interacts with the membranes, crossing them rapidly through diffusion. Furthermore, both DMSO and its metabolites have low toxic potential (Aye et al. 2010).

According to Fauque et al. (2007), the viability test using trypan blue vital dye should be routinely used as a quality control method for cryopreservation of ovarian tissue. At this sense, viability analysis revealed that both vitrification methods can provide a high rate of viable follicles (ranging from 77 to 82% viable PFs), regardless of cryoprotectant used. It confirms that both methods did not cause rupture of the cell membrane. In fact, both vitrification methods used viscous and high concentrated cryoprotectant solutions that presented enough permeation capacity into the ovarian tissue, thus reducing osmotic stress. This fact, associated to the abrupt drop in temperature, causes a direct passage of the media from a liquid to an amorphous state, without the formation of ice crystals, allowing success for follicular conservation (Santos et al. 2007)

Regardless the method or cryoprotectant used, the cell proliferation activity was adequately preserved after vitrification of peccary PFs as confirmed by the Ag-NORs assay. It is known that NORs are segments of DNA that transcribe ribosomal RNA which, in turn, is later translated into protein, forming ribosomes (Brar and Weissman, 2015). For this reason, NORs are directly related to cell proliferation, so the higher the cell proliferative activity, the greater the number of NORs observed (Gossler et al. 2017). In this sense, the vitrified tissue presented the same proliferation capacity as the fresh tissue, since the number of NORs among the treatments was similar. indicating that the proliferative capacity remained active even after the vitrification process. Similar results were observed in donkeys, in which authors found 1.79 ± 1.4 NORs/cell in the fresh control group and 1.89 ± 0.7 NORs/cell in samples vitrified through SSV with a combination of EG and DMSO (Lopes et al. 2018).

Besides using a conventional approach at analyzing aspects as PFs morphology, viability and cell proliferation, this study was pioneer at evaluating the incidence of apoptosis in peccary ovarian tissue after vitrification. Caspases have a central role as an indicator of apoptosis in the intrinsic and extrinsic pathways (Hussein, 2005), especially the caspase-3, which is usually

related to follicle atresia (Johnson and Bridgham, 2002). In fact, this parameter was of fundamental importance to stablish differences regarding the efficiency of the tested treatments. Based on this premise, we evidenced that SSV with EG or CPAs combination, as well as OTC in the use of EG, can preserve the peccary PFs by providing values for the expression of activated caspase-3 similar as those observed for the fresh control group. The increase in apoptosis can probably be caused by physical changes during vitrification and that apoptosis can lead to poor growth and development of follicular vitrified. Necessary to highlight, however, that the use of SSV at these conditions provoked an increase on morphological damage of peccary PFs. Therefore, it is evident the efficiency of OTC at using EG as cryoprotectant for both conserving morphology and preventing apoptosis in peccary PFs.

As in the peccaries, the effectiveness of EG at providing low rates of apoptosis induction after ovary vitrification was previously reported for humans (Abdollahi et al. 2013), murids (Abdi et al. 2015; Jafarabadi et al. 2015), and equine (Gastal et al. 2017). It is known that EG presents a lower molecular weight (62.07 g / mol) than DMSO (78.13 g / mol), which facilitates its penetration into the tissues. Moreover, EG presents a lower toxicity when compared to other CPAs, which may contribute for the PFs preservation after vitrification (Bautista and Kanagawa, 1998). Moreover, it has been shown that DMSO presents a considerable toxicity effect on ovarian cell morphology (Best et al. 2015; Li et al. 2016), but the mechanism by which the toxicity effects of CPAs trigger the activation of apoptotic process in ovarian cells is unclear (Gastal et al. 2017). It is known that DMSO promotes an increase in membrane permemabilidde (Anchardaguy et al., 2002), in which it may have allowed greater entry of EG which may be caused to induce apoptosis.

In conclusion, we indicate the use of a closed system, the OTC, associated with 3 M EG as the cryoprotectant for the vitrification of collared peccary ovarian tissue. The use of this new device allows the preservation of the species and the formation of a germplasm bank, which

will be an important tool for the conservation strategies of both collared peccary and other endangered.

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Capítulo III - Role of growth differentiation factor 9 (GDF-9) in collared peccary folliculogenesis

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Abstract

The aims were to evaluate the expression of growth differentiation factor 9 (GDF-9) receptors in the ovarian cortex and to identify the effects of this factor on the *in vitro* development of preantral follicles (PFs) from collared peccaries. Ovarian fragments were subjected to PCR analysis and were *in vitro* cultured for 1 or 7 days with GDF-9 (0, 50, 100, or 200 ng/mL). The non-cultured (control) and cultured fragments were evaluated for PF survival, activation, viability, and cell proliferation. The PCR analyses identified the expression of BMPR2 (type I receptor) and ALK-5 (type II receptor) in the ovaries. Although there were no differences in the percentage of morphologically normal follicles, only follicles cultured for 7 days with 200 ng/mL GDF-9 had follicular and oocyte diameters that were similar to those observed on the first day ($P < 0.05$). The percentage of growing follicles significantly increased compared to the control in all treatments, especially those cultured with 200 ng/mL GDF-9 for 7 days ($P < 0.05$). In addition, the presence of GDF-9 did not interfere with PF viability ($P > 0.05$), but 200 ng/mL of GDF-9 improved cell proliferation in PFs cultured for 1 or 7 days. In conclusion, we have demonstrated, for the first time, the presence of BMPR2 and ALK-5 receptors for GDF-9 in collared peccary ovaries, and showed that GDF-9, especially at a 200 ng/mL, was actively involved in the *in vitro* development of collared peccary PFs.

Introduction

The collared peccaries (*Pecari tajacu* Linnaeus, 1759) are mammals found in the Americas that play an important role in ecosystems because they act as seed dispersers and as a prey for large carnivores. However, continuous habitat destruction and excessive poaching is reducing collared peccary populations as eastern and southern Argentina (Gongora *et al.* 2013), which means that conservation strategies need to be developed. It was established a protocol for the *in vitro* culture of its ovarian tissue, and demonstrated that preantral follicles (PFs) can activate

and develop after 7 days of culture in TCM199, wich extracellular matrix is positively influenced by the presence of follicle stimulating hormone (FSH) (Lima *et al*. 2017), but it is necessary to observe the influence of other factors as growth differentiation factor 9 (GDF-9). which has already been described in other domestic (Silva *et al*. 2005) and wild mammals (Eckery *et al.* 2002) and the results showed that GDF-9 can affect PF development.

The GDF-9 is expressed and secreted by the oocyte (Chang *et al.* 2002) and acts on the ovary in an autocrine and paracrine manner after binding to specific TGF-β type I (ALK 5) and type II receptors (BMPRII) (Vitt *et al.* 2002). In swine, the same suborder of the collared peccaries, the *in vivo* injection of GDF-9 gene into the ovary promoted follicular activation and growth (Shimizu 2006). Furthermore, GDF-9 has been shown to be expressed by porcine ovaries, especially by the oocyte (Lee *et al.* 2008). It affects *cumulus* expansion and increases the mRNA levels of maternal genes in porcine oocytes during *in vitro* maturation (Lin *et al.* 2013). In view of the above, since it presents promissory results in the pigs, it could present good results also in peccaries

The scientific and ecological importance of collared peccaries along with the effect of GDF-9 in follicular development in other species led us to evaluate the expression of GDF-9 receptors in ovarian cortical tissue and to identify the effects of GDF-9 on its PF *in vitro* development.

