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ALANA AZEVEDO BORGES

ESTABELECIMENTO DE CARIOPLASTOS E CITOPLASTOS DE CATETOS, *Pecari tajacu* (LINNAEUS, 1758), VISANDO A CLONAGEM POR TRANSFERÊNCIA NUCLEAR DE CÉLULA SOMÁTICA EM TAIASSUÍDEOS

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

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Orientadora: Profa. Dra. Alexsandra Fernandes Pereira.

Co-orientador: Prof. Dr. Alexandre Rodrigues Silva.

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Não sei se a vida é curta ou longa para nós, mas sei que nada do que vivemos tem sentido, se não tocarmos o coração das pessoas.
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RESUMO

O direcionamento dos primeiros passos para a realização da transferência nuclear de célula somática (TNCS) em catetos garantirá uma ferramenta efetiva na conservação da espécie, perante sua acelerada diminuição populacional e sua atividade ecológica indispensável para o ecossistema. Para tanto, a presente tese foi dividida em duas etapas (três experimentos por etapa), sendo a primeira etapa o estudo das células doadoras de núcleo ou carioplastos e a segunda etapa, o estudo das células doadoras de citoplasma ou citoplastos. Assim, diante da importância de carioplastos de qualidade reconhecida para a TNCS, nós inicialmente estabelecemos cinco linhagens de fibroblastos de catetos, monitorando a viabilidade, atividade metabólica e estresse oxidativo, de acordo com os efeitos do número de passagens (primeira, terceira e décima) e criopreservação. Embora não haja efeito desses critérios sobre a viabilidade, células em décima passagem tiveram uma redução de seu metabolismo. Adicionalmente, células congeladas/descongeladas tiveram um aumento no número de espécies reativas de oxigênio e potencial de membrana mitocondrial. Além disso, sabendo da importância de manter estas células armazenadas em um biobanco de maneira adequada, nós otimizamos a solução crioprotetora utilizada na congelação lenta de fibroblastos de catetos. Deste modo, a solução composta por 10% de dimetilsulfóxido (DMSO) com 0,2 M de sacarose e 50% de soro fetal bovino (SFB) foi considerada a solução mais eficiente em manter a viabilidade, atividade proliferativa, metabolismo e níveis adequados de estresse oxidativo de células somáticas de catetos, quando comparada a soluções na ausência de sacarose e com 10% de SFB em diferentes combinações. Finalmente, um passo essencial no estabelecimento dos carioplastos para a TNCS consiste na sincronização das células em G_0/G_1 do ciclo celular. Deste modo, nós avaliamos diferentes métodos de sincronização do ciclo celular: (i) supressão de soro (SS) por um a quatro dias, (ii) inibição por contato (IC) por um a três dias e (iii) agentes químicos [DMSO, 6-dimetilaminopurina (6-DMAP), ciclohexamida (CHX), e citocalasina B (CB)] por um a dois dias, em termos de seus efeitos sobre a sincronização em G_0/G_1 e viabilidade. Assim, nós observamos que a IC por três dias foi o método mais eficiente para sincronização do ciclo celular e manutenção da viabilidade de fibroblastos de catetos. Portanto, com estes três experimentos, nós estabelecemos a etapa de carioplastos da TNCS de catetos, obtendo células de qualidade e aptas a serem usadas como doadoras de núcleo. Na segunda etapa, nós, inicialmente, adequamos as condições de maturação *in vitro* (MIV) de oócitos de catetos, avaliando o tempo de MIV e o efeito do fator de crescimento epidermal (EGF) sobre a habilidade meiótica. Assim, nós concluímos que 48 h é o período adequado para a MIV de oócitos quando comparado ao tempo de 24 h, de acordo com o potencial meiótico. Ainda, observou-se que o EGF pode ser utilizado para otimizar o meio de MIV. Finalmente, no terceiro experimento, nós avaliamos a habilidade de desenvolvimento destes oócitos após ativação artificial, usando a ionomicina como ativador primário e comparando diferentes ativadores secundários (6-DMAP, CHX e CB). Nós verificamos que a ativação química usando ionomicina e 6-DMAP foi a mais eficiente combinação, tendo esta tese alcançado como resultado significativo, uma taxa de 27,6% de blastocistos de catetos derivados da ativação oocitária artificial. Em síntese, nós obtivemos carioplastos e citoplastos que poderão ser empregados na TNCS de catetos, deixando a ponto as etapas fundamentais para a clonagem desta espécie. Ainda, destaca-se que os conhecimentos aqui gerados poderão ser aplicados em estudos de fecundação *in vitro*, compreensão do desenvolvimento embrionário, produção de células induzidas à pluripotência, e ensaios de toxicidade. Portanto, este trabalho foi um grande passo para a conservação de catetos.

Palavras-chave: Vida selvagem. Clonagem. Biobancos. Ciclo celular. Ativação oocitária. Produção de embriões.

ABSTRACT

The direction of the first steps for the achievement of somatic cell nuclear transfer (SCNT) in collared peccary will guarantee an effective tool in the conservation of the species, in view of its accelerated population decrease and its essential ecological activity for the ecosystem. Therefore, the present thesis was divided into two steps (three experiments per step), being the first step the study of the donor cells of nucleus or karyoplast and the second stage, the study of the donor cells of cytoplasm or cytoplasts. Thus, in view of the importance of acknowledge quality karyoplast for SCNT, we initially established five cell lines of collared peccary fibroblasts, monitoring viability, metabolic activity and oxidative stress, according to the effects of the number of passages (first, third and tenth) and cryopreservation. Although there is no effect of these criteria on viability, cells in tenth passage had a reduction in their metabolism. Additionally, frozen/thawed cells had an increase in the number of reactive oxygen species and mitochondrial membrane potential. Moreover, knowing the importance of maintaining these cells stored in a biobank properly, we optimize the cryoprotectant solution used in the slow freezing of collared peccary fibroblasts. Thus, the solution composed of 10% dimethyl sulfoxide (DMSO) with 0.2 M sucrose and 50% fetal bovine serum (FBS) was considered the most efficient solution in maintaining the viability, proliferative activity, metabolism and adequate levels of oxidative stress of somatic cell cells, when compared to solutions in the absence of sucrose and with 10% FBS in different combinations. Finally, an essential step in establishing the karyoplast for SCNT is the synchronization of cells in G_0/G_1 of the cell cycle. Thus, we evaluated different cell cycle synchronization methods: (i) serum suppression (SS) for one to four days, (ii) contact inhibition (CI) for one to three days and (iii) chemical agents [DMSO, 6-dimethylaminopurine (6-DMAP), cyclohexamide (CHX), and cytochalasin B (CB)] for one to two days, in terms of their effects on G_0/G_1 synchronization and viability. Thus, we observed that the IC for three days was the most efficient method for synchronizing the cell cycle and maintaining the viability of collared peccary fibroblasts. Consequently, with these three experiments, we have established karyoplast stage of SCNT in collared peccary, obtaining quality cells and able to be used as nuclear donors. In the second stage, we initially adjusted the *in vitro* maturation (IVM) conditions of collared peccary oocytes, evaluating the IVM time and the effect of the epidermal growth factor (EGF) on the meiotic ability. Thus, we concluded that 48 h is the appropriate period for oocyte IVM when compared to 24 h, according to meiotic potential. Still, it was observed that EGF can be used to optimize the IVM medium. Finally, in the third experiment, we evaluated the developmental ability of these oocytes after artificial activation, using ionomycin as the primary activator and comparing different secondary activators (6-DMAP, CHX and CB). We found that chemical activation using ionomycin and 6-DMAP was the most efficient combination, with this thesis achieving as a significant result, a rate of 27.6% of blastocysts of collared peccaries derived from oocyte artificial activation. In summary, we got karyoplast and cytoplasts that may be employed in the SCNT of collared peccary, leaving the point the fundamental steps for the cloning of this species. Furthermore, it is emphasized that the knowledge generated here can be applied for *in vitro* fertilization, studies understanding of embryonic development, production cells induced to pluripotency, and toxicity assessments. Therefore, this work was a great step for the conservation of collared peccaries.

Keywords: Wild life. Cloning. Biobanks. Cell cycle. Oocyte activation. Embryo production.

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LISTA DE ABREVIATURAS E SIGLAS

° C	Grau Celsius
µg	Micrograma
µL	Microlitro
µm	Micrometro
µM	Micromolar
1 PB	First polar body
10th	Tenth
15th	Fiftieth
4th	Fourth
5th	Fifth
6D	6-dimethylaminopurine
6-DMAP	6-dimethylaminopurine
ANOVA	Variance analysis
art.	Artigo
ARTs	Assisted Reproductive Techniques
Av.	Avenida
B	Blastocyst
Bak	Pro-apoptotic
Bcl-xL	Anti-apoptotic
BSA	Bovine Serum Albumin
CA	Califórnia
Ca^{2+}	Íon cálcio
cAMP	3',5' cyclic adenosine monophosphate
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CB	Cytochalasin B
CCM	*Cumulus*-oocyte complex collection medium
CEUA	Committee for Ethics in Animal Use
CHX	Cycloheximide
CI	Contact inhibation
cm	Centímetro
cm^2	Centímetro quadrado

CMXRos	MitoTracker Red®
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de carbono
COC	*Cumulus*-oocyte complex
D0	Day 0
D3	Day 3
D7	Day 7
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eB	Expanded blastocyst
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
et al.	E outros
FBS	Fetal bovine serum
Fig.	Figure
FSH	Follicle-stimulating hormone
g	G force
g	Grama
G	Gauge
Gbif	Biodiversity Information Facility
GC	Growing cells
GI	Grade I
GI	Grade I
GII	Grade II
GIII	Grade III
GIV	Grade IV
GSH	Glutathione
h	Hour
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
H3K18ac	Histone H3 at lysine 18

H3K27me3	Histone H3 at lysine 27
hB	Blastocyst hatching/hatched
hCG	Human Chorionic Gonadotropin Hormone
IA	Inner oocyte area
iB	Initial blastocyst
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IC	Inibição de contato
ICM	Inner cellular mass
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ICSI	Intracytoplasmic sperm injection
IgG	Immunoglobulin G
iSCNT	Intraspecific and interspecific somatic cell nuclear transfer
IU	International units
IUCN	International Union for Conservation of Nature
IVD	*In vitro* development
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
L	Litro
LH	Luteinizing hormone
M	Mol
MG	Minas Gerais
mg	Miligrama
MII	Metaphase II
min	Minute
MIV	Maturação *in vitro*
mL	Mililitro
mM	Milimolar
mm	Milímetro
mm3	Milímetro cúbico
MMA	Ministério do Meio Ambiente
MO	Missouri
MPF	Maturation Promoter Factor

MSD	Merck Sharp & Dohme
MtDNA	DNA mitocondrial
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
n	Número
ng	Nanogram
nm	Nanometro
no	Número
NY	New York
OA	Ooplasm area
OD	Ooplasm diameter
OTC	Ovarian Tissue Cryosystem
P	Probabilidade de significância
PBS	Phosphate buffered saline
PDT	Population doubling time
pH	Hydrogen potential
PI	Propidium iodide
Ptskf	Fibroblast lines derived from adult collared peccaries
PVS	Perivitelline space
Rebio	Reserva Biológica
RN	Rio Grande do Norte
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SCNT	Somatic Cell Nuclear Transfer
SOF	Synthetic Oviductal Fluid
SP	São Paulo
SS	Serum starvation
SS	Supressão de soro
SE	Standard error
TCM 199	Medium 199
TE	Trophectoderm
TNCS	Transferência Nuclear de Célula Somática
UFERSA	Universidade Federal Rural do Semi-Árido
USA	United States

UV	Ultraviolet
VDC	Vitrificação direta em criotubos
vs.	Versus
VSS	Vitrificação em superfície sólida
w/v	Weight per volume
ZPI	Inner oocyte diameter
ZPO	Oocyte diameter
ZPT	Zona pellucida thickness
ΔΨm	Mitochondrial membrane potential

LISTA DE SÍMBOLOS

@	Arroba
®	Marca registrada
%	Porcentagem
+	Soma
<	Menor que
=	Igual
>	Maior que
±	Mais ou menos
×	Multiplicação

SUMÁRIO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

A clonagem por transferência nuclear de célula somática (TNCS) consiste em uma biotécnica de importante papel na conservação da biodiversidade silvestre (PRIYA et al., 2014; WANI et al., 2018). Tal relevância pode ser representada por seus desmembramentos atrelados à multiplicação de reprodutores com melhores características genéticas (HUAN et al., 2015), obtenção de células induzidas à pluripotência (NOVO et al., 2016), investigação de mecanismos do desenvolvimento embrionário (HUANG et al., 2017), e edição genética (LIU et al., 2019). Por essas razões, a TNCS tem se tornado uma alternativa interessante para a conservação de diferentes espécies de mamíferos silvestres, como o elefante asiático (TECHAKUMPHU et al., 2018) e camelo bactriano (WANI et al., 2018), podendo assim ser também aplicada em catetos e em outros taiassuídeos.

Em geral, os catetos são subestimados quanto a sua importância ecológica, na qual na maioria das vezes utilizam-se de argumentos como a sua classificação na União Internacional da Conservação da Natureza (IUCN), a qual afirma que a espécie é classificada como pouco preocupante em nível mundial (GONGORRA et al., 2011). Tal classificação muitas vezes se mostra defasada e não representativa em vários biomas (NAGY-REIS et al., 2019). Assim, a urgência pelo desenvolvimento de biotécnicas que garantam estratégias de conservação para a espécie é um tema relevante. Adicionalmente, os catetos possuem alta adaptação a cativeiros, sendo ainda indivíduos interessantes economicamente pela apreciação da sua carne e couro (BODMER et al., 1990). Diante deste cenário, ter a ponto as etapas iniciais da TNCS é uma alternativa importante, uma vez que as etapas de preparo de células doadoras de núcleo (carioplastos) e citoplasmas receptores (citoplastos) poderão ser também ampliadas em outras biotécnicas, além de permitir a geração de conhecimento na biologia reprodutiva dos catetos e possivelmente ser extrapolada para outros taiassuídeos.

Embora várias pesquisas tenham demonstrado o sucesso da TNCS pelo nascimento de crias nascidas, tanto em mamíferos domésticos (BUEMO et al., 2016), quanto silvestres (FOLCH et al., 2009), a eficiência da técnica ainda é reduzida, sendo atribuída a seleção e a manipulação dos carioplatos (KWONG et al., 2014) e citoplastos (GARCIA-MENGUAL et al., 2008) como alguns dos fatores que devem ser estabelecidos na espécie de interesse. Ainda, é importante ressaltar que as etapas da TNCS possuem íntima relação com as

características biológicas da espécie, sendo importante definir e estabelecer modificações adequadas nos protocolos desenvolvidos (MOHAMMED et al., 2019).

Assim, a otimização da TNCS pela escolha de carioplastos eficientes envolve o conhecimento das alterações sofridas nas células durante seu cultivo *in vitro*, ou seja, o estabelecimento das condições de incubação *in vitro* e a avaliação do potencial de conservação das caraterísticas normais após esse período (SONG et al., 2007). Portanto, ter linhagens celulares definidas e um protocolo de criopreservação adequado que garantam que essas células possam ser armazenadas para uso futuro consiste em um fator determinante para uma maior variabilidade genética e amostragem da espécie (LEÓN-QUINTO et al., 2011). Além disso, o estágio do ciclo celular é um dos fatores mais relevantes para que o carioplasto seja reprogramado eficientemente pelo oócito receptor enucleado (CAMPBELL et al., 1996). Nesse sentido, células devem ser sincronizadas em G_0/G_1 do seu ciclo celular. Contudo, a resposta celular a diferentes protocolos tem sido variável em mamíferos domésticos (GOISSIS et al., 2007) e silvestres (MAHESH et al., 2012), necessitando da avaliação de cada protocolo para uma determinada espécie e tipo celular (WITTAYARAT et al., 2013, VERAGUAS et al., 2017).

Já no que se refere à seleção de citoplastos aptos à TNCS, o estabelecimento de protocolos de maturação *in vitro*, bem como da capacidade de ativação desses oócitos *in vitro* possui também variação em cada espécie, sendo relevante o estudo desses passos em catetos. Portanto, os protocolos de maturação partem principalmente da proximidade taxonômica entre catetos e suínos, levando em consideração as variações de tempo utilizadas em suínos (PEREYRA-BONNET et al., 2008) e suas diferentes suplementações (WU et al., 2017). Além disso, diferentes protocolos de ativação já foram avaliados e variações em respostas foram observadas em espécies domésticas (CHOI et al., 2013) e silvestres (LOI et al., 2011).

Portanto, o objetivo desta tese foi avaliar as condições de manipulação de carioplastos e citoplastos, visando à TNCS em catetos. Especificamente, para atender esse objetivo foram propostos: **i)** desenvolver as condições de manipulação de carioplastos pelo estabelecimento de linhagens celulares, associado à definição de uma solução de criopreservação dessas células e de um método de sincronização do ciclo celular em G_0/G_1 e; **ii)** avaliar diferentes condições de maturação *in vitro* e ativação artificial de oócitos.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Aspectos ecológicos e quantitativo populacional dos catetos

Os catetos (*Pecari tajacu*) também conhecidos como caititu ou porco-do-mato são pertencentes à família Tayassuidae, da ordem Artiodactyla, da subordem Suiformes e da superfamília Suoidea (DESBIEZ et al., 2012). De acordo com estudos genéticos, os catetos podem conter pelo menos dois grandes clados, ou seja, um grupo de organismos originados de um único ancestral comum exclusivo, incluindo espécimes das regiões da América do Norte, Central e do Sul (GONGORA et al., 2006; 2011) com diferenças estruturais cromossômicas, mas com semelhanças fenotípicas (ADEGA et al., 2006; GONGORA et al., 2005).

Os catetos são mamíferos com 17–35 kg, de hábito diurno-crepuscular (EMMONS; FEER, 1997) que possuem hábitos alimentares herbívoros o que proporcionam a eles uma importância ecológica como dispersores de sementes (DESBIEZ et al., 2011). Em geral, os catetos vivem em grupos de 5–30 indivíduos entre machos e fêmeas de várias faixas etárias, caracterizando-se como indivíduos altamente sociáveis e cooperativos (GONGORRA et al., 2011; BIONDO et al., 2014; KEUROGHLIAN et al., 2004). Em um estudo desenvolvido por Byers; Bekoff (1981), os autores observaram um comportamento de cooperação quando vários catetos se alimentaram próximos um do outro, consumindo a mesma planta, sendo observado ainda uma relação de parentesco com alta relação genética e filopatria feminina (BYERS; BEKOFF, 1981). Adicionalmente, a estrutura do rebanho é organizada com os mais jovens centralizados e os adultos, e principalmente fêmeas, em posições mais periféricas, tanto para a remoção de alimentos quanto para proteção de agressões ao grupo (BIONDO et al., 2014).

Quanto à distribuição populacional de catetos, segundo dados da Global Biodiversity Information Facility (GBIF, 2019), há 11.131 ocorrências de indivíduos, registradas por diferentes fontes entre os anos de 1800 a 2020, como observação não especificada (106; 1,0%), observação por máquina (433; 3,9%), amostra de material (14; 0,1%), espécime preservado (3.632; 32,6%), observação humana (6.489; 58,3%), espécime fóssil (21; 0,2%) e fontes desconhecidas (436; 3,9%). Dessas ocorrências, quatorze subespécies de catetos foram observadas (**Quadro 1**), tendo ainda mais quatro espécimes registradas a serem classificadas ou não como subespécies (GBIF, 2019). Nessas ocorrências, um alto número de locais foi identificado, sendo observada uma dispersão dos catetos nos últimos anos pela América Latina, com grande concentração no México. Assim, os catetos podem ser encontrados em

diferentes habitats em virtude de sua resistência a ações antrópicas, sendo encontrados em florestas tropicais úmidas, regiões semiáridas, áreas desertas, e em áreas de temperaturas noturnas menores que 0 °C (GONGORRA et al., 2011).

Quadro 1. Ocorrência e distribuição de subespécies de catetos.

Subespécies de catetos	N° de ocorrências	Países
angulatus (Cope, 1889)	232	EUA, México, Bolívia, Guatemala
bangsi Goldman, 1917	45	Panamá, Colômbia
crassus (Merriam, 1901)	42	México, Guatemala
crusnigrum (Bangs, 1902)	90	Costa Rica, Nicarágua, Panamá
humeralis (Merriam, 1901)	72	México
nanus (Merriam, 1901)	135	México
nelsoni Goldman, 1926	154	México, Guatemala, Belize,
niger (Allen, 1913)	10	Equador
nigrescens Goldman, 1926	9	Honduras, Nicarágua, Guatemala
patira (Kerr, 1792)	41	Venezuela, Suriname, Guiana, Brasil
sonoriensis (Mearns, 1897)	189	México, EUA
tajacu (Linnaeus, 1758)	110	Brasil, Colômbia, Venezuela, Peru, Argentina
torvus (Bangs, 1898)	86	Colômbia, Venezuela, Trindade Tobago
yucatanensis (Merriam, 1901)	122	México, Guatemala

Fonte: Global Biodiversity Information Facility (GBIF, 2019).

Já quanto à distribuição populacional de catetos, segundo a União Internacional para Conservação da Natureza (IUCN, GONGORRA et al., 2011), a espécie é classificada como menos preocupante e com distribuição em vários habitats. Por outro lado, em um estudo apresentado por Desbiez et al. (2011), os autores detalharam a situação da espécie em diferentes biomas brasileiros, classificando os catetos como menos preocupantes, mas também considerando os indivíduos como quase ameaçados na Mata Atlântica em decorrência da fragmentação e perda da qualidade de habitat bem como da caça predatória. Tal detalhamento traz a perspectiva de um possível declínio, possivelmente alterando a classificação da espécie para vulnerável à extinção (DESBIEZ et al., 2011).

Com relação ao Nordeste brasileiro, tem sido observada uma escassez de informações sobre a distribuição de catetos (CASSANO et al., 2017). Especificamente no Rio Grande do Norte, Marinho et al. (2019) apresentaram o primeiro registro dos espécimes de catetos em uma área de ecótono Caatinga-Mata Atlântica. Anteriormente, a espécie era apontada apenas por registros fósseis por Araújo-Júnior e Porpino (2011) e, posteriormente, em 2014, foi sugerido por Faria que a espécie havia sido extinta na região centro-sul do Rio Grande do Norte (Seridó). Adicionalmente, um registro de 2018 por Marinho et al. consideraram que os catetos poderiam ou estar extintos ou em locais intocados, pois não foi registrado nenhum cateto no inventário de amostragem de mamíferos de médio a grande porte do estado.

Apesar destes animais terem uma tolerância a habitas degradados, estes são considerados como indicadores da qualidade ambiental, o que demonstra que a ausência destes animais em paisagens alteradas é sinal de muita perturbação ambiental (DESBIEZ et al., 2011). Em nosso bioma Caatinga foram listadas as principais ameaças aos catetos que são ocasionadas por atividades como a geração de energia eólica, a produção de carvão, utilização de lenha, cultura de mamona e pinhão como fonte de biocombustíveis, ampliação de plantações de sisal, área de pastagem que também agravam o desmatamento e fragmentação do habitat, bem como, a criação extensiva de caprinos, ovinos e bovinos (DESBIEZ et al., 2011).

Portanto, diante da importância ecológica dos catetos e as inúmeras ações antropológicas que ameaçam o quantitativo populacional desta espécie, estratégias de conservação representam ferramentas essenciais para a manutenção da biodiversidade. Nesse contexto, em catetos, estratégias de conservação *in situ* e *ex situ* já foram desenvolvidas por diferentes grupos, objetivando desta forma a conservação da espécie.

2.2 Estratégias de conservação *in situ* aplicadas em catetos

A conservação *in situ* consiste em proteger toda área onde a espécie está inserida, preservando não somente a espécie em si, mas todo o ecossistema o qual a espécie pertence, permanecendo assim todo o habitat natural (MMA, 2020). Em geral, o desenvolvimento de estratégias *in situ* segue os pressupostos constituídos pela Convenção sobre Diversidade Biológica (BRASIL, 2000), no qual estabelecem as condições de áreas protegidas, regulamentações dos recursos biológicos, e uso sustentável dos recursos naturais. Assim, a conservação *in situ* tem como vantagens permitir que espécies continuem seus processos

evolutivos, mantendo as melhores condições para a conservação da vida silvestre (MMA, 2020).

No Brasil, o Ministério do Meio Ambiente estabeleceu as estratégias de conservação *in situ* baseadas na formação de Unidades de Conservação, as quais são definidas como 10% da área de cada bioma destinada para a proteção ambiental. Assim, para catetos, diferentes regiões mantêm unidades de conservação com a espécie, tais como o Parque Nacional de Brasília (ICMBio, 2012), Reserva Biológica do Tinguá, Rio de Janeiro (ICMBio, 2015), e Reserva Biológica do Gurupi na Amazônia Maranhense (ICMBio, 2017). Contudo, somente na Estação Ecológica de Pirapitinga do Bioma Cerrado e na Reserva Biológica União do bioma Mata Atlântica, o cateto é descrito dentre a Lista de Espécies Ameaçadas protegidas da Unidade de Conservação (ICMBio, 2020).

Adicionalmente, a criação comercial de animais silvestres é uma alternativa para produtores rurais de forma sustentável, possibilitando o aproveitamento de áreas e igualmente configurando-se como uma ferramenta ecológica de conservação desses indivíduos Além disso, o manejo dos catetos é previsto pelo Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) pelo Decreto n° 6.099, de 26 de abril de 2007 (nº 02001.005418/2007-11). Portanto, a criação em cativeiro nos locais onde esses animais são encontrados em maior quantidade pode também ser usada de forma econômica e sustentável.

Não somente no Brasil, mas em outros países onde catetos podem ser encontrados, uma série de estudos já foram desenvolvidos *in situ* (**Figura 1**) em áreas preservadas ou em florestas onde estes animais possuem habitats. Adicionalmente, tem sido observado que embora haja uma ampla distribuição desta espécie nas Américas, a maioria dos trabalhos tem sido realizada no Brasil, ressaltando a sua distribuição em diferentes biomas.

Contudo, o número de trabalhos ainda se mostra bem reduzido em virtude provavelmente por a conservação *in situ* ser onerosa, mediante das necessidades de manejo e monitoramento constantes. Além disso, é de extrema importância destacar que a conservação de uma espécie em um ou poucos locais de ocorrência não significa a conservação de toda a sua variabilidade genética (MMA, 2020). Assim, apesar de todas as vantagens da conservação *in situ* ela não mostra uma garantia ampla de preservar a variabilidade genética que é um dos pontos que muitas vezes torna a discussão sobre métodos *ex situ* questionáveis. Adicionalmente, é interessante destacar que a conservação da biodiversidade só poderá ser alcançada por uma soma de estratégias atuando conjuntamente para aliar forças na manutenção da biodiversidade.

LOCAL	FINALIDADE	AUTORES
Texas	Avaliação da população	Hellgren et al. (1995)
Peru	Descrição da biologia reprodutiva de fêmeas	Gottdenker; Bodmer (1998)
Chaco Argentino	Distribuição e interação com fatores humanos	Altrichtera; Boaglio (2003)
Mata Atlântica	Monitoramento da área	Keuroghlian et al. (2004)
Pantanal	Interações competitivas com espécie invasora	Desbies et al. (2009)
Alagoas	Impacto dos catetos na dispersão de sementes	Lazure et al. (2010)
México	Avaliação da população em resposta à caça	Briceño-Méndez et al. (2016)
América do Sul	Influência do espaço geográfico na forma do crânio	Hendges et al. (2016)
Deserto de Sonora Arizona	Comportamento anti-predador	Lundgren; Moeller (2017)
Minas Gerais	Observações sobre história natural, conservação e distribuição regional	Silveira; Pacheco (2018)
Peru	Desenvolvimento embrionário e fetal	Mayor et al. (2019)
Chaco Argentino	Avaliação do estresse durante o desmatamento	Semper-Pascual et al. (2019)

Figura 1. Estratégias de conservação *in situ* desenvolvidas em catetos.

2.3 Estratégias de conservação *ex situ* aplicadas em catetos

A conservação *ex situ* consiste na manutenção de espécies fora de habitats naturais, tendo como principais objetivos a conservação do material genético por tempo indeterminado, permitindo que em um único local haja uma amostragem genética de muitas procedências e garantindo que haja a proteção de uma mesma espécie que tem uma distribuição geográfica muito ampla em um mesmo espaço (MMA, 2020). Neste intuito, a conservação *ex situ* pode ser considerada de duas maneiras: *ex situ in vivo* que se trata de medidas que visem a conservação em uma área restrita onde o animal geralmente é mantido em cativeiros ou fazendas (SONGSASEN; COMIZZOLI, 2019); e a *ex situ in vitro* que engloba biotecnologias reprodutivas ou técnicas de reprodução assistida (ANDRABI; MAXWELL, 2007).

No Brasil, estratégias de conservação *ex situ* são desenvolvidas de acordo com a Convenção sobre Diversidade Biológica (BRASIL, 2000), a qual são considerados componentes de diversidade biológica, estabelecimento de instalações adequadas, medidas para recuperação, regeneração e reintrodução de espécies, e colheita de recursos biológicos. Em catetos, estratégias tanto *in vivo* quanto *in vitro* já foram desenvolvidas, conforme descrição a seguir.

2.3.1 Estratégias de conservação *ex situ in vivo* aplicadas em catetos

A conservação *ex situ in vivo* consiste em estratégias que são realizadas em um ambiente delimitado, que não é o habitat natural da espécie, como fazendas de criação e cativeiros. Em catetos, as estratégias de conservação *ex situ in vivo* estão relacionadas, principalmente, ao desenvolvimento do conhecimento biológico acerca da espécie e em respostas fisiológicas e comportamentais (**Quadro 2**). A partir deste conhecimento tem sido possível desenvolver técnicas de manejo e biotecnologias que sejam mais adequadas para a espécie.

Assim, desde os primeiros estudos buscando conhecer os hábitos e a fisiologia em catetos (HELLDREN et al., 1985), trabalhos relacionados ao conhecimento fisiológico do macho (HELLDREN et al., 1989; FILGUEIRA et al., 2005; PEIXOTO et al., 2012a) e da fêmea (MAYOR et al., 2005; 2006; 2007; BATISTA et al, 2007; GARNERO et al., 2013; MAIA et al., 2014a,b; SILVA et al., 2016; AHUJA-AGUIRRE et al., 2017; MAYOR et al., 2019) têm sido desenvolvidos. Adicionalmente, estudos relacionados à aplicação destes

conhecimentos em técnicas de manejo e ferramentas biotecnológicas têm sido estabelecidos (GARBOR et al., 1997; SOUZA et al., 2009).

Quadro 2. Desenvolvimento de estratégias *ex situ in vivo* em catetos.

Finalidade	Autores
Resposta endócrina e metabólica à anestésico	Helldren et al. (1985)
Comparação do perfil dos níveis de testosterona, características seminais e mensuração testicular em animais de cativeiro e de vida livre	Helldren et al. (1989)
Avaliação de um adequado protocolo anestésico	Gabor et al. (1997)
Avaliação de parâmetros reprodutivos	Pinheiro et al. (2001)
Monitoramento reprodutivo por ultrassonografia e perfil hormonal em fêmeas	Mayor et al. (2005;2012)
Avaliação do primeiro estro pós parto e gestação em indivíduos da Amazônia	Mayor et al. (2006b)
Descrição das características estrais em indivíduos do leste da Amazônia	Mayor et al. (2007a,b)
Biometria e alterações histopatológicas dos testículos	Filgueira et al. (2005)
Padrões de atividades em cativeiro	Venturieri; Pendu (2006)
Patologias do sistema genital feminino em cativeiro	Batista et al. (2007)
Níveis de estresse produzido por densidade populacionais	Montes-Pérez et al. (2009)
Avaliação de protocolo anestésico para eletroejaculação	Souza et al. (2009)
Determinação do ciclo estral por aspectos colpocitológicos e clínicos	Guimarães et al. (2011)
Avaliação do regime de enriquecimento alimentar em cativeiro	Nogueira et al. (2011)
Análise da morfologia e ultrassonografia de órgãos abdominais em machos	Peixoto et al. (2012a)
Variação sazonal na disponibilidade dos frutos	Santos; Nogueira-Filho 2012
Avaliação da atividade do hormônio adrenocorticotrófico	Coradello et al. (2012)
Relação entre a biometria testicular e características seminais	Peixoto et al. (2012b)
Descrição da curva de crescimento de fêmeas criadas em cativeiro	Garnero et al. (2013)
Achados *post-mortem* em cativeiro no Nordeste do Brasil	Batista et al. (2014)

Comportamento social em cativeiro	Biondo et al. (2014)
Monitoramento do ciclo estral em cativeiro no semiárido nordestino	Maia et al. (2014a, b)
Influência de anestésicos sobre a ereção e ejaculação após eletroejaculação	Paiva et al. (2014)
Comportamento sexual no período periovulatório e início da gestação	Silva et al. (2016a)
Relação de dominância em cativeiro	Silva et al. (2016b)
Métodos não invasivos para avaliação da biometria testicular	Peixoto et al. (2016)
Perfis hormonais em diferentes estágios reprodutivos de fêmeas	Ahuja-Aguirre et al. (2017)
Monitoramento reprodutivo por análise dos metabólitos fecais	Mayor et al. (2019)
Variações climáticas sobre características reprodutivas do macho	Maia et al. (2019)
Sincronização do estro e inseminação artificial	Peixoto et al. (2019)
Habilidades anti-predatórias em cativeiro	Faria et al. (2020)

Além disso, trabalhos têm sido realizados para investigar os aspectos reprodutivos, como as relações da biometria testicular e características seminais (PEIXOTO et al., 2012b), monitoramento do estro (MAIA et al., 2014a), sincronização do estro (MAIA et al., 2014b), influência de variações climáticas sobre o macho (MAIA et al., 2019), e inseminação artificial (PEIXOTO et al., 2019). Tais estudos evidenciam a necessidade da continuidade de investigações nesta espécie.

2.3.2 Estratégias de conservação *ex situ in vitro* aplicadas em catetos

As técnicas de conservação *ex situ in vitro* consistem no uso de técnicas de criopreservação para a manutenção de recursos biológicos, bem como seu emprego em biotecnologias assistidas. Em catetos, as primeiras pesquisas foram voltadas para a caracterização fisiológica reprodutiva, tanto em machos (SONNER et al., 2004; CAMPOS et al., 2017) (**Figura 2**), quanto em fêmeas (LIMA et al., 2012; SILVA et al., 2016) (**Figura 3**). Adicionalmente, a conservação de amostras somáticas tem sido realizada com intuito de cultivar (SANTOS et al., 2016) e criopreservar amostras somáticas (BORGES et al., 2017b), visando aplicações futuras.

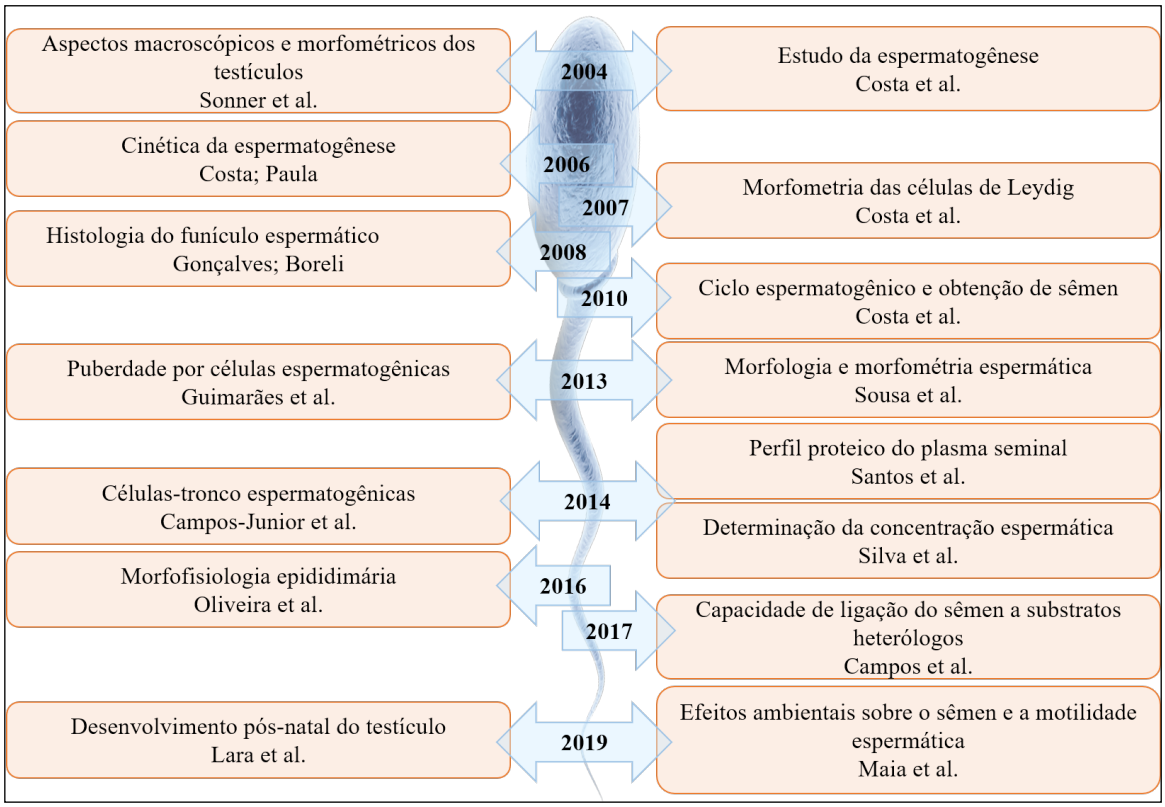

Figura 2. Características reprodutivas de catetos a partir de estudos *in vitro* sobre a morfofisiologia do macho.

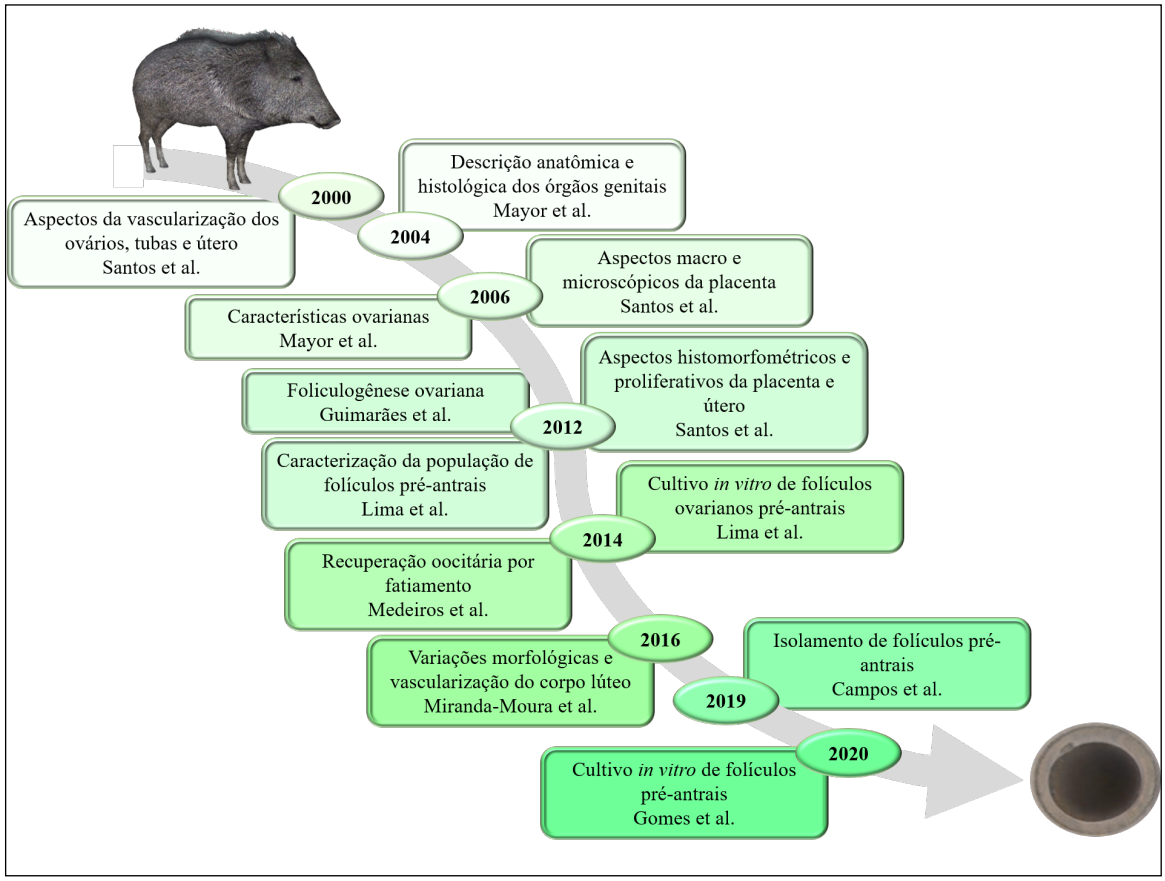

Figura 3. Evolução do conhecimento sobre aspectos reprodutivos de fêmeas de *P. tajacu* investigados *in vitro*.

Paralelamente, estratégias reprodutivas foram empregadas nesses animais (**Quadro 3**), especialmente quanto à conservação de germoplasma masculino (SILVA et al., 2019), e feminino (CAMPOS et al., 2019). Interessantemente, os trabalhos evidenciaram a importância de desenvolver técnicas específicas para esta espécie, uma vez que apesar de muitas vezes o ponto de partida ser protocolos de suínos, devido algumas similaridades com os catetos, não se pode extrapolar o conhecimento acerca dos suínos para a espécie em questão.

Especificamente, em amostras somáticas (**Figura 4**), a partir de 2016, Santos et al. iniciaram os estudos visando o melhor conhecimento sobre o material somático de catetos, a fim de conhecer as características do cultivo *in vitro* de células somáticas desta espécie. Posteriormente, Borges et al. (2017a) identificaram os aspectos do tecido somático e as células que o compõe, sendo possível conhecer as peculiaridades deste tecido e desenvolver um protocolo de criopreservação específico. Ainda em 2017, foi estabelecido a melhor técnica de criopreservação para tecidos somáticos, a qual a vitrificação em superfície sólida foi mais adequada que a vitrificação direta em criotubos (BORGES et al., 2017b) e a melhor solução crioprotetora, a qual consistiu de 3,0 M de etilenoglicol, 0,2 M de sacarose e 10% de soro fetal bovino (BORGES et al., 2018a; BORGES et al., 2018b). Tais estudos permitiram desenvolver o armazenamento adequado de tecidos somáticos da espécie. Adicionalmente, em situações onde os indivíduos encontram-se distantes dos laboratórios de cultivo, técnicas de refrigeração dos tecidos somáticos de catetos foram também avaliados (QUEIROZ NETA et al., 2018).

Quadro 3. Estratégias *ex situ in vitro* aplicadas em catetos.

AVANÇO	AUTORES	AVANÇO	AUTORES
Conservação de germoplasma		**Outras biotécnicas/ferramentas**	
Influência da taxa de descongelação sobre a criopreservação de sêmen	Castelo et al. (2010b)	Análise evolutiva por relações genéticas e moleculares	Theimer; Keim (1994); Gongora; Morgan (2005); Gongora et al. (2006); Adega et al. (2006); Lee et al. (2017)
Efeito da centrifugação e suplementação com sacarídeos sobre a criopreservação de sêmen	Castelo et al. (2010a)	Análise da origem do plexo braquial	Moura et al. (2007)
Conservação de sêmen a 17 °C	Garcia et al. (2012)	Avaliação de protozoários gastrointestinais	Farret et al. (2010)
Uso da água de coco em pó na criopreservação de sêmen	Silva et al. (2012)	Avaliação da variabilidade genética	Silva et al. (2010)
Avaliação de diferentes concentrações de glicerol e gema de ovo na criopreservação de sêmen	Alves et al. (2013)	Aspectos hematológicos	Almeida et al. (2011)
Avaliação de sêmen usando diferentes curvas de congelamento, tamanhos de palheta e taxas de descongelação	Silva et al. (2013)	Ensaio metabólico por glicocorticoides fecais	Coradello et al. (2012)
Conservação por curtos períodos de folículos ovarianos pré-antrais	Lima et al. (2014)	Diferenciação de células-tronco mesenquimais de tecido adiposo	Pessoa et al. (2014)

Uso de diferentes concentrações de *Aloe Vera* na criopreservação de sêmen	Souza et al. (2016)	Descrição histológica da glândula dorsal	Morales et al. (2015)
Vitrificação de tecido ovariano utilizando Ovarian Tissue Cryosystem (OTC)	Campos et al. (2019)	Diferenciação de células-tronco mesenquimais do sangue	Argôlo-Neto et al. (2016)
Vitrificação de tecido ovariano usando diferentes crioprotetores	Lima et al. (2019)	Morfologia e função da retina	Ezra-Elia et al. (2018)
Vitrificação de tecido testicular usando diferentes crioprotetores	Silva et al. (2019)	Suscetibilidade ao vírus da síndrome reprodutiva e respiratória porcina (PRRSV)	Molina-Barrios et al. (2018)

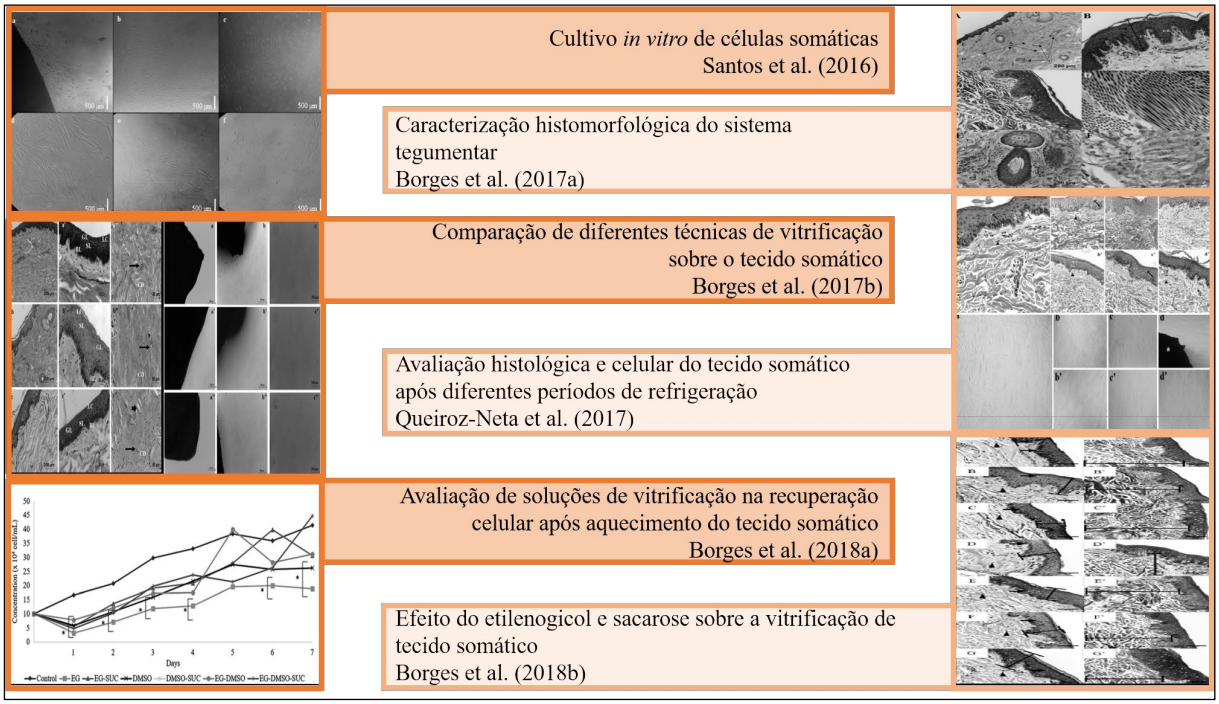

Figura 4. Caminho percorrido no estabelecimento de amostras somáticas de catetos.

Diante deste contexto, é possível entender que ainda há lacunas sobre a pesquisa básica na espécie, bem como a necessidade de novos estudos no desenvolvimento das biotecnologias a serem continuadas. Assim, avanços importantes têm norteados os próximos passos na conservação desta espécie por diferentes grupos de pesquisa.

3 JUSTIFICATIVA

A alta degradação ambiental, especialmente do bioma Caatinga, junto com a redução de mamíferos silvestres que compõem este ambiente, como os catetos, torna urgente o desenvolvimento de estratégias de conservação que possam ser aplicadas a esta espécie. Diante do potencial da transferência nuclear de célula somática (TNCS) na manutenção da biodiversidade, o esclarecimento dos primeiros passos desta técnica em catetos são fundamentais para a aplicação da TNCS visando sua conservação e criação sustentável.

Assim, é essencial o isolamento, caracterização e criopreservação de fibroblastos de catetos que possam ser utilizados como células doadoras de núcleos (carioplastos). Além disso, um protocolo mais efetivo para sincronização do ciclo celular G_0/G_1 garante que a reprogramação celular ocorra com sucesso durante a TNCS. Ainda, para que haja o sucesso na clonagem é necessário que o gameta feminino utilizado como célula receptora do núcleo (citoplasto) esteja preparado para orquestrar a reprogramação embrionária. Partindo desta necessidade, o ambiente de cultivo de oócitos deve garantir a maturação ideal para alcançar o seu potencial meiótico. Subsequentemente, o protocolo de ativação oocitária artificial necessita ser eficiente para proporcionar o desenvolvimento do embrião reconstruído.

Com base na importância destas etapas, este trabalho se preocupou em estabelecer o passo a passo do desenvolvimento inicial da técnica de TNCS, tanto dos carioplastos quanto dos citoplastos.

4 HIPÓTESES CIENTÍFICAS

4.1 Fibroblastos derivados da pele de catetos resultam em linhagens celulares com padrões proliferativos e metabólicos não alterados pelo tempo de cultivo e pela criopreservação;

4.2 A adição de altas concentrações de soro fetal bovino (SFB) na solução de congelação lenta promovem uma melhor conservação de células somáticas de catetos após a descongelação;

4.3 A inibição por contato, independente da duração, promove a sincronização das células somáticas de catetos no estágio de G_0/G_1 do ciclo celular, não afetando ainda a viabilidade destas células;

4.4 Oócitos de catetos necessitam de um tempo de 48 h de maturação *in vitro* para o completo desenvolvimento meiótico, apresentando similaridade com oócitos suínos;

4.5 Oócitos de catetos aumentam sua habilidade meiótica quando maturados na presença de fator de crescimento epidermal (EGF);

4.6 A habilidade de desenvolvimento embrionário de oócitos de catetos após a ativação artificial é dependente do tipo de ativador secundário.

5 OBJETIVOS

5.1 Objetivo Geral

Definir as condições de manipulação de carioplastos e citoplastos, visando à transferência nuclear de célula somática (TNCS) em catetos.

5.2 Objetivos Específicos

5.2.1 Estabelecer linhagens celulares de catetos a partir de ensaios que garantam a qualidade das células para uso em TNCS;

5.2.2 Avaliar a criotolerância de células somáticas de catetos usando um agente intracelular [dimetilsulfóxido (DMSO)] em associação a diferentes crioprotetores extracelulares (sacarose e soro fetal bovino) e suas concentrações;

5.2.3 Comparar a eficiência da sincronização nas fases G_0/G_1 e viabilidade de fibroblastos de catetos, usando as estratégias: inibição por contato *vs.* privação do soro *vs.* agentes químicos [DMSO, cicloheximida (CHX), citocalasina B (CB) ou 6-dimetilaminopurina (6D)] a diferentes tempos de incubação;

5.2.4 Investigar a relação entre o potencial meiótico e os períodos (24 h *vs.* 48 h) de maturação *in vitro* de oócitos derivados de catetos;

5.2.5 Identificar a influência do fator de crescimento epidermal (EGF) no meio de maturação *in vitro* de oócitos de catetos;

5.2.6 Avaliar a ativação artificial de oócitos de catetos usando agentes químicos como ativador primário (ionomicina) e secundários (6D *vs.* 6D + CB *vs.* CHX *vs.* CHX + CB) sobre o desenvolvimento embrionário.