Material and methods

Experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of the Semi-arid Region, Brazil (no. 23091.006525/2016-82) and the Chico Mendes Institute for Biodiversity Conservation, Brazil (no. 37329). Unless otherwise indicated, all chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Promega (Madison, WI, USA), and Thermo Fisher Scientific (Waltham, MA, USA).

Source of the ovaries: Ovaries (n = 12) were collected from adult collared peccaries (2 years old and weighing 22 kg) that were part of the annual programmed slaughter conducted by the Centre of Multiplication of Wild Animals (Mossoró, RN, Brazil; 5°10'S, 37°10'W). The ovaries were washed twice in 70% alcohol and minimum essential medium (MEM). They were then transported to laboratory in MEM and kept for 10 min at 27ºC. In the laboratory, the ovaries were fragmented (3 mm x 3 mm x 1 mm) and then used in the experiments.

Experiment 1: Detection of GDF-9 receptors in ovarian cortical tissue: Isolation of total RNA was performed using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA). Following the manufacturer's instructions, the ovarian fragments were incubated in lysis buffer and 1-thioglycerol buffer, and mixed with 100% isopropanol. An RNA wash solution was added to the mixture and the solution was transferred to a minicolumn coupled with a collection tube so that the RNA could bind to the column. The RNA purification process lasted for 15 min at 25°C, and included RNAse-free and DNase-free treatments. After three washes, the RNA was eluted with RNAse-free water.

Reverse transcription was achieved by adding the following to each 1 µg RNA sample: 1 µL of oligo-dT primer (20 µM) and RNase-free water to make a final volume of 5 µL. The samples were heated to 70°C for 5 min and then placed on ice for 2 min. Following this, 5×Improm II reaction buffer (4 µL; 1×), Improm II reverse transcriptase (1 µL), dNTPs (1 µL, 0.5 mM of each), recombinant RNasin (0.5 µL; 1 U/µL), and $MgCl_2$ (2.4 µL; 3 mM) were added to the mixture. Then RNasefree water was added the mixture to make a final reaction volume of 15 µL. Reverse transcription was performed at 42°C for 60 min and then at 70°C for 15 min. The cDNA products were stored at –80°C until they were used as templates in the RT-PCR procedure.

Transforming Growth Factor Beta Receptor 1 (ALK5), Bone Morphogenetic Protein Receptor Type 2 (BMPR2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were detected using a MasterCycler ep Gradient S (Eppendorf, Hamburg, Germany). The reaction mixture (25 μL total volume) contained the following: 2 × GoTaq® Hot Start Colorless Master Mix (12.5 μL), each primer (2.5 μL; 1 μM, Table 1), and cDNA (1 μL). Template cDNAs were denatured at 94°C for 2 min and the genes were amplified by 30 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min plus a final extension of 8 min at 72°C. The amplified product was subjected to 1% agarose gel electrophoresis using a 100 bp DNA ladder as a reference for fragment size. They were stained with 0.5 μg/mL of ethidium bromide and visualized with a UV transilluminator.

Table 1. Primers used in the real-time PCR analysis of gene detection in collared peccary ovarian cortical tissue.

Gene symbol	**Nucleotide Sequence (5'-3')**	**Product size (bp)**	**GenBank accession number**
ALK5	TCACCATCGAGTGCCAAATGA	468	NM_001038639.1
	GCTGTGGACAGAGCAAGTTT		
BMPR2	ACACCACTCAGTCCACCTCA	499	NM_001204900.1
	GGCAAGAGCTTACCCAGTCA		
GAPDH	TTCCACGGCACAGTCAAGG	394	AJ431207.1
	TCTTCTGGGTGGCAGTGATG		

Specific primers were designed to amplify BMPR2 and ALK5 genes based on swine sequence. Specific primers were designed to amplify *GAPDH* gene based on goat sequence.

Experiment 2: Effects of GDF-9 on preantral follicular development: The ovarian fragments were cultured for 1 day to confirm their PF viability, and for 7 days to evaluate PF development (Lima *et al.* 2018). The fragments were transferred to 24-well culture dishes containing 1.0 mL of TCM199 supplemented with 10 μg/mL insulin, 5.5 μg/mL transferrin, 5.0 ng/mL selenium, 0.23 mM pyruvate, 2.0 mM glutamine, 2.0 mM hypoxantine, and 1.25 mg/mL bovine serum albumin (BSA). The samples were then cultured at 38.5°C and 5% CO_2 in a humidified incubator. The TCM199 was supplemented with different concentrations of GDF-9 (0, 50, 100, or 200 ng/mL), according to Wang and Roy 2004, Martins *et al.* 2008. Each treatment was repeated six times, and the culture medium was replaced every other day.

The non-cultured and cultured fragments were fixed in Carnoy solution for 12 h, dehydrated in an ethanol graduated series, clarified with xylene, embededed in paraffin wax and cut into 7.0 μm sections. Every 5th section was mounted on glass slides that were stained with haematoxylin–eosin. Follicle development stage and survival were assessed microscopically (×400) on serial sections. The follicles were individually classified as morphologically normal or degenerated, and were categorized as primordial or growing follicles (Lima *et al.* 2013). The follicular activation analysis verified the percentages of healthy primordial and growing follicles before (non-cultured control) and after culture. The diameters of the healthy follicles were also measured (Lima *et al.* 2018) and 30 follicles were counted per treatment.

Viability was determined by isolating follicles (n= 30 follicles) from non-cultured and cultured fragments using a scalpel blade and evaluating them with a trypan blue dye (0.4%) under inverted microscopy. The PFs were classified as viable when the oocyte and < 10% of granulosa cells did not show staining or were deemed non-viable when the oocyte and/or > 10% of granulosa cells were stained (Amorim *et al.* 2003).

The follicular cell proliferation rate was evaluated using the silver nitrate (Ag) impregnation method, which was used to quantify the number of nucleolar organizing regions (NOR). Thirty developing follicles were visualized under light microscopy (1000×) and the NORs of the nuclei in visible granulosa cells were counted (Silva *et al.* (2003).

Statistical analysis: Data were expressed as means ± standard error of the means (SEM) and analysed by the Statview 5.0 software (SAS, Cary, NC, USA). Results were submitted to Smirnov-Kolmogorov and Bartlett tests to confirm their normal distribution and homogeneity of variance, respectively. Then the non-cultured samples and those cultured for 1 or 7 days were compared by a two-way variance analysis (ANOVA). The effects of different GDF-9 concentrations on PF morphology, categories distribution, and diameters were evaluated by one-way ANOVA followed by Student's t-test. Follicular viability data were analyzed by dispersion of frequency and a Chi-square test. Differences were significant when $P < 0.05$.

Results

Experiment 1: Detection of GDF-9 receptors in collared peccary ovarian cortical tissue

After the electrophoretic analysis of the amplicons produced by PCR, abundant messages for BMPR2 (type I receptor, 499 bp), and ALK-5 (type II receptor, 468 bp) were clearly observed after amplification of the cDNA from collared peccary ovarian cortex (Fig. 1). Furthermore, GAPDH (394 bp) was also supported as the reference gene.