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CAPÍTULO 2 – POTENTIAL ROLE OF INTRASPECIFIC AND INTERSPECIFIC CLONING IN THE CONSERVATION OF WILD MAMMALS (Artigo de revisão)

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Potential role of intraspecific and interspecific cloning in the conservation of wild mammals

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Summary

Intraspecific and interspecific cloning via somatic cell nuclear transfer (iSCNT) is a biotechnique with great possibilities for wild mammals because it allows for the maintenance of biodiversity by recovering species, nuclear reprogramming for the production of pluripotency-induced cells, and studies related to embryonic development. Nevertheless, many areas in cloning, especially those associated with wild mammals, are still in question because of the difficulty in obtaining cytoplasmic donor cells (or cytoplasts). On the other hand, donor cell nuclei (or karyoplasts) are widely obtained from the skin of living or post-mortem individuals and often maintained in somatic cell banks. Moreover, the creation of karyoplast-cytoplast complexes by fusion followed by activation and embryo development is one of the most difficult steps that require further clarification to avoid genetic failures. Although difficult, cloning different species, such as wild carnivores and ungulates, can be successful via iSCNT with embryo development and the birth of offspring. Thus, novel research in the area that contributes to the conservation of biodiversity and knowledge of the physiology of species continues. The present review presents the failures and successes that occurred with the application of the technique in wild mammals, with the goal of helping future work on cloning via iSCNT.

Keywords: Biodiversity, Biotechnology, Embryo development, SCNT, Somatic cells.

Introduction

The decrease in the biodiversity of wild mammals has been caused mainly by human activity, resulting in an increase in research aimed at the development of conservation strategies (Pereira *et al.,* 2016). In general, several techniques to help with the conservation of wild animals are available, including the formation of biobanks (León-Quinto *et al.,* 2009), artificial insemination (Howard *et al.,* 2016), embryo transfer (Goeritz *et al.,* 2012), in vitro fertilization (Herrick *et al.,* 2010), and cloning using somatic cell nuclear transfer (SCNT, Folch *et al.,* 2009). Because of the low availability of oocytes for SCNT, interspecific cloning using intraspecific and interspecific nuclear transfer techniques (iSCNT) has been shown to be an important tool in conservation (Wani *et al.,* 2017).

The main argument for the application of iSCNT is the rapid decrease in the number of species. Any tool that can avoid this decrease is important. iSCNT preserves and even expands genetic variability when somatic cells of different individuals representative of the

original biodiversity of a population are collected for its use (Loi *et al.,* 2001). In addition, interest in cloning has increased not only for the conservation of endangered species, but also for the multiplication of reproducers with better genetic characteristics (Saini *et al.,* 2015), basic research on cell epigenetic status (Saragusty *et al.,* 2016), embryonic development (González-Grajales *et al.,* 2016), and the production of induced pluripotent cells (Sukparangsi *et al.,* 2018).

Therefore, in all applications of cloning, studies related to the improvement of iSCNT, as well as its wide use in different individuals, are important.

Overview of the iSCNT technique and its limitations

The iSCNT technique involves embryo reconstruction by fusing a nucleus of a donor cell (karyoplast) derived from a wild mammal with an enucleated oocyte (cytoplast) from a domestic mammal of a different species, family, order, or class (Do & Taylor-Robinson, 2014). The nucleus in G0/G1 is exposed to reprogramming by the oocyte, followed by the fusion and activation of the reconstructed embryo (Loi *et al.,* 2011). Subsequently, the resultant embryo can be transplanted into the uterus of a recipient for term development (Pereira & Freitas, 2009).

Different steps are involved in the production of clones via iSCNT. Therefore, it is interesting to highlight the steps of the technique and its peculiarities that can define the success of cloning by iSCNT.

Preparation of cytoplasts

Whether using the oocyte from a domestic or a wild mammal, some fundamental criteria must be met to obtain a cytoplast suitable for cloning, such as oocyte selection, in vitro maturation, and enucleation systems (Loi *et al.,* 2011). In general, follicular size, the oocyte collection method, and the culture environment are factors that can affect the quality of mature oocytes, and different responses to these factors can be observed in wild mammals. In some cervid species, Brahmansani *et al.* (2013) observed that low maturation rates could probably be caused by the slicing. In this method, non-competent oocytes can be recovered since the technique can result in the recovery of structures of small diameter follicles (Rho *et al.,* 2001). Additionally, the quality of ovaries obtained post-mortem may have been one of the factors that reduce the quality of oocytes in these species.

Thus, studies have shown that enriched culture media and ovarian transport conditions may result in good results using ovaries from post-mortem animals for recovery of immature oocytes. Macías-Gárcia *et al.* (2018) verified that oocytes of Iberian red deer (*Cervus elaphus hispanicus*) obtained from ovaries maintained for 16 h in a holding medium increased the oocyte meiotic competence. Moreover, these authors observed that the epidermal growth factor (EGF) demonstrated a differencial effect depending upon oocyte gradind and conditions of ovary transportation. Addittionally, for ovaries derived from Hokkaido sika deer (*Cervus nippon yesoensis*), maturation rates of oocytes were highest when ovaries were kept for 12 h at 20–25 °C, when compared to 24 h (Tulake *et al.,* 2014).

Specifically, with respect to the culture environment, the requirements for both composition and maturation time should be established for the in vitro maturation of each species. In the Indian blackbuck (*Antilope cervicapra*), oocytes cultured in the presence of gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) showed higher rates of expansion of the cumulus oophorus (79.3%) and extrusion of the first polar body (46.1%) compared to oocytes cultured without gonadotropins (60.4% and 33.3%, respectively) (Rao *et al.,* 2010). In the Sika deer (*Cervus nippon hortulorum*), oocytes cultured in medium supplemented with fetal bovine serum (FBS), FSH, LH, cysteamine and EGF resulted in a higher maturation rate (75.4%) compared to medium without supplementation (30.1%; Yin *et al.,* 2013). Already, different hormonal combinations of FSH, LH and 17β-estradiol did not alter the maturation rates in oocytes derived from lions (*Panthera leo*, Fernandez-Gonzalez *et al.,* 2015). In the collared peccary (*Pecari tajacu*), we proved that oocytes need 48 h to achieve maturation instead of 24 h, according to the expansion of the cumulus cells (100% vs. 38.1%), the presence of first polar body (90.5% vs. 52.4%), and the status of the nucleus in the second metaphase (76.2% vs. 52.4%), respectively (Borges *et al.,* 2018c).

In addition to obtaining mature oocytes, the preparation of cytoplasts depends on the method of enucleation of these structures. The amount of ooplasm present in the reconstructed embryo is related to the enucleation technique that removes the nucleus from the oocyte. Matured oocytes can be enucleated in different ways, including squeezing the first polar body and the surrounding cytoplasm through a cleft in the zona pellucida of the oocyte (Lee *et al.,* 2003). Another method is manual removal in which zona-free oocytes are enucleated with a bisection blade that handbisect the metaphase II chromosomes along with a small volume of

the surrounding cytoplasm. Oocytes can also be aspirated using a micromanipulator at the location of the metaphase II chromosomes and the polar body via brief exposure to ultraviolet light (Pereira *et al.,* 2015).

Selection of karyoplasts

To obtain karyoplasts appropriate for cloning, their type and age and the manipulation techniques used are important for their future reprogramming (Kim *et al.,* 2007). Karyoplasts can be obtained from fresh or cryopreserved somatic tissues (Folch *et al.,* 2009, Pan *et al.,* 2014), from an adult (Moulavi *et al.,* 2017) or a fetus (Liu *et al.,* 2018), and in vivo or post-mortem (Pereira *et al.,* 2014). Although the recovery of these cells is not a difficult task, their processing and preservation until use in iSCNT require attention (Pereira *et al.,* 2014). In general, skin cells have been the most used cell type for karyoplasts (Song *et al.,* 2007). The skin has an abundance of cells of interest that may have different efficiencies in cloning, as observed in wild buffalo (*Bubalus arnee*). Saini *et al.* (2015) detected that fibroblasts of this species are easier to reprogram than epithelial cells.

After harvest, cells used as nuclei donors need to be characterized with respect to their culture conditions, cryopreservation, and cell cycle synchronization (Pereira *et al.,* 2014). For these steps, cells are evaluated for the number of passages, nutritional requirements during in vitro culture (Santos *et al.,* 2016), and the damage done during cryopreservation (Song *et al.,* 2007). Thus, karyoplasts have been established in vitro in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS and growth factors (Santos *et al.,* 2016).

Karyoplasts have been routinely cryopreserved by slow freezing (Sharma et al., 2018) using a combination of dimethyl sulfoxide (DMSO), FBS, and sucrose as the cryoprotectant, as observed with Iberian lynx (*Lynx pardinus*, León-Quinto *et al.,* 2014). Although it is more desirable to use a somatic cell bank after tissue culture, the absence of in vitro culture conditions sometimes makes these banks unfeasible, resulting in the immediate formation of the targets for those somatic tissues (Borges *et al.,* 2017a,b; Queiroz Neta *et al.,* 2018). The three somatic tissue conservation techniques used for wild animals are slow-freezing cryopreservation (Mestre-Citrinovitz *et al.,* 2016), vitrification (Borges *et al.,* 2018a,b), and cooling at 4–6°C (Queiroz Neta *et al.,* 2018). In collared peccaries, we compared two techniques of vitrification and we observed that the solid-surface vitrification was found to be more efficient method for vitrifying skin tissue when compared to direct vitrification in

cryovials, probably due tissues not be involved in a large amount of cryoprotectants before passing through a drastic change in temperature during the solid-surface vitrification (Borges *et al.,* 2017b).

Finally, the third step in the preparation of the karyoplasts is cell synchronization in the G0/G1 stage (Gómez *et al.,* 2003; Yelisetti *et al.,* 2016). In general, nuclear reprogramming is controlled by epigenetic modification. For this to occur, the somatic cells must be in G0/G1 to allow the removal of reversible epigenetic changes acquired during cell differentiation (Song *et al.,* 2007). Thus, cells can be subjected to different treatments for synchronization during culture. Inhibition by contact (Moulavi *et al.,* 2017), serum deprivation (Wani *et al.,* 2017), and chemicals that inhibit the cell cycle (Gómez *et al.,* 2003) are methods used for synchronization. Serum deprivation and inhibition by contact are the most commonly used (Moulavi *et al.,* 2017).

Under high confluence or serum privation, fibroblast cells derived from the skin of adult argali (*Ovis ammon*) were efficiently synchronized at G0/G1; nevertheless, cells were in lower proportion in the growing stage (Pan *et al.,* 2014). Authors observed that the highest proportion of cells from the African wild cat (*Felis silvestris lybica*) at G0/G1 was obtained by serum deprivation compared with that obtained by inhibition by contact and the inhibitor roscovitine (Gómez *et al.,* 2003). Leopard (*Panthera pardus*) skin cells treated with chemical inhibitors such as sodium butyrate have a greater propensity to undergo alterations (Yelisetti *et al.,* 2016).

Embryonic reconstruction stages

After the transfer of the nucleus into the enucleated oocyte, the cytoplast-karyoplast complex is subjected to an electric pulse that not only induces the fusion of the somatic cell nucleus with the enucleated oocyte to form a new complex, but also promotes the release of intracellular calcium that initiates cellular activation (Pereira & Freitas, 2009). In general, the successful development of a reconstructed embryo depends on the complex interactions between the cytoplast and the nuclear structure during embryonic development; failures in this interaction can cause problems during early cleavage and embryonic development (González-Grajales *et al.,* 2016).

The activation of the cytoplast-karyoplast complexes guarantees adequate embryonic reconstruction (Yamochi *et al.,* 2013). Because the iSCNT technique reprograms the nucleus of a somatic cell of one species using the oocyte cytoplasm of another species, it is essential that the activation protocol be able to activate the reconstructed embryo (Zhao *et al.,* 2006). Physiologically, a mammalian oocyte is activated during fusion with a sperm, releasing meiotic cell cycle arrest and enabling the resumption of the oocyte meiotic cell cycle (Sparman *et al.,* 2010). Therefore, a well-developed protocol allows a high rate of blastocyst formation by promoting good embryonic development through activation.

Activation protocols, including physical methods such as electrical pulses and alteration of osmolarity, and chemical methods such as calcium-mobilizing compounds like strontium chloride, ionomycin, and ethanol, to promote the initial release of calcium have been evaluated in different species, as sika deer (Yin *et al.,* 2013), alpaca (*Vicugna pacos*) and llama (*Lama glama*, Ruiz *et al.,* 2015), with blastocyst rates of 32.4%, 22.5% and 18.7%, respectively. In general, calcium mobilizers are used in combination with kinase protein inhibitors or protein synthesizers such as cyclohexamide and 6-dimethylaminopurine (6-DMAP). In addition, a cytostatic factor inactivator and microfilament inhibitor such as cytochalasin B are used to prevent extrusion of the second polar body and maintain the diploidy of the presumed embryo (Ruiz *et al.,* 2015).

For red deer (*Cervus elaphus*), electrical activation before chemical activation with ionomycin and 6-DMAP was efficient for the production of clone embryos (32–44%), obtaining genetically healthy calves (Berg *et al.,* 2007). Nevertheless, the same protocol resulted in a low developmental rate (5.7%) of activated oocytes in swamp deer and 0.0% embryos in spotted deer, sambar deer, and Brow-antlered deer after oocyte parthenogenetic activation (Brahmasani *et al.,* 2013). Blackbuck (*Antilope cervicapra*) oocytes activated with ionomycin and 6-DMAP resulted in 58% cleaved embryos and 13% blastocysts (Rao *et al.,* 2010). Therefore, the artificial activation method (chemical, electrical protocols or your combination) can result in different responses among species. In this sense, it is necessary to evaluate the type of artificial activation that promotes the best rates of embryonic development in the species of interest.

In vitro culture systems are essential for early embryonic development and nuclear reprogramming (Gómez *et al.,* 2008; Pereira *et al.,* 2013). Choosing the appropriate culture

medium for each species is considered the initial step in proper embryonic development (Zhao *et al.,* 2006). Lee *et al.* (2003) used somatic cells of the mountain bongo (*Tragelaphus eurycerus isaaci*) and domestic cow (*Bos taurus*) oocytes and observed that a chemically defined, protein-free medium of TCM199 supplemented with FBS supported embryonic development. Nonetheless, there is no one culture medium suitable for all species that allows better embryonic development for a given species under study.

Finally, the effect of epigenetic reprogramming is a very relevant factor in the success of iSCNT (Gómez *et al.,* 2008). Some epigenetic markers were characterized with respect to their function during embryonic reprogramming and their influence on the chromatin structure from post-translational modifications (Song *et al.,* 2007). The overall level of the acetylation of histone H3 at lysine 18 (H3K18ac) and trimethylation of histone H3 at lysine 27 (H3K27me3), and the expression level of some important apoptosis proteins (caspase 3 and caspase 7), and p53 were evaluated. The hyperacetylated state of histones is associated with transcriptionally active domains, while the hypoacetylated state is associated mainly with silenced chromatin regions of histone acetyl transferases and histone deacetylases. The methylation pattern of the DNA is determined by DNA methyltransferases. OCT3/4, NANOG, and CDX2 are very important because of their close association with pluripotency and early embryonic development (Saini *et al.,* 2015).

Advances and perspectives of iSCNT in wild mammals

Several works aimed at cloning different wild mammals have been conducted (Table 1). Among these studies, those that obtained offspring were on wild bovine (Lanza *et al.,* 2000), sheep (Loi *et al.,* 2001), felid (Gómez *et al.,* 2004; Li *et al.,* 2007), canid (Kim *et al.,* 2007; Oh *et al.,* 2008), and goat (Folch *et al.,* 2009). Therefore, several families have proven the success of using iSCNT for the recovery and reintroduction of wild mammals.

An important point to remember is that as the taxonomic distance between donor and recipient species increases, the production of blastocysts decreases because of the decreased ability of somatic cells to be reprogrammed (Priya *et al.,* 2014). In general, enucleated oocytes are from a domestic species that is phylogenetically close to the wild species that donates the nucleus. For example, domestic sheep cytoplasts were able to reprogram argali fibroblast nuclei (Pan *et al.,* 2014) and domestic buffalo cytoplast was able to reprogram wild buffalo karyoplast (Priya *et al.,* 2014).

Table 1 iSCNT in some wild mammals.

Scientific Name	IUCN Category*	Country	Karyoplast	Cytoplast	Outcome	Authors
Carnivores						
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	17% pregnancies, 2 pups	Kim *et al.* (2007)
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	23.5% pregnancies, 4 pups	Oh *et al.* (2008)
Felis margarita	Least Concern	USA	Fibroblast	*Felis catus*	3 pups	Gómez *et al.* (2008)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	28% blastocyst	Gómez *et al.* (2003)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	75% pregnancies, 17 pups	Gómez *et al.* (2004)
Ungulates						
Acinonyx jubatus	Vulnerable	Argentina	Fibroblast	*Felis catus*	27.4% blastocyst	Moro *et al.* (2015)
Acinonyx jubatus venaticus	Vulnerable	Iran	Fibroblast	*Felis catus*	5.9% morula	Moulavi *et al.* (2017)
Bos gaurus	Vulnerable	USA	Fibroblast	*Bos taurus*	25% pregnancies, none term	Lanza *et al.* (2000)
Bos javanicus	Endangered	USA	Fibroblast	*Bos taurus*	17% pregnancies, none term	Sansinena *et al.* (2005)
Bubalus arnee	Endangered	India	Fibroblast	*Bubalus*	38.7% blastocyst	Priya *et al.* (2014)

				bubalis		
Bubalus arnee	Endangered	India	Fibroblast/Epithelial cell	*Bubalus bubalis*	50.6 *vs* 20.5% blastocyst	Saini *et al.* (2015)
Capra ibex	Least Concern	China	Fibroblast	*Capra hircus*	11% blastocyst	Wang *et al.* (2007)
Capra pyrenaica pyrenaica	Absente	Spain	Fibroblast	*Capra pyrenaica*	1 born	Folch *et al.* (2009)
Ovis ammon	Near Threatened	China	Fibroblast/*Cumulus* cell	*Ovis aries*	22.1% blastocyst	Pan *et al.* (2014)
Ovis orientalis musimon	Vulnerable	Italy	Granulosa cell	*Ovis aries*	1 pup	Loi *et al.* (2001)
Tragelaphus eurycerus isaaci	Critically Endangered	USA	Fibroblast	*Bos taurus*	24% blastocyst	Lee *et al.* (2003)
Others species						
Macaca fascicularis	Least Concern	Thailand	Fibroblast	*Bos taurus*	33% blastocyst	Lorthongpanich *et al.* (2008)
Macaca fascicularis	Least Concern	China	Fibroblast	*Macaca fascicularis*	2 born	Liu *et al.* (2018)
Balaenoputera bonaerensis	Data deficient	Japan	*Cumulus* cells	*Bos taurus*/ *Sus scrofa domesticus*	27.5–52.8% cleavage	Ikumi *et al.* (2004)

*IUCN: International Union for Conservation of Nature and Natural Resources. USA: United States of Americ

Carnivores

Some works have shown the advances achieved by iSCNT in wild canine species. These species, including the gray wolf (*Canis lupus*), have gradually become endangered or extinct. Thus, in 2007, with the goal of canid conservation, Kim *et al.* (2007) cultured fibroblasts derived from the ear of an adult female gray wolf that were then used as donor cells of nuclei. Using domestic canine oocytes, the authors produced a pregnancy with cloned embryos of two genetic identities of the cloned wolves, but there were no births. In 2008, Oh *et al.* (2008) obtained three wolf pups from cloned embryos using cells obtained from a male gray wolf 6 h after death and domestic canine oocytes. These studies demonstrated the successful cloning of endangered wild canines.

In felid species, the main oocyte source has been the domestic cat. In species from the Felidae subfamily, some progress has been achieved. Thus, synchronized nuclei were donated by the African wild cat and transferred to enucleated domestic cat oocytes resulting in a high rate of blastocyst formation but no pregnancies (Gómez *et al.,* 2003). In another study in which embryos were constructed using somatic cells derived from the African wild cat and domestic cat oocytes, 75% of the embryos developed to term and 25% underwent fetal resorption or abortion (Gómez *et al.,* 2004). Of the 17 cloned kittens born, seven were stillborn, eight died within hours of delivery or up to 6 weeks of age, and two are alive and healthy. Additionally, some studies on wild felids have shown the establishment of somatic resource banks. There is a bank with somatic samples of 69 individual Iberian lynx, considered the most endangered felid in the world, with the aim of future cloning (León-Quinto *et al.,* 2009; 2014).

In addition, works on the cheetah (*Acinonyx jubatus*), a species of the Pantherinae subfamily, have been performed in South America and Asia. Somatic cells from a cheetah raised in South America were transferred to domestic cat oocytes, and, after embryo aggregation during in vitro culture, high blastocyst formation rates were obtained (16.7%–28.3%) (Moro *et al.,* 2015). Moulavi *et al.* (2017) used nonviable frozen cells derived from frozen tissue from an Asiatic cheetah (*Acinonyx jubatus venaticus*) and in vitro-matured domestic cat oocytes and obtained morula rates of 5.9%. Although no blastocyst was obtained, this study demonstrated that enucleated cat oocytes can partially remodel and reactivate nonviable nuclei of the Asiatic cheetah and support its reprogramming back to the embryonic stage.

Ungulates

Some studies with ungulates have been performed with significant success, especially for species already extinct. The first animal derived from an extinct subspecies was obtained using fibroblasts from skin biopsies collected before the death of the last female *Capra pyrenaica pyrenaica*. After a year under cryopreservation, these cells were used as karyoplasts and fused with the cytoplasts of a domestic goat to reconstruct embryos. The rate of cleaved embryos after 36 h was 47.3%, of which 65.5% were transferred. Five recipients were pregnant at 45 days but only one pregnancy went to term. Unfortunately, a few minutes after birth the animal died from pulmonary complications (Folch *et al.,* 2009).

Experiments were carried out with wild yak (*Bros grunniens*) with the goal of evaluating the parameters that affect the success of iSCNT (Li *et al.,* 2007). Fibroblasts and cumulus cells were used as donor cells, but the cell type and different ages were found to have no significant effect on iSCNT.

In 2017, the birth of a Bactrian camel cloned by iSCNT was first reported (Wani *et al.,* 2017). The fibroblasts used to donate nuclei were obtained from ear skin biopsy samples from an adult male Bactrian camel (*Camelus bactrianus*) and the cytoplast of dromedary camel (*Camelus dromedaries*) was the oocyte recipient. Twenty-six blastocysts were transferred to 23 synchronized dromedary recipients yielding five pregnancies with one going to term. This work has great importance because the Bactrian camel is the eighth most endangered large mammal on Earth.

Finally, the woolly mammoth (*Mammuthus primigenius*) is perhaps the one wild mammal of the ungulates whose cloning arouses the greatest interest. This animal became extinct about 10,000 years ago. However, epithelial and muscular cells from 14,000–15,000-year-old mammoth tissues were cryopreserved, with the goal of producing embryos of this species (Kato *et al.,* 2009). In this study, the authors injected cell nucleus-like structures into mature mouse enucleated oocytes; however, the oocytes did not form pronuclear-like structures at 7 h after injection.

Other species

The ability of bovine enucleated oocytes to support dedifferentiation of nuclei from monkey fibroblasts in interspecies cloned monkey embryos has been observed (Lorthongpanich *et al.,* 2008). These embryos were cultured in conditions different from the medium used for cattle with monkey-specific alterations, but the embryos were not able to develop past 16 cells under any culture condition. Nevertheless, OCT-4 was detected, demonstrating the ability of bovine ooplasm to support dedifferentiation but not embryonic development. Therefore, the culture medium promotes dedifferentiation but is not able to support complete embryonic development (Lorthongpanich *et al.,* 2008). In another work that used porcine cytoplasts and donor cells from a rhesus monkey, it was possible to obtain blastocysts despite the low rate (2.04%) (Zhu *et al.,* 2014). Although being a SCNT study, the cloning of cynomolgus monkeys (*Macaca fascicularis*) is cited here because of recent advances in this species. Thus, in a study on cynomolgus monkeys using SCNT, Liu *et al.* (2018) applied histone demethylase Kdm4d mRNA and histone deacetylase inhibitor trichostatin A after activation. Embryonic development improved followed by a greater number of pregnancies, which resulted in the birth of two monkeys via the SCNT technique using fetal fibroblasts and oocytes of cynomolgus monkeys.

With respect to aquatic mammals, a study performed on the minke whale (*Balaenoputera bonaerensis*) compared different conditions of iSCNT, including the ability of porcine and bovine ooplasms to produce reconstructed embryos and the effects of different donor cell types (viable or nonviable cells) on whale SCNT embryos (Ikumi *et al.,* 2004). The authors concluded that whale iSCNT embryos can develop to at least the four-cell stage, regardless of the survivability of the donor cells and the porcine or bovine ooplasm.

Final considerations

Although cloning has several technical limitations that require greater attention to improve the technique, iSCNT has been applied to numerous species of wild mammals and has achieved positive results with respect to embryonic stages in pregnancies and offspring born. The works cited in this paper have made it possible to analyze the state of the art and to perform specific studies the problems in the technique that can be fixed according to the species being studied.

This review has shown that there is no rule that says several species should be cloned following the same protocol, but that each species has different needs at each stage of the technique. In addition, all the papers referred to in this review point to the need for improvement and study at a certain stage, which will lead to improvement of the technique. Thus, to achieve a satisfactory result with iSCNT, each step involved in cloning must be suitable for the species being studied.

Although iSCNT is not the main tool for the reestablishment of endangered wild mammals, its use to increase the possibilities of reproduction and multiplication of individuals has been proposed. It should be refined so that it can be an alternative when traditional techniques cannot be applied. In addition, cloning helps elucidate the embryonic development of a wild species and the subsequent application of this knowledge.

Finally, this biotechnology can help generate more ways to maintain individual species. Therefore, the improvement of protocols to potentiate this technique is of interest because although it has low efficiency rates, iSCNT shows promise because of the pups of different species that have been born.

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CAPÍTULO 3 – ISOLATION, CHARACTERIZATION, AND CRYOPRESERVATION OF COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES

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Isolation, characterization, and cryopreservation of collared peccary skin-derived fibroblast cell lines

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Running title: Peccary skin-derived fibroblast lines.

Abstract

Background. Biobanking of cell lines is a promising tool of support for wildlife conservation. In particular, the ability to preserve fibroblast cell lines derived from collared peccaries is of significance as these wild mammals are unique to the Americas and play a large role in maintaining the ecosystem. We identified collared peccary fibroblasts by immunofluorescence and evaluated their morphology, growth, and adherence capacity. Further, we monitored the viability and metabolic activity of the fibroblasts to determine the effects of passage number and cryopreservation on establishment of cell lines.

Methods. Skin biopsies were collected from the peripheral ear region from five adult animals in captivity. Initially, cells were isolated from fragments and cultured in the Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 2% antibiotic-antimycotic solution under a controlled atmosphere (38.5°C, 5% CO_2). We evaluated the maintenance of primary cells for morphology, adherence capacity of explants, explants in subconfluence, cell growth, and absence of contamination. Moreover, we identified the fibroblast cells by immunofluorescence. Additionally, to evaluate the influence of the number of passages (first, third, and tenth passage) and cryopreservation on establishment of cell lines, fibroblasts were analysed for the viability, metabolic activity, population doubling time (PDT), levels of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi m$).

Results. All explants (20/20) adhered to the dish in 2.4 days ± 0.5 with growth around the explants in 4.6 days ± 0.7, and subconfluence was observed within 7.8 days ± 1.0. Moreover, by morphology and immunocytochemistry analyses, cells were identified as fibroblasts presenting oval nuclei, a fusiform shape, and positive vimentin staining. No contamination was observed after culture without antibiotics and antifungals for 30 days. While no difference was observed for cell viability after the passages (first vs. third: $P = 0.98$; first vs. tenth: $P = 0.76$; third vs. tenth: $P = 0.85$), metabolic activity was found to be reduced in the tenth passage (23.2% ± 12.1%), when compared to that in the first and third passage (100.0% ± 24.4%, $P = 0.006$). Moreover, the cryopreservation did not influence the viability ($P = 0.11$), metabolic activity ($P = 0.77$), or PDT ($P = 0.11$). Nevertheless, a greater $\Delta\Psi m$ ($P = 0.0001$) was observed for the cryopreserved cells (2.12 ± 0.14) when compared to that in the non-cryopreserved cells (1.00 ±

0.05). Additionally, the cryopreserved cells showed greater levels of intracellular ROS after thawing (1.69 ± 0.38 vs. 1.00 ± 0.22, P = 0.04).

Conclusions. This study is the first report on isolation, characterization, and cryopreservation of fibroblasts from collared peccaries. We showed that adherent cultures were efficient for obtaining fibroblasts, which can be used as donor cells for nuclei for species cloning and other applications.

Subjects: Cell Biology, Veterinary Medicine.
Keywords: Biological characterization, cellular conservation, cryobanking, wild mammals.

Introduction

Collared peccaries (*Pecari tajacu* Linnaeus, 1758) are wild mammals found only in the Americas and show a distribution from southern United States to northern Argentina, inhabiting the most diverse environments (*Santos et al., 2009*). Currently, their population is considered to be stable (*Gongora et al., 2011*); however, a significant reduction of their population has been seen in some biomes, such as the Caatinga (*Desbiez et al., 2012*) and the Atlantic forest (*Lazure et al., 2010*). As excellent seed dispersers (*Redford 1992*), they are very important for the maintenance of our ecosystem, whereas, economically, they have been commercialized for their meat and in leather production (*Santos et al., 2009*). Scientifically, collared peccaries can be used as experimental models for closely related species such as the *Tayassu peccary* and *Catagonus wagneri* that have been listed as "vulnerable" in the IUCN Red List of Threatened Species (*Keuroghlian et al., 2013; Altrichter et al., 2015*).

In this sense, studies related to the conservation of the collared peccary have been intensified, especially aimed at improving the techniques related to the preservation of somatic samples. Using this study, we established a culture condition for explants derived from the skin of adult collared peccaries (*Santos et al., 2016*) and developed a protocol for cryopreservation *(Borges et al., 2017; Borges et al., 2018a; Borges et al., 2018b*) and refrigeration of these explants (*Queiroz Neta et al., 2018*). In order to conduct the cloning experiments on this species by a somatic cell nuclear transfer, as well as to produce induced pluripotent cells, it is necessary to establish properly characterized cell lines.

In general, as observed in other mammals (*Guan et al., 2010; Kwong et al., 2014*), establishment of an adequate cell line is a prerequisite step for the success of cloning and producing induced pluripotent cells (*Borges & Pereira 2019*). For these techniques, fibroblasts and epithelial cells derived from the skin have been widely used (*Jyotsana et al., 2016; Siengdee et al., 2018*). Initially, epithelial and fibroblast cells were grown simultaneously; nevertheless, fibroblasts can more easily adhere as well as detach by trypsinization as compared to the epithelial cells (*Bai et al., 2012; Saadeldin et al., 2019; Siengdee et al., 2018*). In these methods, the culture after the second passage has been considered to contain mainly fibroblasts (*Mehrabani et al., 2014*).

Additionally, for the confirmation of a fibroblast line, it is necessary to verify the possible changes that occur in these cells during culture (*Guan et al., 2010; Song et al., 2007*) and cryopreservation (*Magalhães et al., 2017*). In general, the number of passages throughout an *in vitro* study can modify the cellular epigenetic state, affecting the embryonic development after cloning (*Rodriguez-Osorio et al., 2012; Trokovic et al., 2015*). *Magalhães et al. (2017)* observed a reduced viability and metabolic activity in the cells derived from the skin of the brown brocket deer in the tenth passage. Thus, the establishment of a cell line ensures a complete knowledge of the parameters that confer quality to the nucleus of the donor cell, named the karyoplast (*Guan et al., 2010*). Moreover, identification of the damages occurring during cryopreservation is essential for establishment of a cell line. Cryo-variables may affect several cellular processes, including survival, functionality, and the cytoskeleton, which may compromise the reprogramming ability of the karyoplasts (*Chatterjee et al., 2017*). Therefore, we aimed to isolate, characterize, and cryopreserve the fibroblast cells derived from the skin of the ear of collared peccaries for their future application in cloning strategies by a somatic cell nuclear transfer and production of induced pluripotent cells.

Materials & Methods

Chemicals and media

The Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin solutions were obtained from Gibco-BRL (Carlsbad, USA). Fluorescent probes were purchased from Invitrogen (Carlsbad, USA). Anti-vimentin antibody

and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, USA). The other chemicals were obtained from Sigma-Aldrich (St. Louis, USA). Media were filtered using a 0.22-µm system (Corning, New York, USA) and adjusted to pH of 7.2–7.4.

Bioethics and animals

This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2). All animals belonged to the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil, 5°10'S, 37°10'W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding site (no. 1478912). The breeder stocks 100 collared peccaries on an average, and for this research four females and one male at ages of 26.8 months ± 2.9 months were used.

Ear tissue explant collection and primary culture

Peripheral skin (1 cm^2–2 cm^2) was recovered from the ear sections used to identify collared peccaries kept in captivity. After the collection, a trichotomy of the tissue followed by a sterilization with 70% alcohol was performed. Samples were transported to the laboratory in DMEM supplemented with 2% antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B) at 37°C within 30 min.

In the laboratory, fragments (9.0 mm^3) were washed sequentially under laminar flow in the following media: 1) DMEM supplemented with 10% FBS, and 10% antibiotic-antimycotic solution; 2) alcohol; and 3) DMEM plus 10% FBS, and 2% antibiotic-antimycotic solution. Then, the samples were fragmented (four fragments per animal) and placed in polystyrene culture dishes treated for cell adhesion with the latter medium for cell culture. The skin was cultured at 38.5°C under a controlled environment with 5% CO_2 and 95% air, according to a method described by *Santos et al. (2016)*.

Evaluation of the somatic cells in primary cultures and subcultures

During primary culture, the medium was changed every 24 h. For evaluation of the somatic cells, the primary culture was analyzed before reaching confluency and until it reached a confluency of

70%–80%. Using an inverted microscope (Nikon TS100, Tokyo, Japan), the cells were evaluated for the following parameters: cell morphology, number of adhered samples, number of samples, evident subconfluency, day of sample adherence, day of subconfluent growth of the samples, and total time to reach 70%–80% confluence (*Borges et al., 2017*).

When the cells reached 70%–80% subconfluency, they were subcultured and distributed for other analyses. The 70%–80% subconfluence was defined as the stage when 70%–80% of the culture dishes consisted of somatic cells (*Santos et al., 2016*). Subconfluent cells were washed with PBS then trypsinized with a trypsin/EDTA solution (0.25%/0.2%) for 7 min and centrifuged at 600 x *g* for 10 min. The supernatant was removed, the cell pellet was resuspended in culture medium, and the cell suspension was transferred to another dish for subculturing (*Borges et al., 2018b*). The medium was replaced with a fresh medium every other day and the cells were monitored daily. With the successful passaging of the cultures, the cells are considered a cell line, following the convention of the Society of In Vitro Biology (*Schaeffer, 1990*). The cell line was designated as Ptskf.

Thus, in addition to an evaluation of the maintenance of cells in the primary culture, the subcultured cells were initially evaluated for the confirmation of fibroblasts using morphology and immunofluorescence analyses. Moreover, the possibility of contamination was also evaluated. Subsequently, the influence of the number of passages (first, third, and tenth passage) and the metabolic activity of the cells were analyzed by a viability assay using trypan blue and the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, respectively. Moreover, the cells were also evaluated for the effects of a slow freezing cryopreservation. Other than the above-mentioned tests, growth dynamics by quantification of the population doubling time (PDT), oxidative stress analysis for quantification of intracellular reactive oxygen species (ROS) levels using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), and assessment of the mitochondrial membrane potential ($\Delta\Psi m$) using the fluorescent probe MitoTrackerRed® were performed.

Morphological characterization of the fibroblasts

Morphological characteristics were observed throughout the in vitro culture under light microscopy for cellular and nuclear shapes and cytoplasmic extensions.

Vimentin immunofluorescence

For a morphological confirmation, the cells were subjected to an immunocytochemistry protocol based on the method described by *Amoli et al. (2017)*. Briefly, the cells were fixed using 4% paraformaldehyde for 10 min at 25°C, then washed with chilled PBS. Subsequently, cells were incubated with an antigen-retrieval buffer (100 mM Tris, 5% urea, pH 9.5), and then permeabilized for 1 h in 0.4% Triton X-100. Afterwards, the cells were incubated in 0.1% Tween-20 for 1 h to block non-specific binding of the antibodies. Finally, the cells were immuno-stained with mouse anti-vimentin antibody (ab8979, 1:200) for 24 h at 4°C, and, then incubated with the secondary antibody (goat anti-mouse IgG, Alexa Fluor® 488, ab150113, 1:400) for 1 h at 25°C in the dark. Cells were counter-marked with 1 μg/mL Hoechst for 1 min and observed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan).

Confirmation of the absence of bacterial and fungal contamination

Cells of the third passage were cultured for 30 days in DMEM containing 10% FBS in the absence of an antibiotic-antimycotic solution, at 38.5°C, 5% CO_2 and 95% air. Daily evaluation was performed under light microscopy for the identification of bacterial and fungal contamination.

Influence of the passage number on the quality of fibroblast lines

Initially, the fibroblast cells were analyzed for the effect of the number of passages (first, third, and tenth passage) by a viability assay using trypan blue, according to the method described by *Magalhães et al. (2017)*. We evaluated these three cell passages specifically because both fibroblast and epithelial cells were present in the initial (first) passage, only fibroblasts were visualized at the third passage onwards, and the cells of the tenth passage were used for most of the production of embryonic clones (*Shiga et al., 1999; Kubota et al., 2000*). The evaluations were performed in triplicate for each animal for each passage.

Briefly, the cells were stained with 0.4% trypan blue in PBS and counted on a hemocytometer. Subsequently, the cells were also analyzed for a metabolic activity using the MTT assay, according to the method described by *Borges et al. (2018b)*. A concentration of 5.0×10^4 cells/mL from the first, third, and tenth passages was grown in 12-well polystyrene plates treated for cell adhesion. After 5 days, 1.5 mL of the MTT solution (5 mg/mL in DMEM) was added and the polystyrene culture dishes treated for cell adhesion were incubated for 3 h. The MTT solution was then removed and 1.0 mL of dimethyl sulfoxide (DMSO) was added for 5 min under slow stirring to solubilize the MTT. After the total dissolution of formazan crystals, samples were analyzed in a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) at an absorbance wavelength of 595 nm. The evaluations were performed in triplicate for each animal for each passage.

Influence of cryopreservation on the quality of fibroblast lines

To evaluate the effect of cryopreservation on the quality of fibroblast lines, cells of the third passage of the five animals were subjected to slow freezing in the freezing medium (DMEM supplemented with 10% DMSO as a permeating cryoprotectant and 10% FBS and 0.2 M sucrose as non-permeating cryoprotectants). Cells at a concentration of 5.0×10^4 cells/mL were first exposed to DMSO-FBS solution for 15 min at 4°C, then sucrose solution was added followed by an additional incubation for 15 min at 4°C. The cryovials containing 1.0 mL of cells in the freezing medium were cooled in a Mr. Frosty freezing container (Thermo Scientific, Waltham, USA) at a cooling rate of 1°C/min, and later stored in a freezer at -80°C, reaching -70°C overnight before being transferred into liquid nitrogen (*León-Quinto et al., 2014*).

For thawing, the cryovials were exposed for 1 min at 25°C and immersed in a water bath at 37°C for 3 min–4 min. Then, the cell contents were removed from the cryovials and washed to remove the cryoprotectants. Initially, the first wash was performed with DMEM and 10% FBS containing 0.2 M sucrose at 4°C for 15 min and centrifuged. Subsequently, the second wash was performed using only DMEM and 10% FBS, maintained at 25°C for 15 min, centrifuged, and the cells were recovered for the evaluations as per a method described previously (*Santos et al., 2016*).

After thawing, the non-cryopreserved and the cryopreserved cells were evaluated for growth dynamics by quantification of PDT. The evaluations were performed in triplicate for each animal. Briefly, the growth kinetics was studied for nine days using 3.0×10^4 cells/mL, and cells were counted daily to determine the number of growing cells. Data on the cell growth and density were monitored and recorded, mean values of which were used to plot a growth curve and calculate PDT (*Roth 2006*) using the following formula:

PDT = T ln2/ln (Xe/Xb) where PDT is the time of the culture (in hours), T is the incubation time, Xb is the number of cells at the beginning of the time incubation, Xe is the number of cells at the end of the incubation time, and ln is the Napierian logarithm.

Moreover, for evaluation of an oxidative stress by quantification of the intracellular ROS levels, cells were stained with the fluorescent probe H_2DCFDA, according to a method described by *Santos et al. (2019)*. Thawed cells were washed with PBS and placed into polystyrene culture dishes treated for cell adhesion containing 500 µL of 5 µM H_2DCFDA. The cells obtained after a 70% confluency were incubated at 38.5°C in 5% CO_2 for 30 min. Stained cells were washed with PBS, placed on glass slides, photographed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and fluorescence signal intensity (pixels) was quantified. Ten images (two/animal) obtained were evaluated using the ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the values obtained for the treated samples. Measured mean value of the micrograph for the non-cryopreserved cells was taken as a calibrator. Relative expression levels (arbitrary fluorescence units) were generated by dividing the measured value of each micrograph for the cryopreserved cells by the mean of the calibrator.

Finally, for the assessment of $\Delta\Psi m$, cells were stained using 500 nM of the fluorescent probe MitoTracker Red® (CMXRos), according to a method described by *Santos et al. (2019)*. The procedure, incubation, and evaluation of the ten images (two/animal) were performed as described for the quantification of ROS.

Statistical analysis

All data have been expressed as the mean ± standard error (one animal/one repetition) and were analyzed using the StatView 5.0 software (Graph-Pad Software Incorporation, La Jolla, USA). Normality of all results was verified by the Shapiro-Wilk test and homoscedasticity was verified by the Levene's test. ROS levels, $\Delta\Psi m$, viability, and metabolic activity were altered with arcsine and analysed by variance analysis (ANOVA) followed by the Tukey's test. PDT was compared with ANOVA followed by the unpaired t-test. Statistical significance was set at $P < 0.05$.

Results

Evaluation of the somatic cells in the primary cultures and subcultures

The total culture time was 95 days with an evaluation of cells until the tenth passage. The adhesion of the fragments (Fig. 1A), detachment of cells (Fig. 1B-1C), and proliferative capacity were observed in all the explants until reaching a confluence (and later, a subconfluence) around the adhered fragments (Fig. 1D-1F, Table 1). All explants had adhesion ability and reached subconfluence. Number of days for each explant to reach a 100% tissue adherence (2.4 days ± 0.5 days), to grow around the explants (4.6 days ± 0.7 day), and to reach subconfluence (7.8 days ± 1.0 day), were different.

Morphological characterization of the fibroblasts

In cultures, monolayers of cells with a fibroblast-like morphology were observed (Fig. 1E). The cells had an oval nuclei and extensions with a fusiform shape, showing rapid growth that replaced the epithelial cells.

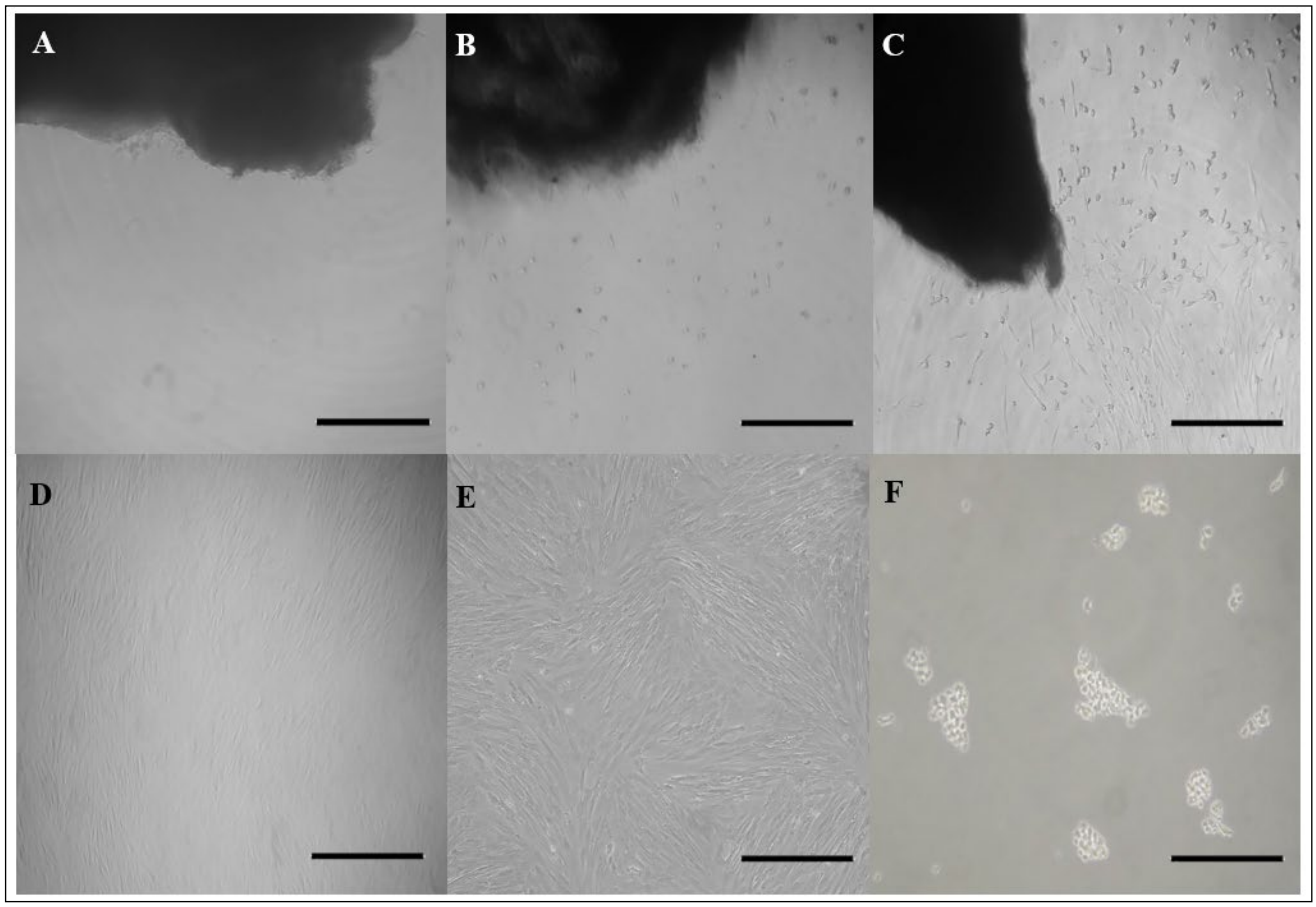

Figure 1 **Outgrowth of fibroblast cells from ear skin samples of collared peccaries.** Exhibit a skin explant cultured in A) day 1, B) day 3 and C) day 5 of primary culture and exhibit a fibroblast population cultured in D) day 15 and E) day 19 of subculture. F) exhibit cells after the trypsinization process. Scale bar = 100 µm.

Table 1 Establishment of primary culture and subcultures of somatic cells derived from collared peccary ear skin.

Animal	No. samples			No. attached samples		
	Initial	Attached (%)	Day all attached explants	Grow to subconfluence (%)	Day all cell grow explants	Subconfluence total time (days)
F1	4	100	2	100	4	6
F2	4	100	2	100	4	5
F3	4	100	1	100	3	9
F4	4	100	3	100	5	10
M1	4	100	4	100	7	9
Mean ± S.E	20	100	2.4 ± 0.5	100	4.6 ± 0.7	7.8 ± 1.0

Vimentin immunofluorescence

Morphology of the fibroblast-like cells in the initial culture was observed by light microscopy, which was further confirmed for the cell type identification as vimentin-labeled fibroblasts under fluorescence microscopy (Fig. 2A-2F). Cells exhibited a high expression of vimentin that marked the cytoplasm completely, and the spindle-like shape and ovoid nucleus was highlighted by the Hoechst labeling. Therefore, the identification of a fibroblast cell was evident.

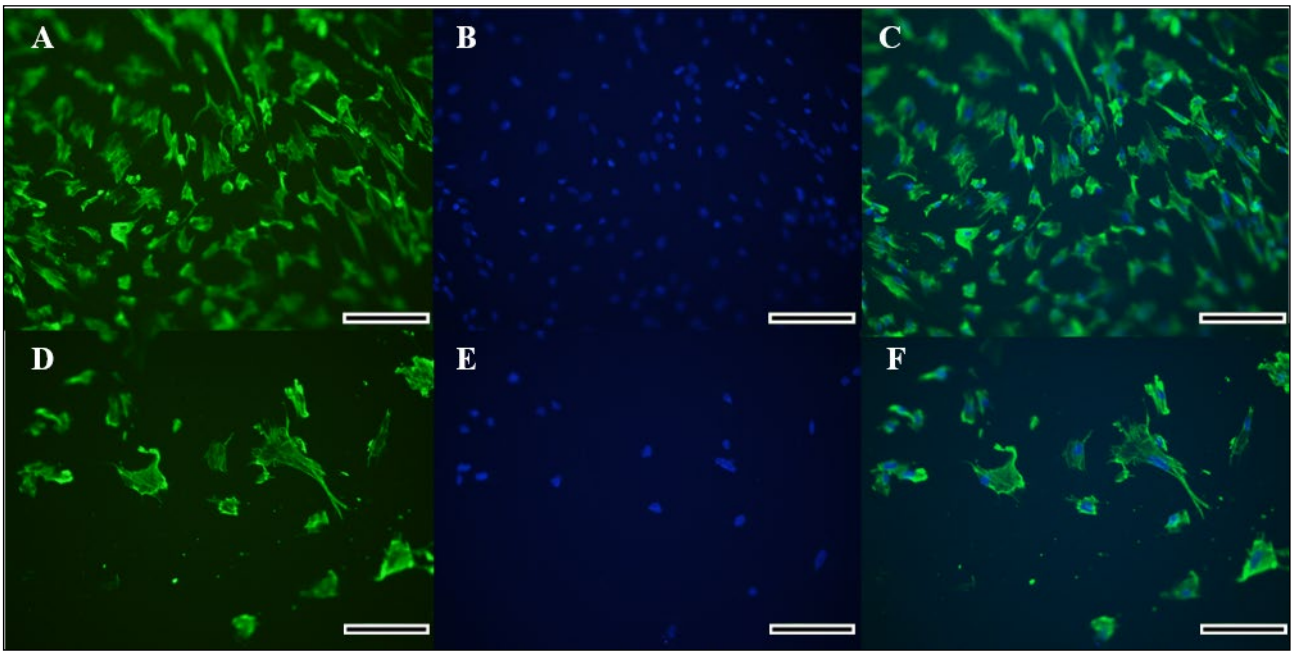

Figure 2 **Immunocytochemical detection of vimetin protein for identification of collared peccary fibroblasts.** A-D) cells stained with vimetin antibody. B-E) nucleus of cells stained by Hoechst. C-F) merged vimetin (green) and Hoechst (blue). a-b-c (x5), d-e-f (x10). Scale bar = 10 µm.

Confirmation of the absence of bacterial and fungal contamination

No sign of contamination (turbidity, colony, or hyphal growth) was observed for 30 days in the culture without antibiotics and antifungals. The culture medium did not show any change in the appearance when observed under a light microscope. We did not observe turbidity or any specific odor. In addition, there was no change in the biological characteristics of growth and proliferation indicating a complete absence of contamination.

Influence of the passage number on the quality of fibroblast lines

No significant difference was observed in the cell viability (74.5% to 84.4%) when evaluated by trypan blue staining after the passages (first vs. third: P =0.98; first vs. tenth: P = 0.76; third vs. tenth: P = 0.85). However, the metabolic activity was reduced in the tenth passage (23.2% ± 12.1%) as compared to that of the first and third passages (100.0% ± 24.4%, P = 0.006).

Influence of cryopreservation on the quality of fibroblast lines

Cryopreservation did not affect the viability when evaluated by trypan blue staining (87.4% ± 0.3% vs. 74.0% ± 5.9%, P = 0.11). Moreover, after two passages of the thawed cells, the viability was 86.4% ± 3.2%. In addition, no difference (P = 0.77) was observed for the metabolic activity between the cryopreserved (85.2% ± 10.0%) and the non-cryopreserved cells (100.0% ± 36.4%).

Moreover, the cryopreserved and the non-cryopreserved cells were compared for growth dynamics (Fig. 3). The growth curve of both groups showed a typical “S-shaped” pattern from the nine-day culture of cells. The latency time was two days, followed by an exponential phase until the fourth day, the stationary phase until the seventh day, and the plateau phase from day eight (Fig. 3A). No difference was observed for the PDT values of the cryopreserved and the non-cryopreserved cells (P = 0.11, Fig. 3B).

Nevertheless, the cryopreserved cells showed greater levels of intracellular ROS (Fig. 4A) in arbitrary fluorescence units when compared to that of the non-cryopreserved cells (1.69 ± 0.38 vs. 1.00 ± 0.22, P = 0.04) (Fig. 4C). In addition, an alteration in the $\Delta\Psi m$ (Fig. 4B) in arbitrary fluorescence units (P = 0.0001) was observed for the cryopreserved cells (2.37 ± 0.07) when compared to that of the non-cryopreserved cells (1.00 ± 0.05) (Fig.4D).

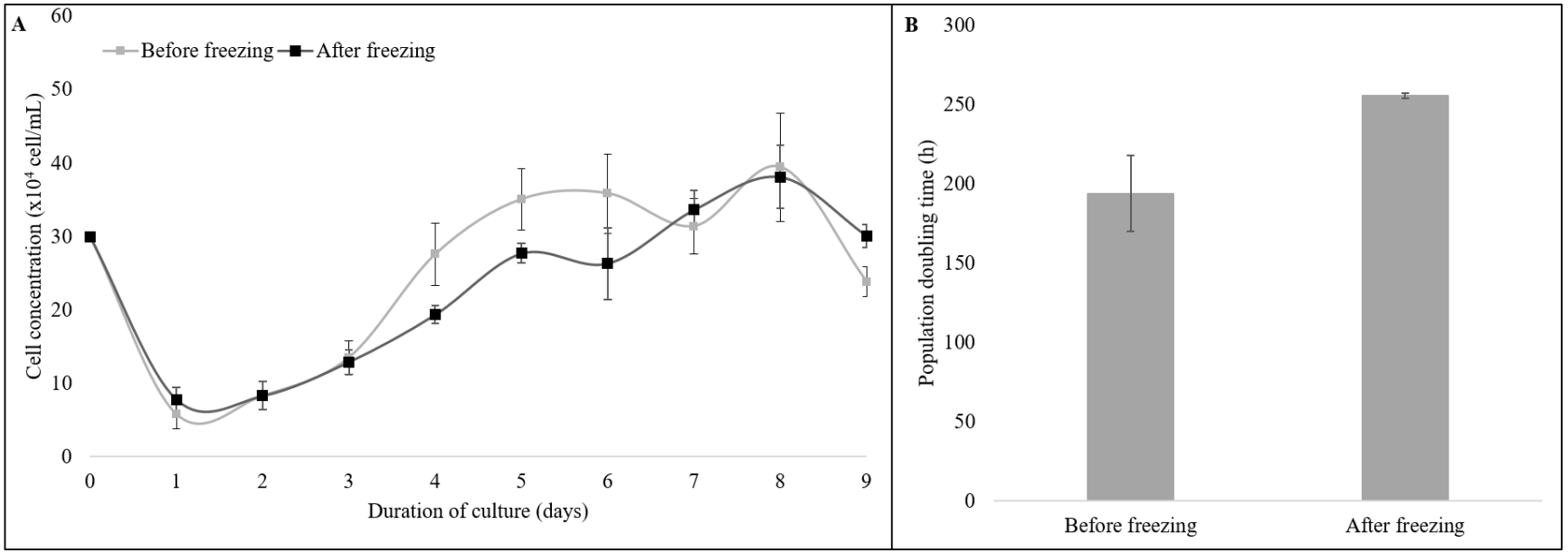

Figure 3 **The growth dynamics of cryopreserved and non-cryopreserved cells derived from collared peccary skin.** A) growth curves of cryopreserved and non-cryopreserved cells. B) values of population doubling time (PDT) after culture for nine days.

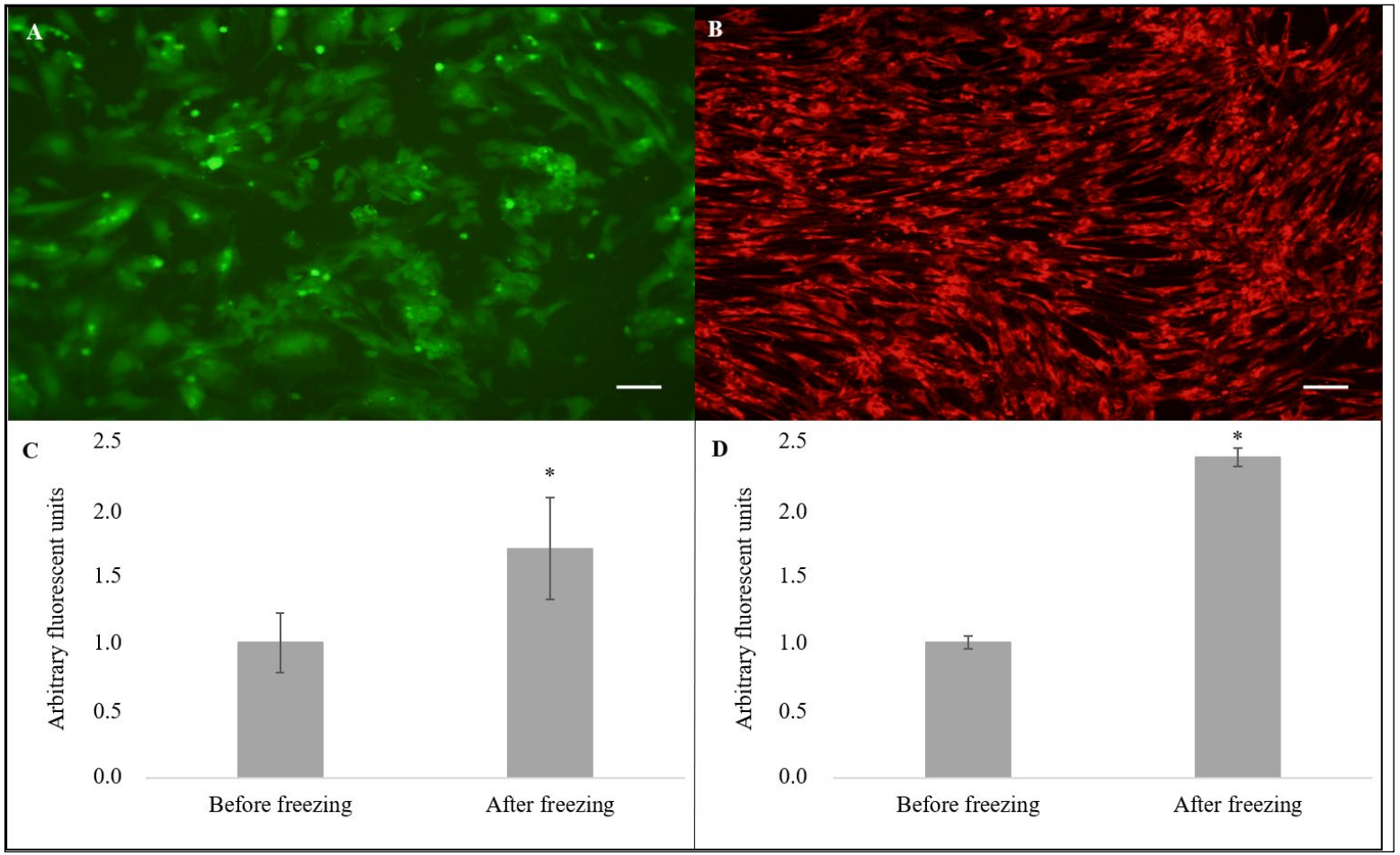

Figure 4 **Evaluation of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (ΔΨm)**. Cell stained using fluorescent probe A) 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and B) MitoTrackerRed® (CMXRos) (x10). Quantification of C) ROS and D) ΔΨm levels. Scale bar = 10 µm. (*) Indicate statistical difference ($P < 0.05$).

Discussion

In this study, we isolated, characterized, and cryopreserved the fibroblast cells derived from the skin of collared peccaries. Moreover, we established the fibroblast cell lines of these animals with an aim to use these cells in cloning experiments by a somatic cell nuclear transfer in the future. The cell line can be considered as the first constituent of the peccary invitrome and a resource for future studies in many disciplines (*Barioch 2018; Bols et al., 2017*). Thus, the ear tissues of collared peccaries can be isolated and grown into fibroblasts in an adherent culture for establishment of cell lines and development of a cryobank. The development of these somatic cell banks has been increasing in the interest of conserving genetic samples of wild mammals to preserve valuable species, and as sources for biological research (*León-Quinto et al., 2009; Mehrabani et al., 2014; Saadeldin et al., 2019; Siengdee et al., 2018*).

All explants adhered to the flask surface within 2 days to 4 days, with cellular growth around the explant within 3 days, and demonstrating confluency within 5 days to 10 days after a culture initiation. These characteristics of explants during *in vitro* culture were similar to the explants derived from other domestic and wild mammals. In studies using tissues from horses, the migration of fibroblast and epithelial-like cells from explants have been observed after 5 days to 7 days of an *in vitro* culture (*Amoli et al., 2017*). In the case of goat-derived tissues, the explants reportedly adhered to the flasks within 5 days to 7 days and the cells became confluent within 3 days to 5 days post adhesion (*Bai et al., 2012*). In the Iranian Sistani cattle-derived tissues, the explants adhered to the culture flasks within 7 days to 14 days and were observed to allow the growth of fibroblast-like cells from the margins of explants (*Gorji et al., 2017*).

For the Luxi cattle-derived tissues, fibroblast-like or epithelial-like cells could be seen migrating from the tissues within 5 days to 12 days post adhesion (*Liu et al., 2008*). In the tissues derived from wild camels, fibroblast-like or epithelial-like cells could be seen migrating from the sides of explants within 8 days to 10 days post adhesion (*Sharma et al., 2018*). In tissues derived from the domestic porcine, a species phylogenetically close to the collared peccaries, all the explants adhered within 3 days to 8 days (*Silvestre, Sánchez & Gómez, 2004*). The similarity among these data can be related to the culture medium because in a majority of these studies, DMEM containing FBS, antibiotic, and antimycotic solution was used (*Magalhães et al., 2017; Saadeldin et al., 2019; Siengdee et al., 2018*). Since

primary culture needs to mimic the *in vivo* environment of the cells (*Guo et al., 2018*), we observed previously (*Santos et al., 2016*) that the medium for growth of somatic cells derived from collared peccaries was DMEM with 10% FBS and 2% antibiotic-antimycotic solution.

We showed that ear explant cultures obtained from the tissues of the collared peccaries were efficient for cell recovery allowing a culture for 95 days and up to ten passages. Moreover, we confirmed that the ear skin was a common source where fibroblasts cells could be separated and eventually used as karyoplasts for cloning purposes (*Luo et al., 2014*). These cells were identified by vimentin, an intermediate filament that indicates the mesenchymal origin of endothelial and fibroblast cells (*Yajing et al., 2018*). Initially, during *in vitro* culture, epithelial and fibroblastic cells grew simultaneously. However, fibroblasts can be trypsinized more rapidly and adhere more easily as compared to the epithelial cells (*Bai et al., 2012; Saadeldin et al., 2019*). Therefore, in this work, cells from the third passage were confirmed as fibroblasts by morphology and immunofluorescence analyses.

The clear medium observed during the 30-day assay demonstrated the ability to allow the growth of the culture without any biological contamination. The propagation of fungi causes turbidity by accumulation of their metabolites. In addition, the colonies can be seen under a light microscope, or sometimes with the naked eye (*Li et al., 2009*). Bacterial contamination can also be identified by the naked eye as turbidity. One simple way to avoid this contamination is to filter the culture medium (*Bai et al., 2012*). Sources of contamination may include, but are not limited to, the equipment, air, culture medium, serum, and explant. Therefore, microbial contaminations are quite frequent in cell culture (*Bai et al., 2012*). Thus, the use of antibiotic and antimycotic combined with careful handling, is essential to ensure the absence of contamination.

After two passages, it was possible to separate fibroblast cells from other primary cells because different cell types exhibit different cellular behavior upon trypsinization. The fibroblast cells detach quicker than epithelial cells (*Gorji et al., 2017*). However, fibroblasts detach in response to trypsin more rapidly than epithelial cells and adhere more quickly (*Bai et al., 2012*). In wild camels, the initial lag phase of 48 h representing the adaptation of fibroblasts and recovery from a protease damage is followed by the exponential phase (*Sharma et al., 2018*). In collared peccaries, the replication of cells begins to slow down after seven days because of contact inhibition, which, in wild camels, has been observed after six

days (*Sharma et al., 2018*). No difference in the cell viability was observed among the first, third, and the tenth passage, corroborating with the studies that used cells from these passages for production of competent cloned embryos (*Shiga et al., 1999; Kubota et al., 2000*).

In contrast, through the metabolic activity test evaluated by the formation of formazan crystals, a significant reduction in the metabolic activity at the tenth passage was observed, indicating a reduced cellular functionality. Similar behavior was observed in cells from the brown brocket deer in which the metabolic activity measured by the MTT assay showed significantly lower values in the tenth passage than the values in the fourth passage (*Magalhães et al., 2017*). Therefore, the number of passages can reduce the metabolic activity rate and cell proliferation, thereby conserving cells of the early passages (*Li et al., 2009*). After several passages, genetic characteristics of the cells can be modified by culture conditions; hence, a minimum number of passages have been recommended to conserve the cellular characteristics (*Mehrabani et al., 2014*). Owing to this reason, the cells were cryopreserved in the third passage for the conservation of the somatic germplasm of collared peccaries.

The cell survival rate after thawing is the most commonly used criteria to evaluate the success of a cryopreservation (*Chatterjee et al., 2017*). The cellular viability and the functional metabolic activity of the cells were maintained after thawing the fibroblasts isolated from the collared peccaries. This factor demonstrates that optimal *in vitro* culture conditions significantly influence the recovery from cellular damages caused by the freezing process (*Gorji et al., 2017*). As for the growth curve, cryopreserved cells presented a very similar profile to that of the non-cryopreserved cells, showing their normal proliferation capacity regardless of the cryopreservation process. The establishment of somatic cell banks using cryopreservation technology is an easy and effective approach towards storing the genetic information of diverse species (*Li et al., 2009*). However, the cells should be handled with the utmost care during cryopreservation to maintain a high-quality cell bank in the long term (*Mehrabani et al., 2014*).

Moreover, epigenetic alterations, such as DNA fragmentation, free radical accumulation, ionic imbalances, apoptosis, biochemical alterations, DNA methylation, and histone modification can be a result of the cryopreservation (*Chatterjee et al., 2017*). These after-effects of cryopreservation may have caused mitochondrial structural abnormalities, thereby

promoting an increased ROS production and H_2O_2 content, increased lipid peroxidation, and increased expression of autophagic proteins harbored by the cells (*Mata et al., 2012*). A failure in the mitochondrial membrane potential is a hallmark of apoptosis, leading to the collapse of the organelle and release of cytochrome-C into the cytoplasm, and ultimately activation of the apoptotic cascade (*Magalhães et al., 2012*). Moreover, a high ΔΨm mitochondrial respiratory chain becomes a significant ROS producer (*Korshunov, Skulachev, &Starkov., 1997*). Therefore, a higher ΔΨm in cryopreserved cells can be linked mainly to an increase in the oxidative stress.

Finally, parameters like cryovariables, including cooling and thawing rates, type and concentration of the cryoprotectant, cell type and shape, and nucleation temperature may affect the success of cryopreservation (*Chatterjee et al., 2017*). This suggests that the optimization of related cryopreservation methods for the collared peccary fibroblasts to minimize an altered ΔΨm and increased levels of intracellular ROS production is essential.

Conclusions

To our knowledge, this study is the very first report on a successful isolation, characterization, and cryopreservation of fibroblast lines derived from adult collared peccaries (Ptskf). We showed that the adherent culture was efficient for obtaining fibroblasts, which can be used as donor cells for nuclei for cloning of this species. Moreover, it was possible to maintain the viability of the cells until the tenth passage. In addition, cryopreservation did not affect the viability, metabolic activity, and proliferative activity of the fibroblasts after slow freezing. However, cryopreservation altered the ROS levels and ΔΨm, indicating necessary optimization of the cryopreservation protocol. Lastly, the establishment of fibroblast cell lines derived from collared peccaries may be a source of experimental models for many biological studies such as nuclear reprogramming and animal cloning.

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CAPÍTULO 4 – CRYOPRESERVATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) SOMATIC CELLS IS IMPROVED BY SUCROSE AND HIGH CONCENTRATIONS OF FETAL BOVINE SERUM

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CRYOPRESERVATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) SOMATIC CELLS IS IMPROVED BY SUCROSE AND HIGH CONCENTRATIONS OF FETAL BOVINE SERUM

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Abstract

BACKGROUND: The formation of somatic cell banks is affected by, amongst other factors, the cryoprotectant solution used. The selection of an effective solution, therefore, is a primary parameter. **OBJECTIVE:** We optimized the cryoprotectant used for collared peccary somatic cell cryopreservation. **MATERIALS AND METHODS:** We categorized cells into different groups based on their cryopreservation and evaluated the morphology, viability, proliferative activity, metabolism, and oxidative stress. One group was cryopreserved in 10% DMSO with 10% fetal bovine serum (DMSO-10FBS), and another with 50% FBS (DMSO-50FBS). The cryopreservation of both groups included the presence of 0.2 M sucrose (DMSO-SUC-10FBS and DMSO-SUC-50FBS). Non- cryopreserved cells and cells cryopreserved with 10% DMSO (DMSO) supplemented with 0.2 M sucrose (DMSO-SUC) were used as controls. **RESULTS:** There was no difference observed in morphology or viability among the groups. Proliferative activity was reduced in DMSO-10FBS when compared to controls. Although cryopreservation reduced metabolism, no difference was observed among solutions. A lower level of reactive oxygen species was observed in cells of DMSO-SUC- 50FBS when compared to other cryoprotectants. Only cells of DMSO-SUC-50FBS had mitochondrial potential similar to non-cryopreserved cells. **CONCLUSION:** 10% DMSO supplemented with 50% FBS and 0.2 M SUC was observed to be the most efficient cryoprotectant for preserving collared peccary somatic cells.

Keywords: Peccaries, extracellular cryoprotectants, slow freezing, cryobanking.

Introduction

Collared peccaries (*Pecari tajacu* Linneaus, 1758), also known as “wild pigs”, are wild mammals belonging to the Tayassuidae family of the order Artiodactyla (21). These animals play an important role in maintaining the global ecosystem where they act as seed dispersers, promote flora renewal, and contribute to the food chain (34). Although globally classified as “Least Concern”, this species is already extinct in eastern and southern Argentina and is declining in some biomes (9), thus necessitating conservation strategies for the maintenance of its population.

One of the conservation tools applied to wild mammals has been the formation of somatic resource banks (6). These banks allow long-term storage of tissues and somatic cells to be employed in different proposals, such as multiplying individuals by somatic cell

nuclear transfer (SCNT) (43) and generating induced pluripotent cells (41). The efficiency of these procedures depends on the quality of cells after slow freezing, which is dependent on the choice of the cryoprotectant, a crucial step for the success of these cryobanks (28). In general, an effective cryoprotectant is the appropriate combination of intracellular cryoprotectants such as dimethyl sulfoxide (DMSO) (25), ethylene glycol (EG) (16), and extracellular cryoprotectants such as sucrose (SUC) (23) and/or fetal bovine serum (FBS) (15).

Specifically, our group has successfully established somatic tissue banks of collared pecarries based on our knowledge of the tissues (2), techniques (3), and solutions required (4, 5). Subsequently, we initiated somatic cell banks of this species, demonstrating that 10% DMSO in the presence of 0.2 M SUC was more efficient in somatic cell slow freezing when compared to EG with 0.2 M SUC, showing a viability of 69.8% and 58.5%, respectively (17). Thus, one way to improve the rates of cell recovery would be to optimize the extracellular agents employed. Studies have shown that FBS, when associated with 10% DMSO and 0.2 M SUC, promoted a beneficial effect in some species (*Lynx pardinus* and *Elephas maximus*) (15, 36). This agent can reduce oxidative stress, caused by excessive production of reactive oxygen species (ROS) during slow freezing (11).

Nevertheless, variations in FBS concentration can be observed in some species with its use at 10% (*Felis silvestris líbica*) (8) and 50% (*Bubalus bubalis*) (18), necessitating the evaluation of the most appropriate concentration, as well as its relationship with SUC. Therefore, we aimed to optimize the cryoprotectant by varying the different extracellular combinations of SUC and FBS. We assessed the impact of the cryoprotectants on the morphology, viability, proliferative activity, metabolism, and oxidative stress of cryopreserved collared peccary cells.

Materials and Methods

Chemicals and media

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), FBS, trypsin-EDTA, Antibiotic-Antimycotic (ATB-ATM) solution and fluorescent probes were obtained from Gibco-BRL (Carlsbad, CA, USA). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was purchased from Grainer (Miami, FL, USA).

Compliance with ethical standards and animals

The experimental protocols and animal handling procedures were performed with the approval of the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92), in compliance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2). A total of four adult collared peccaries, 41.5 ± 1.8 months old, provided by the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W) were used.

Skin biopsy, primary culture, and subcultures

Sections (1-2 cm^2) used for collared peccary identification were taken from peripheral ear tissue using pliers. After collection, tissue samples were washed in 70% ethanol and incubated in DMEM supplemented with 10% FBS and 2% ATB-ATM solution at 37 °C for 30 min.

In the laboratory, tissues were trichotomized, washed in 70% ethanol followed by DMEM supplemented with 10% FBS and 2% ATB-ATM solution. Subsequently, tissue samples were fragmented in 9.0 mm^3 sections and cultured in DMEM supplemented with 10% FBS and 2% ATB-ATM solution at 38.5 °C in an incubator with 5% CO_2 and 95% air (31). During the primary culture, the medium was changed every 24 h. Cells were subcultured after reaching 70-80% confluence until the third passage, followed by cryopreservation. The subconfluence of 70-80% was defined when 70-80% of the petri dishes presented somatic cells (31).

Study design and cell cryopreservation

The morphology, viability, proliferative activity, metabolism, and oxidative stress were evaluated in non-cryopreserved and cryopreserved cells. Cells were cryopreserved in either 10% DMSO with 10% FBS (DMSO-10FBS group), or 50% FBS (DMSO-50FBS group). In both the groups, 0.2 M SUC was added and these were further designated as the DMSO-SUC-10FBS and DMSO-SUC-50FBS groups, respectively. Moreover, non-cryopreserved cells (non-cryopreserved group), those cryopreserved in 10% DMSO (DMSO group) and those cryopreserved with 10% DMSO supplemented with 0.2 M SUC (DMSO-SUC group), were used as controls.

For cryopreservation, cells were subjected to slow cooling in freezing medium (DMEM supplemented with 10% DMSO and extracellular cryoprotectants according to experimental groups). Briefly, cells at a concentration of 1.0×10^4 cells/mL were first exposed to DMSO solution for 15 min at 4 °C in DMEM, followed by the addition of SUC and FBS solutions according to experimental groups and incubated for an additional 15 min at 4 °C. The cryovials containing 1.0 mL of cells in freezing solution were cooled in a Mr. Frosty freezing container (Thermo Scientific Nalgene, Rochester, NY, USA) at a cooling rate of 1 °C/min, and kept in a freezer -80 °C until reaching -70 °C, before being transferred to liquid nitrogen (15).

After 2 weeks, cryovials were removed from liquid nitrogen and kept at room temperature of 25 °C for up to 1 min and then warmed in a water bath at 37 °C for 3-4 min. For the removal of cryoprotectants, the cells were washed twice with DMEM. Cell suspension was centrifuged for 10 min at 400×g and washed again with DMEM and centrifuged according to León-Quinto et al. (15). In the groups containing SUC, the cells were washed with DMEM with 0.2 M SUC, prior to centrifugation as previously described.

Evaluation of morphological characteristics and cell viability

The cells' morphology was evaluated daily using an inverted microscope (Nikon TS100, Tokyo, Japan). The following morphological characteristics were assessed: size, aspect, shape and adhesion (29). The viability analysis was performed by cells stained with trypan blue. For each animal and each group, the viability test was performed in duplicate. Briefly, the cells were centrifuged and suspended in 1.0 mL of the cell culture medium (DMEM supplemented with 10% FBS and 2% ATB-ATM solution); an aliquot of cells was stained with 0.4% trypan blue (in phosphate buffered saline, PBS) in the ratio 1:1 and counted in a Neubauer chamber (39).

Analysis of proliferative activity and metabolism

Proliferative activity was quantified according to the population doubling time (PDT), which was calculated by seeding cells at a density of 1.0×10^4 cells/well in a twenty-four well plate. Cells were trypsinized in duplicate and counted at 24 h intervals for up to 216 h of culture. The mean cell counts were recorded every time and the cell growth curve was delineated. Finally, the PDT was calculated using the following formula (30):

PDT = T ln2/ln (Xe/Xb), where PDT is the time of the culture (in hours), T is the incubation time, Xb is the number of cells at the beginning of the time incubation, Xe is the number of cells at the end of the incubation time, and ln is Napierian logarithm.

For evaluation of metabolism, cells were seeded into 12-well dishes at a density of 5.0×10^4 cells/mL and cultured at 38.5 °C in a humid atmosphere with 5% CO_2 (4). After five days, MTT solution (5 mg/mL in DMEM) was added to each well for 3 h under the same conditions. After incubation, the MTT solution was removed, and DMSO was added to each dish to solubilize the formazan crystals. Finally, the absorbance (595 nm) of the samples was read using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan). The mean values of non-cryopreserved cells were considered 100% as compared to the other groups.

Assessment of oxidative stress

Cellular oxidative stress was evaluated by quantification of intracellular reactive oxygen species (ROS) levels and measurement of mitochondrial membrane potential (ΔΨm). Briefly, intracellular levels of ROS were quantified using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) as a fluorescent probe, according to Santos et al. (32). For this, the cells, independent of the experimental group, were seeded in glass slides and grown *in vitro* until reaching 70% confluence. Subsequently, cells were washed in PBS and incubated with 1.0 mL 5 μM H_2DCFDA at 38.5 °C with 5% CO_2 for 30 min protected from light. After this period, the stained cells were washed twice in PBS and imaged using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The intensity of the signal fluorescence (pixels) was measured using ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the values obtained for the treated samples. Measured mean value of the micrograph for non-cryopreserved cells was used as a calibrator. Relative expression levels (arbitrary fluorescence units) were generated by dividing the measured value of each micrograph for the cryopreserved cells by the mean of the calibrator.

The ΔΨm was assessed using the fluorescent probe MitoTracker Red® (CMXRos) at 500 nm according to Santos et al. (32). The procedure, incubation and evaluation of the images, were performed as described for the quantification of ROS.

Statistical analysis

Data were expressed as mean ± standard error (one animal/one repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporated, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by Tukey test. Significance was set at $P < 0.05$.

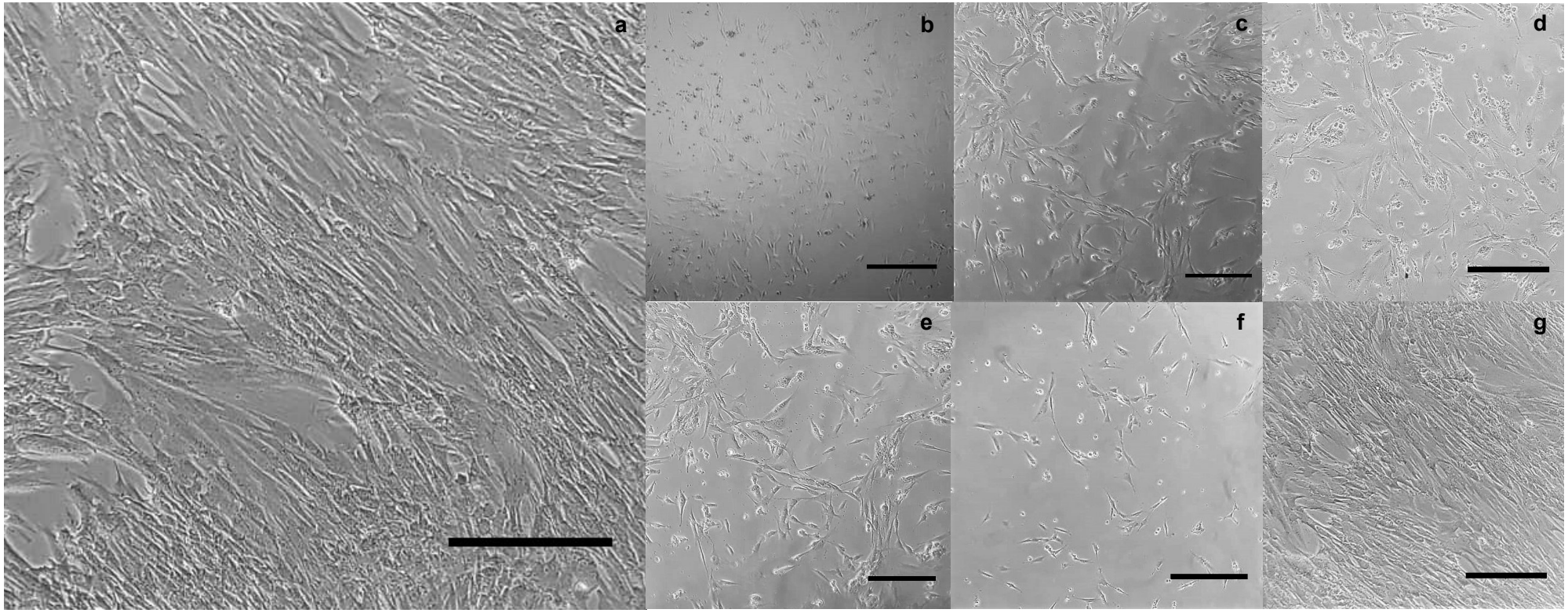
Results

Evaluation of morphological characteristics and cell viability

All cells derived from collared peccary tissue had consistent morphological characteristics in all experimental groups (Fig. 1a-g). Slow cooling did not alter the capacity of the cells to grow and reach 70-80% confluence, presenting cells with similar morphology to fibroblasts, with fusiform aspect, cytoplasmic prolongations, abundant cytoplasm and central nucleus, with an overall large and oval morphology.

Moreover, no difference was observed among groups for cell viability evaluated by trypan blue (Fig. 2, $P > 0.05$). Thus, while non-cryopreserved cells had a viability of 87.0% ± 5.2, cryopreserved cells of different groups showed viability in the range of 62.5% ± 5.2 to 91.7% ± 0.0.

1



2 **Figure 1.** Subcultures of fibroblast-like cells from ear skin samples of collared peccaries. **a)** Non-cryopreserved cells and cell cryopreserved in **b)**

3 DMSO, **c)** DMSO-SUC, **d)** DMSO-10FBS, **e)** DMSO-50FBS, **f)** DMSO-SUC-10FBS, **g)** DMSO-SUC-50FBS. Scale bar = 100 µm.

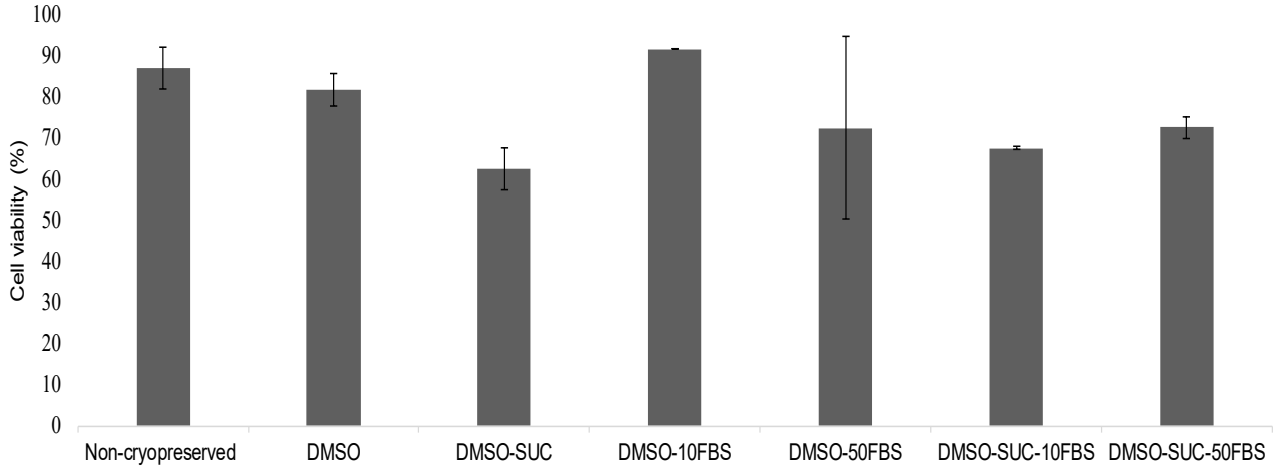


Figure 2. Viability of collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). $P > 0.05$.

Analysis of proliferative activity and metabolism

Although the growth curve did not show a typical "S" shape in all cryopreserved groups, it was possible to observe from the shape of the curve the occurrence of the initial adaptation phase of the cells, followed by exponential growth until reaching the stable phase of decline (Fig. 3a). In addition, non-cryopreserved cells showed a reduction in their growth, however, this reduction stabilized from day 8. No longer observed the decline in the curve, which showed that there was no cell death only the decrease of proliferative activity of cells from this group. Additionally, the proliferative activity evaluated by population doubling time was reduced in cells derived from DMSO-10FBS group (10.5 h ± 0.3) when compared to non-cryopreserved cells (19.4 ± 2.6, Fig. 3b).

Although cryopreservation reduced cell metabolism, no difference was observed among cryoprotectant solutions ($P > 0.05$). Thus, while cryopreserved cells had a metabolic activity of 100.0% ± 0.0, cryopreserved cells among the different groups varied among 63.6% ± 22.6 and 97.7% ± 25.1.

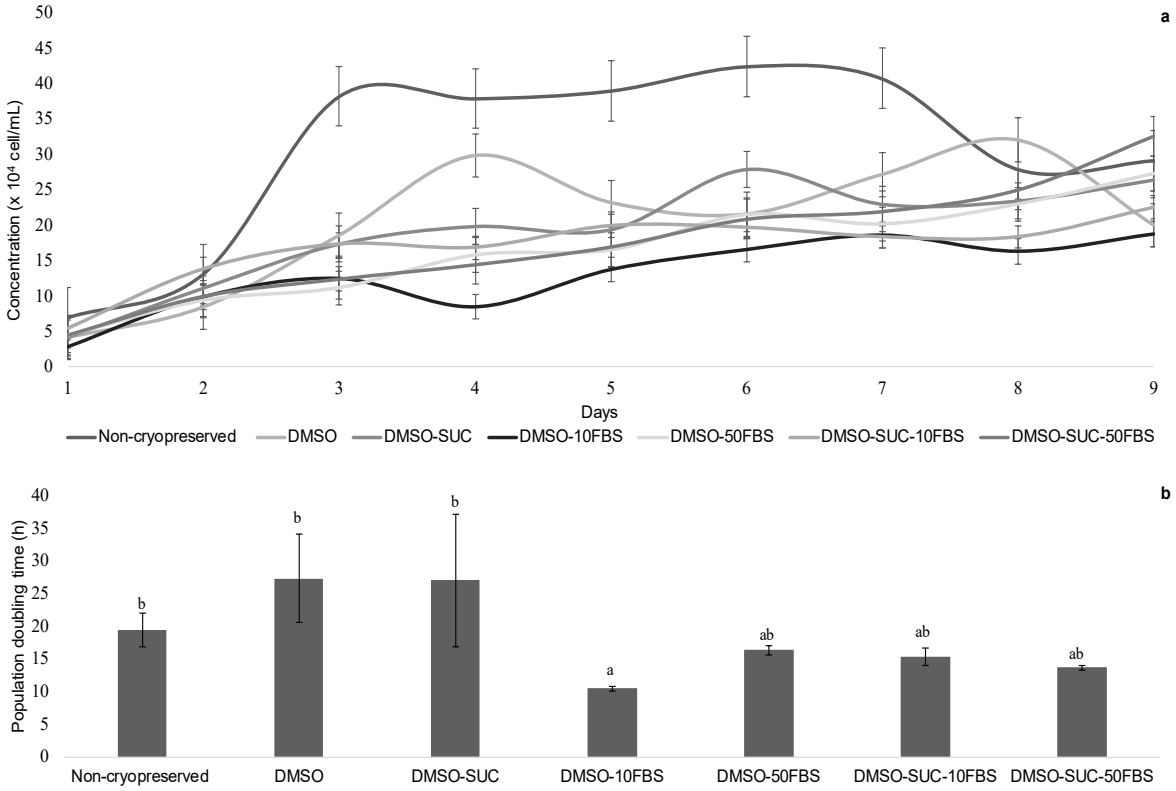


Figure 3. The growth dynamics and metabolism of collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. **a)** Growth curves of cryopreserved and non-cryopreserved cells. **b)** Values of population doubling time (PDT) after culture for nine days. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). Different letters show significant differences among the groups ($P < 0.05$). Bars indicate standard error.

Assessment of oxidative stress

A lower level of ROS was observed in cells derived from DMSO-SUC-50FBS when compared to other cryopreserved groups (Fig 4a and 4c).

Moreover, only cells from the DMSO-SUC-50FBS group had mitochondrial membrane potential similar to the non-cryopreserved cells (Fig 4b and 4d).

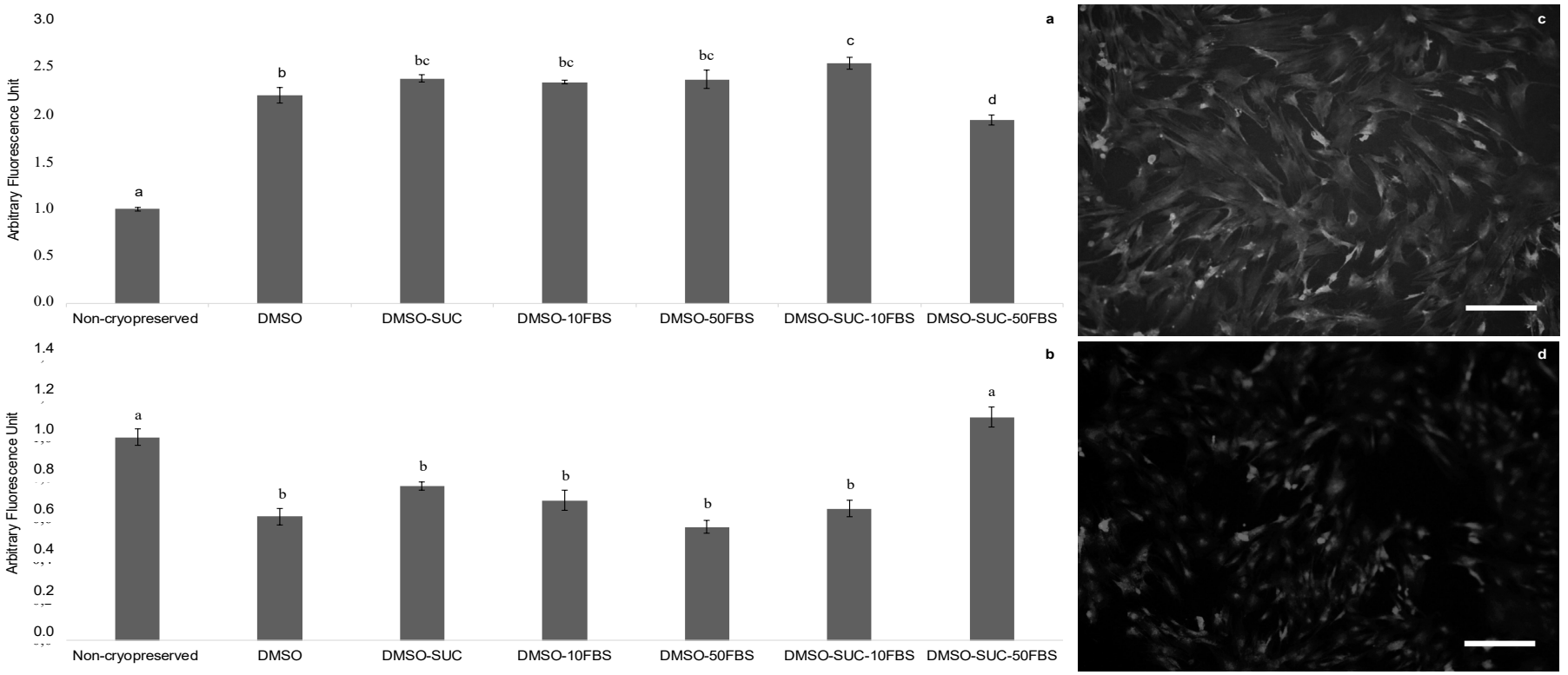


Figure 4. Assessment of oxidative stress in collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). **a)** Quantification of intracellular reactive oxygen species (ROS) levels **b)** Measurement of mitochondrial membrane potential (ΔΨm). **c)** Cells labeled with H_2DCFDA for quantification of ROS levels. **d)** Cells labeled with MitoTracker Red® for measurement of ΔΨm. Different letters within each analysis show significant differences among the groups ($P < 0.05$). Scale bar = 200 µm.

Discussion

The successful formation of a bank of biological resources is correlated to the efficiency of the cryopreservation protocol used. As such, the initial combination of intracellular and extracellular cryoprotectants is key in ensuring cell quality after thawing. In this study, we showed that the combination of 0.2 M SUC with 50% FBS added to the cryoprotectant solution containing 10% DMSO, ensured a better quality of the recovered collared peccary somatic cells, particularly by reducing the oxidative stress caused by slow freezing and by maintaining normal cellular characteristics. Therefore, our results demonstrated three relevant points of cryopreservation of somatic cells: (a) the cells obtained from collared peccary tissue showed characteristics similar to fibroblast cells used as the main donor nucleus in SCNT, (b) some alterations resulting from the cryopreservation may not be observed from the analysis of the viability and metabolic activity of the thawed cells; therefore, more specific assessments such as the analysis of the oxidative stress may be necessary, and (c) the favorable effect of the addition of 50% FBS on the cryoprotectant solution containing 10% DMSO and 0.2 M SUC. Thus, these findings ensure that higher quality cells are employed in future conservation strategies.

In general, cryopreservation protocols can induce lesions such as lipid peroxidation of membranes and damage to DNA in fibroblasts, resulting from the formation of ROS (33). Moreover, the natural antioxidant defense mechanism of the cells is not enough to eliminate excess ROS (11). Therefore, studies have shown that supplementation with antioxidants and/or factors that modulate the cell death process caused by excess ROS may be a potential solution to reduce oxidative stress induced by cryopreservation (14). Additionally, a decrease in $\Delta\Psi m$ has been observed in cells with excess ROS formation (13). Thus, as suggested by Poot et al. (27), we used a fluorescent probe that has an affinity for molecules present in cells. Such probes are responsible for evaluating the relationship among mitochondria and the triggering of apoptotic mechanisms. With this, it was possible to demonstrate that among the cryoprotectant solutions tested, the cells obtained from the DMSO-SUC-50FBS treatment showed high $\Delta\Psi m$ after thawing.

Thus, one compound that has such antioxidant potential is FBS, which has been frequently used in cryoprotectant solutions (12). In general, FBS is routinely added to stabilize cell membranes and adjust intra- and extracellular osmotic pressure; however, the

role of this cryoprotectant as an antioxidant has become increasingly evident, which may be related to its constituents, mostly amino acids, being formed (20). This could be due to the synergistic effect of their constituents, which may have attached to cell membranes, promoting stabilization and avoiding excessive concentration of solutes that may be related to ROS formation (12). In addition, their constituents may be responsible for the increased activity of antioxidant intracellular enzymes (26). Thus, it is likely that the FBS in the DMSO-SUC-FBS solution acted as a source of antioxidants. However, the mechanisms underlying the antioxidant role of FBS under the formation of ROS suffered by cells during cryopreservation are still unclear. Moreover, when the cells were cryopreserved with 10% FBS (DMSO-10FBS and DMSO-SUC-10FBS groups), the fluorescence intensity increased, indicating an increase in ROS production. This suggests that the reduction and/or absence of antioxidative agents in the DMSO, DMSO-10FBS and DMSO-SUC-10FBS groups may be associated with the formation of ROS (24, 42, 44).

This result may be due to FBS counteracting the cytotoxicity induced by DMSO, as demonstrated by Miki et al. (22), when analyzing the ΔΨm of stem cells subjected to a cryoprotectant solution with 10% DMSO in association with 90% FBS, which resulted in a ΔΨm of 1.00. Therefore, treatments with 10% FBS caused the cells to be more exposed to cytotoxicity caused by DMSO during the freeze-thaw cycle. The DMSO penetrates the cell membrane and forms hydrogen bonds with the water molecules present in the cytosol. Thus, the freezing point of the water decreases, and there is a lower probability of the formation of ice crystals inside the cell. Furthermore, the integrity of the membrane is conserved and for this reason, DMSO is an indispensable intracellular cryoprotectant. However, even though its positive effect is observed, its toxicity remains high, suggesting its association with extracellular cryoprotectants such as SUC and FBS. This association can accelerate the cellular dehydration process and balance cytotoxicity. Therefore, the ideal concentrations for achieving this cytotoxic balance must be established so that the concentrations of extracellular cryoprotectants are sufficient to minimize the negative effect of the high toxicity of DMSO. Additionally, the presence of SUC can promote osmotic maintenance of the medium and perform synergistically with the intracellular cryoprotectant, resulting in improved effects when used in combination (37).

The ROS levels of the DMSO-SUC-10FBS group were higher in comparison to the DMSO group, probably due to the use of more cryoprotective agents in their composition. It should be borne in mind that such cryoprotective agents (DMSO-SUC-10FBS), despite promoting cell protection, present a certain toxicity that may be responsible for greater oxidative stress and subsequent increased production of ROS. However, this does not mean that the use of fewer cryoprotective agents is recommended, but rather that a combination promoting balance between the concentration used, toxicity, protection of cells at cryogenic temperatures and lower production of ROS, should be utilized.

In addition, the morphological and confluence aspects observed in the collared cells were similar to those observed by Queiroz Neta et al. (29) and Borges et al. (4) describing collared peccary cells recovered from refrigerated and vitrified tissues, respectively. Thus, proper maintenance of the morphological characteristics of cultured cells may be associated with a decrease in ice crystal formation due to the cryopreservation process, which when exposed to cells, may have deleterious effects on cell characteristics (15).

The viability data showed that cryopreserved cells and non-cryopreserved cells were stable regardless of treatments. This can be attributed to the quality of the 10% DMSO slow freezing technique that is commonly associated with better results for somatic cells derived from wild mammals (23, 35).

Further, the data found on higher viability rates and the performance of cells in vitro culture shown in this study, corroborate the findings of Subramanian et al. (40) and Barnes et al. (1). These authors affirmed that cultures derived from cryopreserved and non-cryopreserved cells have a similar growth performance, since the cell population after culture can recover with rates similar to non-cryopreserved cells.

Finally, all groups showed no change in PDT values except DMSO-10FBS. In general, cells cultured in specific treatments including FBS in their composition required less time to replicate (19). Our PDT rates and metabolism activity evaluated by MTT may be associated with the use of an adequate system of cryopreservation that promoted an optimum cooling rate (10) associated with compounds present in FBS such as transport proteins, adhesion

proteins, enzymes, hormones, growth factors, cytokines, fatty acids, lipids, vitamins, carbohydrates, and nitrogen of non-protein origin (7).

In conclusion, 10% DMSO supplemented with 0.2 M SUC and 50% FBS was the most efficient cryoprotectant solution for conserving collared peccary somatic cells. This work presents a breakthrough for the improvement and establishment of an ideal freezing protocol for collared peccary somatic cells that can be used in future biotechnical techniques such as cloning, since each stage involved should be optimized for the species studied.

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CAPÍTULO 5 – EFFECTS OF INCUBATION TIME AND METHOD OF CELL CYCLE SYNCHRONIZATION ON COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES

(Artigo experimental)

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FATOR DE IMPACTO: 1,586

Effects of incubation time and method of cell cycle synchronization on collared peccary skin-derived fibroblast cell lines*

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Abbreviated title: Cell cycle of peccary fibroblasts

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Abstract

The success of cloning by somatic cell nuclear transfer depends on the efficiency of nuclear reprogramming, with the cycle stage of the donor cell playing a crucial role. Therefore, the aim was to evaluate three different approaches for cell cycle synchronization: (i) serum starvation (SS) for 1 to 4 days, (ii) contact inhibition (CI) for 1 to 3 days, and (iii) using cell cycle regulatory inhibitors (dimethyl sulfoxide, cycloheximide, cytochalasin B, or 6-dimethylaminopurine) for 1 and 2 days, in terms of their effects on synchronization in G_0/G_1 phases and viability of collared peccary skin fibroblasts. Flow cytometry analysis revealed that SS for 4 days (79.0% ± 1.6) and CI for 3 days 78.0% ± 1.4) increased the percentage of fibroblasts in G_0/G_1 compared to growing cells GC, 68.1% ± 8.6). However, SS for 3 and 4 days reduced the viability evaluated by differential staining (81.4% ± 0.03 and 81.6% ± 0.06) compared to growing cells (GC, 95.9% ± 0.06). CI did not affect the viability at any of the analyzed time intervals. No cell cycle inhibitors promoted synchronization in G_0/G_1. These results indicate that CI for 3 days was the most efficient method for cell cycle synchronization in peccary fibroblasts.

Key words: G_0/G_1 phase, karyoplast, somatic cell nuclear transfer, wild mammals.

Introduction

The collared peccaries are wild mammals endemic to the American continent, where they play a crucial ecological role in maintaining their habitats (Desbiez et al., 2012). Among the various interactions of these animals with the ecosystem are their functions as seed dispersers and prey for larger mammals (Desbiez and Keuroghlian, 2009). Although their population is not considered to be of concern internationally (Gongora et al., 2011), in some biomes, such as Caatinga and the Atlantic forest, these individuals' populations are declining (Desbiez et al., 2012), thus requiring conservation strategies that ensure their maintenance in biodiversity.

Fortunately, this species' high adaptation capacity and zootechnical performance in captivity (Briceño-Méndez et al., 2016) suggest that reproductive biotechniques such as cloning by somatic cell nuclear transfer (SCNT) may be successfully used for their multiplication, as well as for research on embryonic development mechanisms of the species. SCNT involves embryo reconstruction by fusing the nucleus of a donor cell (karyoplast) with

an enucleated oocyte (cytoplast), where the karyoplast in cell cycle phase G_0/G_1 is exposed to reprogramming by the oocyte, followed by fusion and activation of the reconstructed embryo (Loi et al., 2011). Usually, the success of SCNT depends on the efficiency of nuclear reprogramming and each species has different needs at each stage of the technique (Borges and Pereira, 2019). Although this process has not yet been completely clarified, it is known that the cell cycle phase of the karyoplast affects the results (Zhao et al., 2016). Therefore, the establishment of an appropriate cell synchronization protocol in G_0/G_1 is a crucial step in the development of SCNT usage in collared peccaries.

In recent years, our group has developed steps to implement the SCNT technique in collared peccaries. This path has been developed since the establishment of the ideal environment for receptor oocytes (cytoplasts, Borges et al., 2018b), and the development of an efficient oocyte artificial activation protocol to provide the development of the reconstructed embryo (Borges et al., 2020b). In parallel, we established somatic tissue banks (Borges et al., 2017, 2018a; Queiroz Neta et al., 2018), somatic cell banks (Lira et al., 2020), and, recently, we established skin-derived fibroblasts lines (Borges et al., 2020a). Therefore, now, we have evaluated the different cell cycle synchronization protocols of these lines, aiming at a higher percentage of cells in G_0/G_1.