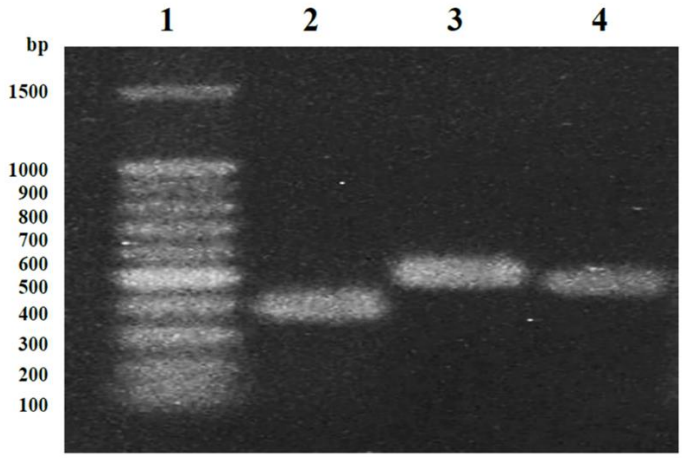

Figure 1. Electrophoretic analysis of amplicons produced by PCR of genes codifying GDF9-receptor subunits (*ALK5* and *BPMR2*) and GAPDH. Lane 1: 100 bp DNA ladder; Lane 2: *GAPDH*; Lanes 3 and 4: *BMPR2* and *ALK5* subunits, respectively, in collared peccaries' ovarian cortex.

Experiment 2: Effects of GDF-9 on collared peccary PF development

A total of 1620 follicles were counted and evaluated. There were no differences in PF morphology (Table 2) among the fresh or cultured samples ($P > 0.05$). The GDF-9 and non-GDF-9 cultures also produced similar numbers of morphologically normal PFs ($P > 0.05$). The morphometry (Table 2) remained unaltered after culturing for 1-day under all the conditions ($P > 0.05$). However, the 7-day culture samples showed a significant reduction in morphometry ($P < 0.05$), and this was being more evident in the samples cultured without GDF-9 ($P < 0.05$). In addition, groups containing GDF-9 at any concentration had similar oocyte diameters to the control group ($P > 0.05$). However, there was a reduction in oocyte diameter in the samples cultured without GDF-9. Furthermore, the follicles cultured for 7 days in 200 ng/mL GDF-9 had follicular and oocyte diameters that were similar to the diameters observed on the first day ($P > 0.05$).

Table 2. Percentages (Mean ± SE) of morphologically normal preantral follicles (MNPF) and the diameters of follicles and oocytes derived from collared peccary ovarian tissues before and after culture for 1 or 7 days, in the absence and presence of GDF-9 at different concentrations (50, 100 or 200 n/mL).

	%MNPF		**Follicle diameter (µm)**		**Oocyte diameter (µm)**	
	Day 1	**Day 7**	**Day 1**	**Day 7**	**Day 1**	**Day 7**
Non-cultured	90.0 ± 3.4		33.4 ± 1.7		23.93 ± 0.8	
0 ng/mL	76.6 ± 12.8	86.0 ± 5.8	32.24 ± 1.2^{A}	24.61 ± 0.4^{bB*}	21.60 ± 0.5^{cA*}	17.62 ± 0.4^{bB*}
50 ng/mL	75.0 ± 6.2	88.3 ± 5.7	31.92 ± 1.0^{A}	27.84 ± 0.8^{aB*}	23.36 ± 0.7^{abcA}	20.88 ± 0.9^{aB*}
100 ng/mL	76.0 ± 9.8	81.1±11.3	32.66 ± 5.5^{A}	26.55 ± 0.5^{aB*}	24.62 ± 0.6^{aA}	17.29 ± 0.4^{bB*}
200 ng/mL	86.1 ± 8.9	90.0 ± 3.2	29.07 ± 3.5	28.03 ± 0.7^{a*}	21.94 ± 0.4^{bc}	20.93 ± 0.6^{a*}

*Asterisk indicates significant difference from the fresh control group; ^{a,b} Superscript lowercase letters indicate differences among treatments into the same culturing day; A,B Superscript capital letters indicate the same treatment in different days.

The histology results showed that the follicles were normal in the non-cultured (Fig. 2A), 1-day cultured (Fig. 2B), and 7-days cultured (Fig. 2C) ovarian cortexes. All the groups also had some atretic follicles, in which the oocyte had a pycnotic nucleus and/or showed ooplasma shrinkage. Occasionally the granulosa cell layers became disorganized, were detached from the basement membrane, and/or included enlarged cells (Fig. 2D).

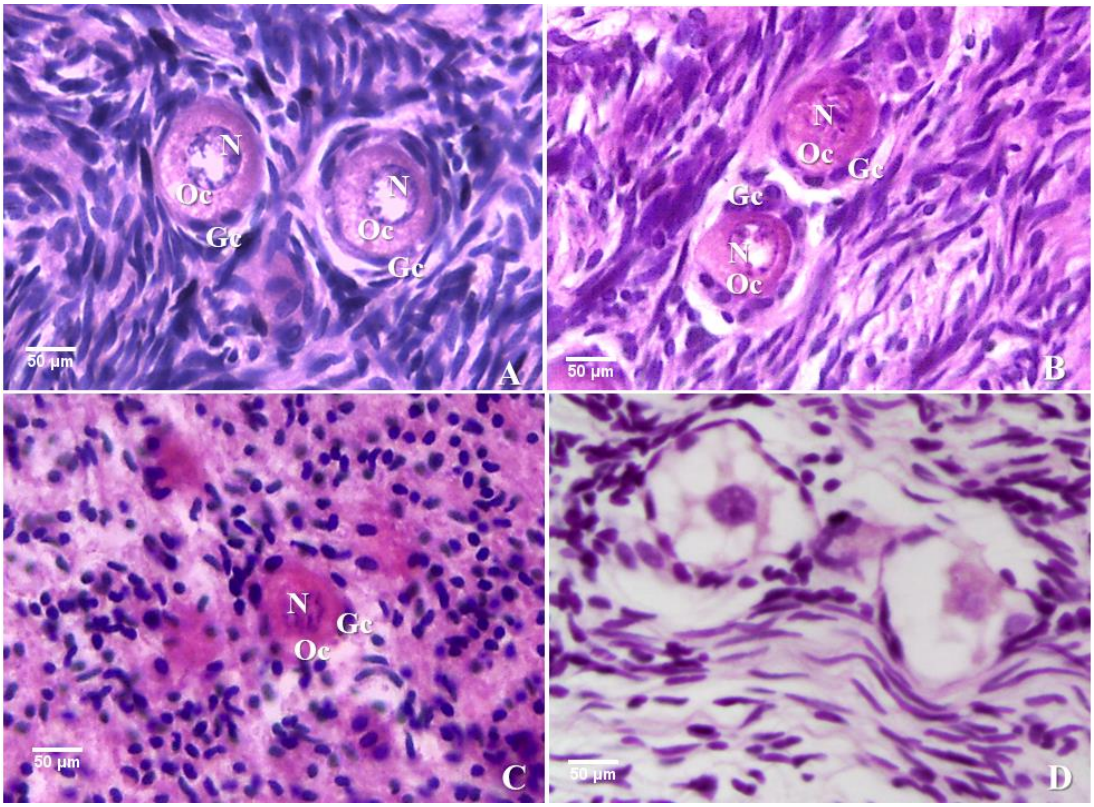

Figure 2. Collared peccaries' morphologically normal follicles from fresh control group (A) and cultured for one (B) or seven days (C) exhibiting an oocyte with homogenous cytoplasm (Oc), central nucleus (N) and intact and organized granulosa cells (GC). Atretic follicle from non-cultured (D) group showing cytoplasmic retraction and disorganization of granulosa cells.