Several cell cycle synchronization protocols in G_0/G_1 stages have been proposed. However, the cellular response to these protocols has been variable in domestic (Goissis et al., 2007) and wild mammals (Mahesh et al., 2012), necessitating the evaluation of each protocol for a given cell type and species (Wittayarat et al., 2013; Veraguas et al., 2017). Three main approaches have been employed for cell synchronization: serum starvation (SS, Kues et al., 2000), contact inhibition (CI, Boquest et al., 1999), and using cell cycle regulatory inhibitors (Goissis et al., 2007). While SS and CI act on the checkpoints by depriving the cells of adequate environmental or nutritional conditions (Kues et al., 2000), cell cycle inhibitors regulate specific biosynthetic processes such as repression or induction of cyclins (dimethyl sulfoxide, DMSO), protein synthesis inhibition (cycloheximide, CHX), cytoskeleton inhibition (cytochalasin B, CB), and protein kinase inhibition (6-dimethylaminopurine, 6-DMAP) to cause prolongation of the G_1 phase (Koo et al., 2009; Kretz et al., 2019).

The aim of this study was to evaluate SS, CI, and cell cycle regulatory inhibitors (DMSO, CHX, CB, or 6-DMAP) on synchronization in G_0/G_1 phases as well as the viability of collared peccary skin fibroblasts, in order to develop efficient cell cycle synchronization protocols for this species. We want to achieve this goal by defining appropriate methodologies for the last stage of the preparation of karyoplast to be used in the cloning of this species.

Materials and methods

Chemicals and bioethics

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA). The experiments were conducted in accordance with the Animal Ethics Committee (CEUA/UFERSA, no. 23091.001072/2015-92) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2).

Establishment and culture of fibroblasts

Skin tissue samples were obtained from the ear notch of four adult male collared peccaries at the Wild Animal Multiplication Center (CEMAS/UFERSA, no. 1478912, BR). The skin samples were cultured, and four fibroblast lines were previously established (Borges et al., 2020a). Subsequently, cells from four lines frozen in 10% DMSO, 10% FBS and 0.2 M sucrose were thawed, and 4^{th} and 5^{th} passage cells were used for this study.

Initially, cells were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution in a humid atmosphere containing 5% CO_2 at 38.5 °C. Prior to initiating cell cycle synchronization protocols, the cells were evaluated for their proliferative activity by obtaining the growth curve and determining the population doubling time (PDT). Cells were seeded in 12-well plates at a concentration of 1.0 x 10^4 cells/mL. Cells from each well were counted at 24 h intervals for up to 216 h of culture. After each interval, the mean cell count was recorded; finally, the cell growth curve was generated, and the PDT was estimated based on these measurements (Roth, 2006).

Additionally, the percentage of living cells was determined by trypan blue exclusion assay (Borges et al., 2018a). Briefly, the cells were stained with 0.4% trypan blue in phosphate buffered saline (PBS) and counted using a hemocytometer.

Cell treatments

Evaluating three cell cycle synchronization methods, we examined the effects of serum starvation (SS), contact inhibition (CI), and cell cycle regulatory inhibitors (DMSO, CHX, CB, and 6-DMAP) treatments on the cell cycle stages of collared peccary fibroblasts in different incubation times. In each treatment group, cells without any treatment and with 70% confluence were used as a control (growing cells, GC). All treatments were performed in duplicate for each animal, producing a total of eight repetitions for each treatment and each incubation time.

Experiment 1 – Effect of serum starvation

SS was performed by replacing the culture medium with DMEM containing 0.5% FBS after the cell confluence had reached 70% in DMEM with 10% FBS (Caamaño et al., 2008). After the start of the SS treatment (day = 0), the stage of fibroblasts from each animal was analyzed on days 1, 2, 3 and 4. The culture medium was changed every 2 days.

Experiment 2 – Effect of cell contact inhibition

The effect of CI was studied for 3 days in culture after confluence had reached 90-100%. During the treatment of CI, the culture medium composed of DMEM and 10% FBS was changed every 2 days (Gómez et al., 2018).

Experiment 3 – Effect of cell cycle regulatory inhibitors

DMSO (0.5%, inhibitor of cytokinesis), CHX (7.5 µg/mL, protein synthesis inhibitor), CB (7.5 µg/mL, inhibitor of cytokinesis), and 6-DMAP (2 mM, protein kinase inhibitor) treatments were performed for 2 days of cell culture after the cell confluence reached 70%. After the start of treatment (day = 0), the stage of fibroblasts from each animal was analyzed on days 1 and 2 (Mahesh et al., 2012).

Cell cycle synchronization analysis

After the treatments, cells were trypsinized, centrifuged at 600×g for 10 min and resuspended in 1.0 mL of cold 70% ethanol for fixation. The cells were then maintained at -20 °C for 5 days. The fixed were washed in PBS for ethanol removal and each sample was centrifuged at 400×g for 10 min. Subsequently, cells were stained with 20 µg/mL of propidium iodide, 50 µg/mL of RNase were added, and samples were incubated at 4 °C for 50 min. After that, the samples were analyzed using Guava Easycyte flow cytometer (Guava Technologies, Stamford, Lincolnshire, United Kingdom). Data were obtained from 15,000 events from each sample. Histograms of red fluorescence vs counts were generated to evaluate the percentages of cells for each cell cycle phase (G_0/G_1, S, G_2/M) as well as the levels of sub G_0/G_1. The proportion of cells in each phase of the cell cycle and sub G_0/G_1 levels was assessed using MODFIT software version 5.0 (Verity, https://www.vsh.com/products/mflt/index.asp).

Cellular viability assessment

A cell aliquot was resuspended in 2 µg/mL of acridine orange and 10 µg/mL of ethidium bromide. Subsequently, the cells were evaluated using fluorescence microscopy at 480 nm, where 300 cells were counted at 200×. The cells were then classified into (i) viable cells, with a uniform light green nucleus; (ii) cells in initial apoptosis, with a non-uniform green nucleus; (iii) cells in late apoptosis, with a non-uniform bright orange nucleus, and (iv) necrotic cells, with a uniform orange nucleus (Kosmider et al., 2004). A fluorescence microscope was used to observe apoptotic changes in the stained cells, which were quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data were expressed as mean ± standard error (one fibroblast line/ one repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by Tukey test. Significance was set at $P<0.05$.

Results

Initially, thawed cells were morphologically normal (Figure 1A), with a percentage of 91.2% ± 1.2 live cells after the thawing. Moreover, the cells showed a sigmoidal curve with the lag phase of adaptation up to day 2 followed by exponential and stationary growth, indicating that these cells were going through various phases of growth. Additionally, the decreasing phase was not observed (Figure 1B). The growth kinetics of the cells was demonstrated by counting total cells during 216 h of culture, resulting in an estimated PDT of 15.3 ± 1.4 h.

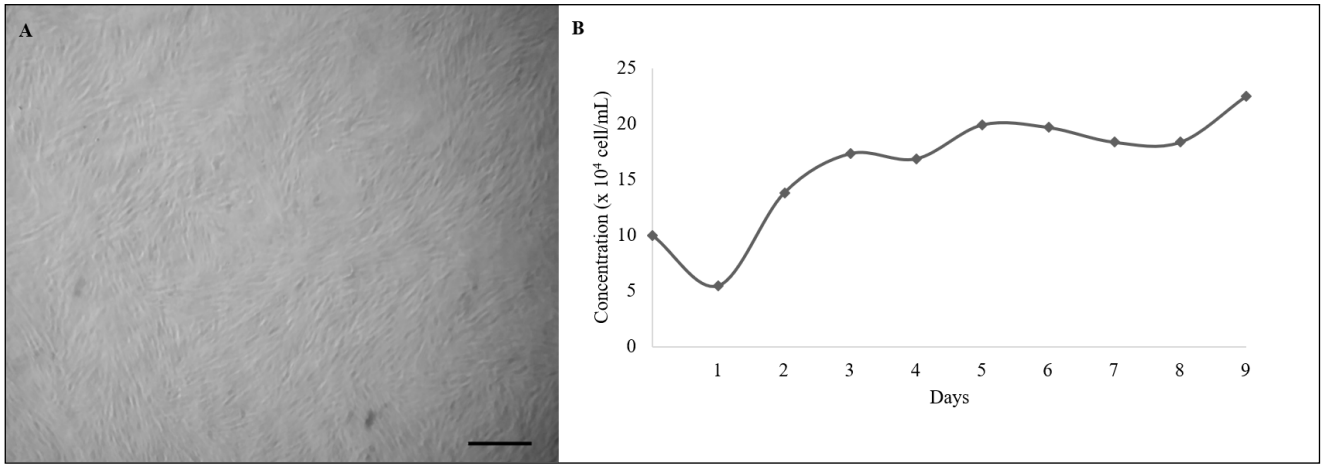

Figure 1. Culture of collared peccary fibroblast cells. (A) Fibroblasts at 70% confluency showing normal morphology. (B) Growth curve of fibroblasts.

Experiment 1 – Effect of serum starvation

After 4 days of SS, the percentage of G_0/G_1 phase cells was significantly higher ($P<0.05$) when compared to GC, which was the control (Figure 2A–B, Table 1). Moreover, there was no change in the other phases of the cell cycle. Although the SS did not cause differences in sub G_0/G_1, when the levels of viability were evaluated by differential staining, 3 and 4 days of SS caused cell damage (Figure 3A).

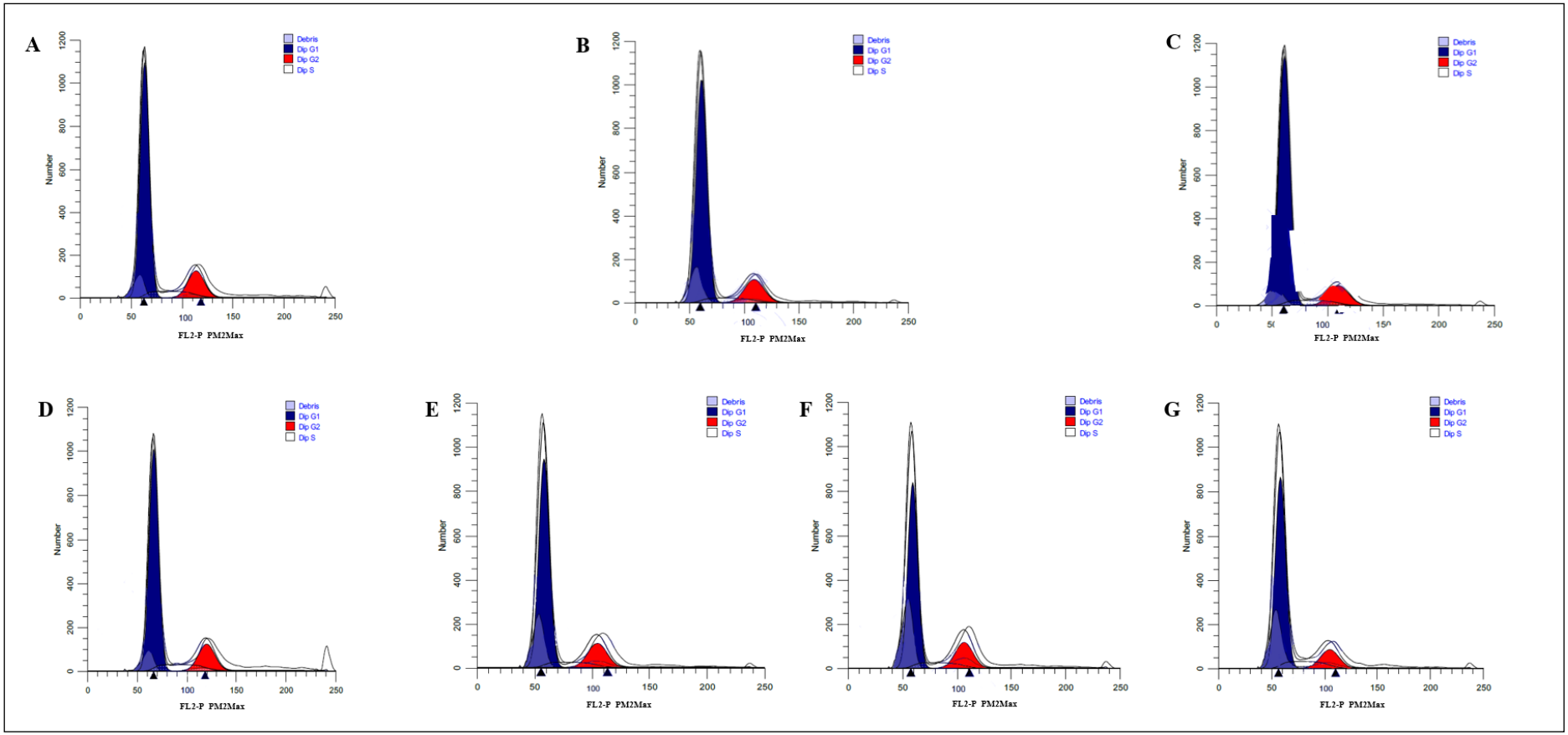

Figure 2. Representative histograms of the flow cytometry analysis of collared peccary fibroblasts. (A) growing cells (GC, control). (B) serum starvation (SS). (C) contact inhibition (CI). (D) dimethylsulfoxide (DMSO). (E) cycloheximide (CHX). (F) cytochalasin B (CB). (G) 6-dimethylaminopurine (6-DMAP).

Table 1. Effect of serum starvation (SS) on the percentage of collared peccary fibroblasts in the G_0/G_1, S and G_2/M phases of the cycle

Conditions	*Cell cycle phase (%)*			
	G_0/G_1	*S*	*G_2/M*	*Sub G_0/G_1*
Growing cells (GC)	68.1 ± 8.5^{a}	17.6 ± 10.3^{a}	14.3 ± 1.8^{a}	15.9 ± 7.2^{a}
1 day SS	78.7 ± 2.3^{ab}	6.7 ± 1.5^{a}	14.6 ± 0.9^{a}	7.0 ± 1.8^{a}
2 day SS	78.1 ± 1.7^{ab}	7.2 ± 0.9^{a}	14.7 ± 1.5^{a}	10.5 ± 3.0^{a}
3 day SS	75.8 ± 2.9^{ab}	6.8 ± 2.2^{a}	17.4 ± 0.7^{a}	19.0 ± 11.3^{a}
4 day SS	79.0 ± 1.6^{b}	5.0 ± 0.8^{a}	16.0 ± 1.1^{a}	5.9 ± 2.2^{a}

Within a column, values with different superscripts differ ($P<0.05$).

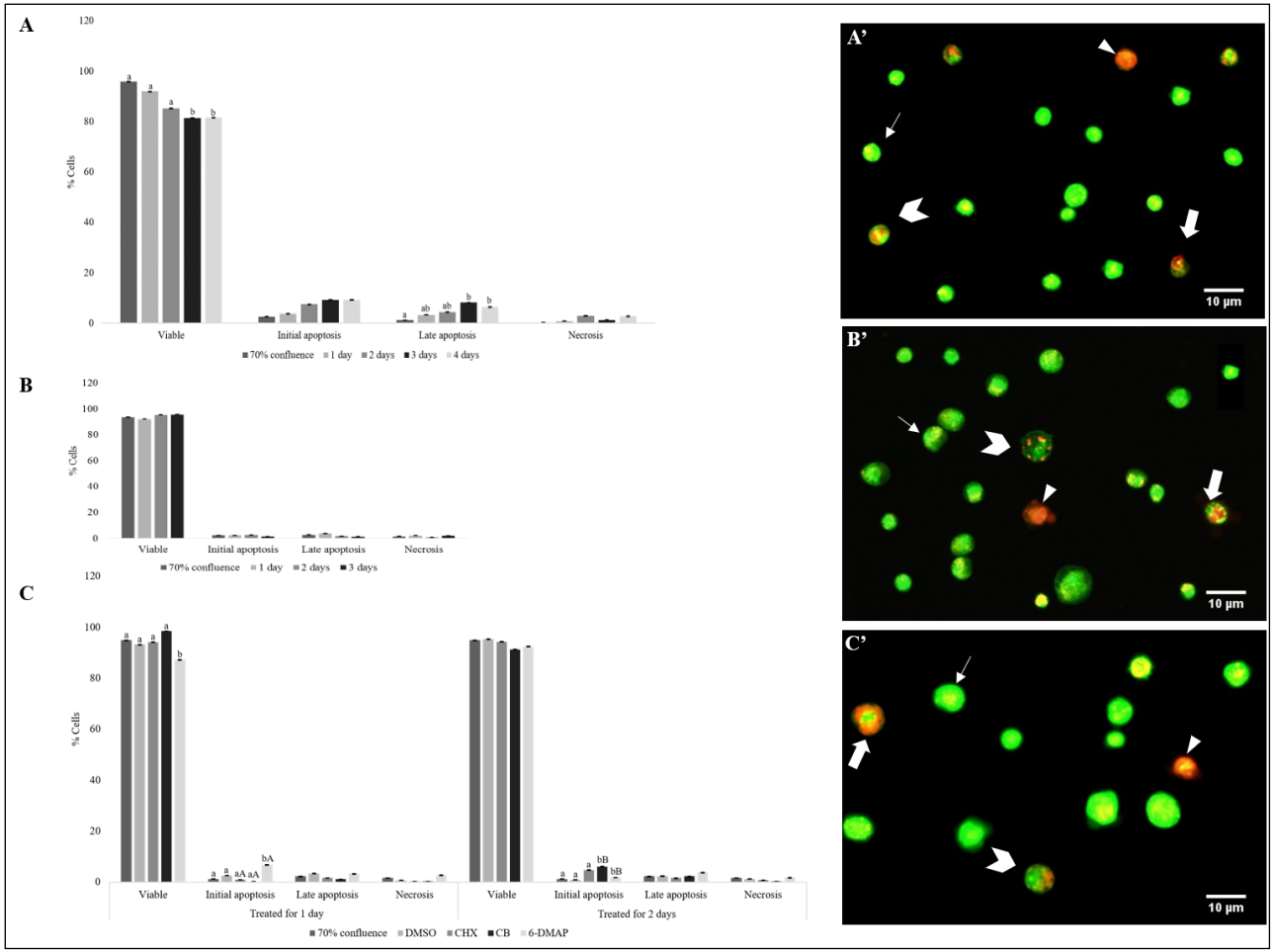

Figure 3. Effect of different synchronization methods on the viability of collared peccary fibroblasts. (A) and (A') Effect of SS on fibroblast viability. (B) and (B') Evaluation of the viability of fibroblasts subjected to CI. (C) and (C') Effect of cell cycle inhibitors on the viability of synchronized fibroblasts. Within a bar at the same time, the values of a,b differ ($P<0.05$). Within a bar at different times, A,B differ ($P<0.05$). Fine arrow: viable cell; arrowhead: cell in initial apoptosis; Fat arrow: cell in late apoptosis; triangle: cell in necrosis.

Experiment 2 – Effect of cell contact inhibition

CI for 3 days significantly increased the proportion of fibroblasts in G_0/G_1 phase when compared to GC (Figure 2C, Table 2) and decreased the proportion of cells in G_2/M phase when compared to GC ($P<0.05$). However, neither evaluated time interval promoted modifications in S cell cycle phase or levels of sub G_0/G_1 ($P>0.05$). Additionally, CI did not affect the cell viability at any of the analyzed intervals ($P>0.05$, Figure 3B).

Experiment 3 – Effect of cell cycle regulatory inhibitors

Cell cycle inhibitors did not promote cell synchronization in G_0/G_1 at the time tested when compared to GC for 1 and 2 days (Figure 2D-G, Table 3). While DMSO did not change any of the evaluated parameters, CHX increased sub G_0/G_1 levels on both days and reduced the percentage of cells in S phase after 2 days when compared to CG ($P<0.05$). After 2 days of treatment with CHX, the proportion of fibroblasts in G_0/G_1 phase was higher when compared to that after 1 day ($P<0.05$).

Moreover, CB reduced the percentage of cells in G_0/G_1 after 1 day, increasing the percentage of cells in S phase when compared to GC ($P<0.05$). Additionally, treatment with 6-DMAP for 1 and 2 days significantly reduced the proportion of fibroblasts in S phase and caused an increase in the proportion of cells in G_2/M phase. 6-DMAP also caused an increase in sub G_0/G_1 levels when compared to GC (Table 3). Finally, after 1 day of treatment of 6-DMAP, a reduction of viable cells was observed when compared to GC, although no reduction was observed after 2 days of treatment ($P<0.05$, Figure 3C).

Table 2. Effect of contact inhibition (CI) on the percentage of collared peccary fibroblasts in the G_0/G_1, S and G_2/M phases of the cycle

Conditions	*Cell cycle phase (%)*			
	G_0/G_1	*S*	*G_2/M*	*Sub G_0/G_1*
Growing cells (GC)	70.8 ± 1.4^{a}	5.9 ± 0.8^{a}	23.3 ± 1.3^{a}	1.1 ± 0.2^{a}
1 day CI	75.6 ± 3.2^{ab}	7.3 ± 1.0^{a}	17.1 ± 2.2^{ab}	2.0 ± 0.7^{a}
2 day CI	75.5 ± 1.8^{ab}	6.9 ± 0.5^{a}	17.6 ± 1.5^{ab}	5.5 ± 3.5^{a}
3 day CI	78.0 ± 1.4^{b}	6.9 ± 1.2^{a}	15.1 ± 1.8^{b}	7.6 ± 5.3^{a}

Within a column, values with different superscripts differ ($P<0.05$).

Table 3. Effect of treatment with dimethyl sulfoxide (DMSO), cycloheximide (CHX), cytochalasin B (CB), or 6-dimethylaminopurin (6-DMAP) on the percentage of collared peccary fibroblasts in the G_0/G_1, S and G_2/M phases of the cycle

Conditions	*Cell cycle phase (%)*							
	Treated for 1 day				*Treated for 2 days*			
	G_0/G_1	*S*	*G_2/M*	*Sub G_0/G_1*	*G_0/G_1*	*S*	*G_2/M*	*Sub G_0/G_1*
Growing cells (GC)	75.4 ± 0.8^{a}	7.1 ± 0.7^{b}	17.5 ± 0.3^{b}	10.9 ± 2.9^{b}	75.4 ± 0.8^{a}	7.1 ± 0.7^{a}	17.5 ± 0.3^{b}	10.9 ± 2.9^{b}
0.5% DMSO	75.7 ± 0.9^{a}	5.9 ± 1.5^{b}	18.4 ± 1.0^{b}	10.9 ± 0.5^{b}	74.1 ± 0.5^{a}	8.0 ± 0.6^{a}	17.9 ± 0.3^{b}	17.7 ± 0.7^{b}
7.5 µg/mL CHX	72.7 ± 0.6^{abB}	9.1 ± 0.5^{abA}	18.2 ± 1.0^{b}	24.3 ± 2.7^{c}	77.4 ± 1.0^{aA}	5.4 ± 0.6^{bB}	17.2 ± 0.9^{b}	25.7 ± 3.3^{a}
7.5 µg/mL CB	68.3 ± 1.6^{bB}	12.5 ± 1.2^{a}	19.2 ± 0.7^{bA}	5.8 ± 2.9^{d}	74.4 ± 0.1^{aA}	9.8 ± 0.5^{a}	15.8 ± 0.5^{bB}	17.2 ± 1.3^{b}
2 mM 6-DMAP	73.5 ± 1.5^{a}	0.0 ± 0.0^{cA}	26.5 ± 1.5^{aA}	46.7 ± 1.1^{aA}	74.6 ± 1.5^{a}	3.7 ± 0.6^{bB}	21.7 ± 1.2^{aB}	27.3 ± 5.7^{aB}

Within a column, ^{a,b} values with different superscripts differ ($P<0.05$). Within a row, ^{A,B} values with different superscripts differ ($P<0.05$).

Discussion

An appropriate cell synchronization protocol in G_0/G_1 is a crucial step in the development of cloning by SCNT in collared peccaries. Previously, our group developed somatic resource banks aiming at their application in the conservation of this species (Borges et al., 2017, 2018a, 2020a,b; Lira et al., 2020; Queiroz Neta et al., 2018a). With this new study, we have developed a suitable protocol for somatic cell synchronization, the last step in the preparation of karyoplasts, i.e., somatic nucleus donor cells. To our knowledge, this is the first work to elucidate nuclear reprogramming in cells of this species.

Here, we identified that using CI for 3 days was more efficient for cell cycle synchronization when compared to SS for 1 to 4 days and treatment with cell cycle inhibitors (DMSO, CHX, CB, or 6-DMAP) for 1 and 2 days. This result may be related to a high cell density where CI favors the regulation of reactive oxygen species (ROS) as well as activates the coactivator-1α (PGC1α), which functions as a key regulator of energy expenditure, involved in reduction of ROS and protection of cells from oxidative stress (Yang et al., 2018). In studies with the Asian golden cat (*Catopuma temminckii*), leopard (*Panthera pardus*), and Siamese cat, researchers observed that CI for 5 days promoted an increase of more than 80% in the percentage of cells in G_0/G_1 when compared to non-synchronized cells, without increase in apoptotic cells (Wittayarat et al., 2013). In swine, domestic species phylogenetically close to the collared peccaries, cells were efficiently synchronized by CI for 3 to 4 days and resulted in 20.4% of zygotes developing into blastocysts after cloning by SCNT (Lee et al., 2019). Moreover, for wood bison (*Bison bison*), the CI was adequate for cell synchronization in G_0/G_1 phase, which resulted in 19.2% of developed blastocysts (Seaby et al., 2013). Therefore, the CI has been efficient in producing clone embryos.

Although SS promoted cell synchronization after 4 days in collared peccaries, this treatment had a negative effect on cell viability after 3 and 4 days. In general, the synchronization of the G_0/G_1 cell cycle of mammalian cells via SS is due to the response to absence or presence of mitogens to continue the cell cycle during the onset of G_1 phase, so when these cells are in absence of mitochondrial growth factors that would be offered by the serum, they accumulate in a state of a 2n DNA content (Coller et al., 2007). In porcine cells, the SS for 1 to 3 days increased the proportion of cells at G_0/G_1 phase up to 77.9-80.2% (Kues et al., 2000). However, these authors also observed cell damage by visualization of DNA

fragmentation. Similar to our study, goat fibroblasts were found to be efficiently synchronized in G_0/G_1 by both CI and SS (Dalman et al., 2010); the number of cells in apoptosis increased by 39.8% in 3 days in SS whereas in cells at CI there was no increased apoptosis (8.7%).

The negative effect of SS was also observed on embryonic development. The cell synchronization using SS resulted in embryos with a higher degree of apoptosis and increased ROS when compared to CI (Lee et al., 2016). Likewise, observed that SS led to high Bak (pro-apoptotic) and low Bcl-xL (anti-apoptotic) expression while the CI showed equilibrium in the increase in Bak and Bcl-xL expression (Park et al., 2010). Hence, the increase in apoptosis in cells synchronized in G_0/G_1 by 4 days with SS may have a negative effect on the later embryonic development in collared peccaries.

In the present study, no cell cycle inhibitors promoted synchronization in G_0/G_1 in collared peccaries and 6-DMAP had negative effects on cell viability with increase in sub G_0/G_1 levels and reduction of viable cells. A study with goral (*Naemorhedus caudatus*) fibroblasts also indicated no cell cycle synchronization in G_0/G_1 using CHX (60.1%), 6-DMAP (3.9%) and CB (10.8%) in cultures for 1 day (Hashem et al., 2006). Porcine fibroblasts treated with CHX after 2 days showed an increased percentage of dead cells when compared to that after 1 day (Goissis et al., 2007). In our study, we observed an increase in cells in sub G_0/G_1 at both times. Also, was evaluated the synchronization in G_0/G_1 for porcine fibroblasts in different passages (5^{th}, 10^{th} and 15^{th}) and observed that CHX when used in cells from the 5^{th} passage was unable to synchronize cells when compared to that observed with cells from the 10^{th} and 15^{th} passages, presenting higher number of cells in G_0/G_1 only in 10^{th} passage fibroblasts (Goissis et al., 2007). Possibly, 10^{th} passage presented a higher proportion of senescent cells, becoming more susceptible to synchronization by this inhibitor because of the lower levels of cyclin D, the target mechanism of CHX in the cell cycle (Goissis et al., 2007). The treated cells in this study were from the 4^{th} and 5^{th} passage; this was probably why the cells did not present a positive synchronization result using CHX.

Furthermore, DMSO did not change any of the evaluated parameters in collared peccary cells. Studies in porcine, brown bear (*Ursus arctos pyrenaicus*), and goral cells have shown that cell cycle synchronization with DMSO is concentration dependent. While for porcine cells, 1% DMSO (88.7%) was more efficient than 0.5% (86.5%) and 2.5% (86.0%)

DMSO after 2 days (Boquest et al., 1999), brown bear cells were efficiently synchronized with DMSO concentrations of 1-3% (79.0-79.1%) after 2 days (Caamaño et al., 2008). Nevertheless, in goral cells, while 0.5% (74.8%) and 1% DMSO (75.2%) caused synchronization in G_0/G_1 after 1 day, no effect was observed using 2.5% DMSO (Hashem et al., 2006). Therefore, these results show that the effect of DMSO is linked to the concentration used and the sensitivity of the species to this chemical. Additionally, although DMSO may be efficient for synchronization in G_0/G_1, the use of high concentrations of this agent may cause negative effects on cells (Caamaño et al., 2008).

In summary, this study identified that both CI for 3 days and SS for 4 days promoted the synchronization of somatic cells in collared peccaries; however, the percentage of viable cells was maintained only in cells synchronized by CI. Therefore, CI for 3 days was considered the most efficient method. With the cells being subjected to less chemical exposure and metabolic stress, CI can be efficiently applied to cell cycle synchronization in collared peccary fibroblasts. Thus, we established the last step of the preparation for the use of these fibroblast as karyoplast for application in somatic cell nuclear transfer of collared peccary, with potential application to conservation of the species.

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CAPÍTULO 6 – *IN VITRO* MATURATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) OOCYTES AFTER DIFFERENT INCUBATION TIMES

(Artigo experimental)

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In vitro maturation of collared peccary (*Pecari tajacu* Linnaeus, 1758) oocytes after different incubation times[1]

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ABSTRACT.- Borges A.A., Santos M.V.O, Queiroz Neta L.B, Oliveira M.F, Silva A.R & Pereira A.F. 2017. **[*In vitro* maturation of collared peccary (*Pecari tajacu* Linnaeus, 1758) oocytes after different incubation times.]** *Pesquisa Veterinária Brasileira 00(0):00-00.* Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil. E-mail: alexsandra.pereira@ufersa.edu.br

Oocyte *in vitro* maturation (IVM) is the first step of the *in vitro* reproductive technologies that enables mature oocytes to be generated *ex vivo* and after used for embryo production. In this sense, the establishment of culture environment, as oocyte incubation time, is essential for the success of the IVM. Therefore, the study was carried out to investigate the relationship between the meiotic potential and the IVM times of collared peccary oocytes, wild mammals of great commercial and ecological interest. Thus, ovaries were collected of females derived from captivity and transported to the laboratory within 1 h of slaughtering. The oocytes derived from follicles (3–6 mm in diameter) were recovered by aspirated and sliced. Good quality oocytes (evenly granulated cytoplasm with a least one layer of surrounding *cumulus* cells) were selected and subjected to culture in TCM 199 supplemented with 10 µg/mL FSH, 10% FBS and 100 µM cysteamine at 38.5°C, 5% CO_2 and maximum humidity for 24 or 48 h. After the incubation period, the nuclear status, the presence of first polar body and the expansion of *cumulus* cells of oocytes were assessed. The data obtained were analyzed by Fisher exact test ($P < 0.05$). A total of four sessions (2–3 females per session) were performed, resulting in eighteen aspirated and sliced ovaries with normal morphological characteristics. An oocyte recovery rate of approximately 83.1% (59/71) was obtained with 3.3 oocytes/ovary and 2.3 viable oocytes/ovary. After different incubation times, differences ($P < 0.05$) were observed between 24 h and 48 h for expansion of the *cumulus* cells (38.1% *vs.* 100%), presence of first polar body (52.4% *vs.* 90.5%) and nuclear status in second metaphase (19.0% *vs.* 76.2%), respectively. In conclusion, 48 h is suitable time for the *in vitro* maturation of oocytes derived from collared peccaries when compared to the time of 24 h,

according to the meiotic potential observed. Additional studies should be conducted to improve the quality of the oocyte culture environment, as medium composition, aiming to obtain viable mature oocytes for other *in vitro* biotechnologies.

INDEX TERMS: Assisted Reproductive Technologies, meiotic competence, nuclear maturation, wild mammals.

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RESUMO.- [Maturação *in vitro* de oócitos de cateto (*Pecari tajacu* Linnaeus, 1758) após diferentes períodos de incubação.] A maturação *in vitro* (MIV) oocitária é a primeira etapa das tecnologias reprodutivas *in vitro* que permite que oócitos maturados sejam gerados *ex vivo* e depois usados para a produção de embriões. Nesse sentido, o estabelecimento do ambiente de cultivo, como o período de incubação de oócitos, é essencial para o sucesso da MIV. Portanto, o estudo foi realizado para investigar a relação entre o potencial meiótico e os períodos de MIV de oócitos derivados de catetos, mamíferos selvagens de grande interesse comercial e ecológico. Para tanto, os ovários foram coletados de fêmeas derivadas de cativeiro e transportados ao laboratório dentro de 1 h após o abate. Os oócitos derivados de folículos (3–6 mm de diâmetro) foram recuperados por aspiração e fatiados. Oócitos de boa qualidade (citoplasma uniformemente granulado com pelo menos uma camada circundante de células *cumulus*) foram selecionados e submetidos ao cultivo em TCM 199 suplementado

com 10 µg/mL de FSH, 10% de SFB e 100 µM de cisteamina a 38,5°C, 5% de CO_2 e umidade máxima por 24 ou 48 h. Após o período de incubação, o estado nuclear, a presença do primeiro corpúsculo polar e a expansão das células do *cumulus* dos oócitos foram avaliados. Os dados obtidos foram analisados pelo teste exato de Fisher ($P < 0,05$). Um total de quatro sessões (2–3 fêmeas por sessão) foi realizado, resultando em dezoito ovários aspirados e fatiados com características morfológicas normais. Uma taxa de recuperação oocitária de aproximadamente 83,1% (59/71) foi obtida com 3,3 oócitos/ovário e 2,3 oócitos viáveis/ovário. Após diferentes períodos de incubação, diferenças ($P < 0,05$) foram observadas entre 24 e 48 h para a expansão das células *cumulus* (38,1% *vs.* 100%), presença de primeiro corpúsculo polar (52,4% *vs.* 90,5%) e estado nuclear na segunda metáfase (19,0% *vs.* 76,2%), respectivamente. Em conclusão, 48 h é o período adequado para a maturação *in vitro* de oócitos derivados de catetos quando comparado ao tempo de 24 h, de acordo com o potencial meiótico observado. Estudos adicionais devem ser conduzidos para melhorar a qualidade do ambiente de cultivo oocitário, como a composição de meio, objetivando obter oócitos maturados viáveis para outras biotecnologias *in vitro*.

TERMOS DE INDEXAÇÃO: Tecnologias Reprodutivas Assistidas, competência meiótica, maturação nuclear, mamíferos silvestres.

Introduction

Oocyte *in vitro* maturation (IVM) is the first step of the *in vitro* reproductive technologies that enables mature oocytes to be generated *ex vivo* (Rahman et al. 2008) and further destined to systems of *in vitro* embryo production (IVEP), as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). All these biotechniques are interesting tools for applying the reproductive management (Kumar et al. 2016) and biodiversity conservation (Moulavi et al. 2017).

The capability of embryonic development depends on successful IVM, since it stimulates the oocyte to achieve the meiotic competence required (Lee et al. 2014) through the nuclear and cytoplasmic events that promote the synthesis of RNA and proteins (Sirard et al. 2006). Therefore, the IVM quality is a determinant factor for the success of the IVEP (Zhang et al. 2015, Park et al. 2017). In this sense, the establishment of culture environment, as

oocyte incubation time, should be clarified, once all IVM events require a specific time that could vary according to the peculiarities of each species (Higaki et al. 2016).

The optimization of IVM conditions for collared peccaries is interesting under various concerns. Although this species is not threatened (IUCN, 2017), their phylogenetic proximity to endangered species as the *Catagonus wagneri* (Keuroghlian et al. 2014), as well as the commercial importance on their meat and leather (Nogueira-Filho et al. 2004), have been leading to the development of studies focused on their reproductive physiology (Maia et al. 2014, Miranda-Moura et al. 2016). Based on these studies, it was possible to know that specifically for females, they have symmetrical and oval ovaries with a relatively smooth surface, which can present irregular appearance due to corpus luteum or follicles (Garcia et al. 2009). Moreover, the ovaries are enveloped by an ovarian bursa (Guimarães et al. 2012), as also observed in swine (Męczyński 1974). Additionally, collared peccaries' estrous cycle lasts 21 days, with a follicular phase of 6 days and 15 days for the luteal phase (Maia et al. 2014). On the other hand, these individuals have a lower ovulation rate (2.3 follicles) when compared to swine (Sowls 1997) and usually produce 1.7–1.9 offspring per female after 141–151 days of gestation (Mayor et al. 2005).

Therefore, in view of the need for studies related to the reproductive biotechniques in collared peccaries, the initial point has been to observe reproductive relationship with close domestic phylogenetic species, as swine and others artiodactyls. Thus, similarities were observed for folliculogenesis (Guimarães et al. 2012), hormonal profiles (Ahuja-Aguirre et al. 2017) and follicular histological characteristics (Lima et al. 2013) between collared peccaries and swine and/or artiodactyla species, as buffalos.

In this context, IVM time used for buffalo oocytes is of 24 h (Kadoom et al. 2014), while maturation time of 48 h is used for porcine oocytes (Pereyra-Bonnet et al. 2008). Moreover, it had observed that a suboptimal culture environment for porcine oocytes may promote nuclear maturation without the structures reaching cytoplasmic maturation (Abeydeera 2002), confirming that the IVM time to be precise and defined (Sirard et al. 2006). Therefore, this is first study was carried out to investigate the relationship between the meiotic potential and the IVM times of collared peccary oocytes. This information will be the first about the IVM of oocytes derived from collared peccary, aiming to direct pathways to

obtain an embryo *in vitro* of this species. This step can serve as a basis for both enlightening studies of embryogenesis and physiological aspects of the animal. In addition, it may be the starting point for advanced biotechnology that can increase zootechnical potential and ways to ensure effective tools that can be used for conservation of phylogenetically near species.

Materials and Methods

Animal ethics and care

The experiment was conducted in accordance with the Committee for Ethics in Animal Use of Federal Rural University of Semi-Arid (CEUA/UFERSA; no. 23091.001072/2015-92) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio; no. 48633-2). The animals used in this research belong to the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding site (no. 1478912). The breeder contains 200 collared peccaries on average. Annually, a programmed slaughter is conducted for population control and the biological material is destined for several experiments.

Reagents and media

All reagents and media used were obtained from Sigma-Aldrich (St. Louis, MO, USA), but fetal bovine serum (FBS) was obtained from Gibco-BRL (Carlsbad, CA, USA). When necessary, the pH was adjusted to 7.2–7.4 for all media. Media were filtered using 0.22 μm system (Corning, NY, USA).

Oocyte collection, selection and in vitro maturation

Ovaries were obtained from nine adult female collared peccaries (age 4–10 years). Eighteen ovaries were removed from the ovarian bursa and transported to the laboratory in 0.9% (w/v) NaCl solution at 37°C for up to 1 h. Antral follicles (3–6 mm) were aspirated with 21 gauge needle attached to a 5.0 mL syringe. After the aspiration, for a greater oocyte recovery, ovaries were sliced with a scalpel blade in 100x20 mm plates. All follicular fluid was pooled in conical tubes for ten minutes. The sediment was diluted in the oocyte collection medium composed of TCM 199 culture medium (M2520) supplemented with 2.2 g/L bicarbonate (S5761), 0.2 mM sodium pyruvate (P45622), 1% antibiotic-antimycotic solution (A5955) and 10% FBS (12657-029). Thus, *cumulus*-oocyte complexes (COCs) were selected

according to the number of *cumulus* cells layers and homogeneity of the cytoplasm, being classified in four grades. Grade 1 (3-5 *cumulus* cells layers and homogeneous cytoplasm); Grade 2 (1-2 *cumulus* cells layers and homogeneous cytoplasm); Grade 3 (partially denuded cytoplasm and slightly heterogeneous); Grade 4 (denuded and heterogeneous cytoplasm), according to Kumar et al. (2016). Only grade 1 and 2 COCs were considered viable to be matured.

After three washes in oocyte collection medium, the COCs were incubated in 100 µL drops of maturation medium composed of TCM 199 with 2.2 g/L bicarbonate, 0.2 mM sodium pyruvate, 1% antibiotic-antimycotic solution and supplemented 10 µg/mL follicle stimulating hormone (Folltropin®-V, Bioniche, Canada), 100 µM cysteamine (M9768) and 10% FBS. All drops were covered with mineral oil and cultured at 38.5°C in humidified atmosphere of 5% CO_2 and divided in two groups, 24 or 48 h.

Assessment of in vitro maturation

After the IVM, COCs were evaluated by the analysis of *cumulus* cells expansion using a stereomicroscope at 20–40X magnification. Thus, oocytes with *cumulus* cells were considered matured (Appeltant et al. 2015). Subsequently, *cumulus* cells were removed by pipetting the oocytes in oocyte collection medium and denuded structures were verified for the presence of the first polar body under stereomicroscope at 20–40X magnification. Thus, oocytes with first polar body were considered matured (Pereyra-Bonnet et al. 2008).

The nuclear status of oocytes was determined by marking it with Hoechst 33342 (B2261). For this, denuded oocytes were fixed in paraformaldehyde buffered in 4% phosphate buffered solution (PBS) for 30 min and then washed in PBS with 0.4% bovine serum albumin (BSA). Afterward, Hoechst 33342 (10 µg/mL) labeling was performed for 15 min and exposed under UV light in fluorescence microscope. Finally, oocytes presenting nucleus in metaphase II (metaphase plate, MII) were considered matured and those in other nuclear phases (prophase I, metaphase I, anaphase I and telophase I) were considered immature (Appeltant et al. 2015).

Data analysis

Data were obtained from four sessions, with two a three females per session, totaling in nine experimental animals. For statistical analysis, the Graphpad Instat 3.06 software (GraphPad Software Inc., La Jolla, CA, USA) was used. Mean numbers in percentage ± standard error of the matured oocytes in two different times (24 *vs*. 48 h) were compared using Fisher exact test ($P < 0.05$) for expansion of *cumulus* cells, presence of the first polar body and nuclear status.

Results

Under gross evaluation, the bursa surrounded all peccaries' ovaries, which were symmetric, presenting an oval shape. Moreover, they had a uniform appearance on most of its surface (Fig. 1 A); nevertheless, some elevations were observed derived from large follicles or the presence of corpus luteum (Fig. 1 B).

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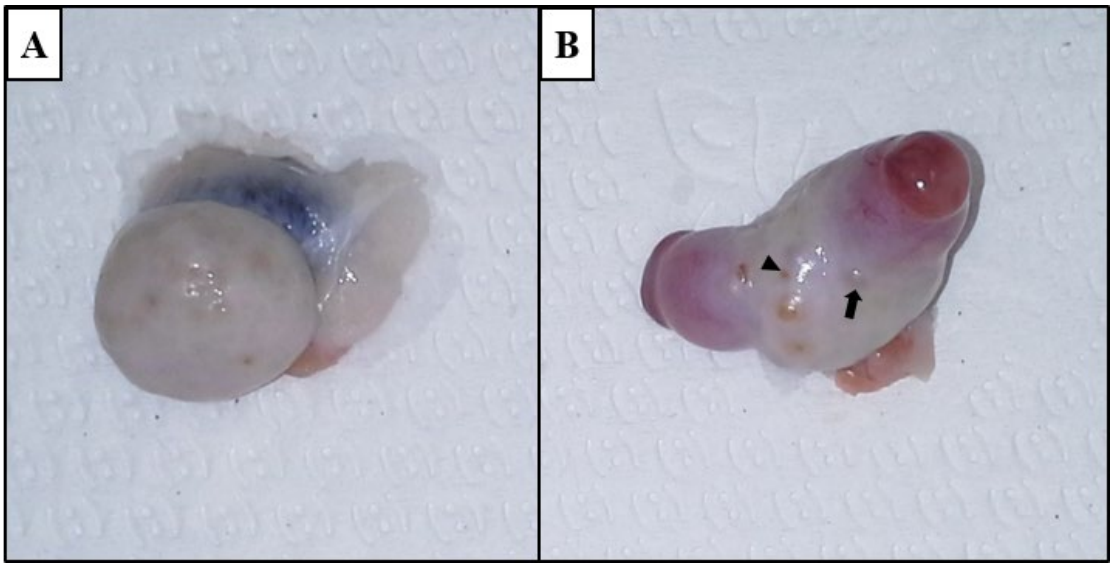

Fig. 1. Macroscopic aspects of collared peccary ovaries. **(A)** Uniform appearance with small follicles. **(B)** Presence of large follicles and corpus luteum. The arrowhead indicates a small follicle and arrow shows a large follicle.

Afterward all sessions, a total of 18 ovaries resulted in 59 immature oocytes from follicles aspirated and sliced follicles with an estimated recovery rate of 83.1% (approximately, 59/71; oocytes recovered/follicles aspirated) and 3.3 oocytes per ovary. Of these, 42 structures (71.2%, 42 structures viable/59 recovered structures) were classified as viable according to the number of *cumulus* cells layers and homogeneity of the cytoplasm, resulting in 2.3 viable oocytes per ovary, (Fig. 2 A).

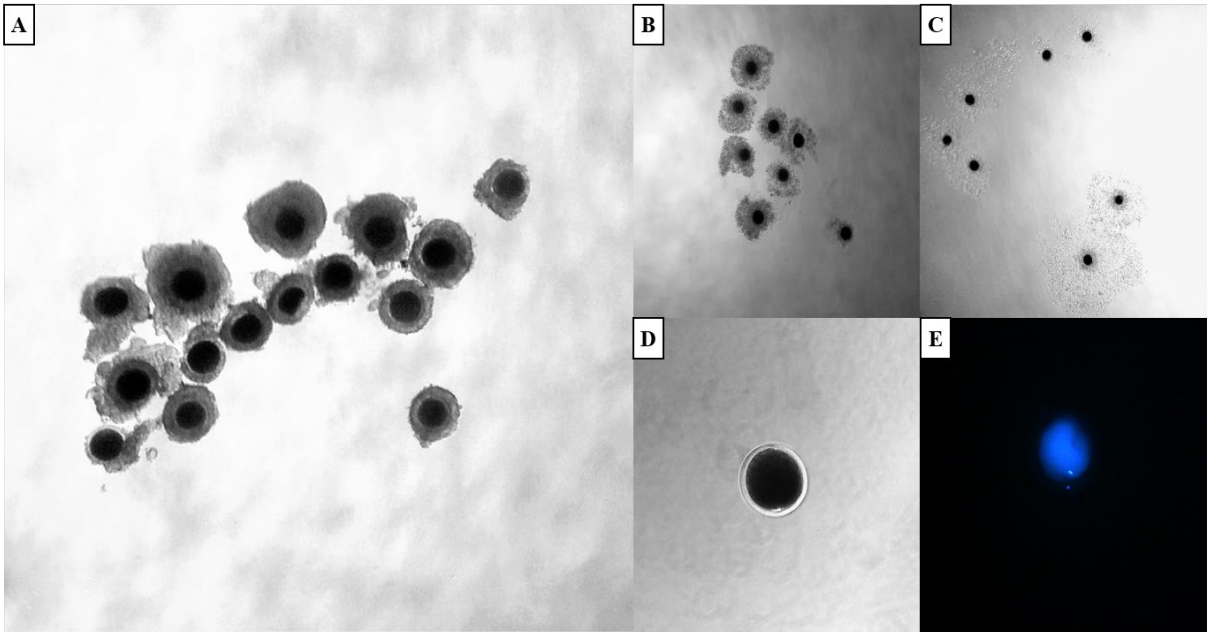

Fig. 2. I*n vitro* maturation of oocytes of collared peccary. **(A)** Immature oocytes before IVM (100X); **(B)** *Cumulus* cell expansion after 24 h IVM (100X); **(C)** *Cumulus* cell expansion after 48 h IVM (100X); **(D)** Oocyte with extrusion of fist polar body (400X); **(E)** Oocyte in MII stage (400X).

After the IVM, the time of 48 h was more adequate than 24 h for the IVM in all evaluated parameters, as *cumulus* cells expansion (P = 0.0002), presence of the first polar body (P = 0.0148) and nuclear status in second metaphase (P = 0.0003) (Table 1). Moreover, a 100% *cumulus* cells expansion was observed for oocytes cultured for 48 h (Fig. 2 C) when compared to 24 h (Fig. 2 B). Additionally, oocytes with fist polar body (Fig. 2 D) and MII presence (Fig. 2 E) were evidenced in more of 50% and 19% of the oocytes, respectively.

Table 1. *In vitro* maturation of collared peccary oocytes after different incubation times.

Evaluation of *in vitro* maturation	**Incubation times (in hours)**	
	24	**48**
Expansion of *cumulus* cells	38.1 ± 1.4 (8/21)[b]	100.0 ± 0.0 (21/21)[a]
Presence of the first polar body	52.4 ± 2.2 (11/21)[b]	90.5 ± 2.0 (19/21)[a]
Nuclear status in second metaphase	19.0 ± 1.4 (4/21)[b]	76.2 ± 1.3 (16/21)[a]

Mean in % ± standard error (*n*). [a,b] in same row differ (P < 0.05).

Discussion

The results indicated that the most appropriate incubation period for collared peccaries' oocytes achieve meiotic competence was 48 h. This time is similar to that observed for porcine oocytes (Pereyra-Bonnet et al. 2008). In this sense, we can infer that similar follicular characteristics described between collared peccaries and swine (Lima et al. 2013) justify this relationship, since the *in vitro* conditions for maturation should mimic the *in vivo* conditions that occur in the females.

Moreover, other peculiar feature of collared peccary oocytes similar to the porcine oocytes is the presence of cytoplasmic lipid droplets (Lima et al. 2013). This aspect is crucial for the establishment of the appropriate requirements for IVM, evidencing that further IVM protocols should be adapted from porcine to peccaries' oocytes. Nevertheless, a factor that still needs to be further investigated by the similarity that collared peccary oocytes had in relation to porcine oocytes is that the IVM in swine oocytes have their altered medium to adapt to the variable requirements of IVM (Grupen 2014) and this step may improve the quality of matured oocytes derived from collared peccaries. Thus, the tracks of such similarities between peccaries and porcine oocytes indicate a starting point, but not the direct application of protocols without further experimentation, considering that differences occur between these species for follicular morphology (Lima et al. 2013).

On the other hand, the follicular development in collared peccaries was also similar to that found for other artiodactyl species regarding the stages of folliculogenesis, i.e. recruitment, selection and dominance (Guimarães et al. 2012) when compared to buffalos (Manik et al. 2002) and camelids (Bausiouni 2007). Nevertheless, despite this similarity, the 24 h IVM time that is frequently used for buffaloes (Kadoom et al. 2014) was not suitable for oocyte maturation in the peccaries. The IVM time to guarantee the formation of the metaphase plate is essential to ensure that the oocytes possess the ability to develop quality embryos (Grupen 2014). In this sense, the 24 h of IVM for collared peccary oocytes was not long enough for the organization of the metaphase plate (19.0%), while the majority of oocytes were able to reach metaphase II in 48 h of IVM (76.2%). As developmental competence is also reached in cytoplasmic maturation, which occurs after meiotic maturation and refers to the ability of the oocyte to be fertilized and to block polyspermy, this type of maturation can be visualized by migration of the cortical granules to the oocyte periphery and

by the expansion of *cumulus* cells (Milakovic et al. 2015). The analysis of *cumulus* cells expansion at 24 and 48 h showed different degrees of expansion (38.1% *vs.* 100.0%, respectively); these may be related to cytoplasmic maturation, being an indicator of successful maturation (Abeydeera 2002). Therefore, 24 h of IVM is not sufficient for IVM of collared peccary oocytes (Fig. 2B).

Several important factors for the IVM success are dependent of the incubation time, which can influence the final quality of the embryonic development (Miyoshi et al. 2002). The signaling pathway of 3',5' cyclic adenosine monophosphate (cAMP) is highly time-dependent and restricted to time mechanisms, which is what triggers germinal vesicle breakdown, so nuclear maturation depends on cAMP so the oocyte can reach metaphase II (Sirard et al. 2006). Likewise, another time dependent factor is the secretion of steroid hormones by COCs derived small and large follicles during IVM. Topfer et al. (2016) observed that oocytes derived from small follicles have a lesser ability to support estradiol synthesis in the first half of IVM (28 h) and to suppress progesterone synthesis at the end of IVM (48 h) compared with oocytes from large follicles. Thus, the steroid hormone secretion by COCs is time-dependent and therefore, the meiotic maturation time should to be precise and defined (Sirard et al. 2006; Topfer et al. 2016).

Moreover, the *cumulus* cells play a bidirectional role for oocyte development. One of the factors that contribute to this role is their mitochondria and mitochondrial DNA, besides contributing to the passage of nutrients and growth factors to the oocyte (Pawlak et al. 2016). Additionally, it is known that the *cumulus* cells play a regulatory role for cAMP, showing great relevance for oocyte maturation (Grupen 2014). Therefore, the clear expansion of *cumulus* cells with 48 h (Fig. 2C) in this study ensured the optimal performance of the cells during IVM. Further adjustments of cAMP levels during maturation may improve fertilization capacity and oocyte development (Appeltant et al. 2015).

Finally, other factors, as follicles size and technique employed for oocyte collection, may influence the IVM conditions (Sun et al. 2001). In this study, 3–6 mm follicles aspirated and matured for 48 h allowed the obtaining of oocytes to reach meiotic competence. Moreover, the recovery rate obtained can be due to the use of two methods of oocytes collection, first the follicular aspiration and then the slicing technique. The slicing technique

allows the increase oocyte recovery, especially when there is limited availability of ovaries (Ferraz et al. 2016). Moreover, viability rate of 71.2% can be attributed to the good development of immature oocytes in the follicular environment (Grupen 2014) and the efficiency of the methods for oocyte recovery.

In conclusion, 48 h IVM for collared peccary is the most suitable incubation period for oocytes reaching the meiotic competence when compared to 24 h time. This was the first study with *in vitro* maturation, in an attempt to improve a protocol of IVEP for this species. Although this time has been defined in the present work, further details for the improvement of IVM, as medium composition, should be considered not only to achieve meiotic competence but also to guarantee appropriate embryonic development.

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CAPÍTULO 7 – PRODUCTION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) PARTHENOGENIC EMBRYOS FOLLOWING DIFFERENT OOCYTE CHEMICAL ACTIVATION AND *IN VITRO* MATURATION CONDITIONS

(Artigo experimental)

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Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions

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Abstract

To optimize the protocols for assisted reproductive techniques (ARTs) in collared peccary (*Pecari tajacu* Linnaeus, 1758), we evaluated various conditions for oocyte *in vitro* maturation (IVM) and chemical activation. Initially, we assessed the IVM rates, *cumulus*-oocyte complex (COC) quality, and oocyte morphometry in the absence or presence of epidermal growth factor (EGF). There was no difference between the COCs matured in absence or presence of EGF for the expansion of *cumulus* cells (97.6% ± 1.2 *vs.* 100% ± 0.0), presence of first polar body (65.9% ± 1.2 *vs.* 70.5% ± 1.8), nuclear status in second metaphase (62.5% ± 11.6 *vs.* 68.4% ± 4.9), cytoplasmic maturation (100.0% ± 0.7 *vs.* 75.0% ± 0.7), reactive oxygen species levels (0.5 ± 0.2 *vs.* 0.3 ± 0.1), and mitochondrial membrane potential (1.1 ± 0.2 *vs.* 1.1 ± 0.1). However, the zona pellucida thickness of matured COCs was reduced in the presence of EGF. Thus, the EGF group was used for further experiments. The oocytes were artificially activated with ionomycin and four secondary activator combinations [6-dimethylaminopurine (6D), 6D and cytochalasin B (6D + CB), cycloheximide (CHX), and CHX and CB (CHX + CB)]. The effect of immature COCs based on *cumulus* cell layers and cytoplasm homogeneity (GI and GII or GIII COCs) on embryonic development and quality was evaluated. There was no difference in the cleavage rates among the groups of secondary activators. The cleavage rates of embryos derived from GI/GII and GIII COCs were greater than 72.2% and 25.0%, respectively. Moreover, treatment with CHX showed a reduction in the cleavage rate of embryos derived from GIII COCs when compared to the cleavage rate of embryos derived from GI/GII COCs ($P < 0.05$). Nevertheless, higher rates of blastocyst/total GI and GII COCs were observed in the 6D group (27.6% ± 0.3) compared to CHX group (6.9% ± 0.3). Additionally, only 6D treatment resulted in the production of embryos derived from GIII COCs (25.0% ± 0.2). The percentage of the ICM/total cell ratio was also greater in blastocysts derived from 6D (42.5% ± 19.0), 6D + CB (37.9% ± 21.9), and CHX + CB (43.8% ± 19.6) groups when compared to CHX (3.6% ± 0.1) group. Thus, the combination of ionomycin and 6D could produce collared peccary embryos by activation of both GI/GII COCs and GIII COCs. These optimized IVM conditions using EGF and chemical activation using ionomycin and 6D in collared peccaries form the first steps for establishing ARTs to conserve this species.

Keywords: Wildlife; Somatic cell nuclear transfer; Artificial activation; Embryo development; Epidermal growth factor.

1. Introduction

In the last decade, a rapid decline in the population of various mammalian species was reported in the South American countries [1]. The collared peccary (*Pecari tajacu* Linnaeus, 1758), a wild pig, is found only in the Americas and is classified as the Least Concern species [2]. However, the species is already extinct in eastern and southern Argentina and is declining in some biomes, such as Caatinga and the Atlantic forest [1]. Generally, this species plays an important role in the ecosystem as a seed dispersing agent. Thus, the maintenance of these animals in captivity is essential for their sustainable use and conservation [3].

The collared peccary populations can be conserved through assisted reproductive techniques (ARTs), such as cloning by somatic cell nuclear transfer (SCNT) and *in vitro* fertilization (IVF). Cloning by SCNT enables the conservation of species as it can be used for increasing the species population [4], basic research [5], and obtaining induced pluripotent cells [6]. Therefore, establishment of all the stages involved in SCNT can aid in the optimal use of this technology for species conservation [5].

There are various steps involved in cloning via SCNT. *In vitro* maturation (IVM) and artificial oocyte activation are the fundamental steps for the development of SCNT technique to obtain cytoplasmic donor cells (or cytoplast) and evaluate their developmental competence. Recently, we had demonstrated that the collared peccary *cumulus*-oocyte complexes (COCs) require maturation times of greater than 24 h for complete meiotic development, which resulted in an IVM rate of 76.2% [7]. However, developmental competence was not evaluated in this study. It is important to understand the development of *in vitro*-matured oocytes to blastocysts.

Moreover, the optimization of IVM medium composition can potentially result in higher IVM rates. In some mammals (swine [8]; sheep [9]; canine [10]), supplementation of IVM medium with 10 ng/mL of epidermal growth factor (EGF) increases the meiotic development and subsequent embryonic development. In pigs, EGF along with gonadotropins enhances oocyte nuclear and cytoplasmic maturation [11]. Although this domestic species is phylogenetically close to collared peccary [12], there are differences in the reproductive features between these species, such as ovulation rate [13, 14] and litter size [14]. Although swine and collared peccary are phylogenetically close, they exhibit substantial phylogenetic

distance. Thus, direct extrapolations between the collared peccary and swine were not possible. Therefore, we aimed to specifically evaluate the effects of EGF on the collared peccary oocyte development. Additionally, different species may exhibit differential responses, as observed in the collared peccary semen cryopreservation. Treatment with 20% low-density lipoproteins (LDL) improved post-thaw sperm motility and plasma membrane integrity of collared peccaries [15], whereas treatment with LDL at concentrations above 10% did not result in a beneficial effect on porcine semen cryopreservation [16].

To the best of our knowledge, we are not aware of any published reports on the establishment of oocyte artificial activation protocols in collared peccaries. Artificial activation of oocytes is also a critical step of SCNT [5] and the activation procedures vary among species (bovine and equine [17]; goat [18]; swine [19]). This indicates that the response to these artificial activation protocols also may vary depending on the species. Previously, Campos Junior et al. [20] artificially activated the collared peccary oocytes using ionomycin and 6-dimethylaminopurine (6D), which resulted in 10% four-cell embryo stage. Increasing the number of oocytes and optimizing the artificial activation protocols may potentially promote higher rates of embryonic development.

An efficient artificial activation protocol promotes *in vitro* embryonic development competencies by decreasing the level of the maturation promoter factor (MPF) through Ca^{2+} oscillation [18]. Thus, different chemical activation methods may be employed using ionomycin as the primary activator, which allows Ca^{2+} transport and induces calcium influx by activation of endogenous entry pathways [21]. Moreover, primary activation can be used in combination with secondary activators, such as protein phosphorylation inhibitors (6D) that inhibit the protein kinase activity and promote mitosis, and protein synthesis inhibitors (cycloheximide or CHX) that inhibit the production of cyclin B, which is a regulatory component of MPF [22]. Additionally, cytochalasin B (CB) has been used to prevent the fragmentation of embryos [23] and increase the rate of diploid embryos as well as to effectively inhibit the secondary polar body extrusion [24].

Generally, COCs used for IVM and chemical activation are selected based on *cumulus* cell layers and cytoplasm homogeneity (Grade I to IV). Only GI and GII COCs are used for IVM, which are considered more appropriate according to morphological classification described

above. However, due to the reduced availability of oocytes in wild mammals, evaluation of development in GIII COCs is important to increase the quantities of oocytes in the subsequent stages of SCNT. In some mammals, similar molecular patterns were observed between GI/GII and GIII COCs [25, 26]. Thus, this study aimed to evaluate various conditions for IVM and chemical activation of collared peccary oocytes.

2. Materials and methods

All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (Opinion Nº 23091.001072/2015-92) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, N° 48633-2). Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Cumulus-oocyte complex (COC) collection

Twenty collared peccaries (n = 20) aged 25.1 ± 2.5 months housed at the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W) and registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, N° 1478912) were used in this study. The animals were stimulated with 600 IU PG600® (400 IU eCG and 200 IU hCG, MSD Saúde Animal, São Paulo, SP, Brazil) before COC collection, following the methods of Peixoto et al. [27] with modifications. At day 4 post-hormone administration, the animals were euthanized and the ovaries were excised. The ovaries were transported to the laboratory within 1 h and maintained in saline solution (NaCl, 0.9%) supplemented with 0.05 mg/mL penicillin at 35–37°C.

At the laboratory, all visible follicles were classified as small (< 0.3 cm), medium (0.3–0.5 cm), or large (> 0.5 cm) [28]. The follicles with 0.2–0.6 cm diameter were aspirated for COC collection using a 21 G needle attached to a 5.0 mL syringe containing COC collection medium (CCM; TCM199 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum (FBS, serum (FBS, Gibco-BRL), and 1% antibiotic-antimycotic solution).

2.2. COC classification and IVM

The COCs were observed under a stereomicroscope and classified into four grades following the classification system proposed by Kumar et al. [29], which was based on the

number of *cumulus* cell layers and homogeneity of the cytoplasm: Grade I (3–5 *cumulus* cells layers and homogeneous cytoplasm); Grade II (1–2 *cumulus* cells layers and homogeneous cytoplasm); Grade III (partially denuded and slightly heterogeneous cytoplasm); Grade IV (denuded and heterogeneous cytoplasm). Only Grade I/II and Grade III COCs were used for IVM in separate drops, according to the experimental design.

The COCs were divided into drops (15–30 COCs per 100 µL) and the drops were covered with mineral oil. The COCs were incubated for 44 h at 38.5°C and 5% CO_2 in a humidified atmosphere. The IVM medium comprised CCM supplemented with 20 µg/mL FSH/LH (Pluset®, Hertape Calier, Juatuba, MG, Brazil), 100 µM cysteamine, and an absence or presence of 10 ng/mL EGF.

2.3. Evaluation of cumulus cells

After IVM, *cumulus* cell expansion was evaluated using a stereomicroscope. The COCs exhibiting *cumulus* cell expansion were considered mature. The *cumulus* cell expansion was scored as follows: score 4, total expansion; score 3, partial expansion; score 2, slight expansion; score 1, minimum expansion; score 0, no expansion [30].

The *cumulus* cells were removed by successive pipetting. The viability of *cumulus* cells was evaluated by staining the cells with trypan blue (0.2%) for 2 min. The viable cells were unstained, whereas the non-viable cells were stained blue. All cells were counted in the 4 outer quadrants of the Neubauer chamber [31].

2.4. Assessment of nuclear maturation

The denuded oocytes were observed under a stereomicroscope to assess the presence of first polar body (1PB). The oocytes exhibiting 1PB were considered mature. To assess the nuclear stage, denuded oocytes were fixed in 4% paraformaldehyde prepared in phosphate buffer saline (PBS) for 30 min. Next, the oocytes were washed with PBS containing 0.4% bovine serum albumin (BSA) fraction V. The cells were then stained with Hoechst 33342 (10 µg/mL) for 15 min and observed under a fluorescent microscope. The oocytes with nucleus at metaphase II (metaphase plate, MII) stage and 1PB were considered mature, while those in other nuclear phases were considered immature.

2.5. Quantification of intracellular ROS levels by a dichlorofluorescein assay

For evaluation of oocyte quality after IVM, reactive oxygen species (ROS) levels in the oocytes were determined using the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Carlsbad, CA, USA), according to Santos et al. [31]. Briefly, denuded oocytes were washed twice with PBS and incubated in 500 µL of 5 µM H_2DCFDA at 38.5°C and 5% CO_2 for 30 min. The stained oocytes were washed twice with PBS and imaged using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The fluorescence signal intensity (pixels) was quantified using the ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the fluorescent intensity values of the treated oocytes. Immature oocytes were assessed as the calibrator. The relative expression levels were calculated by dividing the measured value of each treatment micrograph with the mean value of the calibrator.

2.6. Assessment of mitochondrial membrane potential (ΔΨm) and cytoplasmic maturation

The ΔΨm was measured by incubating the oocytes with MitoTracker Red® (CMXRos, Invitrogen) at 500 nM for 30 min [31]. The staining procedures and evaluation of images were similar to those used for the quantification of ROS levels. Additionally, the cytoplasmic maturation of the labeled oocytes was evaluated based on the mitochondrial distribution as follows: peripheral (immature oocytes), dispersed (mature oocytes), and transitional (between peripheral and dispersed distributions; partially mature oocytes).

2.7. Morphometric evaluation of oocytes after maturation

The denuded oocytes were observed under an inverted microscope for assessing the oocyte morphometric parameters. The images were captured using an inverted microscope (Nikon TS100, Tokyo, Japan) equipped with a camera and image processing software. The morphometric parameters of the captured images were analyzed in the ImageJ software using the scale bar as an arbitrary scale for pixel analysis. The measured morphometric parameters included outer oocyte diameter (ZPO), zona pellucida thickness (ZPT), inner oocyte diameter (ZPI), and ooplasm diameter (OD), as described by Saadeldin et al. [32]. Thus, perivitelline space (PVS) diameter, inner oocyte area (IA), ooplasm area (OA), and PVS area were mathematically calculated based on previous parameters using the following formulae: IA =

3.14 x $(ZPI/2)^2$; OA = 3.14 x $(OD/2)^2$, PVS diameter = ZPI – OD and PVS area = IA – OA. Diameters were measured in μm and areas in μm^2.

2.8. Artificial activation and in vitro development (IVD) of embryos

After IVM, the *cumulus* cells were removed by pipetting the oocytes in the presence of hyaluronidase for up to 2 min. The oocytes with 1PB were activated using 5 μM ionomycin (I24222; Gibco-BRL) prepared in CCM for 4 min at 37°C. Next, the oocytes were washed and incubated with drops of secondary activators at 38.5°C and 5% CO_2 for 3 h, according to the experimental design. Subsequently, the oocytes were washed and incubated in 50 μL drops of IVD medium covered with mineral oil. Synthetic oviductal fluid (SOF) supplemented with 0.2 mM sodium pyruvate, 0.2 mM L-glutamine, 0.34 mM sodium citrate, 2.8 mM myo-inositol, 2% essential amino acid solution, 1% non-essential amino acid solution, 1% antibiotic-antimycotic solution, 5.0 mg/mL BSA, and 2.5% FBS was employed as the IVD medium. The day of activation was considered as D0 and the cells were cultured until D7. On D3, 50% of the culture medium was replaced with fresh medium.

2.9. Evaluation of blastocysts and differential staining of inner cell mass and trophectoderm cells

After seven days of IVD, the blastocyst cells were fixed in ethanol and labeled with Hoechst 33342 (10 μg/mL) and propidium iodide (PI) (10 μg/mL) for 15 min at 38.5°C. Next, the cells were washed twice with PBS and placed on glass slides in glycerol droplets. A coverslip was placed over the droplet and the images were captured under UV light at 330–385 nm using a fluorescent microscope. The embryos were visualized individually and the nuclei were counted in the captured fluorescent images. The nuclei of the inner cellular mass (ICM) appeared blue as they were labeled only with Hoechst 33342, while those of the trophectoderm (TE) appeared red or pink as they were labeled with both PI and Hoechst 33342. The numbers of ICM, TE, and total cells were quantified using the ImageJ software [33].

2.10. Experimental design

To evaluate various conditions for IVM and chemical activation of collared peccary oocytes, two experiments were performed. Initially, we assessed the IVM rates, COC quality, and oocyte morphometric parameters after IVM in the absence or presence of 10 ng/mL of

EGF (experiment 1). Furthermore, we activated the oocytes with ionomycin and four secondary activator combinations (6D, 6D and CB, CHX, and CHX and CB). The effect of immature COC grades (GI and GII or GIII COCs) on the embryonic development and quality was evaluated (experiment 2). For the second experiment, only the oocytes grown under optimal IVM conditions were used.

2.10.1. Experiment 1: effects of EGF supplementation on IVM of collared peccary COCs

The COCs were washed with the medium and four replicates (two females per replicate) were used to assess the effect of EGF on IVM. GI and GII COCs derived from eight females were matured in the absence (EGF negative group) or presence of 10 ng/mL of EGF (EGF positive group). Meiotic competence was measured by visualization of the 1PB and nuclear stage (MII). Moreover, oocytes were evaluated for cytoplasmic maturation based on mitochondrial distribution using MitoTracker Red®. Additionally, the *cumulus* cells were assessed and the ROS levels and ΔΨm were quantified. The matured oocytes were compared by morphometric evaluation according to the ZPO, ZPT, OD, PVS area, and PVS diameter.

2.10.2. Experiment 2: effects of secondary activators and COC *quality on the development of collared peccary parthenogenetic embryos*

After 44 h of IVM, matured GI/GII and GIII COCs derived from twelve females distributed in three replicates were artificially activated and cultured for seven days. The COCs were artificially activated with ionomycin and four secondary activator combinations: 1.9 mM 6D (6D group), 1.9 mM 6D and 7.5 µg/mL CB (6D + CB group), 10 µg/mL CHX (CHX group), and 10 µg/mL CHX and 7.5 µg/mL CB (CHX + CB group). On day 3 of IVD, the total cleavage rate and number of embryos containing 2 cells, 3–7 cells, or 8 or more cells were quantified. On day 7 of IVD, the total blastocyst formation rate and the developmental phase [initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB), and blastocyst hatching/hatched (hB)] were quantified. To evaluate the quality, the total numbers of cells were counted by differentiating the ICM and TE.

2.11. Statistical analysis

All data are expressed as the mean ± standard error and were analyzed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). The normality of the data was verified using the Shapiro-Wilk test, while the homoscedasticity was verified with Levene's test. The

morphometric oocyte data were examined by variance analysis (ANOVA) and Tukey's test. The levels of ROS and ΔΨm, as well as the number of blastomers were transformed with arcsine and analyzed by ANOVA followed by Tukey's test. All other data were compared with Kruskal-Wallis test and Dunn (multiple comparisons) test. The difference was considered statistically significant when the P value was less than 0.05.

3. Results

In total, 630 visible follicles (31.5 follicles per female on average) were obtained upon stimulation of 40 ovaries (Fig. 1A) derived from 20 collared peccaries with PG600®. The number of medium follicles (385 ± 14.1) was higher than that of small follicles (113 ± 1.9, P = 0.03) and large follicles (132 ± 5.4, P = 0.04). The number of small and large follicles was similar (P > 0.05).

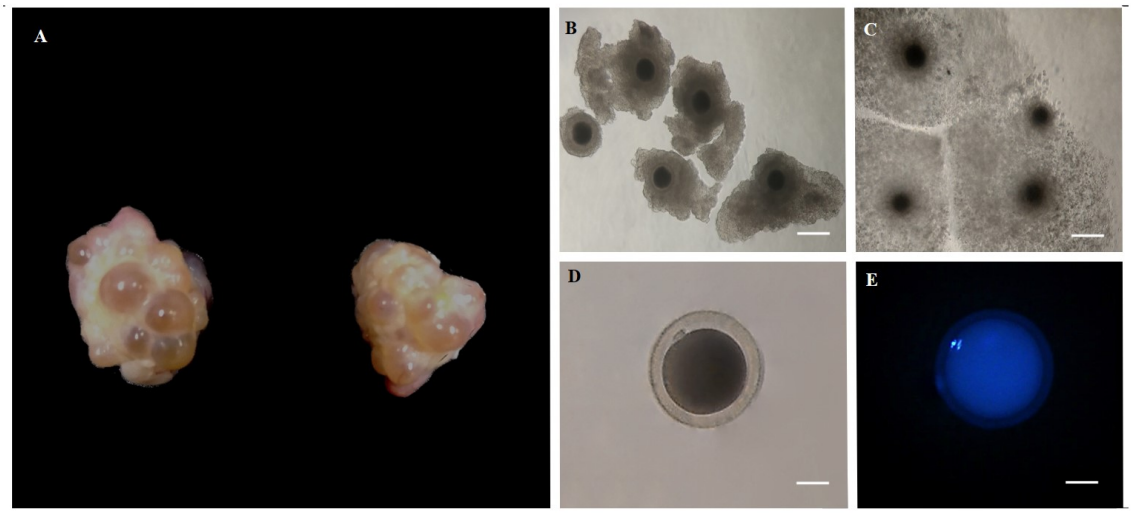

Fig. 1. Representative images of **(A)** ovaries of collared peccary obtained after hormonal induction with PG 600®; **(B)** *Cumulus*-oocyte complex recovered with homogeneous cytoplasm and more one layer of *cumulus* cells; **(C)** COCs matured after 44 h IVM; **(D)** Denuded oocyte with presence of 1PB; **(E)** Visualization of the metaphasic plate (MII).

Moreover, all ovaries were used to acquire 333 ± 10.7 immature oocytes (Fig. 1B) resulting in a recovery rate of 52.9% ± 4.2 (333/630) and 16.2 ± 2.8 oocytes per female. Of these, 253 ± 8.5 (76.0%) structures were classified as GI and GII (12.1 ± 2.2 COCs per female), 37.0 ± 3.3 (11.1%) structures as GIII (3.1 ± 0.8 COCs per female), and 43.0 ± 1.8 (12.9%) structures as GIV (2.8 ± 1.0 COCs per female), according to the cytoplasm homogeneity and the number of *cumulus* cell layers.

Moreover, 333 ± 10.7 immature oocytes (Fig. 1B) were obtained from all ovaries with a recovery rate of 52.9% ± 4.2 (333/630) and 16.2 ± 2.8 oocytes per female. Of these, 253 ± 8.5 (76.0%) oocytes were classified as GI and GII (12.1 ± 2.2 COCs per female), 37.0 ± 3.3 (11.1%) as GIII (3.1 ± 0.8 COCs per female), and 43.0 ± 1.8 (12.9%) as GIV (2.8 ± 1.0 COCs per female) based on the cytoplasm homogeneity and the number of *cumulus* cell layers.

3.1. Experiment 1: effects of EGF supplementation on IVM of collared peccary COCs

The effect of EGF on the IVM of collared peccary COCs is shown in Table 1 and 2. Initially, there was no difference in the IVM rates of COCs exhibiting *cumulus* cell expansion (Fig. 1C) between EGF positive and negative groups. All COCs exhibited *cumulus* cell expansion scores of 3 and 4 and the *cumulus* cell viability was greater than 76.0% for both EGF positive and negative groups. Moreover, there was no difference in the IVM rates of COCs selected on the basis of 1PB presence (Fig. 1D), MII assessments (Fig. 1E), and cytoplasmic maturation evaluated based on mitochondrial distribution. Additionally, the intracellular ROS levels (Fig. 2A, C, and D) and $\Delta\Psi m$ in the matured COCs were similar between the EGF positive and EGF negative groups.

There was no difference for most of the evaluated oocyte morphometric assessment parameters. However, the thickness of the zona pellucida in the matured oocytes reduced in the presence of EGF (Table 2). Thus, the IVM medium supplemented with EGF was used for experiment 2.

Table 1. Effects of EGF supplementation on *in vitro* maturation of collared peccary *cumulus*-oocyte complex (COC).

Group	Evaluation of *cumulus* cells				Nuclear maturation		Cytoplasmic maturation mitochondrial distribution		
	Expansion and grade of expansion, %			Viability, %	1PB, %	MII, %	Peripheral, %	Transition, %	Dispersed, %
	Total	Grade 3	Grade 4						
With EGF	100.0 ± 0.0 (44/44)	63.6 ± 3.0 (28/44)	29.5 ± 0.3 (13/44)	76.0 ± 3.1 (784/1031)	70.5 ± 1.8 (31/44)	68.4 ± 4.9 (13/19)	0.0 ± 0.0 (0/9)	25.0 ± 0.0 (2/8)	75.0 ± 0.7 (6/8)
Without EGF	97.6 ± 1.2 (40/41)	70.7 ± 2.5 (29/41)	29.3 ± 2.1 (12/41)	79.4 ± 1.4 (765/963)	65.9 ± 1.2 (27/41)	62.5 ± 11.6 (10/16)	0.0 ± 0.0 (0/9)	0.0 ± 0.0 (0/9)	100.0 ± 0.0 (9/9)

No differences were observed among groups (P > 0.05). 1PB, first polar body, MII, metaphase II.

Table 2. Morphometric assessment (in μm and μm^2) in of matured COCs derived from collared peccaries (mean ± standard error).

Groups	Outer oocyte diameter (ZPO)	Zona pellucida thickness (ZPT)	Ooplasm diameter (OD)	Perivitelline space (PVS) area	Perivitelline space (PVS) diameter
With EGF	373.9 ± 4.4 (37)	33.3 ± 1.2 (37)[a]	284.3 ± 4.2 (37)	7312.6 ± 823.1 (35)	16.2 ± 2.1 (35)
Without EGF	382.2 ± 5.3 (34)	38.6 ± 1.5 (34)[b]	285.6 ± 3.0 (34)	6284.0 ± 390.9 (27)	13.8 ± 0.8 (27)

[a,b]: Values with different superscript letters within columns are significantly different ($P < 0.05$).

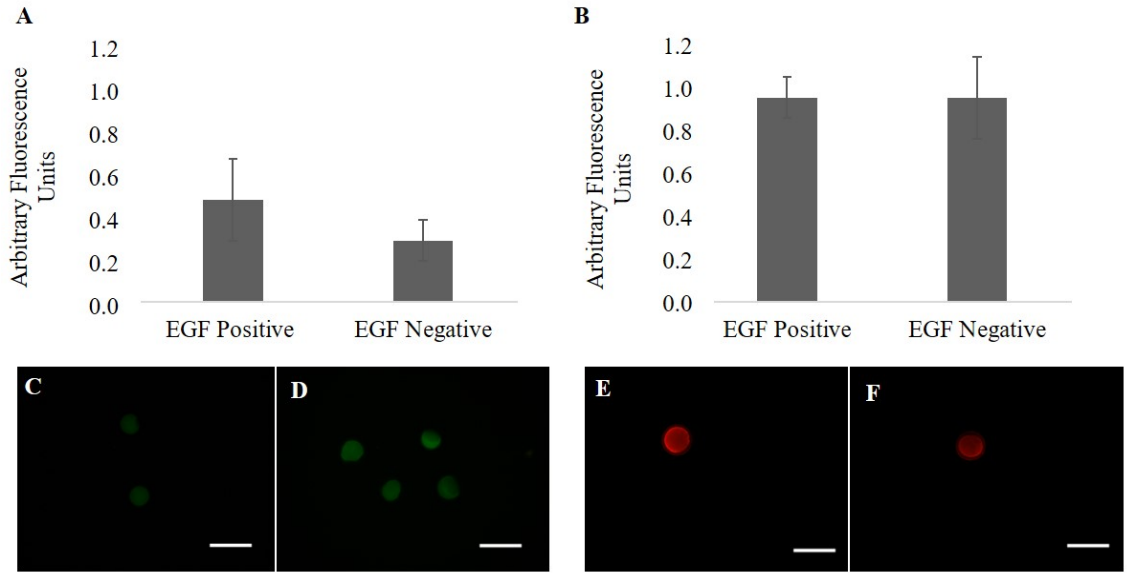

Fig. 2. Oocytes of collared peccaries after maturation evaluated as intracellular concentration of reactive oxygen species (ROS) levels and mitochondrial membrane potential (ΔΨm). **(A)** Measurement of fluorescence of ROS levels in matured oocytes in the presence of EGF (EGF positive group) or in the absence (EGF negative group) ($P > 0.05$). **(B)** Quantification of mitochondrial membrane potential in oocytes matured in the presence of EGF (EGF positive group) or in the absence (EGF negative group) ($P > 0.05$). **(C)** Oocytes labelled with fluorescent probe H_2DCFDA for ROS quantification with EGF (EGF positive group) and **(D)** without EGF (EGF negative group). **(E)** Oocytes labelled with fluorescent probe MitoTracker Red® for ΔΨm with EGF (EGF positive group) and **(F)** without EGF (EGF negative group).

3.2. Experiment 2: effects of secondary activators and COC *quality on the development of collared peccary parthenogenetic embryos*

Assessment of embryonic development is described in Table 3 and 4. The cleavage rate (Fig. 3A–C) after 3 days of IVD was similar in the evaluated groups. The cleavage rates were greater than 72.2% and 25.0% for the embryos derived from GI/GII and GIII COCs, respectively. Moreover, there was no reduction in the cleavage rates of embryos derived from GI/GII and GIII COCs among the 6D and CHX + CB groups (Table 3).

Table 3. Embryonic development of collared peccary GI/GII and GIII *cumulus*-oocyte complex activated with ionomycin in combination with different secondary activators.

Groups	No. COCs		Cleavage, %		Blastocyst/Oocyte, %		Blastocyst /Cleaved, %	
	GI/GII	GIII	GI/GII COCs	GIII COCs	GI/GII COCs	GIII COCs	GI/GII COCs	GIII COCs
6D	34	4	82.9 ± 2.4 (29)	75.0 ± 0.4 (3)	23.5 ± 0.3 (8)^{a}	25.0 ± 0.2 (1)^{a}	27.6 ± 0.3 (8)^{a}	33.3 ± 0.3^{a} (1)
6D + CB	36	9	72.2 ± 1.4 (26)	77.8 ± 0.7 (7)	16.7 ± 0.5 (6)^{a,b}	0.0 ± 0.0 (0)^{b}	23.1 ± 0.5 (6)^{a,b}	0.0 ± 0.0 (0)^{b}
CHX	37	4	78.4 ± 2.2 (29)^{A}	25.0 ± 0.2 (1)^{B}	5.4 ± 0.2 (2)^{b}	0.0 ± 0.0 (0)^{b}	6.9 ± 0.3 (2)^{b}	0.0 ± 0.0 (0)^{b}
CHX + CB	34	7	76.5 ± 2.2 (26)	57.1 ± 0.4 (4)	17.6 ± 0.3 (6)^{a,b}	0.0 ± 0.0 (0)^{b}	23.1 ± 0.2 (6)^{a,b}	0.0 ± 0.0 (0)^{b}

^{a,b}: Values with different superscript letters within columns are significantly different ($P < 0.05$). ^{A,B}: Values with different superscript letters within rows are significantly different ($P < 0.05$). 6D group: 6-DMAP; 6D + CHX group: 6-DMAP and cytochalasin B; CHX group: cycloheximide; CHX + CB group: cycloheximide and cytochalasin B.

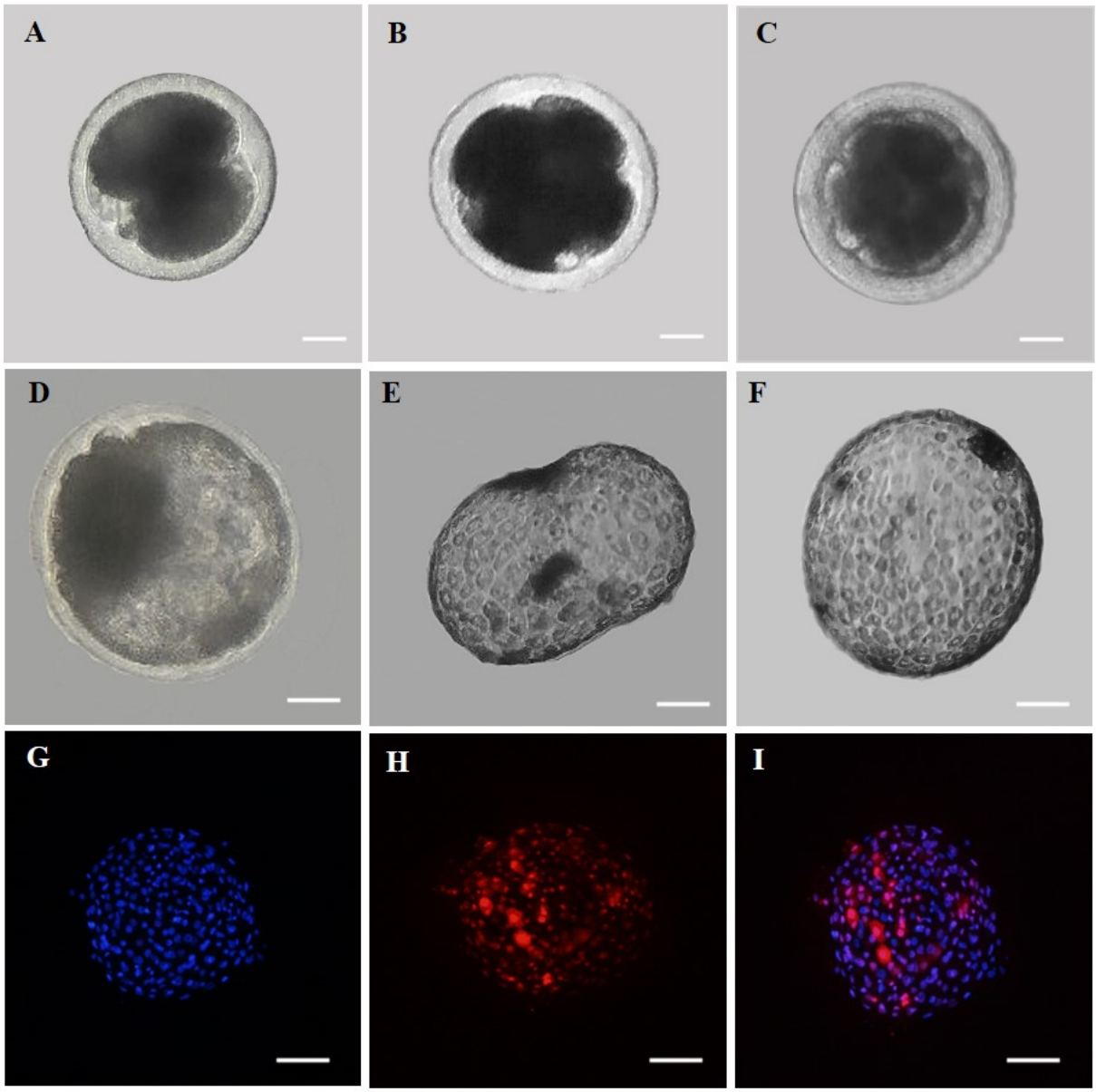

Fig. 3. Kinetics of embryonic development in collared peccaries. After 3 days of IVD different embryos with **(A)** 2 cells; **(B)** 4 cells, **(C)** more than 8 cells. Evaluation of embryonic development on day 7 of IVD **(D)** blastocyst; **(E)** blastocyst hatching; **(F)** blastocyst hatched already without zona pellucida. Counting the total number of blastocyst **(G)** cells inner mass labelled with Hoechst 33342. **(H)** trophectoderm cells stained with propidium iodide. **(I)** merge total cells.

Nevertheless, the 6D group exhibited higher rates of blastocyst/total cell in the embryos derived from GI/GII COCs than CHX group (Table 3). Moreover, only 6D treatment resulted

in the production of embryos derived from GIII COCs (25.0% ± 0.2). Further, the percentages of ICM/total cell ratio in blastocysts derived from 6D, 6D + CB, and CHX + CB group COCs were higher than those in blastocysts derived from CHX group COCs. Interestingly, the total number of blastocyst cells (Fig. 3G–H) derived from 6D + CB and CHX + CB oocyte groups was similar (Table 4).

Table 4. Cell count of *cumulus-oocyte complexes* (COCs) as viable and activated with ionomycin in combination with different secondary activators.

Secondary activators	No. of embryos	ICM, %	TE, %	Total	%ICM/Total
6D	5	78.8 ± 35.2	93.2 ± 41.7	172 ± 76.9[a,b]	42.5 ± 19.0[a]
6D + CB	3	33.7 ± 19.4	65.0 ± 37.5	98.7 ± 57.0[a]	37.9 ± 21.9[a]
CHX	2	7.0 ± 0.1	188.0 ± 0.1	195 ± 0.1[b]	3.6 ± 0.1[b]
CHX + CB	5	40.2 ± 18.0	57.6 ± 25.8	97.8 ± 43.7[a]	43.8 ± 19.6[a]

[a,b]: Values with different superscript letters within columns are significantly different ($P < 0.05$). ICM: internal cellular mass; TE: trophectoderm; %ICM/Total: internal cellular mass/ total cell ratio.

In relation to embryo development kinetics (Fig. 3D–F), or the proportion of embryos at different stages of development, there was no significant difference among the groups in the percentage of embryos classified with 2, 3–7, or ≥ 8 cells (Fig. 4A–B). On D7, the percentages of iB, B, eB, or hB embryos were similar among the treatment groups (Fig. 4C, $P > 0.05$).

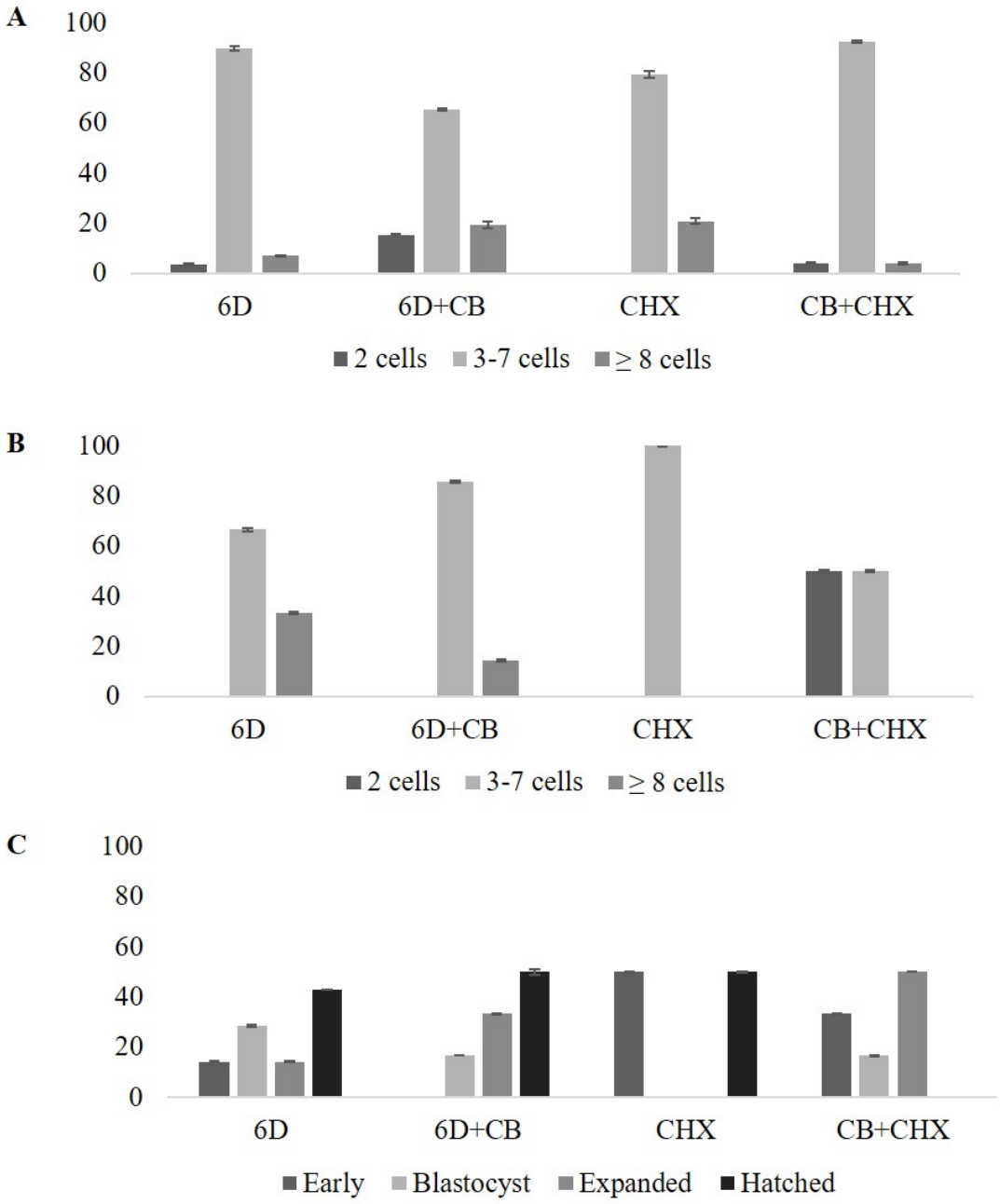

Fig. 4. Embryonic development of classified activated with ionomycin in combination with different secondary activators. Embryonic development in 3 days with 2 cells, 3–7 cells and 8 cells after activation with ionomycin in combination with secondary activators in **(A)** GI/GII

(P > 0.05) and **(B)** GIII COCs (P > 0.05). **(C)** Percentage of embryos in D7 classified in different stages of blastocyst after activation of GI/GII COCs (P > 0.05).

4. Discussion

Establishment of SCNT is an important strategy to develop this technology as a conservation tool for collared peccary. In this study, we optimized the protocols for improving matured oocyte quality and artificial oocyte activation, which are important for the success of SCNT. Recently [34, 35, 36, 37], our group has developed strategies for the establishment of donor nuclei (or karyoplast), which form a database of somatic resources for the species. We have established the conditions for obtaining cytoplasts and evaluated their competence after artificial oocyte activation using different protocols.

Initially, we evaluated the effects of EGF on the IVM of collared peccary oocytes. We, for the first time, demonstrated that the zona pellucida thickness in oocytes matured with EGF was lower than that in oocytes matured without EGF. The thickness of the zona pellucida is an indicator of the success of hatching and implantation of embryos [38]. The elasticity and thinning of zona pellucida are essential for the hatching process. Thick zona pellucida is associated with low-quality embryos. Increased thickness of zona pellucida may be caused by an inappropriate *in vitro* environment [38]. Zhou et al. [39] demonstrated that thinning solutions enhance the nuclear maturation of oocytes that previously had thick zona pellucida. The study hypothesized that thinner zona pellucida could facilitate first polar body extrusion. Khanmohammadi et al. [38] observed that a suitable culture medium promotes optimal environment that allows zona pellucida thinning and subsequently facilitates embryonic implantation. Furthermore, morphometric evaluation of matured oocytes has been used to estimate the oocyte developmental competence [32].

The expansion of *cumulus* cells was observed in almost all oocytes derived from both EGF positive and negative groups (> 97.8%). A similar response was also observed in swine oocytes [8]. Further, both groups exhibited high rates of *cumulus* cell expansion after maturation. There was no difference in the viability of *cumulus* cells, with values higher than 76% for all groups. This response may have occurred due to the presence of gonadotrophins and/or serum in the IVM medium, which can interfere with the stimulation induced by EGF [40]. Additionally, FSH may act through EGF receptors to exert a synergistic effect on

cytoplasmic maturation [11] and may interfere with the effects of EGF on *cumulus* cell expansion.

The quality of matured oocytes is important for the evaluation of meiotic competence. Thus, we evaluated the ROS levels and ΔΨm in matured oocytes and observed no difference between the groups for both parameters. Generally, the decrease in ROS levels is accompanied with an increase in glutathione (GSH) levels, a natural antioxidant that reduces ROS in oocytes [41], where ROS generation depends on ΔΨm [31]. Fan et al. [41] observed that treating the goat oocytes with EGF and cysteamine resulted in lower ROS levels in matured oocytes. In our study, both groups were cultured in a medium containing cysteamine, and EGF probably did not enhance its activity.

Similarly, EGF did not increase the IVM rates of oocytes at MII phase and have 1PB. Although Kishida et al. [42] observed a higher rate of nuclear maturation in porcine oocytes when EGF was present in the medium (10 ng/mL), another study demonstrated that EGF does not influence the nuclear maturation [43]. This may be due to the interaction of EGF with different supplements of the IVM medium, which are highly variable in each study. Further, EGF was involved primarily in the maintenance of embryonic quality where EGF-activated pathways promote greater competence of the oocyte to support embryonic development. Therefore, we suggest the use of EGF in the IVM medium of collared peccary oocytes for future experiments.

In this study, we observed that 6D was the most optimal secondary activator for collared peccary oocytes. However, the combination of 6D and CB did not have a beneficial effect on embryonic development. Further, CHX did not exhibit good potential for oocyte activation, which was improved only in combination with CB. Several studies have demonstrated the ability of 6D, CHX, and CB to inhibit meiotic resumption and second polar body extrusion in porcine oocytes [44, 45]. Moreover, the higher efficiency of 6D compared to CHX was also observed in porcine oocytes [46]. Although 6D and CHX exerted positive effect on oocyte activation and morula-blastocyst formation rates, 6D was more effective than CHX on both matured and immature oocytes [46]. This may be due to the mechanism of action of CHX, which may result in the inhibition of proteins involved in embryonic development [47], whereas 6D specifically inhibits protein kinases [48]. Additionally, Khadijah et al. [49] and

Zhang et al. [50] demonstrated that the combination of ionomycin and 6D was better than the combination of ionomycin and CHX for embryonic development in cattle (7.1% *vs.* 2.2%) and rabbit oocytes (8.6% *vs.* 1.2%).

Moreover, the embryonic development rates in the presence of ionomycin and 6D for collared peccary oocytes were higher than those observed for swine oocytes [51] evaluated based on the cleavage percentage (82.9% *vs.* 47.2%), blastocyst percentage (23.5% *vs.* 11.3%), and ICM/total cell ratio (42.5% *vs.* 24.8%). This indicates that despite similarities in the responses of protocols used between species, some differences are observed, suggesting the importance of establishing a species-specific protocol. Additionally, Campos Junior et al. [20] activated the collared peccary oocyte with the combination of ionomycin and 6D and obtained up to two-cell (40%) and 4-cell (10%) embryos. Thus, this is the first study that achieved the blastocyst stage of embryonic development in collared peccaries through artificial oocyte activation.

The low performance of CHX was observed in both artificial oocyte activation of collared peccaries and blastocyst quality. Although our results demonstrated a higher total number of cells in the 6D and CHX groups, CHX group exhibited a higher number of trophectoderm cells and a very low number of cells of the internal mass, which would later impair the embryonic development of the CHX group that had a low blastocyst rate compared to the other groups. The CHX + CB group exhibited higher ICM/total cell rates compared to the CHX group. This may be because CHX has no effect on blocking the extrusion of the second polar body. Thus, parthenotes treated with CHX were largely haploid and exhibited low blastocyst development, which does not improve blastocyst formation further [52]. The combination of 6D and CB was more effective in inhibiting the extrusion of the second polar body, which promotes the development of diploid embryos with higher developmental competence compared to haploid embryos [53].

The combination of ionomycin and 6D exhibited optimal performance as this was the only combination that produced embryos from GIII oocytes. As CB enhances the effect of CHX on GI and GII oocytes, its mechanism cannot be as efficient as 6D because the combination of CHX and CB could not promote the development of GIII oocytes to blastocysts. However, matured GIII oocytes were activated and developed into embryonic blastocyst only in the 6D

group. Therefore, embryonic development depends on the efficiency of the activation protocol [53]. Thus, use of oocytes with heterogeneous cytoplasm and less than one layer of *cumulus* cells may be suitable when there is low availability of oocytes.

In conclusion, EGF can be used to supplement the maturation medium to obtain a greater quality of matured oocytes and to improve embryonic development. Moreover, the combination of ionomycin and 6D could produce collared peccary embryos from the activation of both GI/GII and GIII COCs. These optimized IVM conditions and artificial activation in collared peccaries represent the first steps for cloning this species through ARTs.

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6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A presente tese obteve resultados significativos no desenvolvimento da clonagem por transferência nuclear de célula somática (TNCS) em catetos, uma vez que nós estabelecemos a obtenção de células doadoras de núcleo ou carioplastos e células doadoras de citoplasma ou citoplastos. No que se refere aos estudos dos carioplastos, inicialmente, nós descrevemos o primeiro trabalho de estabelecimento e caracterização de fibroblastos de catetos adultos durante o cultivo *in vitro* e criopreservação. Posteriormente, nós estabelecemos que a combinação de 10% de dimetilsulfóxido associado com 0,2 M de sacarose e 50% de soro fetal bovino foi a mais eficiente solução de criopreservação para a formação de bancos de células somáticas de catetos. Adicionalmente, nós definimos como melhor protocolo de sincronização do ciclo dessas células em G_0/G_1 o método de inibição por contato por três dias. Portanto, nós estabelecemos a etapa de carioplastos da TNCS de catetos, obtendo células de qualidade e aptas a serem usadas como doadoras de núcleo.

Já no que se refere aos estudos dos citoplastos, inicialmente, nós otimizamos as condições de maturação *in vitro* de oócitos, observando o tempo de 48 h e a presença de 10 ng/mL de fator de crescimento epidermal (EGF) como adequados para uma maior competência meiótica destas estruturas. Além disso, nós verificamos que a ativação química usando ionomicina e 6-dimetilaminopurina (6-DMAP) foi a mais eficiente combinação, tendo esta tese alcançado como resultado significativo, uma taxa de 27,6% de blastocistos de catetos derivados da ativação oocitária artificial.

Em síntese, nós obtivemos carioplastos e citoplastos que poderão ser empregados na TNCS de catetos, deixando a ponto as etapas fundamentais para a clonagem desta espécie. Ainda, destaca-se que os conhecimentos aqui gerados poderão ser aplicados em estudos para produção de células induzidas à pluripotência; ensaios de toxicidade e farmacológicos; fecundação *in vitro*; injeção intracitoplasmática de espermatozoides; compreensão da biologia reprodutiva e do desenvolvimento embrionário; obtenção de células embrionárias totipotentes; avaliações de criotolerância do oócito e do embrião. Portanto, este trabalho foi um grande passo para a conservação de taiassuídeos, especialmente dos catetos.

ANEXO A – VERSÃO DO ARTIGO PUBLICADO NA ZYGOTE

POTENTIAL ROLE OF INTRASPECIFIC AND INTERSPECIFIC CLONING IN THE CONSERVATION OF WILD MAMMALS

QUALIS: Qualis 2013-2016: B1/ Novo Qualis: B2

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Potential role of intraspecific and interspecific cloning in the conservation of wild mammals

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Review

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Summary

Intraspecific and interspecific cloning via somatic cell nuclear transfer (iSCNT) is a biotechnique with great possibilities for wild mammals because it allows the maintenance of biodiversity by recovering species, nuclear reprogramming for the production of pluripotency-induced cells, and studies related to embryonic development. Nevertheless, many areas in cloning, especially those associated with wild mammals, are still in question because of the difficulty in obtaining cytoplasmic donor cells (or cytoplasts). Conversely, donor cell nuclei (or karyoplasts) are widely obtained from the skin of living or post-mortem individuals and often maintained in somatic cell banks. Moreover, the creation of karyoplast–cytoplast complexes by fusion followed by activation and embryo development is one of the most difficult steps that requires further clarification to avoid genetic failures. Although difficult, cloning different species, such as wild carnivores and ungulates, can be successful via iSCNT with embryo development and the birth of offspring. Thus, novel research in the area that contributes to the conservation of biodiversity and knowledge of the physiology of species continues. The present review presents the failures and successes that occurred with the application of the technique in wild mammals, with the goal of helping future work on cloning via iSCNT.