Regarding activation (Fig. 3), the PFs derived from non-cultured tissues were predominantly, morphologically normal at the primordial stage. After 1-day, an increase in the growing follicles percentage was observed for all treatments compared to the fresh control

group (P < 0.05). An increase was also observed after 7-days (P < 0.05), and this was more evident in samples cultured with 200 ng/mL GDF-9 (P < 0.05).

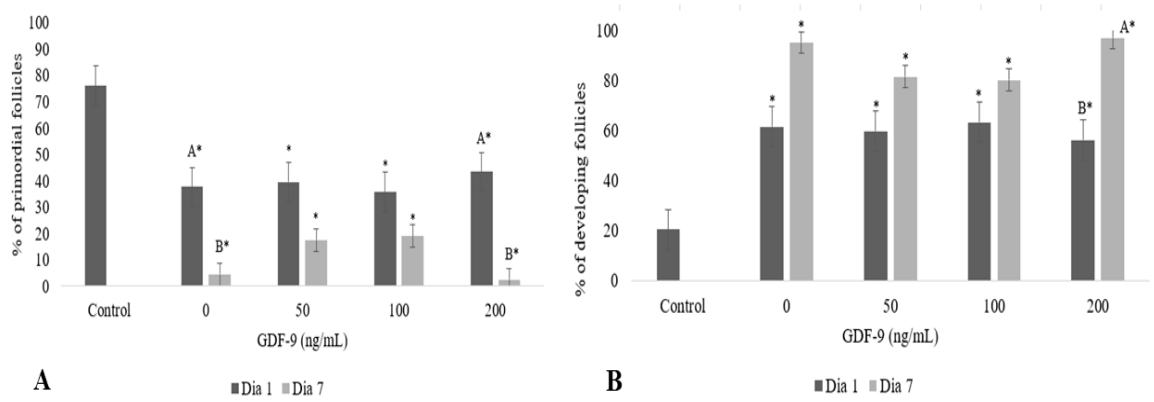

Figure 3. Percentages of collared peccaries' morphologically normal primordial (A) and growing (B) follicles in fresh control group or after in vitro culture for 1 or 7 days with different concentrations of GDF-9 (0, 50, 100, 200 ng/mL).

*Asterisk indicates significant difference from the fresh control group; [a,b] Superscript lowercase letters indicate significant differences among different treatments at the same culturing day; A,B Superscript capital letters indicate differences into the same treatment according to different days of culture (P < 0.05).

The viability of the PFs (Fig. 4A) was efficiently maintained in all treatments compared to the fresh control group (P > 0.05), except for those cultured in 50 ng/mL GDF-9 for 1-day where the PF viability decreased (P < 0.05). In addition, the treatment containing 200 ng/mL GDF-9 improved cell proliferation in PFs cultured for 1 or 7 days compared to the fresh control group (P < 0.05; Figure 4B).

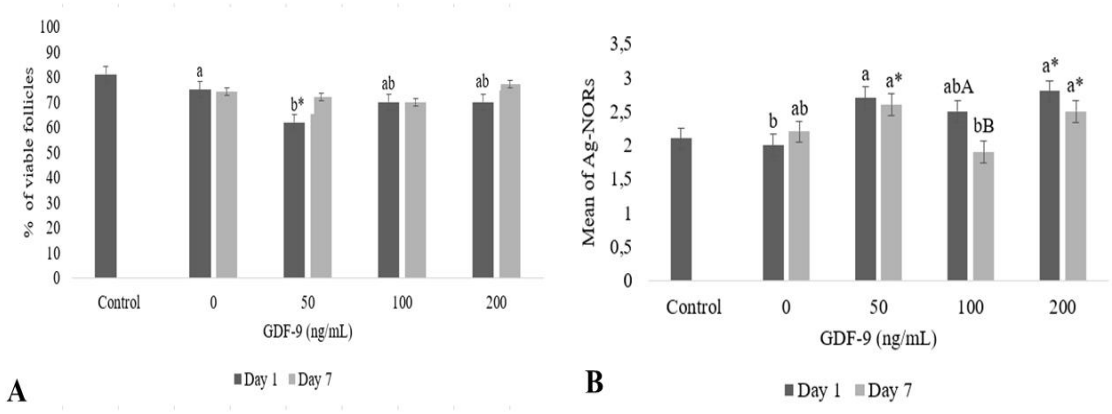

Figure 4. Mean values for viability (A) and mean of Ag-NORs (B) of collared peccaries' preantral follicles in fresh control group or after in vitro culture for 1 or 7 days with different concentrations of GDF-9 (0, 50, 100, 200 ng/mL).

*Asterisk indicates significant difference from the fresh control group; [a,b] Superscript lowercase letters indicate significant differences among different treatments at the same culturing day; A,B Superscript capital letters indicate differences into the same treatment according to different days of culture ($P < 0.05$).

Discussion

In order to elucidate folliculogenesis mechanisms and to improve peccary protocols, the presence of GDF-9 receptors was investigated for the first time as well as its role in the CIV of peccary FPs.

ALK5 is expressed by the oocytes, the theca, and the granulosa cells (Juneja *et al.* 1996), whereas BMPRII is expressed mainly in granulosa cells (Silva *et al.* 2005). The GDF-9 binds to BMPRII, which activates ALK5 receptors by phosphorylation of an intercellular regulatory region. When ALK5 is activated, it phosphorylates cytoplasmic proteins from the family of mother against decapentaplegic (MAD)-related proteins (SMADs), including SMADS 1, 2, 3, 5, and 8. Once activated, the SMADs interact with another SMAD molecule, called SMAD4, which is a common partner for all SMADs and is known as common SMAD (SMAD-Co). The SMAD/SMAD-CoS complex is finally translocated to the cell nucleus where it interacts with

specific transcription factors that regulate the expression of target genes (Gillcrist *et al.* 2008, Peng *et al.* 2013).

In swine, GDF-9 may participate in follicle formation during the fetal stage and helps regulate follicle development from the primordial to the antral stage (Quinn *et al.* 2004). Sun *et al.* (2010) confirmed that GDF-9 receptors exist at all stages of porcine follicle development, which suggests that early follicular development can be controlled by the ligands of these receptors. However, further studies on peccaries, are needed to determine the expression of GDF-9 receptors during the different stages of PF development.

The presence of GDF-9 receptors in the ovarian cortex suggests that it may play an important role in peccary follicular development. This has also been observed in other mammals where it can act as a paracrine factor (Sun *et al.* 2010, Lin *et al.* 2014). In this sense, the addition of 200 ng/mL of GDF-9 to the medium had a positive effect on PF activation, growth, and cell proliferation during the *in vitro* culture of peccary ovarian tissue. The percentage of normal follicles in the non-cultured fragments was not significantly different from the samples after 1 or 7 days of culture at different GDF-9 concentrations. Therefore, it is probable that the presence of GDF-9 does not influence the percentage of normal follicles in peccaries. Indeed, a positive effect on PF survival has only been shown in goats, when the GDF-9, also at 200 ng/mL, was mixed with 10 ng/mL insulin (Dipaz-Berrocal *et al.* 2017). The association of GDF-9 with other substances, such as BMP-15 (Kedem *et al.* 2011), FSH (Vasconcelos *et al*. 2013), or insulin (Dipaz-Berrocal *et al.* 2017), has been shown to improve follicular development in other species and associative effects should be investigated in peccaries. Alternatively, high GDF-9 concentrations (500 ng/mL GDF-9) can also improve PF survival, as has been recently reported for mice (Cook-Andersen *et al.* 2016).

The presence of 200 ng/mL GDF-9 in the TCM199 was able to maintain the peccary follicle and oocyte diameters in the 7-day culture samples. There have been no previous reports

about TCM199 supplemented with FSH affecting follicular growth in this species (Lima *et al.* 2018). Therefore, we suggest that GDF-9 may be involved in the initial growth of peccary PFs. Huang *et al.* (2009) confirmed that this role is due to its mitogenic activity, as has been proven in several species, such as rats (Vitt *et al.* 2000), humans (Ota *et al.* 2006), cattle (Spicer *et al.* 2006), goats (Martins *et al.* 2008), mice (Sasseville *et al.* 2010), and sheep (Peng *et al.* 2010).

Many substances are involved in PF activation, and this probably includes GDF-9 (Peng *et al.* 2010). In peccaries, the number of primordial follicles was reduced concomitantly with the increase in the number of developing follicles. This was more evident in the 200 ng/mL GDF-9 treatment. Yet, in the absence of GDF-9 the decrease in the percentage of primordial on day 1 was not accompanied by the percentage of follicles growing at day 7.

These results agree with those reported for rodents (Wang and Roy 2004) and humans (Hreinsson *et al.* 2002) where 200 ng/mL of GDF-9 promoted the recruitment of primordial follicles to the growing stages. Furthermore, GDF-9 mRNA was previously reported to be expressed in swine neonatal ovaries, which suggests that GDF-9 is present in the primordial follicles of this species (Sun *et al*. 2010). GDF-9 is present in the primordial follicles of sheep (Bodensteiner *et al.* 1999), marsupials (Eckery *et al.* 2002), goats (Silva *et al.* 2005), humans (Oron *et al.* 2010), bovines (Hosoe *et al.* 2011), buffaloes (Abdel-Ghani *et al.* 2016), and canines (Garcia *et al.* 2018), which indicates that this oocyte factor may play an important role in follicular activation.

The membrane integrity of the peccary PFs was preserved regardless of the presence of GDF-9 in the medium. These results suggest that there are species-specific differences in PF requirements because in goats (Martins *et al*. 2008) and humans (Orisaka *et al.* 2006), GDF-9 at high concentrations acted as a survival factor. It prevents atresia, which progressively occurs over time in culture, and inhibits apoptosis in follicles and stromal cells (Peng *et al.* 2010). These differences may be because in peccaries, the supplementation of TCM199 with insulin,

transferrin, selenium, BSA, pyruvate, glutamine, and hypoxanthine is enough to maintain PF survival and integrity, at least when they are cultured for a short period of time.

The histological indication that peccary PFs were activated was confirmed by the AgNOR technique because there was an increase in the number of NORs after the samples had been cultured in media containing 50 and 200 ng/mL GDF-9. This result is similar to the results reported for rats where GDF-9 may stimulate the proliferation of granulosa cells and the progression of the primordial follicles to subsequent stages (Vitt *et al.* 2000). Furthermore, injecting the porcine GDF-9 gene resulted in an increase in the number of primary, secondary, and tertiary follicles and a decrease in the number of primordial follicles in swine ovaries. This showed that the activity of this factor promoted cell proliferation and follicular development (Shimizu *et al.* 2004).

In summary, we have shown that GDF-9 receptors are present in the ovarian cortex of collared peccaries and we demonstrated that 200 ng / mL GDF-9 was actively involved in the in vitro development of catheter PFs. The results from this study improve our understanding of initial folliculogenesis in peccaries, and could be used to improve the protocols for PF *in vitro* culturing. This would help preserve female genetic material derived from collared peccaries and from other threatened closely-related species, such as the white-lipped (*Tayassu pecari*) and the Chacoan (*Catagonus wagneri*) peccaries.

Statement of Interest: The authors have no conflicts of interest to declare

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Capítulo IV - Establishment of an effective protocol for the isolation of ovarian preantral follicles derived from collared peccaries (*Pecari tajacu*)

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Running headline: Isolation of peccaries' ovarian follicles

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Artigo submetido Zygote

Qualis B1

Summary

We compare the efficiency of mechanical and enzymatic methods alone or combination for the isolation of ovarian preantral follicles (PFs) from collared peccaries. The ovaries from 6 females were subjected to the different methods investigated. For the enzymatic method, ovary fragments were exposed to collagenase Type IV in TCM-HEPES medium; the mechanical procedure was based on ovarian cortex dissociation by using a scalpel blade. The residual solution obtained after the mechanical isolation was subjected to the enzymatic procedure. The number of isolated PFs was quantified and classified as primordial, primary, or secondary; their viability was assessed using trypan blue dye assay. To confirm the results, PFs derived from the most efficient method were evaluated for integrity using scanning electron microscopy (SEM) and subjected to a 24 h in vitro culture for subsequent evaluation of viability by using fluorescent probes. A higher number of PFs ($P < 0.05$) was obtained from the enzymatic method (961.7 ± 132.9) in comparison to the mechanical method (434.3 ± 88.9), but no difference was observed between the two methods and their combination (743.2 ± 92.8). The trypan blue assay showed that the enzymatic method (98.7 ± 0.6%) provided the highest percentage of viable follicles ($P < 0.05$). Furthermore, SEM confirmed the ultrastructural integrity of the surface architecture of peccary PFs isolated by the enzymatic procedure; epifluorescence microscopy was used to confirm their viability (86.0%). In conclusion, we suggest that the enzymatic method investigated here is useful for the isolation of viable ovarian PFs from collared peccaries.

Keywords: *Tayassu tajacu*; wildlife; folliculogenesis; ovary; biobanking

Introduction

The collared peccary is a type of "wild pig" whose population is globally classified as one of least concern. In terms of extinction risk, the peccaries occupy the status of little concern in practically all the biomes, being classified as stable species, however, the species is already extinct in eastern and southern Argentina and is declining in some biomes (Gongorra *et al*., 2011). For Latin American communities, peccaries are economically important because of the consumption of its meat and use in leather production by the international market (Silva *et al*., 2017). In addition, the species is currently being used for the development of assisted reproductive techniques that have the potential to be applied for the conservation of closely related endangered species (Silva *et al*., 2017), such as the Chacoan peccary (*Catagonus wagneri*).

To establish strategies for conservation of the germplasm of female collared peccaries, their preantral follicle (PF) population was first estimated to be 33,273.45 follicles per ovary (Lima *et al*., 2013). Subsequently, some efforts for the cryopreservation of peccaries' ovarian tissue were conducted and more than 70% of the morphologically normal and viable PFs could be preserved (Lima *et al*., 2012; Moreira *et al*., 2017). Recently, the efficiency of an *in vitro* culture system for peccary ovarian tissue was demonstrated (Lima *et al.,* 2018). However, studies conducted on swine, the domestic species more closely related to peccaries (Cavalcante-Filho *et al.,* 1998), have highlighted the need for culturing isolated PFs so that they can reach subsequent developmental stages (Sirotkin *et al*., 2017, Lima *et al*, 2017)—a strategy not yet attempted on peccaries.