Introduction

The decrease in the biodiversity of wild mammals has been caused mainly by human activity, resulting in an increase in research aimed at the development of conservation strategies (Pereira *et al.*, 2016). In general, several techniques to help with the conservation of wild animals are available, including the formation of biobanks (León-Quinto *et al.*, 2009), artificial insemination (Howard *et al.*, 2016), embryo transfer (Goeritz *et al.*, 2012), *in vitro* fertilization (Herrick *et al.*, 2010), and cloning using somatic cell nuclear transfer (SCNT, Folch *et al.*, 2009). Because of the low availability of oocytes for SCNT, interspecific cloning using intraspecific and interspecific nuclear transfer techniques (iSCNT) has been shown to be an important tool in conservation (Wani *et al.*, 2017).

The main argument for the application of iSCNT is the rapid decrease in the number of species. Any tool that can avoid this decrease is important. iSCNT preserves and even expands genetic variability when somatic cells of different individuals representative of the original biodiversity of a population are collected for its use (Loi *et al.*, 2001). In addition, interest in cloning has increased not only for the conservation of endangered species, but also for the multiplication of reproducers with better genetic characteristics (Saini *et al.*, 2015), basic research on cell epigenetic status (Saragusty *et al.*, 2016), embryonic development (González-Grajales *et al.*, 2016), and the production of induced pluripotent cells (Sukparangsi *et al.*, 2018).

Therefore, in all applications of cloning, studies related to the improvement of iSCNT, as well as its wide use in different individuals, are important.

Overview of the iSCNT technique and its limitations

The iSCNT technique involves embryo reconstruction by fusing a nucleus of a donor cell (karyoplast) derived from a wild mammal with an enucleated oocyte (cytoplast) from a domestic mammal of a different species, family, order, or class (Do & Taylor-Robinson, 2014). The nucleus in G0/G1 is exposed to reprogramming by the oocyte, followed by the fusion and activation of the reconstructed embryo (Loi *et al.*, 2011). Subsequently, the resultant embryo can be transplanted into the uterus of a recipient for term development (Pereira & Freitas, 2009).

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Different steps are involved in the production of clones via iSCNT. Therefore, it is interesting to highlight the steps of the technique and its peculiarities that can define the success of cloning by iSCNT.

Preparation of cytoplasts

Whether using the oocyte from a domestic or a wild mammal, some fundamental criteria must be met to obtain a cytoplast suitable for cloning, such as oocyte selection, *in vitro* maturation, and enucleation systems (Loi *et al.*, 2011). In general, follicular size, the oocyte collection method, and the culture environment are factors that can affect the quality of mature oocytes, and different responses to these factors can be observed in wild mammals. In some cervid species, Brahmasani *et al.* (2013) observed that low maturation rates could probably be caused by slicing. In this method, non-competent oocytes can be recovered, as the technique can result in the recovery of structures of small diameter follicles (Rho *et al.*, 2001). Additionally, the quality of ovaries obtained post-mortem may have been one of the factors that reduce the quality of oocytes in these species.

Therefore, studies have shown that enriched culture medium and ovarian transport conditions may result in good results using ovaries from post-mortem animals for recovery of immature oocytes. Macías-García *et al.* (2018) verified that oocytes of Iberian red deer (*Cervus elaphus hispanicus*) obtained from ovaries maintained for 16 h in a holding medium increased the oocyte meiotic competence. Moreover, these authors observed that the epidermal growth factor (EGF) demonstrated a differential effect depending upon oocyte grading and conditions of ovary transportation. Additionally, for ovaries derived from Hokkaido sika deer (*Cervus nippon yesoensis*), maturation rates of oocytes were highest when ovaries were kept for 12 h at 20–25°C, when compared with 24 h (Tulake *et al.*, 2014).

Specifically, with respect to the culture environment, the requirements for both composition and maturation time should be established for the *in vitro* maturation of each species. In the Indian blackbuck (*Antilope cervicapra*), oocytes cultured in the presence of gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) showed higher rates of expansion of the cumulus oophorus (79.3%) and extrusion of the first polar body (46.1%) compared with oocytes cultured without gonadotropins (60.4% and 33.3%, respectively) (Rao *et al.*, 2010). In the sika deer (*Cervus nippon hortulorum*), oocytes cultured in medium supplemented with fetal bovine serum (FBS), FSH, LH, cysteamine and EGF resulted in a higher maturation rate (75.4%) compared with medium without supplementation (30.1%; Yin *et al.*, 2013). Already, different hormonal combinations of FSH, LH and 17β-estradiol did not alter the maturation rates in oocytes derived from lions (*Panthera leo*; Fernandez-Gonzalez *et al.*, 2015). In the collared peccary (*Pecari tajacu*), we proved that oocytes need 48 h to achieve maturation instead of 24 h, according to the expansion of the cumulus cells (100% vs. 38.1%), the presence of first polar body (90.5% vs. 52.4%), and the status of the nucleus in the second metaphase (76.2% vs. 52.4%), respectively (Borges *et al.*, 2018c).

In addition to obtaining mature oocytes, the preparation of cytoplasts depends on the method of enucleation of these structures. The amount of ooplasm present in the reconstructed embryo is related to the enucleation technique that removes the nucleus from the oocyte. Matured oocytes can be enucleated in different ways, including squeezing the first polar body and the surrounding cytoplasm through a cleft in the zona pellucida of the oocyte (Lee *et al.*, 2003). Another method is manual removal in which zona-free oocytes are enucleated with a bisection blade that hand bisect the metaphase II chromosomes along with a small volume of the surrounding cytoplasm. Oocytes can also be aspirated using a micromanipulator at the location of the metaphase II chromosomes and the polar body via brief exposure to ultraviolet light (Pereira *et al.*, 2015).

Selection of karyoplasts

To obtain karyoplasts appropriate for cloning, their type and age and the manipulation techniques used are important for their future reprogramming (Kim *et al.*, 2007). Karyoplasts can be obtained from fresh or cryopreserved somatic tissues (Folch *et al.*, 2009, Pan *et al.*, 2014), from an adult (Moulavi *et al.*, 2017) or a fetus (Liu *et al.*, 2018), and *in vivo* or post-mortem (Pereira *et al.*, 2014). Although the recovery of these cells is not a difficult task, their processing and preservation until use in iSCNT require attention (Pereira *et al.*, 2014). In general, skin cells have been the most used cell type for karyoplasts (Song *et al.*, 2007). The skin has an abundance of cells of interest that may have different efficiencies in cloning, as observed in wild buffalo (*Bubalus arnee*). Saini *et al.* (2015) detected that fibroblasts of this species are easier to reprogram than epithelial cells.

After harvest, cells used as nuclei donors need to be characterized with respect to their culture conditions, cryopreservation, and cell cycle synchronization (Pereira *et al.*, 2014). For these steps, cells are evaluated for the number of passages, nutritional requirements during *in vitro* culture (Santos *et al.*, 2016), and the damage done during cryopreservation (Song *et al.*, 2007). Thus, karyoplasts have been established *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS and growth factors (Santos *et al.*, 2016).

Karyoplasts have been routinely cryopreserved by slow freezing (Sharma *et al.*, 2018) using a combination of dimethyl sulfoxide (DMSO), FBS, and sucrose as the cryoprotectant, as observed with Iberian lynx (*Lynx pardinus*, León-Quinto *et al.*, 2014). Although it is more desirable to use a somatic cell bank after tissue culture, the absence of *in vitro* culture conditions sometimes makes these banks unfeasible, resulting in the immediate formation of the targets for those somatic tissues (Borges *et al.*, 2017a,b; Queiroz Neta *et al.*, 2018). The three somatic tissue conservation techniques used for wild animals are slow-freezing cryopreservation (Mestre-Citrinovitz *et al.*, 2016), vitrification (Borges *et al.*, 2018a,b), and cooling at 4–6°C (Queiroz Neta *et al.*, 2018). In collared peccaries, we compared two techniques of vitrification and we observed that solid-surface vitrification was found to be a more efficient method for vitrifying skin tissue when compared with direct vitrification in cryovials, probably due to tissues not being involved in large amounts of cryoprotectants before passing through a drastic change in temperature during the solid-surface vitrification (Borges *et al.*, 2017b).

Finally, the third step in the preparation of the karyoplasts is cell synchronization in the G0/G1 stage (Gómez *et al.*, 2003; Yelisetti *et al.*, 2016). In general, nuclear reprogramming is controlled by epigenetic modification. For this to occur, the somatic cells must be in G0/G1 to allow the removal of reversible epigenetic changes acquired during cell differentiation (Song *et al.*, 2007). Therefore, cells can be subjected to different treatments for synchronization during culture. Inhibition by contact (Moulavi *et al.*, 2017), serum deprivation (Wani *et al.*, 2017), and chemicals that inhibit the cell cycle (Gómez *et al.*, 2003) are methods used for synchronization. Serum deprivation and inhibition by contact are the most commonly used (Moulavi *et al.*, 2017).

Under high confluence or serum privation, fibroblast cells derived from the skin of adult argali (*Ovis ammon*) were efficiently

Table 1. iSCNT in some wild mammals

Scientific name	IUCN Category*	Country	Karyoplast	Cytoplast	Outcome	Authors
Carnivores						
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	17% pregnancies, 2 pups	Kim *et al.* (2007)
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	23.5% pregnancies, 4 pups	Oh *et al.* (2008)
Felis margarita	Least Concern	USA	Fibroblast	*Felis catus*	3 pups	Gómez *et al.* (2008)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	28% blastocyst	Gómez *et al.* (2003)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	75% pregnancies, 17 pups	Gómez *et al.* (2004)
Ungulates						
Acinonyx jubatus	Vulnerable	Argentina	Fibroblast	*Felis catus*	27.4% blastocyst	Moro *et al.* (2015)
Acinonyx jubatus venaticus	Vulnerable	Iran	Fibroblast	*Felis catus*	5.9% morula	Moulavi *et al.* (2017)
Bos gaurus	Vulnerable	USA	Fibroblast	*Bos taurus*	25% pregnancies, none term	Lanza *et al.* (2000)
Bos javanicus	Endangered	USA	Fibroblast	*Bos taurus*	17% pregnancies, none term	Sansinena *et al.* (2005)
Bubalus arnee	Endangered	India	Fibroblast	*Bubalus bubalis*	38.7% blastocyst	Priya *et al.* (2014)
Bubalus arnee	Endangered	India	Fibroblast/Epithelial cell	*Bubalus bubalis*	50.6 *vs* 20.5% blastocyst	Saini *et al.* (2015)
Capra ibex	Least Concern	China	Fibroblast	*Capra hircus*	11% blastocyst	Wang *et al.* (2007)
Capra pyrenaica	Absente	Spain	Fibroblast	*Capra pyrenaica*	1 born	Folch *et al.* (2009)
Ovis ammon	Near Threatened	China	Fibroblast/Cumulus cell	*Ovis aries*	22.1% blastocyst	Pan *et al.* (2014)
Ovis orientalis musimon	Vulnerable	Italy	Granulosa cell	*Ovis aries*	1 pup	Loi *et al.* (2001)
Tragelaphus eurycerus isaaci	Critically Endangered	USA	Fibroblast	*Bos taurus*	24% blastocyst	Lee *et al.* (2003)
Others species						
Balaenoptera bonaerensis	Data deficient	Japan	Cumulus cells	*Bos taurus/Sus scrofa domesticus*	27.5–52.8% cleavage	Ikumi *et al.* (2004)
Macaca fascicularis	Least Concern	Thailand	Fibroblast	*Bos taurus*	33% blastocyst	Lorthongpanich *et al.* (2008)

*IUCN: International Union for Conservation of Nature and Natural Resources. USA: United States of America.

synchronized at G0/G1; nevertheless, cells were in lower proportion in the growing stage (Pan *et al.*, 2014). Authors observed that the highest proportion of cells from the African wild cat (*Felis silvestris lybica*) at G0/G1 was obtained by serum deprivation compared with that obtained by inhibition by contact and the inhibitor roscovitine (Gómez *et al.*, 2003). Leopard (*Panthera pardus*) skin cells treated with chemical inhibitors such as sodium butyrate have a greater propensity to undergo alterations (Yelisetti *et al.*, 2016).

Embryonic reconstruction stages

After the transfer of the nucleus into the enucleated oocyte, the cytoplast–karyoplast complex is subjected to an electric pulse that not only induces the fusion of the somatic cell nucleus with the enucleated oocyte to form a new complex, but also promotes the release of intracellular calcium that initiates cellular activation (Pereira & Freitas, 2009). In general, the successful development of a reconstructed embryo depends on the complex interactions between the cytoplast and the nuclear structure during embryonic development; failures in this interaction can cause problems during early cleavage and embryonic development (González-Grajales *et al.*, 2016).

The activation of the cytoplast–karyoplast complexes guarantees adequate embryonic reconstruction (Yamochi *et al.*, 2013). Because the iSCNT technique reprograms the nucleus of a somatic cell of one species using the oocyte cytoplasm of another species, it is essential that the activation protocol be able to activate the reconstructed embryo (Zhao *et al.*, 2006). Physiologically, a mammalian oocyte is activated during fusion with a sperm, releasing meiotic cell cycle arrest and enabling the resumption of the oocyte meiotic cell cycle (Sparman *et al.*, 2010). Therefore, a well developed protocol allows a high rate of blastocyst formation by promoting good embryonic development through activation.

Activation protocols, including physical methods such as electrical pulses and alteration of osmolarity, and chemical methods such as calcium-mobilizing compounds like strontium chloride, ionomycin, and ethanol, to promote the initial release of calcium have been evaluated in different species, as sika deer (Yin *et al.*, 2013), alpaca (*Vicugna pacos*) and llama (*Lama glama*, Ruiz *et al.*, 2015), with blastocyst rates of 32.4%, 22.5% and 18.7%, respectively. In general, calcium mobilizers are used in combination with kinase protein inhibitors or protein synthesizers such as cycloheximide and 6-dimethylaminopurine (6-DMAP). In addition, a cytostatic factor inactivator and microfilament inhibitor such as cytochalasin B are used to prevent extrusion of the second polar body and maintain the diploidy of the presumed embryo (Ruiz *et al.*, 2015).

For red deer (*Cervus elaphus*), electrical activation before chemical activation with ionomycin and 6-DMAP was efficient for the production of clone embryos (32–44%), obtaining genetically healthy calves (Berg *et al.*, 2007). Nevertheless, the same protocol resulted in a low developmental rate (5.7%) of activated oocytes in swamp deer and 0.0% embryos in spotted deer, sambar deer, and brow-antlered deer after oocyte parthenogenetic activation (Brahmasani *et al.*, 2013). Blackbuck (*Antilope cervicapra*) oocytes activated with ionomycin and 6-DMAP resulted in 58% cleaved embryos and 13% blastocysts (Rao *et al.*, 2010). Therefore, the artificial activation method (chemical, electrical protocols or your combination) can result in different responses among species. In this sense, it is necessary to evaluate the type of artificial activation that promotes the best rates of embryonic development in the species of interest.

In vitro culture systems are essential for early embryonic development and nuclear reprogramming (Gómez *et al.*, 2008; Pereira *et al.*, 2013). Choosing the appropriate culture medium for each species is considered the initial step in proper embryonic development (Zhao *et al.*, 2006). Lee *et al.* (2003) used somatic cells of the mountain bongo (*Tragelaphus eurycerus isaaci*) and domestic cow (*Bos taurus*) oocytes and observed that a chemically defined, protein-free medium of TCM199 supplemented with FBS supported embryonic development. Nonetheless, there is no one culture medium suitable for all species that allows better embryonic development for a given species under study.

Finally, the effect of epigenetic reprogramming is a very relevant factor in the success of iSCNT (Gómez *et al.*, 2008). Some epigenetic markers were characterized with respect to their function during embryonic reprogramming and their influence on the chromatin structure from post-translational modifications (Song *et al.*, 2007). The overall level of the acetylation of histone H3 at lysine 18 (H3K18ac) and trimethylation of histone H3 at lysine 27 (H3K27me3), and the expression level of some important apoptosis proteins (caspase 3 and caspase 7), and p53 were evaluated. The hyperacetylated state of histones is associated with transcriptionally active domains, while the hypoacetylated state is associated mainly with silenced chromatin regions of histone acetyl transferases and histone deacetylases. The methylation pattern of the DNA is determined by DNA methyltransferases. OCT3/4, NANOG, and CDX2 are very important because of their close association with pluripotency and early embryonic development (Saini *et al.*, 2015).

Advances and perspectives of iSCNT in wild mammals

Several works aimed at cloning different wild mammals have been conducted (Table 1). Among these studies, those that obtained offspring were on wild bovine (Lanza *et al.*, 2000), sheep (Loi *et al.*, 2001), felid (Gómez *et al.*, 2004; Li *et al.*, 2007), canid (Kim *et al.*, 2007; Oh *et al.*, 2008), and goat (Folch *et al.*, 2009). Therefore, several families have proven the success of using iSCNT for the recovery and reintroduction of wild mammals.

An important point to remember is that as the taxonomic distance between donor and recipient species increases, the production of blastocysts decreases because of the decreased ability of somatic cells to be reprogrammed (Priya *et al.*, 2014). In general, enucleated oocytes are from a domestic species that is phylogenetically close to the wild species that donates the nucleus. For example, domestic sheep cytoplasts were able to reprogram me argali fibroblast nuclei (Pan *et al.*, 2014) and domestic buffalo cytoplast was able to reprogram me wild buffalo karyoplast (Priya *et al.*, 2014).

Carnivores

Some works have shown the advances achieved by iSCNT in wild canine species. These species, including the grey wolf (*Canis lupus*), have gradually become endangered or extinct. Therefore, in 2007, with the goal of canid conservation, Kim *et al.* (2007) cultured fibroblasts derived from the ear of an adult female grey wolf that were then used as donor cells of nuclei. Using domestic canine oocytes, the authors produced a pregnancy with cloned embryos of two genetic identities of the cloned wolves, but there were no births. In 2008, Oh *et al.* (2008) obtained three wolf pups from cloned embryos using cells obtained from a male grey wolf 6 h after death and domestic canine oocytes. These studies demonstrated the successful cloning of endangered wild canines.

In felid species, the main oocyte source has been the domestic cat. In species from the Felidae subfamily, some progress has been achieved. Therefore, synchronized nuclei were donated by the African wild cat and transferred to enucleated domestic cat oocytes resulting in a high rate of blastocyst formation but no pregnancies (Gómez *et al.*, 2003). In another study in which embryos were constructed using somatic cells derived from the African wild cat and domestic cat oocytes, 75% of the embryos developed to term and 25% underwent fetal resorption or abortion (Gómez *et al.*, 2004). Of the 17 cloned kittens born, seven were stillborn, eight died within hours of delivery or up to 6 weeks of age, and two are currently alive and healthy. Additionally, some studies on wild felids have shown the establishment of somatic resource banks. There is a bank with somatic samples of 69 individual Iberian lynx, considered the most endangered felid in the world, with the aim of future cloning (León-Quinto *et al.*, 2009; 2014).

In addition, works on the cheetah (*Acinonyx jubatus*), a species of the Pantherinae subfamily, have been performed in South America and Asia. Somatic cells from a cheetah raised in South America were transferred to domestic cat oocytes, and, after embryo aggregation during *in vitro* culture, high blastocyst formation rates were obtained (16.7–28.3%) (Moro *et al.*, 2015). Moulavi *et al.* (2017) used non-viable frozen cells derived from frozen tissue from an Asiatic cheetah (*Acinonyx jubatus venaticus*) and *in vitro*-matured domestic cat oocytes and obtained morula rates of 5.9%. Although no blastocyst was obtained, this study demonstrated that enucleated cat oocytes can partially remodel and reactivate nonviable nuclei of the Asiatic cheetah and support its reprogramming back to the embryonic stage.

Ungulates

Some studies with ungulates have been performed with significant success, especially for species already extinct. The first animal

derived from an extinct subspecies was obtained using fibroblasts from skin biopsies collected before the death of the last female *Capra pyrenaica pyrenaica*. After a year under cryopreservation, these cells were used as karyoplasts and fused with the cytoplasts of a domestic goat to reconstruct embryos. The rate of cleaved embryos after 36 h was 47.3%, of which 65.5% were transferred. Five recipients were pregnant at 45 days but only one pregnancy went to term. Unfortunately, a few minutes after birth the animal died from pulmonary complications (Folch *et al.*, 2009).

Experiments were carried out with wild yak (*Bros grunniens*) with the goal of evaluating the parameters that affect the success of iSCNT (Li *et al.*, 2007). Fibroblasts and cumulus cells were used as donor cells, but the cell type and different ages were found to have no significant effect on iSCNT.

In 2017, the birth of a Bactrian camel cloned by iSCNT was first reported (Wani *et al.*, 2017). The fibroblasts used to donate nuclei were obtained from ear skin biopsy samples from an adult male Bactrian camel (*Camelus bactrianus*) and the cytoplast of dromedary camel (*Camelus dromedaries*) was the oocyte recipient. Twenty-six blastocysts were transferred to 23 synchronized dromedary recipients yielding five pregnancies with one going to term. This work has great importance because the Bactrian camel is the eighth most endangered large mammal on Earth.

Finally, the woolly mammoth (*Mammuthus primigenius*) is perhaps the one wild mammal of the ungulates whose cloning arouses the greatest interest. This animal became extinct about 10,000 years ago. However, epithelial and muscular cells from 14,000–15,000-year-old mammoth tissues were cryopreserved, with the goal of producing embryos of this species (Kato *et al.*, 2009). In this study, the authors injected cell nucleus-like structures into mature mouse enucleated oocytes; however, the oocytes did not form pronuclear-like structures at 7 h after injection.

Other species

The ability of bovine enucleated oocytes to support dedifferentiation of nuclei from monkey fibroblasts in interspecies cloned monkey embryos has been observed (Lorthongpanich *et al.*, 2008). These embryos were cultured in conditions different from the medium used for cattle with monkey-specific alterations, but the embryos were not able to develop past 16 cells under any culture condition. Nevertheless, OCT-4 was detected, demonstrating the ability of bovine ooplasm to support dedifferentiation but not embryonic development. Therefore, the culture medium promotes dedifferentiation but is not able to support complete embryonic development (Lorthongpanich *et al.*, 2008). In another work that used porcine cytoplasts and donor cells from a rhesus monkey, it was possible to obtain blastocysts despite the low rate (2.04%) (Zhu *et al.*, 2014). Although being a SCNT study, the cloning of cynomolgus monkeys (*Macaca fascicularis*) is cited here because of recent advances in this species. Thus, in a study on cynomolgus monkeys using SCNT, Liu *et al.* (2018) applied histone demethylase Kdm4d mRNA and histone deacetylase inhibitor trichostatin A after activation. Embryonic development improved followed by a greater number of pregnancies, which resulted in the birth of two monkeys via the SCNT technique using fetal fibroblasts and oocytes of cynomolgus monkeys.

With respect to aquatic mammals, a study performed on the minke whale (*Balaenoptera bonaerensis*) compared different conditions of iSCNT, including the ability of porcine and bovine ooplasms to produce reconstructed embryos and the effects of different donor cell types (viable or nonviable cells) on whale SCNT embryos (Ikumi *et al.*, 2004). The authors concluded that whale iSCNT embryos can develop to at least the four-cell stage, regardless of the survivability of the donor cells and the porcine or bovine ooplasm.

Final considerations

Although cloning has several technical limitations that require greater attention to improve the technique, iSCNT has been applied to numerous species of wild mammals and has achieved positive results with respect to embryonic stages in pregnancies and offspring born. The works cited in this paper have made it possible to analyze the state of the art and to perform specific studies the problems in the technique that can be fixed according to the species being studied.

This review has shown that there is no rule that says several species should be cloned following the same protocol, but that each species has different needs at each stage of the technique. In addition, all the papers referred to in this review point to the need for improvement and study at a certain stage, which will lead to improvement of the technique. Thus, to achieve a satisfactory result with iSCNT, each step involved in cloning must be suitable for the species being studied.

Although iSCNT is not the main tool for the reestablishment of endangered wild mammals, its use to increase the possibilities of reproduction and multiplication of individuals has been proposed. It should be refined so that it can be an alternative when traditional techniques cannot be applied. In addition, cloning helps elucidate the embryonic development of a wild species and the subsequent application of this knowledge.

Finally, this biotechnology can help generate more ways to maintain individual species. Therefore, the improvement of protocols to potentiate this technique is of interest because although it has low efficiency rates, iSCNT shows promise because of the pups of different species that have been born.

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Ethical standards. Not applicable

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ANEXO B – VERSÃO DO ARTIGO PUBLICADO NA PEERJ:

ISOLATION, CHARACTERIZATION, AND CRYOPRESERVATION OF COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES

QUALIS: Quadriênio 2013-2016: Não classificado pela Medicina Veterinária/ Novo Qualis: A2

FATOR DE IMPACTO: 2,353

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Isolation, characterization, and cryopreservation of collared peccary skin-derived fibroblast cell lines

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ABSTRACT

Background: Biobanking of cell lines is a promising tool of support for wildlife conservation. In particular, the ability to preserve fibroblast cell lines derived from collared peccaries is of significance as these wild mammals are unique to the Americas and play a large role in maintaining the ecosystem. We identified collared peccary fibroblasts by immunofluorescence and evaluated their morphology, growth and adherence capacity. Further, we monitored the viability and metabolic activity of the fibroblasts to determine the effects of passage number and cryopreservation on establishment of cell lines.

Methods: Skin biopsies were collected from the peripheral ear region from five adult animals in captivity. Initially, cells were isolated from fragments and cultured in the Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 2% antibiotic–antimycotic solution under a controlled atmosphere (38.5 °C, 5% CO_2). We evaluated the maintenance of primary cells for morphology, adherence capacity of explants, explants in subconfluence, cell growth and absence of contamination. Moreover, we identified the fibroblast cells by immunofluorescence. Additionally, to evaluate the influence of the number of passages (first, third and tenth passage) and cryopreservation on establishment of cell lines, fibroblasts were analysed for the viability, metabolic activity, population doubling time (PDT), levels of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi m$).

Results: All explants (20/20) adhered to the dish in 2.4 days ± 0.5 with growth around the explants in 4.6 days ± 0.7, and subconfluence was observed within 7.8 days ± 1.0. Moreover, by morphology and immunocytochemistry analyses, cells were identified as fibroblasts which presented oval nuclei, a fusiform shape and positive vimentin staining. No contamination was observed after culture without antibiotics and antifungals for 30 days. While there was no difference observed for cell viability after the passages (first vs. third: $P = 0.98$; first vs. tenth: $P = 0.76$; third vs. tenth: $P = 0.85$), metabolic activity was found to be reduced in the tenth passage (23.2 ± 12.1%) when compared to that in the first and third passage (100.0 ± 24.4%, $P = 0.006$). Moreover, the cryopreservation did not influence the viability

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(P = 0.11), metabolic activity (P = 0.77), or PDT (P = 0.11). Nevertheless, a greater ΔΨm (P = 0.0001) was observed for the cryopreserved cells (2.12 ± 0.14) when compared to that in the non-cryopreserved cells (1.00 ± 0.05). Additionally, the cryopreserved cells showed greater levels of intracellular ROS after thawing (1.69 ± 0.38 vs. 1.00 ± 0.22, P = 0.04).

Conclusions: This study is the first report on isolation, characterization and cryopreservation of fibroblasts from collared peccaries. We showed that adherent cultures were efficient for obtaining fibroblasts, which can be used as donor cells for nuclei for species cloning and other applications.

Subjects Cell Biology, Conservation Biology, Veterinary Medicine
Keywords Biological characterization, Cellular conservation, Cryobanking, Wild mammals

INTRODUCTION

Collared peccaries (*Pecari tajacu* Linnaeus, 1758) are wild mammals found only in the Americas and show a distribution from southern United States to northern Argentina, inhabiting the most diverse environments (*Santos et al., 2009*). Currently, their population is considered to be stable (*Gongora et al., 2011*); however, a significant reduction of their population has been seen in some biomes, such as the Caatinga (*Desbiez et al., 2012*) and the Atlantic forest (*Lazure et al., 2010*). As excellent seed dispersers (*Redford, 1992*), they are very important for the maintenance of our ecosystem, whereas, economically, they have been commercialized for their meat and in leather production (*Santos et al., 2009*). Scientifically, collared peccaries can be used as experimental models for closely related species such as the *Tayassu peccary* and *Catagonus wagneri* that have been listed as "vulnerable" in the IUCN Red List of Threatened Species (*Keuroghlian et al., 2013*; *Altrichter et al., 2015*; *Gongora et al., 2011*).

In this sense, studies related to the conservation of the collared peccary have been intensified, especially aimed at improving the techniques related to the preservation of somatic samples. Using this study, we established a culture condition for explants derived from the skin of adult collared peccaries (*Santos et al., 2016*) and developed a protocol for cryopreservation (*Borges et al., 2017*, *2018a*, *2018b*) and refrigeration of these explants (*Queiroz Neta et al., 2018*). In order to conduct the cloning experiments on this species by a somatic cell nuclear transfer, as well as to produce induced pluripotent cells, it is necessary to establish properly characterized cell lines.

In general, as observed in other mammals (*Guan et al., 2010*; *Kwong et al., 2014*), establishment of an adequate cell line is a prerequisite step for the success of cloning and producing induced pluripotent cells (*Borges & Pereira, 2019*). For these techniques, fibroblasts and epithelial cells derived from the skin have been widely used (*Jyotsana et al., 2016*; *Siengdee et al., 2018*). Initially, epithelial and fibroblast cells were grown simultaneously; nevertheless, fibroblasts can more easily adhere as well as detach by trypsinization as compared to the epithelial cells (*Bai et al., 2012*; *Saadeldin et al., 2019*; *Siengdee et al., 2018*). In these methods, the culture after the second passage has been considered to contain mainly fibroblasts (*Mehrabani et al., 2014*).

Additionally, for the confirmation of a fibroblast line, it is necessary to verify the possible changes that occur in these cells during culture (*Guan et al., 2010*; *Song et al., 2007*) and cryopreservation (*Magalhães et al., 2017*). In general, the number of passages throughout an in vitro study can modify the cellular epigenetic state, affecting the embryonic development after cloning (*Rodriguez-Osorio et al., 2012*; *Trokovic et al., 2015*). *Magalhães et al. (2017)* observed reduced viability and metabolic activity in the cells derived from the skin of the brown brocket deer in the tenth passage. Thus, the establishment of a cell line ensures a complete knowledge of the parameters that confer quality to the nucleus of the donor cell, named the karyoplast (*Guan et al., 2010*). Moreover, identification of damage occurring during cryopreservation is essential for establishment of a cell line. Cryo-variables may affect several cellular processes, including survival, functionality and the cytoskeleton, which may compromise the reprogramming ability of the karyoplasts (*Chatterjee et al., 2017*). Therefore, we aimed to isolate, characterize and cryopreserve the fibroblast cells derived from the skin of the ear of collared peccaries for their future application in cloning strategies by a somatic cell nuclear transfer and production of induced pluripotent cells.

MATERIALS AND METHODS

Chemicals and media

The Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and amphotericin solutions were obtained from Gibco-BRL (Carlsbad, CA, USA). Fluorescent probes were purchased from Invitrogen (Carlsbad, CA, USA). Anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488, Warrington, PA, USA) were purchased from Abcam (Cambridge, USA). The other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Media were filtered using a 0.22-µm system (Corning, New York, USA) and adjusted to pH of 7.2–7.4.

Bioethics and animals

This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92) and the Chico Mendes Institute for Biodiversity Conservation (no. 48633-2; ICMBio, Brasilia, Brazil). All animals belonged to the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil, 5°10′S, 37°10′W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding site (no. 1478912). The breeder stocks 100 collared peccaries on an average, and for this research four females and one male at ages of 26.8 months ± 2.9 months were used.

Ear tissue explant collection and primary culture

Peripheral skin (1–2 cm^2) was recovered from the ear sections used to identify collared peccaries kept in captivity. After the collection, a trichotomy of the tissue followed by a sterilization with 70% alcohol was performed. Samples were transported to the laboratory in DMEM supplemented with 2% antibiotic–antimycotic solution (10,000 units/mL

penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B) at 37 °C within 30 min.

In the laboratory, fragments (9.0 mm^3) were washed sequentially under laminar flow in the following media: (1) DMEM supplemented with 10% FBS and 10% antibiotic–antimycotic solution; (2) alcohol; and (3) DMEM plus 10% FBS and 2% antibiotic–antimycotic solution. Then, the samples were fragmented (four fragments per animal) and placed in polystyrene culture dishes treated for cell adhesion with the latter medium for cell culture. The skin was cultured at 38.5 °C under a controlled environment with 5% CO_2 and 95% air, according to a method described by *Santos et al. (2016)*.

Evaluation of the somatic cells in primary cultures and subcultures

During primary culture, the medium was changed every 24 h. For evaluation of the somatic cells, the primary culture was analyzed before reaching confluency and until it reached a confluency of 70–80%. Using an inverted microscope (Nikon TS100, Tokyo, Japan), the cells were evaluated for the following parameters: cell morphology, number of adhered samples, number of samples, evident subconfluency, day of sample adherence, day of subconfluent growth of the samples, and total time to reach 70–80% confluence (*Borges et al., 2017*).

When the cells reached 70–80% subconfluency, they were subcultured and distributed for other analyses. The 70–80% subconfluence was defined as the stage when 70–80% of the culture dishes consisted of somatic cells (*Santos et al., 2016*). Subconfluent cells were washed with PBS then trypsinized with a trypsin/EDTA solution (0.25%/0.2%) for 7 min and centrifuged at 600×*g* for 10 min. The supernatant was removed, the cell pellet was resuspended in culture medium, and the cell suspension was transferred to another dish for subculturing (*Borges et al., 2018b*). The medium was replaced with fresh medium every other day and the cells were monitored daily. With the successful passaging of the cultures, the cells are considered a cell line, following the convention of the Society of In Vitro Biology (*Schaeffer & Terminology Committee Chair Tissue Culture Association, 1990*). The cell line was designated as Ptskf.

Thus, in addition to an evaluation of the maintenance of cells in the primary culture, the subcultured cells were initially evaluated for the confirmation of fibroblasts using morphology and immunofluorescence analyses. Moreover, the possibility of contamination was also evaluated. Subsequently, the influence of the number of passages (first, third and tenth passage) and the metabolic activity of the cells were analyzed by a viability assay using trypan blue and the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, respectively. Moreover, the cells were also evaluated for the effects of a slow freezing cryopreservation. Other than the above-mentioned tests, growth dynamics by quantification of the population doubling time (PDT), oxidative stress analysis for quantification of intracellular reactive oxygen species (ROS) levels using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), and assessment of the mitochondrial membrane potential ($\Delta\Psi m$) using the fluorescent probe MitoTrackerRed® were performed.

Morphological characterization of the fibroblasts

Morphological characteristics were observed throughout the in vitro culture under light microscopy for cellular and nuclear shapes and cytoplasmic extensions.

Vimentin immunofluorescence

For a morphological confirmation, the cells were subjected to an immunocytochemistry protocol based on the method described by *Amoli et al. (2017)*. Briefly, the cells were fixed using 4% paraformaldehyde for 10 min at 25 °C, then washed with chilled PBS. Subsequently, cells were incubated with an antigen-retrieval buffer (100 mM Tris, 5% urea, pH 9.5), and then permeabilized for 1 h in 0.4% Triton X-100. Afterwards, the cells were incubated in 0.1% Tween-20 for 1 h to block non-specific binding of the antibodies. Finally, the cells were immuno-stained with mouse anti-vimentin antibody (ab8979, 1:200) for 24 h at 4 °C, and, then incubated with the secondary antibody (goat anti-mouse IgG, Alexa Fluor® 488, Warrington, PA, USA, ab150113, 1:400) for 1 h at 25 °C in the dark. Cells were counter-marked with one µg/mL Hoechst for 1 min and observed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan).

Confirmation of the absence of bacterial and fungal contamination

Cells of the third passage were cultured for 30 days in DMEM containing 10% FBS in the absence of an antibiotic–antimycotic solution, at 38.5 °C, 5% CO_2 and 95% air. Daily evaluation was performed under light microscopy for the identification of bacterial and fungal contamination.

Influence of the passage number on the quality of fibroblast lines

Initially, the fibroblast cells were analyzed for the effect of the number of passages (first, third and tenth passage) by a viability assay using trypan blue, according to the method described by *Magalhães et al. (2017)*. We evaluated these three cell passages specifically because both fibroblast and epithelial cells were present in the initial (first) passage, only fibroblasts were visualized at the third passage onwards, and the cells of the tenth passage were used for most of the production of embryonic clones (*Shiga et al., 1999*; *Kubota et al., 2000*). The evaluations were performed in triplicate for each animal for each passage.

Briefly, the cells were stained with 0.4% trypan blue in PBS and counted on a hemocytometer. Subsequently, the cells were also analyzed for a metabolic activity using the MTT assay, according to the method described by *Borges et al. (2018b)*. A concentration of 5.0×10^4 cells/mL from the first, third and tenth passages was grown in 12-well polystyrene plates treated for cell adhesion. After 5 days, 1.5 mL of the MTT solution (five mg/mL in DMEM) was added and the polystyrene culture dishes treated for cell adhesion were incubated for 3 h. The MTT solution was then removed and 1.0 mL of dimethyl sulfoxide (DMSO) was added for 5 min under slow stirring to solubilize the MTT. After the total dissolution of formazan crystals, samples were analyzed in a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan) at an absorbance

wavelength of 595 nm. The evaluations were performed in triplicate for each animal for each passage.

Influence of cryopreservation on the quality of fibroblast lines

To evaluate the effect of cryopreservation on the quality of fibroblast lines, cells of the third passage of the five animals were subjected to slow freezing in the freezing medium (DMEM supplemented with 10% DMSO as a permeating cryoprotectant and 10% FBS and 0.2 M sucrose as non-permeating cryoprotectants). Cells at a concentration of 5.0×10^4 cells/mL were first exposed to DMSO–FBS solution for 15 min at 4 °C, then sucrose solution was added followed by an additional incubation for 15 min at 4 °C. The cryovials containing 1.0 mL of cells in the freezing medium were cooled in a Mr. Frosty freezing container (Thermo Scientific, Waltham, MA, USA) at a cooling rate of 1 °C/min, and later stored in a freezer at −80 °C, reaching −70 °C overnight before being transferred into liquid nitrogen (*León-Quinto et al., 2014*).

For thawing, the cryovials were exposed for 1 min at 25 °C and immersed in a water bath at 37 °C for 3–4 min. Then, the cell contents were removed from the cryovials and washed to remove the cryoprotectants. Initially, the first wash was performed with DMEM and 10% FBS containing 0.2 M sucrose at 4 °C for 15 min and centrifuged. Subsequently, the second wash was performed using only DMEM and 10% FBS, maintained at 25 °C for 15 min, centrifuged, and the cells were recovered for the evaluations as per a method described previously (*Santos et al., 2016*).

After thawing, the non-cryopreserved and the cryopreserved cells were evaluated for growth dynamics by quantification of PDT. The evaluations were performed in triplicate for each animal. Briefly, the growth kinetics was studied for nine days using 3.0×10^4 cells/mL, and cells were counted daily to determine the number of growing cells. Data on the cell growth and density were monitored and recorded, mean values of which were used to plot a growth curve and calculate PDT (*Roth, 2006*) using the following formula:

PDT = $T \ln_2 / \ln (X_e/X_b)$ where PDT is the time of the culture (in hours), T is the incubation time, X_b is the number of cells at the beginning of the time incubation, X_e is the number of cells at the end of the incubation time, and ln is the Napierian logarithm.

Moreover, for evaluation of an oxidative stress by quantification of the intracellular ROS levels, cells were stained with the fluorescent probe H_2DCFDA, according to a method described by *Santos et al. (2019)*. Thawed cells were washed with PBS and placed into polystyrene culture dishes treated for cell adhesion containing 500 µL of 5 µM H_2DCFDA. The cells obtained after a 70% confluency were incubated at 38.5 °C in 5% CO_2 for 30 min. Stained cells were washed with PBS, placed on glass slides, photographed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and fluorescence signal intensity (pixels) was quantified. Ten images (two/animal) obtained were evaluated using the ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the values obtained for the treated samples. Measured mean value of the micrograph for the non-cryopreserved cells

was taken as a calibrator. Relative expression levels (arbitrary fluorescence units) were generated by dividing the measured value of each micrograph for the cryopreserved cells by the mean of the calibrator.

Finally, for the assessment of ΔΨm, cells were stained using 500 nM of the fluorescent probe MitoTracker Red® (CMXRos), according to a method described by *Santos et al. (2019)*. The procedure, incubation, and evaluation of the ten images (two/animal) were performed as described for the quantification of ROS.

Statistical analysis

All data have been expressed as the mean ± standard error (one animal/one repetition) and were analyzed using the StatView 5.0 software (Graph-Pad Software Incorporation, La Jolla, CA, USA). Normality of all results was verified by the Shapiro–Wilk test and homoscedasticity was verified by the Levene's test. ROS levels, ΔΨm, viability, and metabolic activity were altered with arcsine and analysed by variance analysis (ANOVA) followed by the Tukey's test. PDT was compared with ANOVA followed by the unpaired *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Evaluation of the somatic cells in the primary cultures and subcultures

The total culture time was 95 days with an evaluation of cells until the tenth passage. The adhesion of the fragments (Fig. 1A), detachment of cells (Figs. 1B and 1C), and proliferative capacity were observed in all the explants until reaching a confluence (and later, a subconfluence) around the adhered fragments (Figs. 1D–1F; Table 1). All explants had adhesion ability and reached subconfluence. Number of days for each explant to reach a 100% tissue adherence (2.4 days ± 0.5 days), to grow around the explants (4.6 days ± 0.7 day), and to reach subconfluence (7.8 days ± 1.0 day), were different.

Morphological characterization of the fibroblasts

In cultures, monolayers of cells with a fibroblast-like morphology were observed (Fig. 1E). The cells had an oval nuclei and extensions with a fusiform shape, showing rapid growth that replaced the epithelial cells.

Vimentin immunofluorescence

Morphology of the fibroblast-like cells in the initial culture was observed by light microscopy, which was further confirmed for the cell type identification as vimentin-labeled fibroblasts under fluorescence microscopy (Figs. 2A–2F). Cells exhibited a high expression of vimentin that marked the cytoplasm completely, and the spindle-like shape and ovoid nucleus was highlighted by the Hoechst labeling. Therefore, the identification of a fibroblast cell was evident.

Confirmation of the absence of bacterial and fungal contamination

No sign of contamination (turbidity, colony, or hyphal growth) was observed for 30 days in the culture without antibiotics and antifungals. The culture medium did not show any change in the appearance when observed under a light microscope. We did not observe

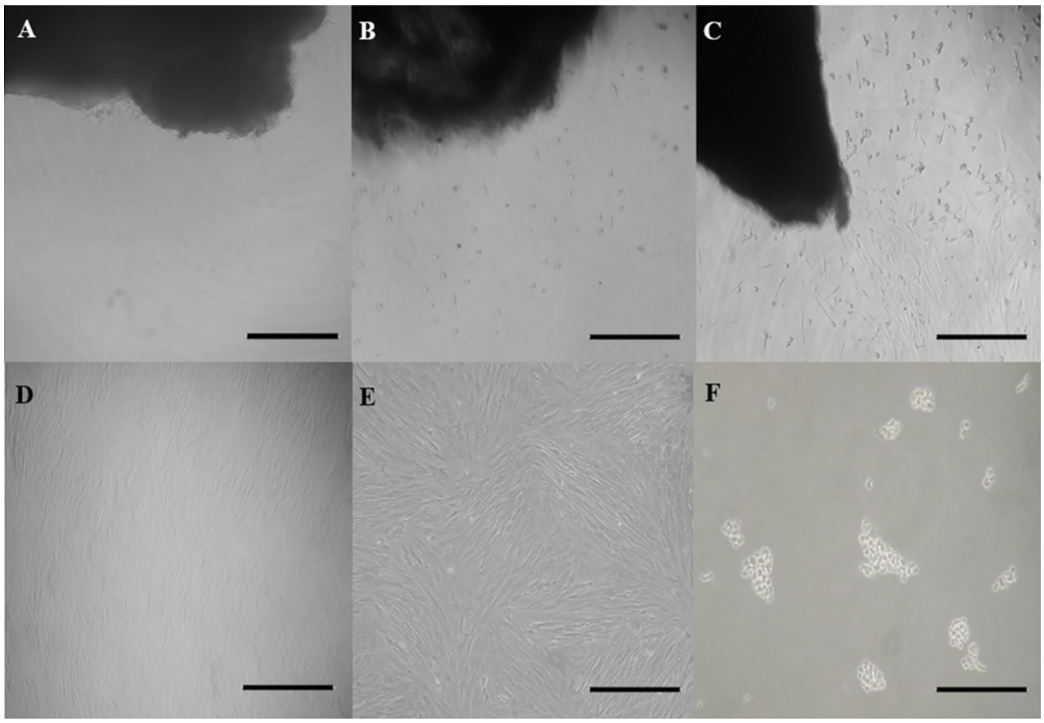

Figure 1 Outgrowth of fibroblast cells from ear skin samples of collared peccaries. Exhibit a skin explant cultured in (A) day 1, (B) day 3 and (C) day 5 of primary culture and exhibit a fibroblast population cultured in (D) day 15 and (E) day 19 of subculture and (F) exhibit cells after the trypsinization process. Scale bar = 100 μm. Full-size DOI: 10.7717/peerj.9136/fig-1

Table 1 Establishment of primary culture and subcultures of somatic cells derived from collared peccary ear skin.

Animal	No. samples			No. attached samples		
	Initial	Attached (%)	Day all attached explants	Grow to subconfluence (%)	Day all cell grow explants	Subconfluence total time (days)
F1	4	100	2	100	4	6
F2	4	100	2	100	4	5
F3	4	100	1	100	3	9
F4	4	100	3	100	5	10
M1	4	100	4	100	7	9
Mean ± S.E.	20	100	2.4 ± 0.5	100	4.6 ± 0.7	7.8 ± 1.0

turbidity or any specific odor. In addition, there was no change in the biological characteristics of growth and proliferation indicating a complete absence of contamination.

Influence of the passage number on the quality of fibroblast lines

No significant difference was observed in the cell viability (74.5–84.4%) when evaluated by trypan blue staining after the passages (first vs. third: P =0.98; first vs. tenth: P = 0.76; third

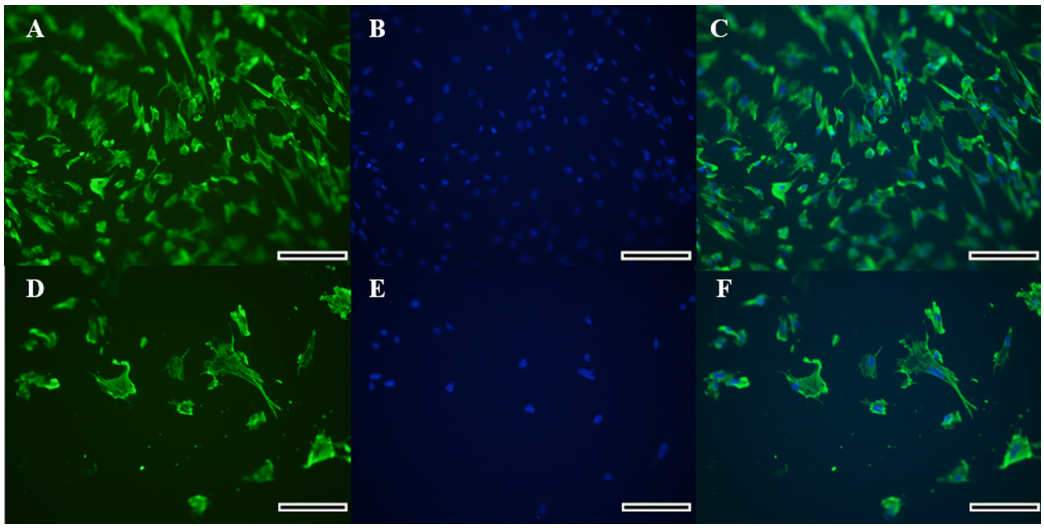

Figure 2 Immunocytochemical detection of vimetin protein for identification of collared peccary fibroblasts. (A–D) Cells stained with vimetin antibody. (B–E) Nucleus of cells stained by Hoechst. (C–F) Merged vimetin (green) and Hoechst (blue). A–C (×5), D–F (×10). Scale bar = 10 μm.
Full-size DOI: 10.7717/peerj.9136/fig-2

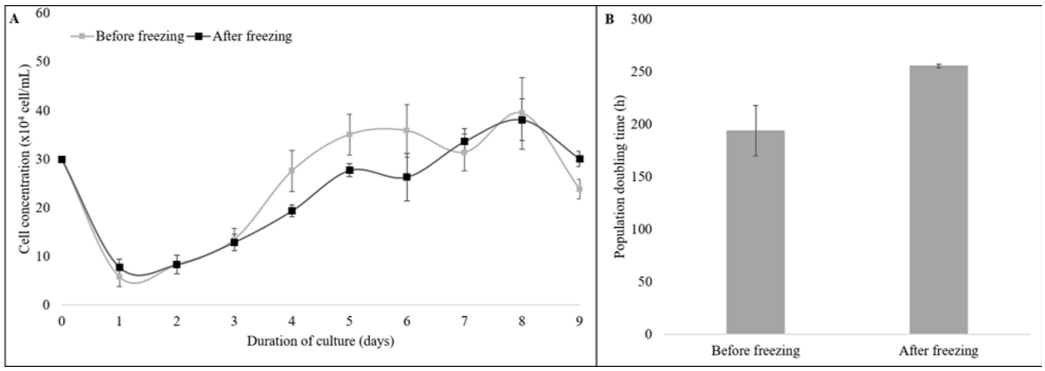

Figure 3 The growth dynamics of cryopreserved and non-cryopreserved cells derived from collared peccary skin. (A) Growth curves of cryopreserved and non-cryopreserved cells. (B) Values of population doubling time (PDT) after culture for nine days.
Full-size DOI: 10.7717/peerj.9136/fig-3

vs. tenth: $P = 0.85$). However, the metabolic activity was reduced in the tenth passage (23.2 ± 12.1%) as compared to that of the first and third passages (100.0 ± 24.4%, $P = 0.006$).

Influence of cryopreservation on the quality of fibroblast lines

Cryopreservation did not affect the viability when evaluated by trypan blue staining (87.4 ± 0.3% vs. 74.0 ± 5.9%, $P = 0.11$). Moreover, after two passages of the thawed cells, the viability was 86.4 ± 3.2%. In addition, no difference ($P = 0.77$) was observed for the metabolic activity between the cryopreserved (85.2 ± 10.0%) and the non-cryopreserved cells (100.0 ± 36.4%).

Moreover, the cryopreserved and the non-cryopreserved cells were compared for growth dynamics (Fig. 3). The growth curve of both groups showed a typical "S-shaped"

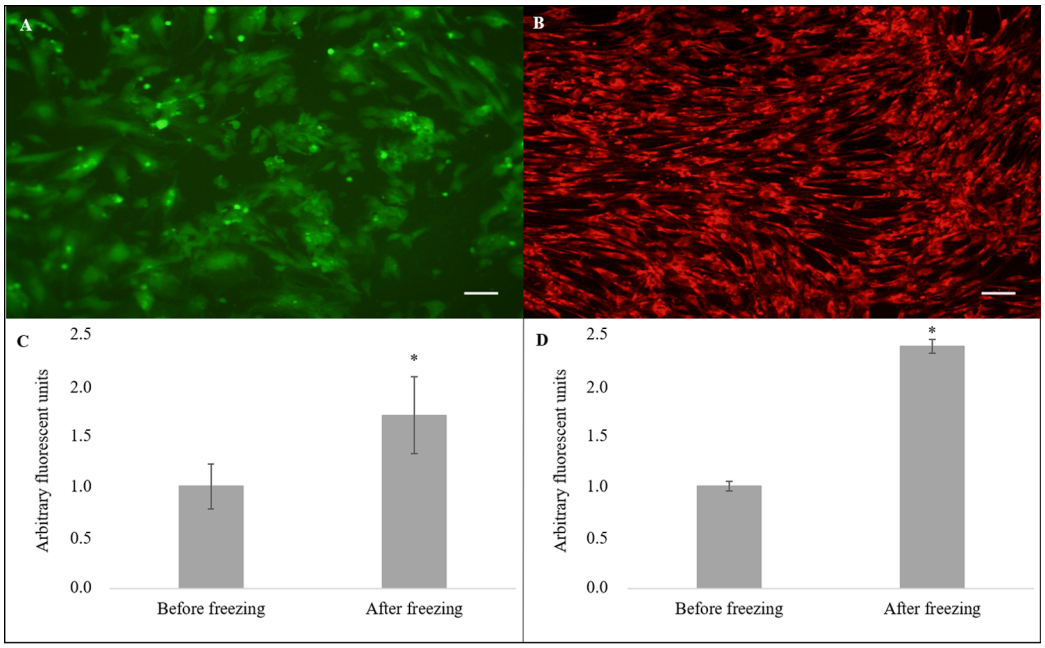

Figure 4 Evaluation of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (ΔΨm). Cell stained using fluorescent probe (A) 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and (B) MitoTrackerRed® (CMXRos) (×10). Quantification of (C) ROS and (D) ΔΨm levels. Scale bar = 10 µm. (*) Indicate statistical difference ($P < 0.05$).

Full-size DOI: 10.7717/peerj.9136/fig-4

pattern from the 9-day culture of cells. The latency time was 2 days, followed by an exponential phase until the fourth day, the stationary phase until the 7th day, and the plateau phase from day eight (Fig. 3A). No difference was observed for the PDT values of the cryopreserved and the non-cryopreserved cells ($P = 0.11$, Fig. 3B).

Nevertheless, the cryopreserved cells showed greater levels of intracellular ROS (Fig. 4A) in arbitrary fluorescence units when compared to that of the non-cryopreserved cells (1.69 ± 0.38 vs. 1.00 ± 0.22, $P = 0.04$) (Fig. 4C). In addition, an alteration in the ΔΨm (Fig. 4B) in arbitrary fluorescence units ($P = 0.0001$) was observed for the cryopreserved cells (2.37 ± 0.07) when compared to that of the non-cryopreserved cells (1.00 ± 0.05) (Fig. 4D).

DISCUSSION

In this study, we isolated, characterized, and cryopreserved the fibroblast cells derived from the skin of collared peccaries. Moreover, we established the fibroblast cell lines of these animals with an aim to use these cells in cloning experiments by a somatic cell nuclear transfer in the future. The cell line can be considered as the first constituent of the peccary invitrome and a resource for future studies in many disciplines (*Barioch, 2018*; *Bols et al., 2017*). Thus, the ear tissues of collared peccaries can be isolated and grown into fibroblasts in an adherent culture for establishment of cell lines and development of a cryobank. The development of these somatic cell banks has been increasing in the interest of conserving genetic samples of wild mammals to preserve valuable species, and as

sources for biological research (*León-Quinto et al., 2009*; *Mehrabani et al., 2014*; *Saadeldin et al., 2019*; *Siengdee et al., 2018*).

All explants adhered to the flask surface within 2–4 days, with cellular growth around the explant within 3 days, and demonstrating confluency within 5–10 days after a culture initiation. These characteristics of explants during in vitro culture were similar to the explants derived from other domestic and wild mammals. In studies using tissues from horses, the migration of fibroblast and epithelial-like cells from explants have been observed after 5–7 days of an in vitro culture (*Amoli et al., 2017*). In the case of goat-derived tissues, the explants reportedly adhered to the flasks within 5–7 days and the cells became confluent within 3–5 days post adhesion (*Bai et al., 2012*). In the Iranian Sistani cattle-derived tissues, the explants adhered to the culture flasks within 7–14 days and were observed to allow the growth of fibroblast-like cells from the margins of explants (*Gorji et al., 2017*).

For the Luxi cattle-derived tissues, fibroblast-like or epithelial-like cells could be seen migrating from the tissues within 5–12 days post adhesion (*Liu et al., 2008*). In the tissues derived from wild camels, fibroblast-like or epithelial-like cells could be seen migrating from the sides of explants within 8–10 days post adhesion (*Sharma et al., 2018*). In tissues derived from the domestic porcine, a species phylogenetically close to the collared peccaries, all the explants adhered within 3–8 days (*Silvestre, Sánchez & Gómez, 2004*). The similarity among these data can be related to the culture medium because in a majority of these studies, DMEM containing FBS, antibiotic and antimycotic solution was used (*Magalhães et al., 2017*; *Saadeldin et al., 2019*; *Siengdee et al., 2018*). Since primary culture needs to mimic the in vivo environment of the cells (*Guo et al., 2018*), we observed previously (*Santos et al., 2016*) that the medium for growth of somatic cells derived from collared peccaries was DMEM with 10% FBS and 2% antibiotic–antimycotic solution.

We showed that ear explant cultures obtained from the tissues of the collared peccaries were efficient for cell recovery allowing a culture for 95 days and up to 10 passages. Moreover, we confirmed that the ear skin was a common source where fibroblasts cells could be separated and eventually used as karyoplasts for cloning purposes (*Luo et al., 2014*). These cells were identified by vimentin, an intermediate filament that indicates the mesenchymal origin of endothelial and fibroblast cells (*Yajing et al., 2018*). Initially, during in vitro culture, epithelial and fibroblastic cells grew simultaneously. However, fibroblasts can be trypsinized more rapidly and adhere more easily as compared to the epithelial cells (*Bai et al., 2012*; *Saadeldin et al., 2019*). Therefore, in this work, cells from the third passage were confirmed as fibroblasts by morphology and immunofluorescence analyses.

The clear medium observed during the 30-day assay demonstrated the ability to allow the growth of the culture without any biological contamination. The propagation of fungi causes turbidity by accumulation of their metabolites. In addition, the colonies can be seen under a light microscope, or sometimes with the naked eye (*Li et al., 2009*). Bacterial contamination can also be identified by the naked eye as turbidity. One simple way to avoid this contamination is to filter the culture medium (*Bai et al., 2012*). Sources of

contamination may include, but are not limited to, the equipment, air, culture medium, serum and explant. Therefore, microbial contaminations are quite frequent in cell culture (*Bai et al., 2012*). Thus, the use of antibiotic and antimycotic combined with careful handling, is essential to ensure the absence of contamination.

After two passages, it was possible to separate fibroblast cells from other primary cells because different cell types exhibit different cellular behavior upon trypsinization. The fibroblast cells detach quicker than epithelial cells (*Gorji et al., 2017*). However, fibroblasts detach in response to trypsin more rapidly than epithelial cells and adhere more quickly (*Bai et al., 2012*). In wild camels, the initial lag phase of 48 h representing the adaptation of fibroblasts and recovery from a protease damage is followed by the exponential phase (*Sharma et al., 2018*). In collared peccaries, the replication of cells begins to slow down after 7 days because of contact inhibition, which, in wild camels, has been observed after 6 days (*Sharma et al., 2018*). No difference in the cell viability was observed among the first, third and the tenth passage, corroborating with the studies that used cells from these passages for production of competent cloned embryos (*Shiga et al., 1999*; *Kubota et al., 2000*).

In contrast, through the metabolic activity test evaluated by the formation of formazan crystals, a significant reduction in the metabolic activity at the tenth passage was observed, indicating a reduced cellular functionality. Similar behavior was observed in cells from the brown brocket deer in which the metabolic activity measured by the MTT assay showed significantly lower values in the tenth passage than the values in the fourth passage (*Magalhães et al., 2017*). Therefore, the number of passages can reduce the metabolic activity rate and cell proliferation, thereby conserving cells of the early passages (*Li et al., 2009*). After several passages, genetic characteristics of the cells can be modified by culture conditions; hence, a minimum number of passages have been recommended to conserve the cellular characteristics (*Mehrabani et al., 2014*). Owing to this reason, the cells were cryopreserved in the third passage for the conservation of the somatic germplasm of collared peccaries.

The cell survival rate after thawing is the most commonly used criteria to evaluate the success of a cryopreservation (*Chatterjee et al., 2017*). The cellular viability and the functional metabolic activity of the cells were maintained after thawing the fibroblasts isolated from the collared peccaries. This factor demonstrates that optimal in vitro culture conditions significantly influence the recovery from cellular damages caused by the freezing process (*Gorji et al., 2017*). As for the growth curve, cryopreserved cells presented a very similar profile to that of the non-cryopreserved cells, showing their normal proliferation capacity regardless of the cryopreservation process. The establishment of somatic cell banks using cryopreservation technology is an easy and effective approach towards storing the genetic information of diverse species (*Li et al., 2009*). However, the cells should be handled with the utmost care during cryopreservation to maintain a high-quality cell bank in the long term (*Mehrabani et al., 2014*).

Moreover, epigenetic alterations, such as DNA fragmentation, free radical accumulation, ionic imbalances, apoptosis, biochemical alterations, DNA methylation and

histone modification can be a result of the cryopreservation (*Chatterjee et al., 2017*). These after-effects of cryopreservation may have caused mitochondrial structural abnormalities, thereby promoting an increased ROS production and H_2O_2 content, increased lipid peroxidation and increased expression of autophagic proteins harbored by the cells (*Mata et al., 2012*). A failure in the mitochondrial membrane potential is a hallmark of apoptosis, leading to the collapse of the organelle and release of cytochrome-C into the cytoplasm and ultimately activation of the apoptotic cascade (*Magalhães et al., 2012*). Moreover, a high ΔΨm mitochondrial respiratory chain becomes a significant ROS producer (*Korshunov, Skulachev & Starkov, 1997*). Therefore, a higher ΔΨm in cryopreserved cells can be linked mainly to an increase in the oxidative stress.

Finally, parameters like cryovariables, including cooling and thawing rates, type and concentration of the cryoprotectant, cell type and shape and nucleation temperature may affect the success of cryopreservation (*Chatterjee et al., 2017*). This suggests that the optimization of related cryopreservation methods for the collared peccary fibroblasts to minimize an altered ΔΨm and increased levels of intracellular ROS production is essential.

CONCLUSIONS

To our knowledge, this study is the very first report on a successful isolation, characterization and cryopreservation of fibroblast lines derived from adult collared peccaries (Ptskf). We showed that the adherent culture was efficient for obtaining fibroblasts, which can be used as donor cells for nuclei for cloning of this species. Moreover, it was possible to maintain the viability of the cells until the tenth passage. In addition, cryopreservation did not affect the viability, metabolic activity and proliferative activity of the fibroblasts after slow freezing. However, cryopreservation altered the ROS levels and ΔΨm, indicating necessary optimization of the cryopreservation protocol. Lastly, the establishment of fibroblast cell lines derived from collared peccaries may be a source of experimental models for many biological studies such as nuclear reprogramming and animal cloning.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Alana Azevedo Borges conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Gabriela Pereira de Oliveira Lira performed the experiments, prepared figures and/or tables, and approved the final draft.
- Lucas Emanuel Nascimento performed the experiments, prepared figures and/or tables, and approved the final draft.
- Maria Valéria de Oliveira Santos performed the experiments, prepared figures and/or tables, and approved the final draft.
- Moacir Franco de Oliveira conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Alexandre Rodrigues Silva conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Alexsandra Fernandes Pereira conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2).

Data Availability

The following information was supplied regarding data availability:

The raw data are available in a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.9136#supplemental-information.

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ANEXO C – VERSÃO DO ARTIGO PUBLICADO NA CRYO-LETTERS

CRYOPRESERVATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) SOMATIC CELLS IS IMPROVED BY SUCROSE AND HIGH CONCENTRATIONS OF FETAL BOVINE SERUM

QUALIS: Quadriênio 2013-2016: B1/ Novo Qualis: A2

FATOR DE IMPACTO: 0,694

CryoLetters **41 (5)**, 271-279 (2020)

CRYOPRESERVATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) SOMATIC CELLS IS IMPROVED BY SUCROSE AND HIGH CONCENTRATIONS OF FETAL BOVINE SERUM

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Abstract

BACKGROUND: The formation of somatic cell banks is affected by, amongst other factors, the cryoprotectant solution used. The selection of an effective solution, therefore, is a primary parameter. **OBJECTIVE:** We optimized the cryoprotectant used for collared peccary somatic cell cryopreservation. **MATERIALS AND METHODS:** We categorized cells into different groups based on their cryopreservation and evaluated the morphology, viability, proliferative activity, metabolism, and oxidative stress. One group was cryopreserved in 10% DMSO with 10% fetal bovine serum (DMSO-10FBS), and another with 50% FBS (DMSO-50FBS). The cryopreservation of both groups included the presence of 0.2 M sucrose (DMSO-SUC-10FBS and DMSO-SUC-50FBS). Non-cryopreserved cells and cells cryopreserved with 10% DMSO (DMSO) supplemented with 0.2 M sucrose (DMSO-SUC) were used as controls. **RESULTS:** There was no difference observed in morphology or viability among the groups. Proliferative activity was reduced in DMSO-10FBS when compared to controls. Although cryopreservation reduced metabolism, no difference was observed among solutions. A lower level of reactive oxygen species was observed in cells of DMSO-SUC-50FBS when compared to other cryoprotectants. Only cells of DMSO-SUC-50FBS had mitochondrial potential similar to non-cryopreserved cells. **CONCLUSION:** 10% DMSO supplemented with 50% FBS and 0.2 M SUC was observed to be the most efficient cryoprotectant for preserving collared peccary somatic cells.

Keywords: Peccaries, extracellular cryoprotectants, slow freezing, cryobanking.

INTRODUCTION

Collared peccaries (*Pecari tajacu* Linneaus, 1758), also known as “wild pigs”, are wild mammals belonging to the Tayassuidae family of the order Artiodactyla (21). These animals play an important role in maintaining the global ecosystem where they act as seed dispersers, promote flora renewal, and contribute to the food chain (34). Although globally classified as “Least Concern”, this species is already extinct in eastern and southern Argentina and is declining in some biomes (9), thus necessitating conservation strategies for the maintenance of its population.

One of the conservation tools applied to wild mammals has been the formation of somatic resource banks (6). These banks allow

long-term storage of tissues and somatic cells to be employed in different proposals, such as multiplying individuals by somatic cell nuclear transfer (SCNT) (43) and generating induced pluripotent cells (41). The efficiency of these procedures depends on the quality of cells after slow freezing, which is dependent on the choice of the cryoprotectant, a crucial step for the success of these cryobanks (28). In general, an effective cryoprotectant is the appropriate combination of intracellular cryoprotectants such as dimethyl sulfoxide (DMSO) (25), ethylene glycol (EG) (16), and extracellular cryoprotectants such as sucrose (SUC) (23) and/or fetal bovine serum (FBS) (15).

Specifically, our group has successfully established somatic tissue banks of collared pecarries based on our knowledge of the tissues (2), techniques (3), and solutions required (4, 5). Subsequently, we initiated somatic cell banks of this species, demonstrating that 10% DMSO in the presence of 0.2 M SUC was more efficient in somatic cell slow freezing when compared to EG with 0.2 M SUC, showing a viability of 69.8% and 58.5%, respectively (17). Thus, one way to improve the rates of cell recovery would be to optimize the extracellular agents employed. Studies have shown that FBS, when associated with 10% DMSO and 0.2 M SUC, promoted a beneficial effect in some species (*Lynx pardinus* and *Elephas maximus*) (15, 36). This agent can reduce oxidative stress, caused by excessive production of reactive oxygen species (ROS) during slow freezing (11).

Nevertheless, variations in FBS concentration can be observed in some species with its use at 10% (*Felis silvestris líbica*) (8) and 50% (*Bubalus bubalis*) (18), necessitating the evaluation of the most appropriate concentration, as well as its relationship with SUC. Therefore, we aimed to optimize the cryoprotectant by varying the different extracellular combinations of SUC and FBS. We assessed the impact of the cryoprotectants on the morphology, viability, proliferative activity, metabolism, and oxidative stress of cryopreserved collared peccary cells.

MATERIALS AND METHODS

Chemicals and media

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), FBS, trypsin-EDTA, Antibiotic-Antimycotic (ATB-ATM) solution and fluorescent probes were obtained from Gibco-BRL (Carlsbad, CA, USA). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was purchased from Grainer (Miami, FL, USA).

Compliance with ethical standards and animals

The experimental protocols and animal handling procedures were performed with the approval of the Animal Ethics Committee of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92), in compliance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2). A total of four adult collared peccaries, 41.5 ± 1.8 months old, provided by the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W) were used.

Skin biopsy, primary culture, and subcultures

Sections (1-2 cm^2) used for collared peccary identification were taken from peripheral ear tissue using pliers. After collection, tissue samples were washed in 70% ethanol (38) and incubated in DMEM supplemented with 10% FBS and 2% ATB-ATM solution at 37 °C for 30 min.

In the laboratory, tissues were trichotomized, washed in 70% ethanol followed by DMEM supplemented with 10% FBS and 2% ATB-ATM solution. Subsequently, tissue samples were fragmented in 9.0 mm^3 sections and cultured in DMEM supplemented with 10% FBS and 2% ATB-ATM solution at 38.5 °C in an incubator with 5% CO_2 and 95% air (31). During the primary culture, the medium was changed every 24 h. Cells were subcultured after reaching 70-80% confluence until the third passage, followed by cryopreservation. The subconfluence of 70-80% was defined when 70-80% of the Petri dishes presented somatic cells (31).

Study design and cell cryopreservation

The morphology, viability, proliferative activity, metabolism, and oxidative stress were evaluated in non-cryopreserved and cryopreserved cells. Cells were cryopreserved in either 10% DMSO with 10% FBS (DMSO-10FBS group), or 50% FBS (DMSO-50FBS group). In both the groups, 0.2 M SUC was added and these were further designated as the DMSO-SUC-10FBS and DMSO-SUC-50FBS groups, respectively. Moreover, non-

cryopreserved cells (non-cryopreserved group), those cryopreserved in 10% DMSO (DMSO group) and those cryopreserved with 10% DMSO supplemented with 0.2 M SUC (DMSO-SUC group), were used as controls.

For cryopreservation, cells were subjected to slow cooling in freezing medium (DMEM supplemented with 10% DMSO and extracellular cryoprotectants according to experimental groups). Briefly, cells at a concentration of 1.0×10^4 cells/mL were first exposed to DMSO solution for 15 min at 4 °C in DMEM, followed by the addition of SUC and FBS solutions according to experimental groups and incubated for an additional 15 min at 4 °C. The cryovials containing 1.0 mL of cells in freezing solution were cooled in a Mr. Frosty freezing container (Thermo Scientific Nalgene, Rochester, NY, USA) at a cooling rate of 1 °C/min, and kept in a freezer -80 °C until reaching -70 °C, before being transferred to liquid nitrogen (15).

After 2 weeks, the cryovials were removed from liquid nitrogen and kept at room temperature of 25 °C for up to 1 min and then warmed in a water bath at 37 °C for 3-4 min. For the removal of cryoprotectants, the cells were washed twice with DMEM. Cell suspension was centrifuged for 10 min at 400×g and washed again with DMEM and centrifuged according to León-Quinto et al. (15). In the groups containing SUC, the cells were washed with DMEM with 0.2 M SUC, prior to centrifugation as previously described.

Evaluation of morphological characteristics and cell viability

The cells' morphology was evaluated daily using an inverted microscope (Nikon TS100, Tokyo, Japan). The following morphological characteristics were assessed: size, aspect, shape and adhesion (29). The viability analysis was performed by cells stained with trypan blue. For each animal and each group, the viability test was performed in duplicate. Briefly, the cells were centrifuged and suspended in 1.0 mL of the cell culture medium (DMEM supplemented with 10% FBS and 2% ATB-ATM solution); an aliquot of cells was stained with 0.4% trypan blue (in phosphate buffered saline, PBS) in the ratio 1:1 and counted in a Neubauer chamber (39).

Analysis of proliferative activity and metabolism

Proliferative activity was quantified according to the population doubling time (PDT), which was calculated by seeding cells at a density of 1.0×10^4 cells/well in a 24-well plate. Cells were trypsinized in duplicate and counted at 24 h intervals for up to 216 h of culture. The mean cell counts were recorded every time and the cell growth curve was delineated. Finally, the PDT was calculated using the following formula (30):

$$PDT = T \ln 2 / \ln (Xe/Xb),$$

where PDT is the time of the culture (in hours), T is the incubation time, Xb is the number of cells at the beginning of the time incubation, Xe is the number of cells at the end of the incubation time, and ln is Napierian logarithm.

For evaluation of metabolism, cells were seeded into 12-well dishes at a density of 5.0×10^4 cells/mL and cultured at 38.5 °C in a humid atmosphere with 5% CO_2 (4). After five days, MTT solution (5 mg/mL in DMEM) was added to each well for 3 h under the same conditions. After incubation, the MTT solution was removed, and DMSO was added to each dish to solubilize the formazan crystals. Finally, the absorbance (595 nm) of the samples was read using a spectrophotometer (Shimadzu® UV-mini-1240, Kyoto, Japan). The mean values of non-cryopreserved cells were considered 100% as compared to the other groups.

Assessment of oxidative stress

Cellular oxidative stress was evaluated by quantification of intracellular reactive oxygen species (ROS) levels and measurement of mitochondrial membrane potential ($\Delta\Psi m$). Briefly, intracellular levels of ROS were quantified using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) as a fluorescent probe, according to Santos et al. (32).

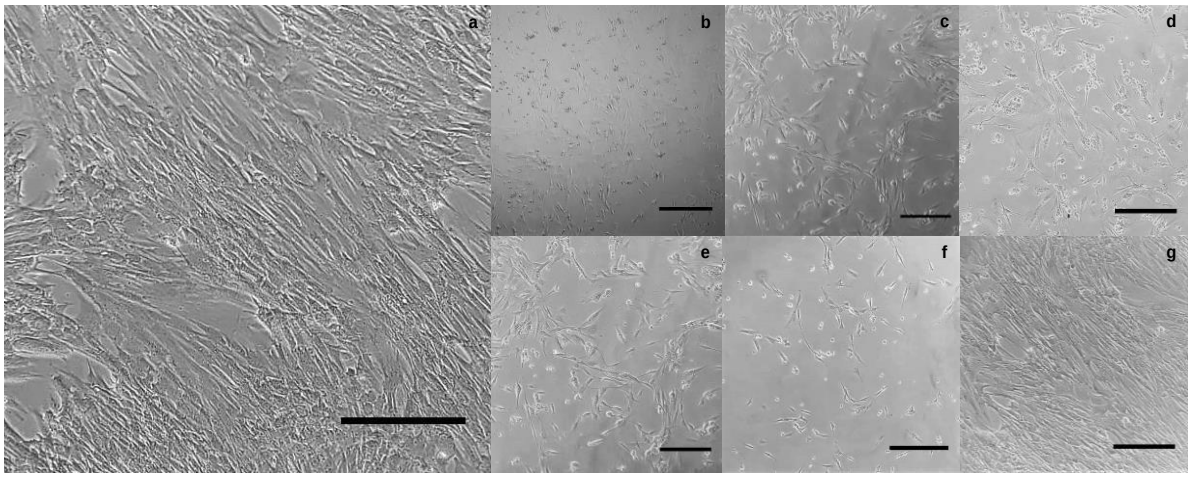

Figure 1. Subcultures of fibroblast-like cells from ear skin samples of collared peccaries. **a)** Non-cryopreserved cells and cell cryopreserved in **b)** DMSO, **c)** DMSO-SUC, **d)** DMSO-10FBS, **e)** DMSO-50FBS, **f)** DMSO-SUC-10FBS, **g)** DMSO-SUC-50FBS. Scale bar = 100 µm.

For this, the cells, independent of the experimental group, were seeded in glass slides and grown in vitro until reaching 70% confluence. Subsequently, cells were washed in PBS and incubated with 1.0 mL 5 µM H_2DCFDA at 38.5 °C with 5% CO_2 for 30 min protected from light. After this period, the stained cells were washed twice in PBS and imaged using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The intensity of the signal fluorescence (pixels) was measured using ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the values obtained for the treated samples. The measured mean value of the micrograph for non-cryopreserved cells was used as a calibrator. Relative expression levels (arbitrary fluorescence units) were generated by dividing the measured value of each micrograph for the cryopreserved cells by the mean of the calibrator.

The ΔΨm was assessed using the fluorescent probe MitoTracker Red® (CMXRos) at 500 nm according to Santos et al. (32). The procedure, incubation and evaluation of the images, were performed as described for the quantification of ROS.

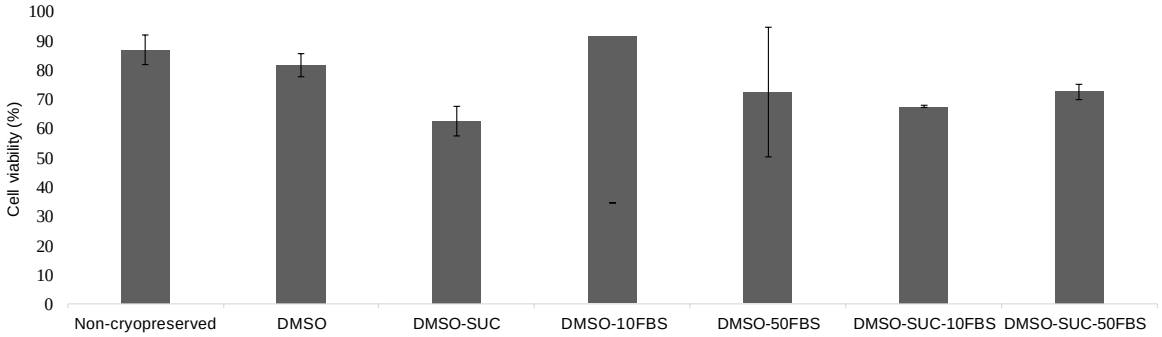

Figure 2. Viability of collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). $P > 0.05$.

Statistical analysis

Data were expressed as mean ± standard error (one animal/one repetition) and analyzed using the GraphPad software (Graph-Pad Software Incorporated, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by Tukey test. Significance was set at $P < 0.05$.

RESULTS

Evaluation of morphological characteristics and cell viability

All cells derived from collared peccary tissue had consistent morphological characteristics in all experimental groups (Fig. 1a-g). Slow cooling did not alter the capacity of the cells to grow and reach 70-80% confluence, presenting cells with similar morphology to fibroblasts; with fusiform aspect, cytoplasmic prolongations, abundant cytoplasm and central nucleus, and with an overall large and oval morphology.

Moreover, no difference was observed among groups for cell viability evaluated by trypan blue (Fig. 2, $P > 0.05$). Thus, while non-cryopreserved cells had a viability of 87.0 ± 5.2%, cryopreserved cells of different groups showed viabilities in the range of 62.5 ± 5.2% to 91.7 ± 0.0%.

Analysis of proliferative activity and metabolism

Although the growth curve did not show a typical "S" shape in all cryopreserved groups, it was possible to observe from the shape of the curve the occurrence of the initial adaptation phase of the cells, followed by exponential growth until reaching the stable phase of decline (Fig. 3a). In addition, non-cryopreserved cells

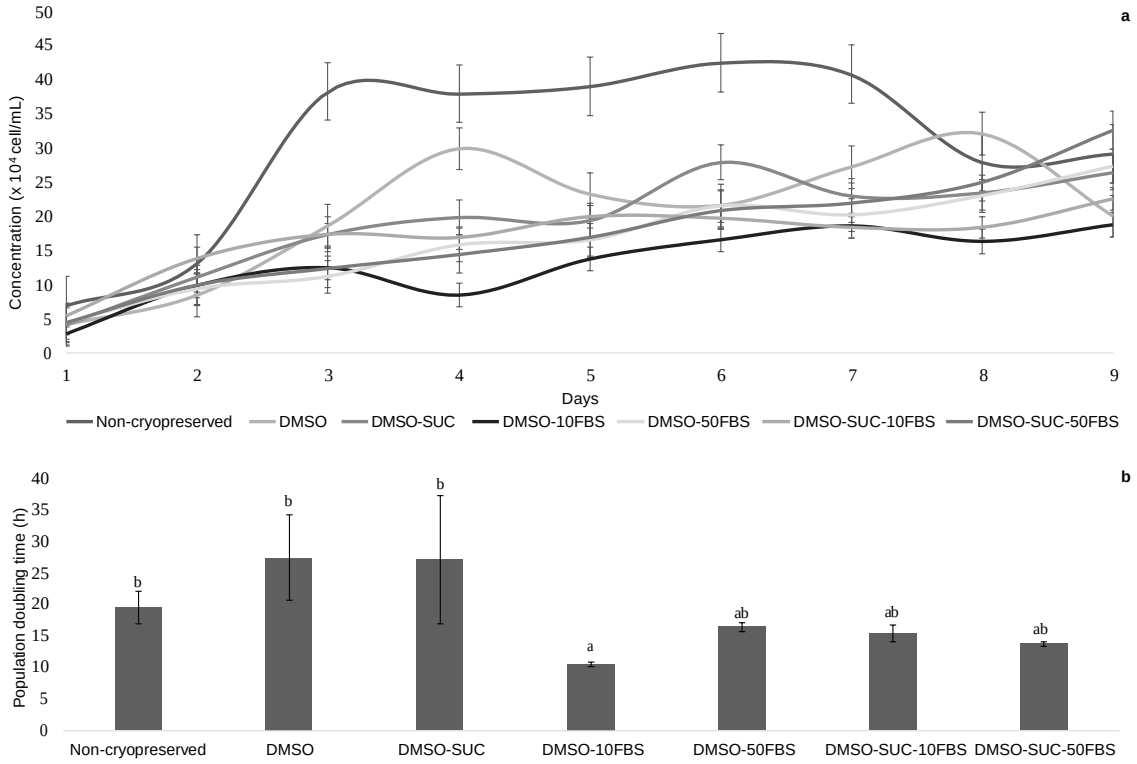

Figure 3. The growth dynamics and metabolism of collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. **a)** Growth curves of cryopreserved and non-cryopreserved cells. **b)** Values of population doubling time (PDT) after culture for nine days. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). Different letters show significant differences among the groups ($P < 0.05$). Bars indicate standard error.

showed a reduction in their growth; however, this reduction stabilized from day 8. Thereafter the decline in the curve was no longer observed, which showed that there was no cell death only the decrease of proliferative activity of cells from this group. Additionally, the proliferative activity evaluated by population doubling time was reduced in cells derived from DMSO-10FBS group (10.5 h ± 0.3) when compared to non-cryopreserved cells (19.4 ± 2.6, Fig. 3b)

Although cryopreservation reduced cell metabolism, no difference was observed among cryoprotectant solutions ($P > 0.05$). Thus, while cryopreserved cells had a metabolic activity of 100.0 ± 0.0%, cryopreserved cells among the different groups varied among 63.6 ± 22.6% and 97.7± 25.1%.

Assessment of oxidative stress

A lower level of ROS was observed in cells derived from DMSO-SUC-50FBS when compared to other cryopreserved groups (Fig 4a and 4c).

Moreover, only cells from the DMSO-SUC-50FBS group had mitochondrial membrane potential similar to the non-cryopreserved cells (Fig 4b and 4d).

DISCUSSION

The successful formation of a bank of biological resources is correlated to the efficiency of the cryopreservation protocol used. As such, the initial combination of intracellular and extracellular cryoprotectants is key in ensuring cell quality after thawing. In this study, we showed that the combination of 0.2 M SUC with 50% FBS added to the cryoprotectant solution containing 10% DMSO, ensured a better quality of the recovered collared peccary somatic cells, particularly by reducing the oxidative stress caused by slow freezing and by maintaining normal cellular characteristics. Therefore, our results demonstrated three relevant points of cryopreservation of somatic cells: (a) the cells obtained from collared peccary tissue showed characteristics similar to fibroblast cells used as the main donor nucleus in SCNT; (b) some alterations resulting from the cryopreservation may not be observed from the analysis of the viability and metabolic activity of the thawed cells; therefore, more specific assessments such as the analysis of the oxidative stress may be necessary; and (c) the favorable effect of the addition of 50% FBS on the cryoprotectant solution containing 10% DMSO and 0.2 M SUC. Thus, these findings ensure that

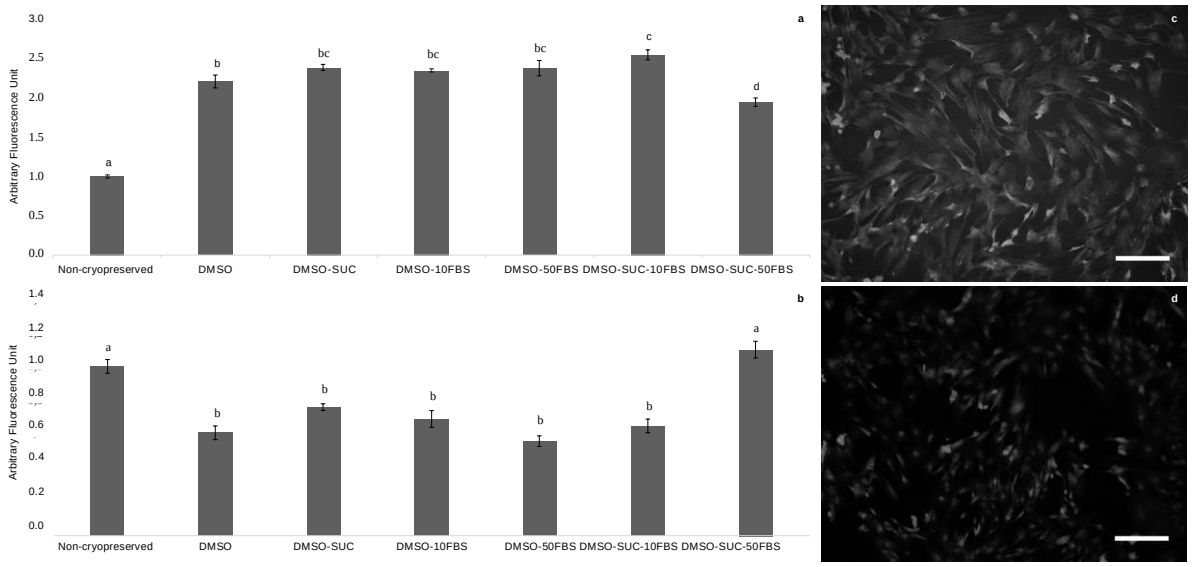

Figure 4. Assessment of oxidative stress in collared peccary non-cryopreserved and cryopreserved cells using different extracellular cryoprotectants. Non-cryopreserved group, 10% DMSO (DMSO group), 10% DMSO and 0.2 M SUC (DMSO-SUC group), 10% DMSO and 10% FBS (DMSO-10FBS group), 10% DMSO and 50% FBS (DMSO-50FBS group), 10% DMSO, 0.2 M SUC and 10% FBS (DMSO-SUC-10FBS group), 10% DMSO, 0.2 M SUC and 50% FBS (DMSO-SUC-10FBS group). **a)** Quantification of intracellular reactive oxygen species (ROS) levels **b)** Measurement of mitochondrial membrane potential (ΔΨm). **c)** Cells labeled with H_2DCFDA for quantification of ROS levels. **d)** Cells labeled with MitoTracker Red® for measurement of ΔΨm. Different letters within each analysis show significant differences among the groups ($P < 0.05$). Scale bar = 200 µm.

higher quality cells are employed in future conservation strategies.

In general, cryopreservation protocols can induce lesions such as lipid peroxidation of membranes and damage to DNA in fibroblasts, resulting from the formation of ROS (33). Moreover, the natural antioxidant defense mechanism of the cells is not enough to eliminate excess ROS (11). Therefore, studies have shown that supplementation with antioxidants and/or factors that modulate the cell death process caused by excess ROS may be a potential solution to reduce oxidative stress induced by cryopreservation (14). Additionally, a decrease in ΔΨm has been observed in cells with excess ROS formation (13). Thus, as suggested by Poot et al. (27), we used a fluorescent probe that has an affinity for molecules present in cells. Such probes are responsible for evaluating the relationship among mitochondria and the triggering of apoptotic mechanisms. With this, it was possible to demonstrate that among the cryoprotectant solutions tested, the cells obtained from the DMSO-SUC-50FBS treatment showed high ΔΨm after thawing.

Thus, one compound that has such antioxidant potential is FBS, which has been frequently used in cryoprotectant solutions (12). In general, FBS is routinely added to stabilize cell membranes and adjust intra- and extracellular osmotic pressure. However, the role of this cryoprotectant as an antioxidant has become increasingly evident, which may be related to its constituents, mostly amino acids (20). This could be due to the synergistic effect of their constituents, which may have attached to cell membranes, promoting stabilization and avoiding excessive concentration of solutes that may be related to ROS formation (12). In addition, their constituents may be responsible for the increased activity of antioxidant intracellular enzymes (26). Thus, it is likely that the FBS in the DMSO-SUC-FBS solution acted as a source of antioxidants. However, the mechanisms underlying the antioxidant role of FBS under the formation of ROS suffered by cells during cryopreservation are still unclear. Moreover, when the cells were cryopreserved with 10% FBS (DMSO-10FBS and DMSO-SUC-10FBS groups), the fluorescence intensity increased, indicating an increase in ROS production. This suggests that the reduction and/or absence of antioxidative agents in the DMSO, DMSO-10FBS and DMSO-SUC-10FBS groups may be associated with the formation of ROS (24, 42, 44).

This result may be due to FBS counteracting the cytotoxicity induced by DMSO, as demonstrated by Miki et al. (22), when analyzing the ΔΨm of stem cells subjected to a cryoprotectant solution with 10% DMSO in association with 90% FBS, which resulted in a ΔΨm of 1.00. Therefore, treatments with 10% FBS caused the cells to be more exposed to cytotoxicity caused by DMSO during the freeze-thaw cycle. The DMSO penetrates the cell membrane and forms hydrogen bonds with the water molecules present in the cytosol. Thus, the freezing point of the water decreases, and there is a lower probability of the formation of ice crystals inside the cell. Furthermore, the integrity of the membrane is conserved and for this reason, DMSO is an indispensable intracellular cryoprotectant. However, even though its positive effect is observed, its toxicity remains high, suggesting its association with extracellular cryoprotectants such as SUC and FBS. This association can accelerate the cellular dehydration process and balance cytotoxicity. Therefore, the ideal concentrations for achieving this cytotoxic balance must be established so that the concentrations of extracellular cryoprotectants are sufficient to minimize the negative effect of the high toxicity of DMSO. Additionally, the presence of SUC can promote osmotic maintenance of the medium and perform synergistically with the intracellular cryoprotectant, resulting in improved effects when used in combination (37).

The ROS levels of the DMSO-SUC-10FBS group were higher in comparison to the DMSO group, probably due to the use of more cryoprotective agents in their composition. It should be borne in mind that such cryoprotective agents (DMSO-SUC-10FBS), despite promoting cell protection, present a certain toxicity that may be responsible for greater oxidative stress and subsequent increased production of ROS. However, this does not mean that the use of fewer cryoprotective agents is recommended, but rather that a combination promoting balance between the concentration used, toxicity, protection of cells at cryogenic temperatures and lower production of ROS, should be utilized.

In addition, the morphological and confluence aspects observed in the collared cells were similar to those observed by Queiroz Neta et al. (29) and Borges et al. (4) describing collared peccary cells recovered from

refrigerated and vitrified tissues, respectively. Thus, proper maintenance of the morphological characteristics of cultured cells may be associated with a decrease in ice crystal formation due to the cryopreservation process, which when exposed to cells, may have deleterious effects on cell characteristics (15).

The viability data showed that cryopreserved cells and non-cryopreserved cells were stable regardless of treatments. This can be attributed to the quality of the 10% DMSO slow freezing technique that is commonly associated with better results for somatic cells derived from wild mammals (23, 35).

Further, the higher viability rates and the in vitro culture performance of cells shown in this study, corroborate the findings of Subramanian et al. (40) and Barnes et al. (1). These authors affirmed that cultures derived from cryopreserved and non-cryopreserved cells have a similar growth performance, since the cell population after culture can recover with rates similar to non-cryopreserved cells.

Finally, all groups showed no change in PDT values except DMSO-10FBS. In general, cells cultured in specific treatments including FBS in their composition required less time to replicate (19). Our PDT rates and metabolism activity evaluated by MTT may be associated with the use of an adequate system of cryopreservation that promoted an optimum cooling rate (10) associated with compounds present in FBS such as transport proteins, adhesion proteins, enzymes, hormones, growth factors, cytokines, fatty acids, lipids, vitamins, carbohydrates, and nitrogen of non-protein origin (7).

In conclusion, 10% DMSO supplemented with 0.2 M SUC and 50% FBS was the most efficient cryoprotectant solution for conserving collared peccary somatic cells. This work presents a breakthrough for the improvement and establishment of an ideal freezing protocol for collared peccary somatic cells that can be used in future biotechnical techniques such as cloning, since each stage involved should be optimized for the species studied.

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ANEXO D – VERSÃO DO ARTIGO PUBLICADO NA PESQUISA VETERINÁRIA BRASILEIRA

IN VITRO MATURATION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) OOCYTES AFTER DIFFERENT INCUBATION TIMES

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In vitro maturation of collared peccary (*Pecari tajacu* Linnaeus, 1758) oocytes after different incubation times[1]

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ABSTRACT.- Borges A.A., Santos M.V.O, Queiroz Neta L.B, Oliveira M.F., Silva A.R. & Pereira A.F. 2018. [***In vitro* maturation of collared peccary (*Pecari tajacu* Linnaeus, 1758) oocytes after different incubation times.] Maturação *in vitro* de oócitos de cateto (*Pecari tajacu* Linnaeus, 1758) após diferentes períodos de incubação**. *Pesquisa Veterinária Brasileira 38(9):.* Universidade Federal Rural do Semi-Árido, Av. Francisco Mota 572, Mossoró, RN 59625-900, Brazil. E-mail: alexsandra.pereira@ufersa.edu.br

Oocyte *in vitro* maturation (IVM) is the first step of the *in vitro* reproductive technologies that enables mature oocytes to be generated *ex vivo* and after used for embryo production. In this sense, the establishment of culture environment, as oocyte incubation time, is essential for the success of the IVM. Therefore, the study was carried out to investigate the relationship between the meiotic potential and the IVM times of collared peccary oocytes, wild mammals of great commercial and ecological interest. Thus, ovaries were collected of females derived from captivity and transported to the laboratory within 1 hour of slaughtering. The oocytes derived from follicles (3-6mm in diameter) were recovered by aspirated and sliced. Good quality oocytes (evenly granulated cytoplasm with a least one layer of surrounding *cumulus* cells) were selected and subjected to culture in TCM 199 supplemented with 10µg/mL FSH, 10% FBS and 100µM cysteamine at 38.5°C, 5% CO_2 and maximum humidity for 24 or 48 hours. After the incubation period, the nuclear status, the presence of first polar body and the expansion of *cumulus* cells of oocytes were assessed. The data obtained were analyzed by Fisher exact test ($P<0.05$). A total of four sessions (2-3 females per session) were performed, resulting in eighteen aspirated and sliced ovaries with normal morphological characteristics. An oocyte recovery rate of about 83.1% (59/71) was obtained with 3.3 oocytes/ovary and 2.3 viable oocytes/ovary. After different incubation times, differences ($P<0.05$) were observed in 24 and 48 hours for expansion of the *cumulus* cells (38.1% *vs.* 100%), presence of first polar body (52.4% *vs.* 90.5%) and nuclear status in second metaphase (19.0% *vs.* 76.2%), respectively. In conclusion, 48 hours is suitable time for the *in vitro* maturation of oocytes derived from collared peccaries when compared to the time of 24 hours, according to the meiotic potential observed. Additional studies should be conducted to improve the quality of the oocyte culture environment, as medium composition, aiming to obtain viable mature oocytes for other *in vitro* biotechnologies.

INDEX TERMS: Collared peccary, *Pecari tajacu,* oocytes, incubation, reproduction, meiotic competence, nuclear maturation, wild mammals.

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RESUMO.- [**Maturação *in vitro* de oócitos de cateto (*Pecari tajacu* Linnaeus, 1758) após diferentes períodos de incubação**.] A maturação *in vitro* (MIV) oocitária é a primeira etapa das tecnologias reprodutivas *in vitro* que permite que oócitos maturados sejam gerados *ex vivo* e depois usados para a produção de embriões. Nesse sentido, o estabelecimento do ambiente de cultivo, como o período de incubação de oócitos, é essencial para o sucesso da MIV.

Portanto, o estudo foi realizado para investigar a relação entre o potencial meiótico e os períodos de MIV de oócitos derivados de catetos, mamíferos selvagens de grande interesse comercial e ecológico. Para tanto, os ovários foram coletados de fêmeas derivadas de cativeiro e transportados ao laboratório dentro de 1 h após o abate. Os oócitos derivados de folículos (3-6mm de diâmetro) foram recuperados por aspiração e fatiados. Oócitos de boa qualidade (citoplasma uniformemente granulado com pelo menos uma camada circundante de células *cumulus*) foram selecionados e submetidos ao cultivo em TCM 199 suplementado com 10µg/mL de FSH, 10% de SFB e 100µM de cisteamina a 38,5°C, 5% de CO_2 e umidade máxima por 24 e 48 h. Após o período de incubação, o estado nuclear, a presença do primeiro corpúsculo polar e a expansão das células do *cumulus* dos oócitos foi avaliada. Os dados obtidos foram analisados pelo teste exato de Fisher (P<0,05). Um total de quatro sessões (2-3 fêmeas por sessão) foi realizado, resultando em dezoito ovários aspirados e fatiados com características morfológicas normais. Uma taxa de recuperação oocitária de aproximadamente 83,1% (59/71) foi obtida com 3,3 oócitos/ovário e 2,3 oócitos viáveis/ovário. Após diferentes períodos de incubação, diferenças (P<0,05) foram observadas entre 24 e 48 h para a expansão das células *cumulus* (38,1% *vs.* 100%), presença de primeiro corpúsculo polar (52,4% *vs.* 90,5%) e estado nuclear na segunda metáfase (19,0% *vs.* 76,2%), respectivamente. Em conclusão, 48 h é o período adequado para a maturação *in vitro* de oócitos derivados de catetos quando comparado ao tempo de 24 h, de acordo com o potencial meiótico observado. Estudos adicionais devem ser conduzidos para melhorar a qualidade do ambiente de cultivo oocitário, como a composição de meio, objetivando obter oócitos maturados viáveis para outras biotecnologias *in vitro*.

TERMOS DE INDEXAÇÃO: Maturação, oócitos, cateto, *Pecari tajacu,* incubação, reprodução, competência meiótica, maturação nuclear, mamíferos silvestres.

INTRODUCTION

Oocyte *in vitro* maturation (IVM) is the first step of the *in vitro* reproductive technologies that enables mature oocytes to be generated *ex vivo* (Rahman et al. 2008) and further destined to systems of *in vitro* embryo production (IVEP), as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). All these biotechniques are interesting tools for applying the reproductive management (Kumar et al. 2016) and biodiversity conservation (Moulavi et al. 2017).

The capability of embryonic development depends on successful IVM, since it stimulates the oocyte to achieve the meiotic competence required (Lee et al. 2014) through the nuclear and cytoplasmic events that promote the synthesis of RNA and proteins (Sirard et al. 2006). Therefore, the IVM quality is a determinant factor for the success of the IVEP (Zhang et al. 2015, Park et al. 2017). In this sense, the establishment of culture environment, as oocyte incubation time, should be clarified, once all IVM events require a specific time that could vary according to the peculiarities of each species (Higaki et al. 2017).

The optimization of IVM conditions for collared peccaries is interesting under various concerns. Although this species is not threatened (IUCN, 2015), their phylogenetic proximity to endangered species as the *Catagonus wagneri* (Keuroghlian et al. 2014), as well as the commercial importance on their meat and leather (Nogueira-Filho et al. 2004), have been leading to the development of studies focused on their reproductive physiology (Maia et al. 2014, Miranda-Moura et al. 2016). Based on these studies, it was possible to know that specifically for females, they have symmetrical and oval ovaries with a relatively smooth surface, which can present irregular appearance due to corpus luteum or follicles (Garcia et al. 2009). Moreover, the ovaries are enveloped by an ovarian bursa (Guimarães et al. 2012), as also observed in swine (Męczyński 1974). Additionally, collared peccaries' estrous cycle lasts 21 days, with a follicular phase of 6 days and 15 days for the luteal phase (Maia et al. 2014). On the other hand, these individuals have a lower ovulation rate (2.3 follicles) when compared to swine (Sowls 1997) and usually produce 1.7-1.9 offspring per female after 141-151 days of gestation (Mayor et al. 2005).

Therefore, in view of the need for studies related to the reproductive biotechniques in collared peccaries, the initial point has been to observe reproductive relationship with close domestic phylogenetic species, as swine and others artiodactyls. Thus, similarities were observed for folliculogenesis (Guimarães et al. 2012), hormonal profiles (Ahuja-Aguirre et al. 2017) and follicular histological characteristics (Lima et al. 2013) between collared peccaries and swine and/or artiodactyla species, as buffalos.

In this context, IVM time used for buffalo oocytes is of 24 h (Kadoom et al. 2014), while maturation time of 48 h is used for porcine oocytes (Pereyra-Bonnet et al. 2008). Moreover, it had observed that a suboptimal culture environment for porcine oocytes may promote nuclear maturation without the structures reaching cytoplasmic maturation (Abeydeera 2002), confirming that the IVM time to be precise and defined (Sirard et al. 2006). Therefore, this is first study was carried out to investigate the relationship between the meiotic potential and the IVM times of collared peccary oocytes. This information will be the first about the IVM of oocytes derived from collared peccary, aiming to direct pathways to obtain an embryo *in vitro* of this species. This step can serve as a basis for both enlightening studies of embryogenesis and physiological aspects of the animal. In addition, it may be the starting point for advanced biotechnology that can increase zootechnical potential and ways to ensure effective tools that can be used for conservation of phylogenetically near species.

MATERIALS AND METHODS

Animal ethics and care. The experiment was conducted in accordance with the Committee for Ethics in Animal Use of Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 23091.001072/2015-92) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2). The animals used in this research belong to the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil, 5°10'S, 37°10'W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding site (no. 1478912). The breeder contains 200 collared peccaries on average. Annually, a programmed slaughter is conducted for population control and the biological material is destined for several experiments.

Reagents and media. All reagents and media used were obtained from Sigma-Aldrich (St. Louis, MO, USA), but fetal bovine serum

(FBS) was obtained from Gibco-BRL (Carlsbad, CA, USA). When necessary, the pH was adjusted to 7.2-7.4 for all media. Media were filtered using 0.22µm system (Corning, NY, USA).

Oocyte collection, selection and *in vitro* maturation. Ovaries were obtained from nine adult female collared peccaries (age 4-10 years). Eighteen ovaries were removed from the ovarian bursa and transported to the laboratory in 0.9% (w/v) NaCl solution at 37°C for up to 1 h. Antral follicles (3-6 mm) were aspirated with 21 gauge needle attached to a 5.0 mL syringe. After the aspiration, for a greater oocyte recovery, ovaries were sliced with a scalpel blade in 100x20 mm plates. All follicular fluid was pooled in conical tubes for ten minutes. The sediment was diluted in the oocyte collection medium composed of TCM 199 culture medium (M2520) supplemented with 2.2g/L bicarbonate (S5761), 0.2 mM sodium pyruvate (P45622), 1% antibiotic-antimycotic solution (A5955) and 10% FBS (12657-029). Thus, *cumulus*-oocyte complexes (COCs) were selected according to the number of *cumulus* cells layers and homogeneity of the cytoplasm, being classified in four grades. Grade 1 (3-5 *cumulus* cells layers and homogeneous cytoplasm); Grade 2 (1-2 *cumulus* cells layers and homogeneous cytoplasm); Grade 3 (partially denuded cytoplasm and slightly heterogeneous); Grade 4 (denuded and heterogeneous cytoplasm), according to Kumar et al. (2016). Only grade 1 and 2 COCs were considered viable to be matured.

After three washes in oocyte collection medium, the COCs were incubated in 100 µL drops of maturation medium composed of TCM 199 with 2.2g/L bicarbonate, 0.2mM sodium pyruvate, 1% antibiotic-antimycotic solution and supplemented 10µg/mL follicle stimulating hormone (Folltropin®-V, Bioniche, Canada), 100µM cysteamine (M9768) and 10% FBS. All drops were covered with mineral oil and cultured at 38.5°C in humidified atmosphere of 5% CO_2 and divided in two groups, 24 and 48 h.