The current methods for the isolation of PFs can be classified as enzymatic and/or mechanical. The enzymatic method consists of exposing the ovarian tissue to the action of enzymes, such as collagenase, as reported for swine (Choi *et al*., 2008). However, using this method, the basal membrane or theca cells are damaged in many of the isolated PFs, especially

when the incubation time is not strictly controlled (Rossetto *et al*., 2011). On the other hand, the mechanical method has the advantage of maintaining the integrity of the follicular and basal membrane structure, as well as the interactions among the oocytes, granulosa cells, and theca cells. Despite being slower and more laborious than the enzymatic method and allowing the recovery of a low number of follicles, it has been recommended for the isolation of porcine PFs (Ahn *et al*., 2012). To optimize the isolation of PFs, a combination of enzymatic and mechanical methods was successfully applied for cattle (Figueiredo *et al*., 1993), human (Dong *et al*., 2014), and sheep (Sadeghnia *et al*., 2016). After isolation, PFs are usually evaluated for viability and development through *in vitro* culturing followed by evaluation with fluorescent probes, but electron microscopy has now emerged as an effective method to provide accurate details of the ultrastructure of PFs (Klein *et al*., 2012).

To establish a protocol for the isolation of collared peccaries'PFs, the purpose of the present study was to compare the efficiency of mechanical and enzymatic methods, alone or in combination, in conserving the morphology and viability of peccary PFs.

Materials and methods

The ethics committee of the UFERSA approved the experimental protocols, as well as the animal care procedures used (no. 23091.006525/2016-82). The study was authorized by the Chico Mendes Institute for Biodiversity (SISBio no. 37329). All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless indicated otherwise.

Source of ovaries

The ovaries (n = 12 ovaries) from 6 adult (~3.5 years old) collared peccaries were collected. The animals belonged to the Center of Wild Animal Multiplication (IBAMA Register No. 1478912), UFERSA, Mossoró, RN, Brazil (5°10′S, 37°10′W). This center presently shelters

200 collared peccaries that are used in studies that focus on the development of productive management practices. A programmed slaughter is conducted every year for population control, and the carcasses are used in various studies in the fields of morphology, histology, physiology, pathology, etc. For our study, immediately post-mortem, the ovaries were washed in 70% alcohol and TCM-HEPES and then transported to the laboratory.

Comparison of protocols for the isolation of PFs

For all the ovaries, the corpora lutea and antral follicles, if present, were removed. The ovarian cortexes from the same individual were randomly assigned to the mechanical or enzymatic methods for isolation of PFs.

For the enzymatic procedure (Figueiredo *et al*., 1993, adapted), the ovarian cortex was divided into small fragments (1 × 1 × 1 mm), which were then suspended in TCM-HEPES after the addition of 0.5 mg/mL collagenase Type IV for 20 min at 37°C in a water bath. Every 5 min, the suspensions were gently homogenized 40 times with a Pasteur pipette. After 20 min, the collagenase activity was blocked through the addition of 10% fetal bovine serum (FBS) to the suspensions that were gently homogenized using a Pasteur pipette; the suspensions were then added to TCM-HEPES with 1% BSA and incubated for 10 min. The suspensions were filtered first through 500 μm and then through 200 μm nylon mesh filters, and the filtrates were centrifuged at 1600 × *g* for 5 min and evaluated.

For mechanical isolation, the ovarian cortex was placed in a Petri dish and subjected to dissociation by using a scalpel blade. The suspension was then transferred to a 50 mL containing 5 mL of TCM-HEPES and gently homogenized 40 times with a Pasteur pipette. The suspensions were then filtered first through 500 μm and then through 200 μm nylon mesh filters. The filtrates were then centrifuged at 1600 × *g* for 5 min and evaluated (Figueiredo *et al*., 1993).

For the combination of the enzymatic and mechanic isolations, the remaining filtrate from the nylon mesh filters used during mechanical isolation was submitted to the enzymatic procedure as previously described.

Initial analysis

After each method, the number of isolated PFs in the suspension was quantified and classified according to the method of Hulshof *et al*. (1994) by using an inverted microscope (LEICA, Episcopic Fluorescent Attachment EFA Halogen Lamp Set, Leica Microsystems Inc., Bannockburn, USA). In summary, the primordial follicles were considered to be those that had an immature oocyte in the center of the follicle surrounded by a layer of pre-granulosa cells in a pavement format; primary follicles, those with an immature oocyte in the center of the follicle surrounded by a layer of granulosa cells arranged cubically; and secondary follicles, those with an immature oocyte surrounded by two or more layers of cubic granulosa cells in the presence of theca cells (Hulshof *et al*., 1994). Furthermore, the denuded oocytes (follicles without granulosa cells or partially surrounded by them) were also counted (Lopes *et al*., 2009).

For viability analysis, the suspension obtained after isolation procedures was centrifuged for 10 min, and 10 μL trypan blue (0.4%) (Sigma Chemical Co., St. Louis, MO, USA) was added to the precipitate at 25°C. After 5 min, the samples were evaluated under an inverted microscope (Eclipse TS-100F, Nikon Corporation, Tokyo, Japan), and PFs were classified as viable, if unstained, and not viable, if stained blue (Lopes *et al*., 2009).

Scanning electron microscopy analysis

To evaluate the ultrastructural integrity of the isolated PFs, only those obtained by the most effective method were fixed in 2% glutaraldehyde. After fixation, the PFs were recovered using a pipette and mounted onto stubs with the aid of a carbon tape. For metallization, the stubs were

placed on a metallizer (Q150T ES; Quorum Technologies, Guelph, Ontario, Canada) and metalized with a 20 nm layer of gold. SEM (Quanta 450-FEG; FEI, Hillsboro, OR, USA) observations of at least 10 different PFs were conducted according to the methodology described by Bustos-Obregon & Fléchon (1975).

In vitro culture of PFs

To confirm the viability of isolated PFs, only those obtained by the most effective isolation method were subjected to a 24 h in vitro culture (Santos *et al.,* 2006). Twenty intact follicles were selected and individually cultured in 20 µL of the culture medium with mineral oil for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (adapted by Demeestere *et al*., 2002). The basic culture medium consisted of TCM199 (pH 7.2–7.4) supplemented with ITS (insulin 10 µg/mL, transferrin 5.5 µg/mL, and selenium 5.0 ng/mL), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/mL BSA.

After culture, the isolated PFs were incubated in the holding medium that contained a mixture of propidium iodide (2 µM) and Hoechst 33342 (40 µM) for 10 minutes at 37°C to detect follicular viability and to enable the counting of nuclei, respectively. After labeling, the stained follicles were placed on a glass microscope slide and examined under an epifluorescence microscope equipped with a digital camera. Oocytes and granulosa cells were classified as ‘degenerating’ if the chromatin was stained positively with propidium iodide and as ‘viable’ if the chromatin was unlabeled with propidium iodide. The percentages of viable granulosa cells were calculated in relation to the total number of Hoechst-positive nuclei. Follicles with a viable oocyte surrounded by ≥ 90% viable granulosa cells were considered viable (Santos *et al*., 2006).

Statistical analysis

Data were expressed as mean ± standard error and were initially subjected to Smirnov–Kolmogorov and Bartlett tests to confirm the normal distribution and homogeneity of variance, respectively. Analysis of variance (ANOVA) was then carried out using the Statview (5.0) software, and Fisher's PLSD test was applied for comparison of the isolation methods (enzymatic, mechanical, or combined). Comparisons among the treatments in relation to the percentage of viable PFs were conducted using Chi-square test. Values were considered statistically significant at $P < 0.05$.

Results

Compared to the mechanical method (434.3± 88.9 PFs), the enzymatic method (961.7 ± 132.9 PFs) provided a higher number of isolated PFs ($P < 0.05$), but the number of isolated PFs did not differ from the combined method (743.2 ± 92.8 PFs) (Table 1). The highest number of isolated follicles was classified as primary, regardless of the method used (Table 1). It was possible to verify the presence of denuded oocytes in all the methods, but there was no statistical difference between them (mechanical: 10.3 ± 6.1; enzymatic: 3.2 ± 3.2; combined: 10.3 ± 6.1 oocyte). From this initial analysis, the highest percentage of viable PFs ($P < 0.05$), as determined by the trypan blue assay, was obtained by the enzymatic method (98.7 ± 0.6%) in comparison to the mechanical (89.2 ± 1.6%) or the combined (90.2 ± 1.9%) method.

We confirmed the integrity of PFs obtained by the enzymatic method by using SEM that provided images of the follicular surface (Fig. 1). From these images, we were able to observe the structural elements to the theca cells and granulosa cells, where the oocytes remained intact in the PFs evaluated. The theca layer was distributed as a continuous thin layer with a smooth surface appearance. The granulosa cells formed a compact layer around the oocyte, whose surface was with few pores.

Table 1. Mean values (mean ± SEM) for collared peccaries' ovarian preantral follicles isolated by the mechanical or enzymatic methods, or the association of both (n=6 ovarian pairs).

Follicular Classification	Isolation method		
	Mechanical	Enzymatic	Association
Primordial	62.5 ± 14.7[b]	80.5 ± 26.6[b]	67.5 ± 15.6[b]
Primary	461.0 ± 88.6[a]	702.7 ± 137.7[a]	718.8 ± 134.0[a]
Secondary	15.3 ± 6.5[b]	14.2 ± 8.9[b]	17.0 ± 6.2[b]
Total	434.3± 88.9[B]	961.7 ± 132.9[A]	743.2 ± 92.8[AB]
(Range)	(150 – 740)	(470 – 1.447)	(418 – 955)

[a,b]Superscript lowercase letters compare follicular categories into each treatment; [A,B] Superscript capital letters compare methods regarding the total number of isolated follicles ($P < 0.05$).

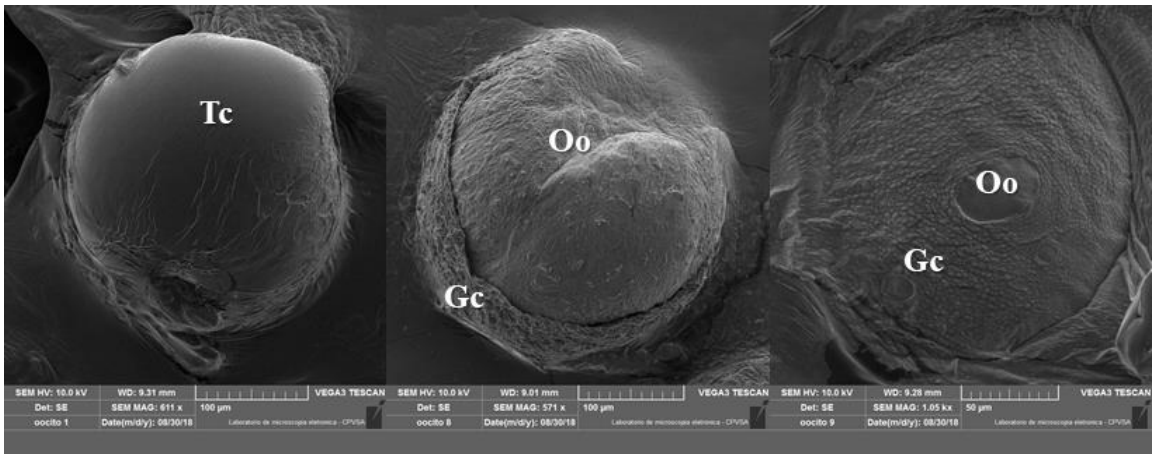

Figure 01 – Scanning electron microscopy images showing intact structures on the surface of collared peccaries' ovarian preantral follicles isolated by the enzymatic method. Note that it is possible to observe the theca cells (Tc) layer surface, the compacted granulosa cells (GC) and the oocyte (Oo).

Visualization through epifluorescence microscopy indicated that 86% of the PFs isolated by the enzymatic procedure remained viable after the 24 h in vitro culture (Fig. 2).

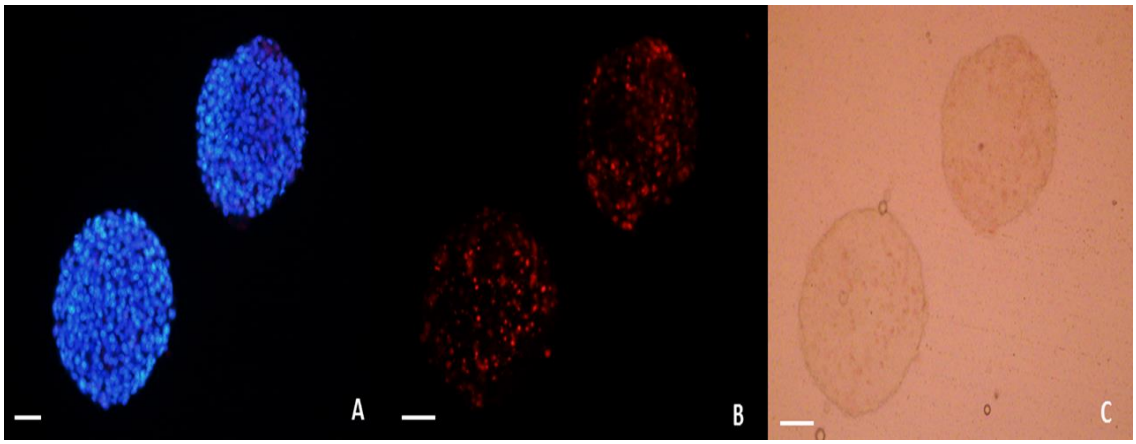

Figure 02 - Representative images of collared peccaries' ovarian preantral follicles submitted to a short in vitro culture for 24h after enzymatic isolation. Note the presence of viable follicles marked in blue by the Hoechst probe (A) with the absence of the propidium iodide impregnation (B), and also the follicles evaluated under the clear field (C).

Discussion

To improve the technologies for the recovery and storage of the germplasm of female collared peccaries, we demonstrate, for the first time, the application of protocols for the isolation of PFs from this species. Although viable PFs can be obtained by any of the methods tested here, the enzymatic procedure proved to be the most efficient. This fact highlights the effective action of the enzyme collagenase that promotes efficient dissociation of PFs from peccaries, which ovaries are surrounded by a layer of fibrous connective tissue (Mayor *et al.*, 2006).

Although the domestic swine presents a follicular population (>400,000 PFs per ovarian pair; Gosden and Telfer, 1987) many times higher than that of peccaries (~60,000 PFs per ovarian pair; Lima *et al.*, 2013), the application of the enzymatic method was more effective in

peccaries than in the prepubertal gilts from which only 157.8 ± 26.6 PFs were isolated (Choi *et al*., 2008). This is probably due to differences in the ovarian structures since the ovaries of prepubertal gilts seem to be more fibrous (Smitz and Cortvrindt, 2001) than those of peccaries (Lima *et al.*, 2013), which hinders successful enzymatic isolation. Besides providing the highest number of isolated PFs from peccaries, the enzymatic method also provided a higher percentage of viable follicles (98.7 ± 0.6%) than that by the mechanical (89.2 ± 1.6%) or the combined (90.2 ± 1.9%) methods, as evaluated using the trypan blue assay. Such values were even higher than those reported for domestic swine in which 74% viable follicles were recovered after enzymatic isolation (Ahn *et al.*, 2012).

The collagenase used in the present study was a partially purified bacterial enzyme (*Clostridium histolyticum*) that could attack native collagen without affecting related proteins and damaging the epithelial tissue (Mandl *et al*., 1958). The efficiency of type IV collagenase enzyme has previously been proven in bovine (Figueiredo *et al*., 1993), caprine (Machado *et al*., 2002), porcine (Choi *et al*., 2008), human (Lierman *et al*., 2014), and murine (Young *et al*., 2017) tissues. In addition, the crucial factors of this enzyme are its concentration and duration of digestion (Shuttleworth *et al*., 2002). In the present study, the enzyme incubation time was 20 min, which was sufficient to isolate a large number of viable peccary PFs, as has been previously reported for rabbits (Nicosia *et al*., 1975), bovines (Figueiredo *et al*., 1993), equines (Telfer and Watson, 2000), porcines (Choi *et al*., 2008), and humans (Lierman *et al*., 2014), in all of which, the incubation time should not exceed 30 min. Moreover, the addition of up to 5% FBS in the collagenase solution seemed to ensure better preservation of the follicle cytostructure since FBS forms a protective layer on the follicular membrane (Nicosia *et al.*, 1975). In the present experiment, 10% FBS was used, which was enough to prevent the basal membrane digestion in some follicles.

Compared to the enzymatic method, we highlighted that it is possible to isolate viable peccary PFs by the mechanical method, even if a lower amount was provided. Probably, the scalpel blade used for this purpose was not efficient enough to dissociate the connective tissue, and thus, released less follicles (434.3 ± 88.9) than those obtained for prepubertal gilts (599.160 ± 74.089; Alves *et al.,* 2012). Furthermore, it is worth noting that the mechanical method is a cheap and easily accessible procedure that can be applied at any laboratory. In addition, it is evident that the combination of mechanical and enzymatic methods can improve the efficiency of the mechanical method, and thus, increase the number of viable follicles isolated from peccaries as previously reported for buffalos (Santos *et al.,* 2006), humans (Dong *et al.*, 2014), and sheep (Sadeghnia *et al.,* 2016).

By comparing the follicular categories, we verified that most of the isolated follicles were primary, regardless of the method used. This is an interesting result, because it has been established previously that most of the peccary follicle population is composed of primordial (91.56%) follicles, followed by primary (6.29%) and secondary (2.15%) follicles (Lima *et al.*, 2013). This discrepancy is possibly because of the primordial follicles being more intimately embedded in the tunica albuginea, which may make it difficult for the follicles to dissociate, as previously reported for other species, including caprine (Machado *et al.*, 2002), swine (Kerong *et al.*, 2007), and humans (Dong *et al.*, 2014).

We also highlighted the presence of denuded oocytes after the isolation of follicles in all the tested methods. This can be attributed to the action of collagenase or mechanical instruments that can destroy the membranes of many follicles during the isolation, thus, denuding them (Dong *et al.,* 2014). In the present experiment, an average of 3.2 ± 3.2 oocytes was observed in the enzymatic method with no differences observed between methods ($P > 0.05$). In bovines, however, some studies have shown that the presence of denuded oocytes is

the most evident in the enzymatic method, suggesting that the enzyme can rupture the follicular membrane (Figueiredo *et al.,* 1993), which did not occur in the present study.

As an additional attempt to confirm the integrity of PFs isolated by the enzymatic method, which was the most efficient method that we observed here, we conducted SEM analysis that provided unprecedent detailed ultrastructural images of the surface of peccary PFs. Transmission electron microscopy, associated with other in vitro analytical studies, has a well-recognized diagnostic–prognostic role in the assessment of ovarian follicle and oocyte viability (Notolla *et al.,* 2011). However, SEM is not commonly used to verify the PF viability. In the present study, SEM provided important information regarding the maintenance of the PF surface architecture after enzymatic isolation. The ultrastructural view of peccary PF surface structures presented general similarities with those that have been described previously for mice, although the double theca layer usually described for this species (Notolla *et al.,* 2011) was not observed in the peccary PFs evaluated here. In addition, we highlighted the presence of numerous microvilli on the peccary oocyte surface that are probably involved in apposition and fusion of the sperm and oocyte membranes during fertilization (Runge *et al.,* 2007). Moreover, the presence of some pores, as found in peccary PFs, has also been reported for bovines in which the increase in the number of pores is strictly involved with the process of oocyte maturation (Suzuki *et al.,* 1994).

To support the interpretations of the SEM results, the follicles isolated by the enzymatic method were cultured for 24 h. This short-culture has been shown to be an effective procedure in the evaluation of follicular viability since the follicular damage appears only after a few hours, when the follicle returns to its physiological conditions in which case follicular growth does not occur anymore (Vanhoute *et al.,* 2004). In the present study, peccary PF viability was confirmed using fluorescent probes that confirmed the presence of an intact basal membrane around isolated follicles, similar to that for domestic swine (Ahn *et al.,* 2012), sheep

(Lakshminarayana *et al.,* 2014), and mice (Zhang *et al.,* 2017), suggesting that the isolation method did not interfere with the integrity of the follicle.

In conclusion, we demonstrated that the enzymatic method is an efficient procedure for the isolation of peccary ovarian PFs; however, we highlighted that it is alternatively possible to obtain viable follicles by the mechanical method or by combining both methods. The information generated here presents ample evidence for the positive effect on the availability of competent oocytes in terms of future applicability for in vitro development and fertilization. Such techniques, in association with cryopreservation, will contribute to the conservation and multiplication of germplasm derived from valuable endangered tayassuids.

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Statement of Interest

The authors have no conflicts of interest to declare

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CONCLUSÕES GERAIS

-A vitrificação usando o método OTC com etilenoglicol (3M) como crioprotetor é eficiente para preservação do tecido ovariano de catetos;

-Existem receptores BMPR2 e ALK-5 para o GDF-9 no cortex ovariano de cateto;

-O GDF-9 na concentração de 200 ng/mL é importante para o desenvolvimento *in vitro* de folículos ovarianos de catetos;

-Método enzimático é um procedimento eficiente para o isolamento de folículos pré-antrais de catetos.

PERSPECTIVAS

Com o estudo de vitrificação foi possivel observar uma nova perspectiva para a criopreservação de tecido ovariano com resultados promissores, permitindo a conservação do material genético feminino, visando no futuro a preservação da espécie, bem como, para um melhor aproveitamento do seu potencial reprodutivo. Contudo, o procedimento pode ser aprimorado, para tanto, pode ser teste outras concentrações e tipos de agentes crioprotetores, bem como, temperatura de aquecimento.

Ainda, o protocolo de cultivo *in vitro* de tecido ovariano de cateto foi aprimorado com a utilização do GDF-9, melhorando os resultados das taxas de ativação desses foliculos, bem como, permitindo a possibilidade de estudos futuros para a associação do GDF-9 com outras substâncias. Ainda, com um protocolo de cultivo *in vitro* pode ser uma ferramenta importante para avaliar a eficiência da criopreservação, uma vez que lesões foliculares não detectadas pós-descongelação podem evoluir e serem manifestadas após cultivo.

Adicionalmente, a presente tese abordou um método eficiente de isolamento folicular em catetos, possibilitando assim, o isolamento de inúmeros folículos viáveis, para que possa ser aplicado futuramente em sistema de cultivo isolado, possibilitando o crescimento até a maturação e aplicações em outras biotécnicas.