Assessment of *in vitro* maturation. After the IVM, COCs were evaluated by the analysis of *cumulus* cells expansion using a stereomicroscope at 20-40x magnification. Thus, oocytes with *cumulus* cells were considered matured (Appeltant et al. 2015). Subsequently, *cumulus* cells were removed by pipetting the oocytes in oocyte collection medium and denuded structures were verified for the presence of the first polar body under stereomicroscope at 20-40X magnification. Thus, oocytes with first polar body were considered matured (Pereyra-Bonnet et al. 2008).

The nuclear status of oocytes was determined by marking it with Hoechst 33342 (B2261). For this, denuded oocytes were fixed in paraformaldehyde buffered in 4% phosphate buffered solution (PBS) for 30 min and then washed in PBS with 0.4% bovine serum albumin (BSA). Afterward, Hoechst 33342 (10µg/mL) labeling was performed for 15 min and exposed under UV light in fluorescence microscope. Finally, oocytes presenting nucleus in metaphase II (metaphase plate, MII) were considered matured and those in other nuclear phases (prophase I, metaphase I, anaphase I and telophase I) were considered immature (Appeltant et al. 2015).

Data analysis. Data were obtained from four sessions, with 2-3 females per session, totaling in nine experimental animals. For statistical analysis, the Graphpad Instat 3.06 software (GraphPad Software Inc., La Jolla, CA, USA) was used. Mean numbers in percentage ± standard error of the matured oocytes in two different times (24 *vs*. 48 h) were compared using Fisher exact test ($P<0.05$) for expansion of *cumulus* cells, presence of the first polar body and nuclear status.

RESULTS

Under gross evaluation, the bursa surrounded all peccaries' ovaries, which were symmetric, presenting an oval shape. Moreover, they had a uniform appearance on most of its surface (Fig.1A); nevertheless, some elevations were observed derived from large follicles or the presence of corpus luteum (Fig.1B).

Afterward all sessions, a total of 18 ovaries resulted in 59 immature oocytes from follicles aspirated and sliced follicles with an estimated recovery rate of 83.1% (approximately, 59/71; oocytes recovered/follicles aspirated) and 3.3 oocytes per ovary. Of these, 42 structures (71.2%, 42 structures viable/59 recovered structures) were classified as viable according to the number of *cumulus* cells layers and homogeneity of the cytoplasm, resulting in 2.3 viable oocytes per ovary, (Fig.2A).

After the IVM, the time of 48 h was more adequate than 24 h for the IVM in all evaluated parameters, as *cumulus* cells

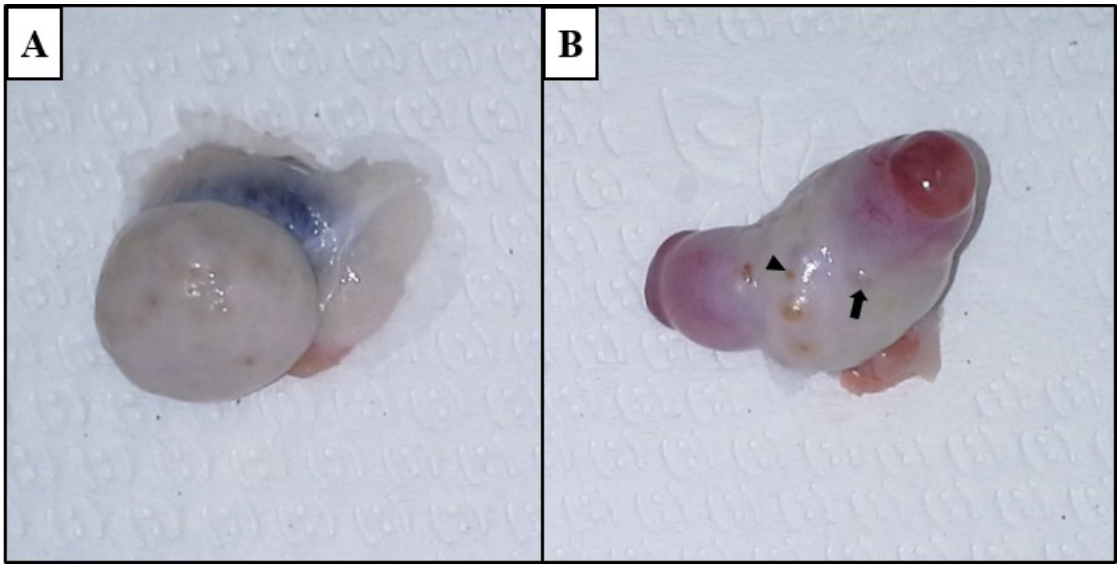

Fig.1. Macroscopic aspects of collared peccary ovaries. (**A**) Uniform appearance with small follicles. (**B**) Presence of large follicles and corpus luteum. The arrowhead indicates a small follicle and arrow shows a large follicle.

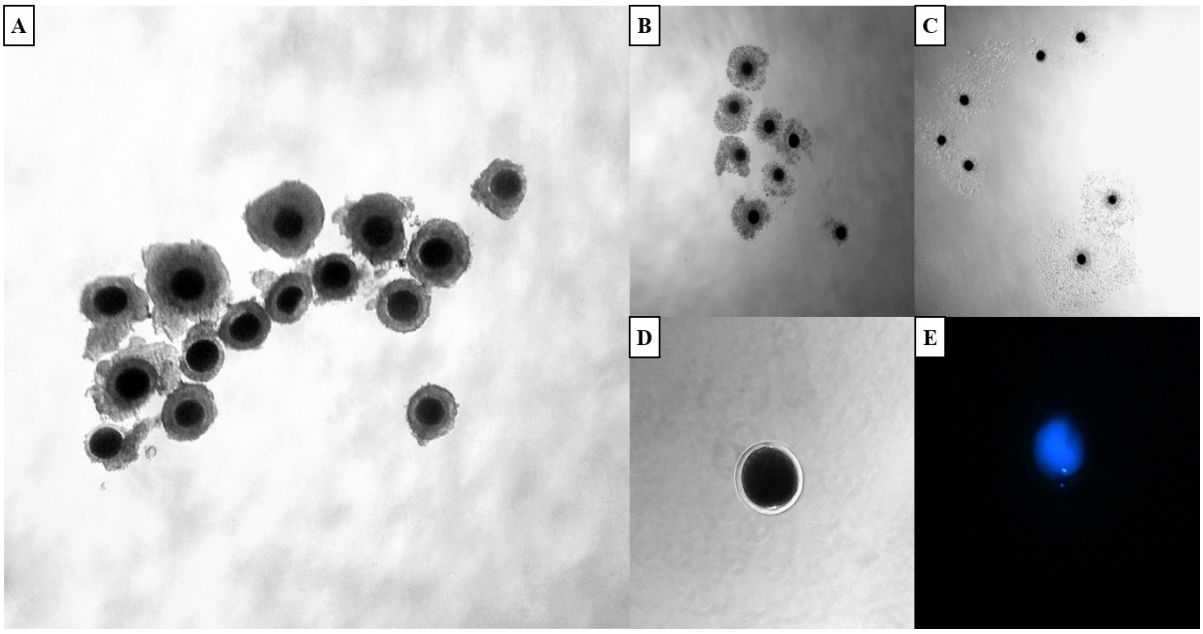

Fig.2. *In vitro* maturation (IVM) of oocytes of collared peccary. (**A**) Immature oocytes before IVM. Obj.10x. (**B**) *Cumulus* cell expansion after 24 h IVM, Obj.10x. (**C**) *Cumulus* cell expansion after 48 h IVM. Obj.10x. (**D**) Oocyte with extrusion of fist polar body. Obj.40x. (**E**) Oocyte in MII stage. Obj.40x.

Table 1. *In vitro* maturation of collared peccary oocytes after different incubation times

Evaluation of *in vitro* maturation	Incubation times (in hours)	
	24	48
Expansion of *cumulus* cells	38.1 ± 1.4 (8/21)[b]	100.0 ± 0.0 (21/21)[a]
Presence of the first polar body	52.4 ± 2.2 (11/21)[b]	90.5 ± 2.0 (19/21)[a]
Nuclear status in second metaphase	19.0 ± 1.4 (4/21)[b]	76.2 ± 1.3 (16/21)[a]

Mean in % ± standard error (*n).* [a,b] In same row differ (P<0.05).

expansion (P=0.0002), presence of the first polar body (P=0.0148) and nuclear status in second metaphase (P=0.0003) (Table 1). Moreover, a 100% *cumulus* cells expansion was observed for oocytes cultured for 48 h (Fig.2C) when compared to 24 h (Fig.2B). Additionally, oocytes with fist polar body (Fig.2D) and MII presence (Fig.2E) were evidenced in more of 50% and 19% of the oocytes, respectively.

DISCUSSION

The results indicated that the most appropriate incubation period for collared peccaries' oocytes achieve meiotic competence was 48 h. This time is similar to that observed for porcine oocytes (Pereyra-Bonnet et al. 2008). In this sense, we can infer that similar follicular characteristics described between collared peccaries and swine (Lima et al. 2013) justify this relationship, since the *in vitro* conditions for maturation should mimic the *in vivo* conditions that occur in the females.

Moreover, other peculiar feature of collared peccary oocytes similar to the porcine oocytes is the presence of cytoplasmic lipid droplets (Lima et al. 2013). This aspect is crucial for the establishment of the appropriate requirements for IVM, evidencing that further IVM protocols should be adapted from porcine to peccaries' oocytes. Nevertheless, a factor that still needs to be further investigated by the similarity that collared peccary oocytes had in relation to porcine oocytes is that the IVM in swine oocytes have their altered medium to adapt to the variable requirements of IVM (Grupen 2014) and this step may improve the quality of matured oocytes derived from collared peccaries. Thus, the tracks of such similarities between peccaries and porcine oocytes indicate a starting point, but not the direct application of protocols without further experimentation, considering that differences occur between these species for follicular morphology (Lima et al. 2013).

On the other hand, the follicular development in collared peccaries was also similar to that found for other artiodactyl species regarding the stages of folliculogenesis, i.e. recruitment, selection and dominance (Guimarães et al. 2012) when compared to buffalos (Manik et al. 2002) and camelids (Basiouni 2007). Nevertheless, despite this similarity, the 24 h IVM time that is frequently used for buffaloes (Kadoom et al. 2014) was not suitable for oocyte maturation in the peccaries. The IVM time to guarantee the formation of the metaphase plate is essential to ensure that the oocytes possess the ability to develop quality embryos (Grupen 2014). In this sense, the 24 h of IVM for collared peccary oocytes was not long enough for the organization of the metaphase plate (19.0%), while the majority of oocytes were able to reach metaphase II in 48 h of IVM (76.2%). As developmental competence is also reached in cytoplasmic maturation, which occurs after meiotic maturation and refers to the ability of the oocyte to

be fertilized and to block polyspermy, this type of maturation can be visualized by migration of the cortical granules to the oocyte periphery and by the expansion of *cumulus* cells (Milakovic et al. 2015). The analysis of *cumulus* cells expansion at 24 and 48 h showed different degrees of expansion (38.1% *vs.* 100.0%, respectively); these may be related to cytoplasmic maturation, being an indicator of successful maturation (Abeydeera 2002). Therefore, 24 h of IVM is not sufficient for IVM of collared peccary oocytes (Fig. 2B).

Several important factors for the IVM success are dependent of the incubation time, which can influence the final quality of the embryonic development (Miyoshi et al. 2002). The signaling pathway of 3',5' cyclic adenosine monophosphate (cAMP) is highly time-dependent and restricted to time mechanisms, which is what triggers germinal vesicle breakdown, so nuclear maturation depends on cAMP so the oocyte can reach metaphase II (Sirard et al. 2006). Likewise, another time dependent factor is the secretion of steroid hormones by COCs derived small and large follicles during IVM. Topfer et al. (2016) observed that oocytes derived from small follicles have a lesser ability to support estradiol synthesis in the first half of IVM (28 h) and to suppress progesterone synthesis at the end of IVM (48 h) compared with oocytes from large follicles. Thus, the steroid hormone secretion by COCs is time-dependent and therefore, the meiotic maturation time should to be precise and defined (Sirard et al. 2006, Topfer et al. 2016).

Moreover, the *cumulus* cells play a bidirectional role for oocyte development. One of the factors that contribute to this role is their mitochondria and mitochondrial DNA, besides contributing to the passage of nutrients and growth factors to the oocyte (Pawlak et al. 2016). Additionally, it is known that the *cumulus* cells play a regulatory role for cAMP, showing great relevance for oocyte maturation (Grupen 2014). Therefore, the clear expansion of *cumulus* cells with 48 h (Fig. 2C) in this study ensured the optimal performance of the cells during IVM. Further adjustments of cAMP levels during maturation may improve fertilization capacity and oocyte development (Appeltant et al. 2015).

Finally, other factors, as follicles size and technique employed for oocyte collection, may influence the IVM conditions (Sun et al. 2001). In this study, 3–6 mm follicles aspirated and matured for 48 h allowed the obtaining of oocytes to reach meiotic competence. Moreover, the recovery rate obtained can be due to the use of two methods of oocytes collection, first the follicular aspiration and then the slicing technique. The slicing technique allows the increase oocyte recovery, especially when there is limited availability of ovaries (Ferraz et al. 2016). Moreover, viability rate of 71.2% can be attributed to the good development of immature oocytes in the follicular environment (Grupen 2014) and the efficiency of the methods for oocyte recovery.

In conclusion, 48 h IVM for collared peccary is the most suitable incubation period for oocytes reaching the meiotic competence when compared to 24 h time. This was the first study with *in vitro* maturation, in an attempt to improve a protocol of IVEP for this species. Although this time has been defined in the present work, further details for the improvement of IVM, as medium composition, should be considered not only to achieve meiotic competence but also to guarantee appropriate embryonic development.

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ANEXO E – VERSÃO DO ARTIGO PUBLICADO NA THERIOGENOLOGY

PRODUCTION OF COLLARED PECCARY (*Pecari tajacu* LINNAEUS, 1758) PARTHENOGENIC EMBRYOS FOLLOWING DIFFERENT OOCYTE CHEMICAL ACTIVATION AND *IN VITRO* MATURATION CONDITIONS

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Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions

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ABSTRACT

To optimize the protocols for assisted reproductive techniques (ARTs) in collared peccary (*Pecari tajacu* Linnaeus, 1758), we evaluated various conditions for oocyte *in vitro* maturation (IVM) and chemical activation. Initially, we assessed the IVM rates, *cumulus*-oocyte complex (COC) quality, and oocyte morphometry in the absence or presence of epidermal growth factor (EGF). There was no difference between the COCs matured in absence or presence of EGF for the expansion of *cumulus* cells (97.6% ± 1.2 *vs.* 100% ± 0.0), presence of first polar body (65.9% ± 1.2 *vs.* 70.5% ± 1.8), nuclear status in second metaphase (62.5% ± 11.6 *vs.* 68.4% ± 4.9), cytoplasmic maturation (100.0% ± 0.7 *vs.* 75.0% ± 0.7), reactive oxygen species levels (0.5 ± 0.2 *vs.* 0.3 ± 0.1), and mitochondrial membrane potential (1.1 ± 0.2 *vs.* 1.1 ± 0.1). However, the zona pellucida thickness of matured COCs was reduced in the presence of EGF. Thus, the EGF group was used for further experiments. The oocytes were artificially activated with ionomycin and four secondary activator combinations [6-dimethylaminopurine (6D), 6D and cytochalasin B (6D + CB), cycloheximide (CHX), and CHX and CB (CHX + CB)]. The effect of immature COCs based on *cumulus* cell layers and cytoplasm homogeneity (GI and GII or GIII COCs) on embryonic development and quality was evaluated. There was no difference in the cleavage rates among the groups of secondary activators. The cleavage rates of embryos derived from GI/GII and GIII COCs were greater than 72.2% and 25.0%, respectively. Moreover, treatment with CHX showed a reduction in the cleavage rate of embryos derived from GIII COCs when compared to the cleavage rate of embryos derived from GI/GII COCs ($P < 0.05$). Nevertheless, higher rates of blastocyst/total GI and GII COCs were observed in the 6D group (27.6% ± 0.3) compared to CHX group (6.9% ± 0.3). Additionally, only 6D treatment resulted in the production of embryos derived from GIII COCs (25.0% ± 0.2). The percentage of the ICM/total cell ratio was also greater in blastocysts derived from 6D (42.5% ± 19.0), 6D + CB (37.9% ± 21.9), and CHX + CB (43.8% ± 19.6) groups when compared to CHX (3.6% ± 0.1) group. Thus, the combination of ionomycin and 6D could produce collared peccary embryos by activation of both GI/GII COCs and GIII COCs. These optimized IVM conditions using EGF and chemical activation using ionomycin and 6D in collared peccaries form the first steps for establishing ARTs to conserve this species.

1. Introduction

In the last decade, a rapid decline in the population of various mammalian species was reported in the South American countries [1]. The collared peccary (*Pecari tajacu* Linnaeus, 1758), a wild pig, is found only in the Americas and is classified as the Least Concern

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species [2]. However, the species is already extinct in eastern and southern Argentina and is declining in some biomes, such as Caatinga and the Atlantic forest [1]. Generally, this species plays an important role in the ecosystem as a seed dispersing agent. Thus, the maintenance of these animals in captivity is essential for their sustainable use and conservation [3].

The collared peccary populations can be conserved through assisted reproductive techniques (ARTs), such as cloning by somatic cell nuclear transfer (SCNT) and *in vitro* fertilization (IVF). Cloning by SCNT enables the conservation of species as it can be used for increasing the species population [4], basic research [5], and obtaining induced pluripotent cells [6]. Therefore, establishment of all the stages involved in SCNT can aid in the optimal use of this technology for species conservation [5].

There are various steps involved in cloning via SCNT. *In vitro* maturation (IVM) and artificial oocyte activation are the fundamental steps for the development of SCNT technique to obtain cytoplasmic donor cells (or cytoplast) and evaluate their developmental competence. Recently, we had demonstrated that the collared peccary *cumulus*-oocyte complexes (COCs) require maturation times of greater than 24 h for complete meiotic development, which resulted in an IVM rate of 76.2% [7]. However, developmental competence was not evaluated in this study. It is important to understand the development of *in vitro*-matured oocytes to blastocysts.

Moreover, the optimization of IVM medium composition can potentially result in higher IVM rates. In some mammals (swine [8]; sheep [9]; canine [10]), supplementation of IVM medium with 10 ng/mL of epidermal growth factor (EGF) increases the meiotic development and subsequent embryonic development. In pigs, EGF along with gonadotropins enhances oocyte nuclear and cytoplasmic maturation [11]. Although this domestic species is phylogenetically close to collared peccary [12], there are differences in the reproductive features between these species, such as ovulation rate [13,14] and litter size [14]. Although swine and collared peccary are phylogenetically close, they exhibit substantial phylogenetic distance. Thus, direct extrapolations between the collared peccary and swine were not possible. Therefore, we aimed to specifically evaluate the effects of EGF on the collared peccary oocyte development. Additionally, different species may exhibit differential responses, as observed in the collared peccary semen cryopreservation. Treatment with 20% low-density lipoproteins (LDL) improved post-thaw sperm motility and plasma membrane integrity of collared peccaries [15], whereas treatment with LDL at concentrations above 10% did not result in a beneficial effect on porcine semen cryopreservation [16].

To the best of our knowledge, we are not aware of any published reports on the establishment of oocyte artificial activation protocols in collared peccaries. Artificial activation of oocytes is also a critical step of SCNT [5] and the activation procedures vary among species (bovine and equine [17]; goat [18]; swine [19]). This indicates that the response to these artificial activation protocols also may vary depending on the species. Previously, Campos Junior et al. [20] artificially activated the collared peccary oocytes using ionomycin and 6-dimethylaminopurine (6D), which resulted in 10% four-cell embryo stage. Increasing the number of oocytes and optimizing the artificial activation protocols may potentially promote higher rates of embryonic development.

An efficient artificial activation protocol promotes *in vitro* embryonic development competencies by decreasing the level of the maturation promoter factor (MPF) through Ca^{2+} oscillation [18]. Thus, different chemical activation methods may be employed using ionomycin as the primary activator, which allows Ca^{2+} transport and induces calcium influx by activation of endogenous entry pathways [21]. Moreover, primary activation can be used in combination with secondary activators, such as protein phosphorylation inhibitors (6D) that inhibit the protein kinase activity and promote mitosis, and protein synthesis inhibitors (cycloheximide or CHX) that inhibit the production of cyclin B, which is a regulatory component of MPF [22]. Additionally, cytochalasin B (CB) has been used to prevent the fragmentation of embryos [23] and increase the rate of diploid embryos as well as to effectively inhibit the secondary polar body extrusion [24].

Generally, COCs used for IVM and chemical activation are selected based on *cumulus* cell layers and cytoplasm homogeneity (Grade I to IV). Only GI and GII COCs are used for IVM, which are considered more appropriate according to morphological classification described above. However, due to the reduced availability of oocytes in wild mammals, evaluation of development in GIII COCs is important to increase the quantities of oocytes in the subsequent stages of SCNT. In some mammals, similar molecular patterns were observed between GI/GII and GIII COCs [25,26]. Thus, this study aimed to evaluate various conditions for IVM and chemical activation of collared peccary oocytes.

2. Materials and methods

All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (Opinion N° 23091.001072/2015–92) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, N° 48633–2). Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Cumulus-oocyte complex (COC) collection

Twenty collared peccaries (n = 20) aged 25.1 ± 2.5 months housed at the Centre of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10′S, 37°10′W) and registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, N° 1478912) were used in this study. The animals were stimulated with 600 IU PG600® (400 IU eCG and 200 IU hCG, MSD Saúde Animal, São Paulo, SP, Brazil) before COC collection, following the methods of Peixoto et al. [27] with modifications. At day 4 post-hormone administration, the animals were euthanized and the ovaries were excised. The ovaries were transported to the laboratory within 1 h and maintained in saline solution (NaCl, 0.9%) supplemented with 0.05 mg/mL penicillin at 35–37 °C.

At the laboratory, all visible follicles were classified as small (<0.3 cm), medium (0.3–0.5 cm), or large (>0.5 cm) [28]. The follicles with 0.2–0.6 cm diameter were aspirated for COC collection using a 21 G needle attached to a 5.0 mL syringe containing COC collection medium (CCM; TCM199 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum (FBS, serum (FBS, Gibco-BRL), and 1% antibiotic-antimycotic solution).

2.2. COC classification and IVM

The COCs were observed under a stereomicroscope and classified into four grades following the classification system proposed by Kumar et al. [29], which was based on the number of *cumulus* cell layers and homogeneity of the cytoplasm: Grade I (3–5 *cumulus* cells layers and homogeneous cytoplasm); Grade II (1–2 *cumulus* cells layers and homogeneous cytoplasm); Grade III (partially denuded and slightly heterogeneous cytoplasm); Grade IV (denuded and heterogeneous cytoplasm). Only Grade I/II and Grade III COCs were used for IVM in separate drops, according to the experimental design.

The COCs were divided into drops (15–30 COCs per 100 μL) and

the drops were covered with mineral oil. The COCs were incubated for 44 h at 38.5 °C and 5% CO_2 in a humidified atmosphere. The IVM medium comprised CCM supplemented with 20 μg/mL FSH/LH (Pluset®, Hertape Calier, Juatuba, MG, Brazil), 100 μM cysteamine, and an absence or presence of 10 ng/mL EGF.

2.3. Evaluation of cumulus cells

After IVM, *cumulus* cell expansion was evaluated using a stereomicroscope. The COCs exhibiting *cumulus* cell expansion were considered mature. The *cumulus* cell expansion was scored as follows: score 4, total expansion; score 3, partial expansion; score 2, slight expansion; score 1, minimum expansion; score 0, no expansion [30].

The *cumulus* cells were removed by successive pipetting. The viability of *cumulus* cells was evaluated by staining the cells with trypan blue (0.2%) for 2 min. The viable cells were unstained, whereas the non-viable cells were stained blue. All cells were counted in the 4 outer quadrants of the Neubauer chamber [31].

2.4. Assessment of nuclear maturation

The denuded oocytes were observed under a stereomicroscope to assess the presence of first polar body (1 PB). The oocytes exhibiting 1 PB were considered mature. To assess the nuclear stage, denuded oocytes were fixed in 4% paraformaldehyde prepared in phosphate buffer saline (PBS) for 30 min. Next, the oocytes were washed with PBS containing 0.4% bovine serum albumin (BSA) fraction V. The cells were then stained with Hoechst 33342 (10 μg/mL) for 15 min and observed under a fluorescent microscope. The oocytes with nucleus at metaphase II (metaphase plate, MII) stage and 1 PB were considered mature, while those in other nuclear phases were considered immature.

2.5. Quantification of intracellular ROS levels by a dichlorofluorescein assay

For evaluation of oocyte quality after IVM, reactive oxygen species (ROS) levels in the oocytes were determined using the fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Carlsbad, CA, USA), according to Santos et al. [31]. Briefly, denuded oocytes were washed twice with PBS and incubated in 500 μL of 5 μM H_2DCFDA at 38.5 °C and 5% CO_2 for 30 min. The stained oocytes were washed twice with PBS and imaged using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The fluorescence signal intensity (pixels) was quantified using the ImageJ software (version 1.49v, Java 1.8.0_201, Wayen Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; website: http://rsb.info.nih.gov/ij/download.html). The background signal intensity was subtracted from the fluorescent intensity values of the treated oocytes. Immature oocytes were assessed as the calibrator. The relative expression levels were calculated by dividing the measured value of each treatment micrograph with the mean value of the calibrator.

2.6. Assessment of mitochondrial membrane potential ($\Delta\Psi m$) and cytoplasmic maturation

The ΔΨm was measured by incubating the oocytes with Mito-Tracker Red® (CMXRos, Invitrogen) at 500 nM for 30 min [31]. The staining procedures and evaluation of images were similar to those used for the quantification of ROS levels. Additionally, the cytoplasmic maturation of the labeled oocytes was evaluated based on the mitochondrial distribution as follows: peripheral (immature oocytes), dispersed (mature oocytes), and transitional (between peripheral and dispersed distributions; partially mature oocytes).

2.7. Morphometric evaluation of oocytes after maturation

The denuded oocytes were observed under an inverted microscope for assessing the oocyte morphometric parameters. The images were captured using an inverted microscope (Nikon TS100, Tokyo, Japan) equipped with a camera and image processing software. The morphometric parameters of the captured images were analyzed in the ImageJ software using the scale bar as an arbitrary scale for pixel analysis. The measured morphometric parameters included outer oocyte diameter (ZPO), zona pellucida thickness (ZPT), inner oocyte diameter (ZPI), and ooplasm diameter (OD), as described by Saadeldin et al. [32]. Thus, perivitelline space (PVS) diameter, inner oocyte area (IA), ooplasm area (OA), and PVS area were mathematically calculated based on previous parameters using the following formulae: $IA = 3.14 \times (ZPI/2)^2$; $OA = 3.14 \times (OD/2)^2$, PVS diameter = ZPI − OD and PVS area = IA − OA. Diameters were measured in μm and areas in μm^2.

2.8. Artificial activation and in vitro development (IVD) of embryos

After IVM, the *cumulus* cells were removed by pipetting the oocytes in the presence of hyaluronidase for up to 2 min. The oocytes with 1 PB were activated using 5 μM ionomycin (I24222; Gibco-BRL) prepared in CCM for 4 min at 37 °C. Next, the oocytes were washed and incubated with drops of secondary activators at 38.5 °C and 5% CO_2 for 3 h, according to the experimental design. Subsequently, the oocytes were washed and incubated in 50 μL drops of IVD medium covered with mineral oil. Synthetic oviductal fluid (SOF) supplemented with 0.2 mM sodium pyruvate, 0.2 mM L-glutamine, 0.34 mM sodium citrate, 2.8 mM myo-inositol, 2% essential amino acid solution, 1% non-essential amino acid solution, 1% antibiotic-antimycotic solution, 5.0 mg/mL BSA, and 2.5% FBS was employed as the IVD medium. The day of activation was considered as D0 and the cells were cultured until D7. On D3, 50% of the culture medium was replaced with fresh medium.

2.9. Evaluation of blastocysts and differential staining of inner cell mass and trophectoderm cells

After seven days of IVD, the blastocyst cells were fixed in ethanol and labeled with Hoechst 33342 (10 μg/mL) and propidium iodide (PI) (10 μg/mL) for 15 min at 38.5 °C. Next, the cells were washed twice with PBS and placed on glass slides in glycerol droplets. A coverslip was placed over the droplet and the images were captured under UV light at 330–385 nm using a fluorescent microscope. The embryos were visualized individually and the nuclei were counted in the captured fluorescent images. The nuclei of the inner cellular mass (ICM) appeared blue as they were labeled only with Hoechst 33342, while those of the trophectoderm (TE) appeared red or pink as they were labeled with both PI and Hoechst 33342. The numbers of ICM, TE, and total cells were quantified using the ImageJ software [33].

2.10. Experimental design

To evaluate various conditions for IVM and chemical activation of collared peccary oocytes, two experiments were performed. Initially, we assessed the IVM rates, COC quality, and oocyte morphometric parameters after IVM in the absence or presence of 10 ng/mL of EGF (experiment 1). Furthermore, we activated the oocytes with ionomycin and four secondary activator combinations (6D, 6D and CB, CHX, and CHX and CB). The effect of immature COC grades (GI and GII or GIII COCs) on the embryonic development and

quality was evaluated (experiment 2). For the second experiment, only the oocytes grown under optimal IVM conditions were used.

2.10.1. Experiment 1: effects of EGF supplementation on IVM of collared peccary COCs

The COCs were washed with the medium and four replicates (two females per replicate) were used to assess the effect of EGF on IVM. GI and GII COCs derived from eight females were matured in the absence (EGF negative group) or presence of 10 ng/mL of EGF (EGF positive group). Meiotic competence was measured by visualization of the 1 PB and nuclear stage (MII). Moreover, oocytes were evaluated for cytoplasmic maturation based on mitochondrial distribution using MitoTracker Red®. Additionally, the *cumulus* cells were assessed and the ROS levels and ΔΨm were quantified. The matured oocytes were compared by morphometric evaluation according to the ZPO, ZPT, OD, PVS area, and PVS diameter.

2.10.2. Experiment 2: effects of secondary activators and COC quality on the development of collared peccary parthenogenetic embryos

After 44 h of IVM, matured GI/GII and GIII COCs derived from twelve females distributed in three replicates were artificially activated and cultured for seven days. The COCs were artificially activated with ionomycin and four secondary activator combinations: 1.9 mM 6D (6D group), 1.9 mM 6D and 7.5 μg/mL CB (6D + CB group), 10 μg/mL CHX (CHX group), and 10 μg/mL CHX and 7.5 μg/mL CB (CHX + CB group). On day 3 of IVD, the total cleavage rate and number of embryos containing 2 cells, 3–7 cells, or 8 or more cells were quantified. On day 7 of IVD, the total blastocyst formation rate and the developmental phase [initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB), and blastocyst hatching/hatched (hB)] were quantified. To evaluate the quality, the total numbers of cells were counted by differentiating the ICM and TE.

2.11. Statistical analysis

All data are expressed as the mean ± standard error and were analyzed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). The normality of the data was verified using the Shapiro-Wilk test, while the homoscedasticity was verified with Levene's test. The morphometric oocyte data were examined by variance analysis (ANOVA) and Tukey's test. The levels of ROS and ΔΨm, as well as the number of blastomers were transformed with arcsine and analyzed by ANOVA followed by Tukey's test. All other data were compared with Kruskal-Wallis test and Dunn (multiple comparisons) test. The difference was considered statistically significant when the P value was less than 0.05.

3. Results

In total, 630 visible follicles (31.5 follicles per female on average) were obtained upon stimulation of 40 ovaries (Fig. 1A) derived from 20 collared peccaries with PG600®. The number of medium follicles (385 ± 14.1) was higher than that of small follicles (113 ± 1.9, P = 0.03) and large follicles (132 ± 5.4, P = 0.04). The number of small and large follicles was similar (P > 0.05).

Moreover, 333 ± 10.7 immature oocytes (Fig. 1B) were obtained from all ovaries with a recovery rate of 52.9% ± 4.2 (333/630) and 16.2 ± 2.8 oocytes per female. Of these, 253 ± 8.5 (76.0%) oocytes were classified as GI and GII (12.1 ± 2.2 COCs per female), 37.0 ± 3.3 (11.1%) as GIII (3.1 ± 0.8 COCs per female), and 43.0 ± 1.8 (12.9%) as GIV (2.8 ± 1.0 COCs per female) based on the cytoplasm homogeneity and the number of *cumulus* cell layers.

3.1. Experiment 1: effects of EGF supplementation on IVM of collared peccary COCs

The effect of EGF on the IVM of collared peccary COCs is shown in Tables 1 and 2. Initially, there was no difference in the IVM rates of COCs exhibiting *cumulus* cell expansion (Fig. 1C) between EGF positive and negative groups. All COCs exhibited *cumulus* cell expansion scores of 3 and 4 and the *cumulus* cell viability was greater than 76.0% for both EGF positive and negative groups. Moreover, there was no difference in the IVM rates of COCs selected on the basis of 1 PB presence (Fig. 1D), MII assessments (Fig. 1E), and cytoplasmic maturation evaluated based on mitochondrial distribution. Additionally, the intracellular ROS levels (Fig. 2A, C, and D) and ΔΨm in the matured COCs were similar between the EGF positive and EGF negative groups.

There was no difference for most of the evaluated oocyte morphometric assessment parameters. However, the thickness of the zona pellucida in the matured oocytes reduced in the presence of EGF (Table 2). Thus, the IVM medium supplemented with EGF was used for experiment 2.

3.2. Experiment 2: effects of secondary activators and COC quality on the development of collared peccary parthenogenetic embryos

Assessment of embryonic development is described in Tables 3 and 4. The cleavage rate (Fig. 3A–C) after 3 days of IVD was similar

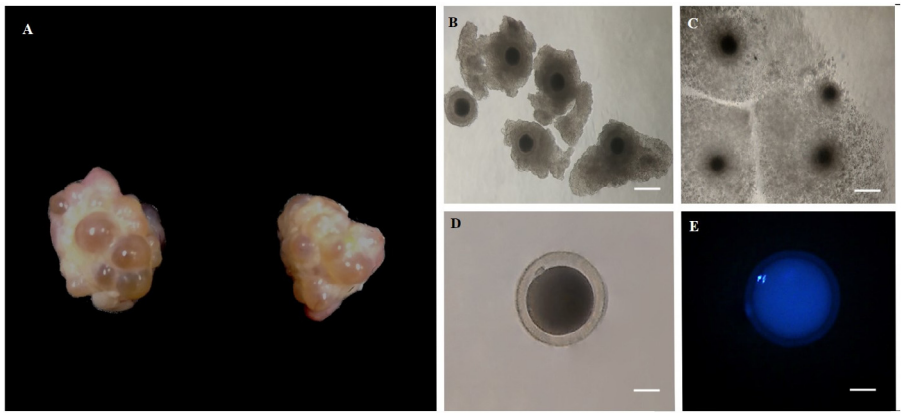

Fig. 1. Representative images of **(A)** ovaries of collared peccary obtained after hormonal induction with PG600®; **(B)** *Cumulus*-oocyte complex (COC) recovered with homogeneous cytoplasm and more one layer of *cumulus* cells; **(C)** COCs matured after 44 h IVM; **(D)** Denuded oocyte with presence of 1 PB; **(E)** Visualization of the metaphasic plate (MII).

Table 1
Effects of EGF supplementation on *in vitro* maturation of collared peccary *cumulus*-oocyte complexes (COCs).

Group	Evaluation of *cumulus* cells				Nuclear maturation		Cytoplasmic maturation mitochondrial distribution		
	Expansion and grade of expansion, %			Viability, %	1 PB,%	MII,%	Peripheral, %	Transition, %	Dispersed, %
	Total	Grade 3	Grade 4						
EGF positive	100.0 ± 0.0 (44/44)	63.6 ± 3.0 (28/44)	29.5 ± 0.3 (13/44)	76.0 ± 3.1 (784/1031)	70.5 ± 1.8 (31/44)	68.4 ± 4.9 (13/19)	0.0 ± 0.0 (0/9)	25.0 ± 0.0 (2/8)	75.0 ± 0.7 (6/8)
EGF negative	97.6 ± 1.2 (40/41)	70.7 ± 2.5 (29/41)	29.3 ± 2.1 (12/41)	79.4 ± 1.4 (765/963)	65.9 ± 1.2 (27/41)	62.5 ± 11.6 (10/16)	0.0 ± 0.0 (0/9)	0.0 ± 0.0 (0/9)	100.0 ± 0.0 (9/9)

No differences were observed among groups (P > 0.05). 1 PB, first polar body, MII, metaphase II.

Table 2
Morphometric assessment (in μm and μm^2) in of matured COCs derived from collared peccaries (mean ± standard error).

Groups	Outer oocyte diameter (ZPO)	Zona pellucida thickness (ZPT)	Ooplasm diameter (OD)	Perivitelline space (PVS) area	Perivitelline space (PVS) diameter
EGF positive	373.9 ± 4.4 (37)	33.3 ± 1.2 (37)[a]	284.3 ± 4.2 (37)	7312.6 ± 823.1 (35)	16.2 ± 2.1 (35)
EGF negative	382.2 ± 5.3 (34)	38.6 ± 1.5 (34)[b]	285.6 ± 3.0 (34)	6284.0 ± 390.9 (27)	13.8 ± 0.8 (27)

[a,b]: Values with different superscript letters within columns are significantly different (P < 0.05).

in the evaluated groups. The cleavage rates were greater than 72.2% and 25.0% for the embryos derived from GI/GII and GIII COCs, respectively. Moreover, there was no reduction in the cleavage rates of embryos derived from GI/GII and GIII COCs among the 6D and CHX + CB groups (Table 3).

Nevertheless, the 6D group exhibited higher rates of blastocyst/total cell in the embryos derived from GI/GII COCs than CHX group (Table 3). Moreover, only 6D treatment resulted in the production of embryos derived from GIII COCs (25.0% ± 0.2). Further, the percentages of ICM/total cell ratio in blastocysts derived from 6D, 6D + CB, and CHX + CB group COCs were higher than those in blastocysts derived from CHX group COCs. Interestingly, the total number of blastocyst cells (Fig. 3G and H) derived from 6D + CB and CHX + CB oocyte groups was similar (Table 4).

In relation to embryo development kinetics (Fig. 3D–F), or the proportion of embryos at different stages of development, there was no significant difference among the groups in the percentage of embryos classified with 2, 3–7, or ≥8 cells (Fig. 4A and B). On D7, the percentages of iB, B, eB, or hB embryos were similar among the treatment groups (Fig. 4C, P > 0.05).

4. Discussion

Establishment of SCNT is an important strategy to develop this technology as a conservation tool for collared peccary. In this study, we optimized the protocols for improving matured oocyte quality and artificial oocyte activation, which are important for the success of SCNT. Recently [34–37], our group has developed strategies for the establishment of donor nuclei (or karyoplast), which form a database of somatic resources for the species. We have established the conditions for obtaining cytoplasts and evaluated their competence after artificial oocyte activation using different protocols.

Initially, we evaluated the effects of EGF on the IVM of collared peccary oocytes. We, for the first time, demonstrated that the zona pellucida thickness in oocytes matured with EGF was lower than that in oocytes matured without EGF. The thickness of the zona pellucida is an indicator of the success of hatching and implantation of embryos [38]. The elasticity and thinning of zona pellucida are essential for the hatching process. Thick zona pellucida is associated with low-quality embryos. Increased thickness of zona

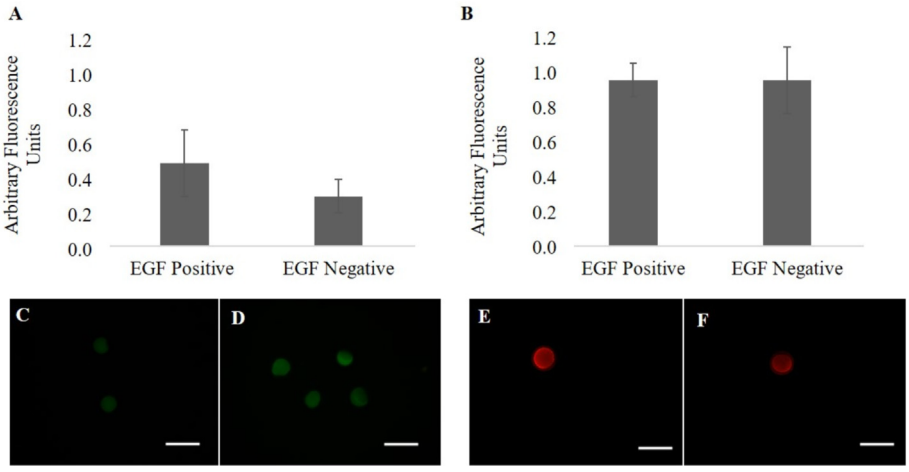

Fig. 2. Oocytes of collared peccaries after maturation evaluated as intracellular concentration of reactive oxygen species (ROS) levels and mitochondrial membrane potential (ΔΨm). **(A)** Measurement of fluorescence of ROS levels in matured oocytes in the presence of EGF (EGF positive group) or in the absence (EGF negative group) (P > 0.05). **(B)** Quantification of mitochondrial membrane potential in oocytes matured in the presence of EGF (EGF positive group) or in the absence (EGF negative group) (P > 0.05). **(C)** Oocytes labeled with fluorescent probe H_2DCFDA for ROS quantification with EGF (EGF positive group) and **(D)** without EGF (EGF negative group). **(E)** Oocytes labeled with fluorescent probe MitoTracker Red® for ΔΨm with EGF (EGF positive group) and **(F)** without EGF (EGF negative group).

Table 3
Embryonic development of collared peccary GI/GII and GIII *cumulus*-oocyte complexes (COCs) activated with ionomycin in combination with different secondary activators.

Groups	No. COCs		Cleavage, %		Blastocyst/Oocyte, %		Blastocyst/Cleaved, %	
	GI/GII	GIII	GI/GII COCs	GIII COCs	GI/GII COCs	GIII COCs	GI/GII COCs	GIII COCs
6D	34	4	82.9 ± 2.4(29)	75.0 ± 0.4(3)	23.5 ± 0.3(8)^{a}	25.0 ± 0.2 (1)^{a}	27.6 ± 0.3(8)^{a}	33.3 ± 0.3^{a} (1)
6D + CB	36	9	72.2 ± 1.4(26)	77.8 ± 0.7(7)	16.7 ± 0.5(6)^{a,b}	0.0 ± 0.0(0)^{b}	23.1 ± 0.5(6)^{a,b}	0.0 ± 0.0 (0)^{b}
CHX	37	4	78.4 ± 2.2(29)^{A}	25.0 ± 0.2(1)^{B}	5.4 ± 0.2(2)^{b}	0.0 ± 0.0(0)^{b}	6.9 ± 0.3(2)^{b}	0.0 ± 0.0 (0)^{b}
CHX + CB	34	7	76.5 ± 2.2(26)	57.1 ± 0.4(4)	17.6 ± 0.3(6)^{a,b}	0.0 ± 0.0(0)^{b}	23.1 ± 0.2(6)^{a,b}	0.0 ± 0.0 (0)^{b}

^{a,b}: Values with different superscript letters within columns are significantly different ($P < 0.05$). ^{A,B}: Values with different superscript letters within rows are significantly different ($P < 0.05$). 6D group: 6D; 6D + CHX group: 6D and cytochalasin B; CHX group: cycloheximide; CHX + CB group: cycloheximide and cytochalasin B.

Table 4
Cell count of *cumulus*-oocyte complexes (COCs) as viable and activated with ionomycin in combination with different secondary activators.

Secondary activators	No. of embryos	ICM, %	TE, %	Total	%ICM/Total
6D	5	78.8 ± 35.2	93.2 ± 41.7	172 ± 76.9^{a,b}	42.5 ± 19.0^{a}
6D + CB	3	33.7 ± 19.4	65.0 ± 37.5	98.7 ± 57.0^{a}	37.9 ± 21.9^{a}
CHX	2	7.0 ± 0.1	188.0 ± 0.1	195 ± 0.1^{b}	3.6 ± 0.1^{b}
CHX + CB	5	40.2 ± 18.0	57.6 ± 25.8	97.8 ± 43.7^{a}	43.8 ± 19.6^{a}

^{a,b}: Values with different superscript letters within columns are significantly different ($P < 0.05$). ICM: internal cellular mass; TE: trophectoderm; %ICM/total: internal cellular mass/total cell ratio.

pellucida may be caused by an inappropriate *in vitro* environment [38]. Zhou et al. [39] demonstrated that thinning solutions enhance the nuclear maturation of oocytes that previously had thick zona pellucida. The study hypothesized that thinner zona pellucida could facilitate first polar body extrusion. Khanmohammadi et al. [38] observed that a suitable culture medium promotes optimal environment that allows zona pellucida thinning and subsequently facilitates embryonic implantation. Furthermore, morphometric evaluation of matured oocytes has been used to estimate the oocyte developmental competence [32].

The expansion of *cumulus* cells was observed in almost all oocytes derived from both EGF positive and negative groups (>97.8%). A similar response was also observed in swine oocytes [8]. Further, both groups exhibited high rates of *cumulus* cell expansion after maturation. There was no difference in the viability of *cumulus* cells, with values higher than 76% for all groups. This response may have occurred due to the presence of gonadotrophins and/or serum

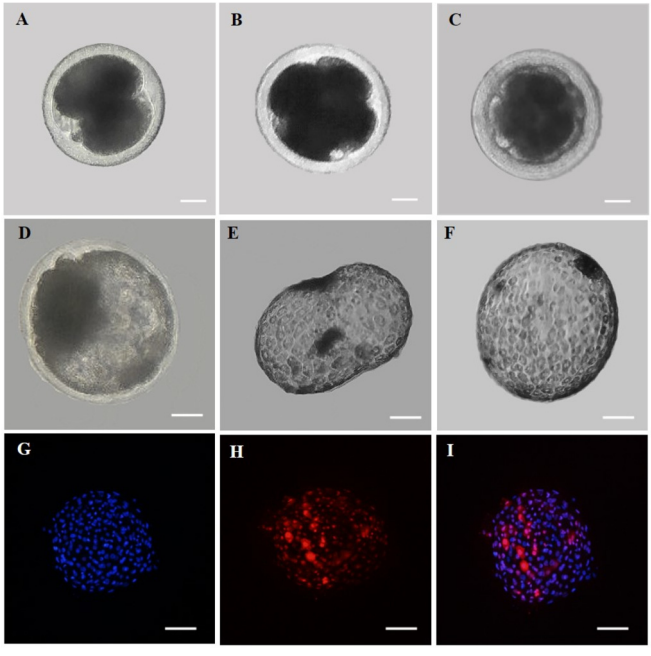

Fig. 3. Kinetics of embryonic development in collared peccaries. After 3 days of IVD different embryos with **(A)** 2 cells; **(B)** 4 cells, **(C)** more than 8 cells. Evaluation of embryonic development on day 7 of IVD **(D)** blastocyst; **(E)** blastocyst hatching; **(F)** blastocyst hatched already without zona pellucida. Counting the total number of blastocyst **(G)** cells inner mass labeled with Hoechst 33342. **(H)** trophectoderm cells stained with propidium iodide. **(I)** merge total cells.

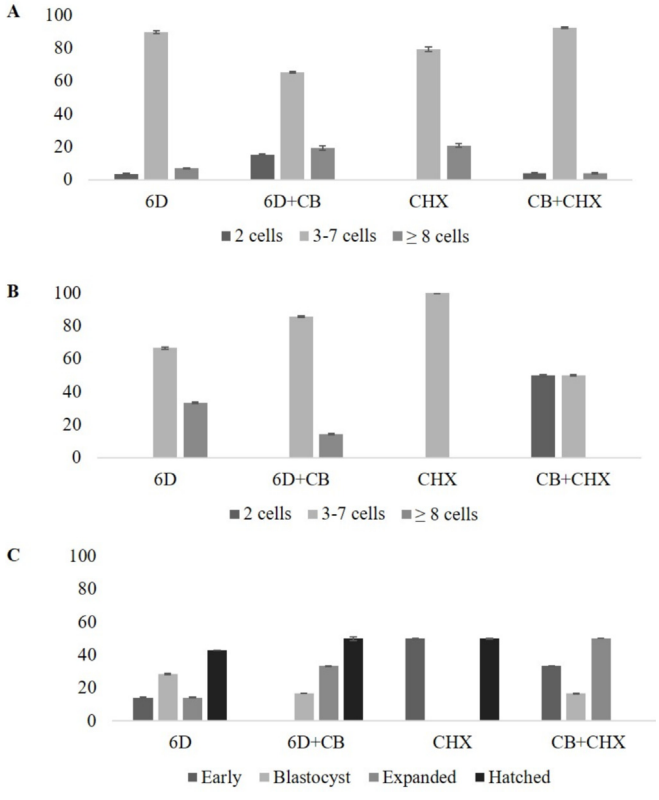

Fig. 4. Embryonic development of classified activated with ionomycin in combination with different secondary activators. Embryonic development in 3 days with 2 cells, 3–7 cells and 8 cells after activation with ionomycin in combination with secondary activators in **(A)** GI/GII ($P > 0.05$) and **(B)** GIII COCs ($P > 0.05$). **(C)** Percentage of embryos in D7 classified in different stages of blastocyst after activation of GI/GII COCs ($P > 0.05$).

in the IVM medium, which can interfere with the stimulation induced by EGF [40]. Additionally, FSH may act through EGF receptors to exert a synergistic effect on cytoplasmic maturation [11] and may interfere with the effects of EGF on *cumulus* cell expansion.

The quality of matured oocytes is important for the evaluation of meiotic competence. Thus, we evaluated the ROS levels and ΔΨm in matured oocytes and observed no difference between the groups for both parameters. Generally, the decrease in ROS levels is accompanied with an increase in glutathione (GSH) levels, a natural antioxidant that reduces ROS in oocytes [41], where ROS generation depends on ΔΨm [31]. Fan et al. [41] observed that treating the goat oocytes with EGF and cysteamine resulted in lower ROS levels in matured oocytes. In our study, both groups were cultured in a medium containing cysteamine, and EGF probably did not enhance its activity.

Similarly, EGF did not increase the IVM rates of oocytes at MII phase and have 1 PB. Although Kishida et al. [42] observed a higher rate of nuclear maturation in porcine oocytes when EGF was present in the medium (10 ng/mL), another study demonstrated that EGF does not influence the nuclear maturation [43]. This may be due to the interaction of EGF with different supplements of the IVM medium, which are highly variable in each study. Further, EGF was involved primarily in the maintenance of embryonic quality where EGF-activated pathways promote greater competence of the oocyte to support embryonic development. Therefore, we suggest the use of EGF in the IVM medium of collared peccary oocytes for future experiments.

In this study, we observed that 6D was the most optimal secondary activator for collared peccary oocytes. However, the combination of 6D and CB did not have a beneficial effect on embryonic development. Further, CHX did not exhibit good potential for oocyte activation, which was improved only in combination with CB. Several studies have demonstrated the ability of 6D, CHX, and CB to inhibit meiotic resumption and second polar body extrusion in porcine oocytes [44,45]. Moreover, the higher efficiency of 6D compared to CHX was also observed in porcine oocytes [46]. Although 6D and CHX exerted positive effect on oocyte activation and morula-blastocyst formation rates, 6D was more effective than CHX on both matured and immature oocytes [46]. This may be due to the mechanism of action of CHX, which may result in the inhibition of proteins involved in embryonic development [47], whereas 6D specifically inhibits protein kinases [48]. Additionally, Khadijah et al. [49] and Zhang et al. [50] demonstrated that the combination of ionomycin and 6D was better than the combination of ionomycin and CHX for embryonic development in cattle (7.1% *vs.* 2.2%) and rabbit oocytes (8.6% *vs.* 1.2%).

Moreover, the embryonic development rates in the presence of ionomycin and 6D for collared peccary oocytes were higher than those observed for swine oocytes [51] evaluated based on the cleavage percentage (82.9% *vs.* 47.2%), blastocyst percentage (23.5% *vs.* 11.3%), and ICM/total cell ratio (42.5% *vs.* 24.8%). This indicates that despite similarities in the responses of protocols used between species, some differences are observed, suggesting the importance of establishing a species-specific protocol. Additionally, Campos Junior et al. [20] activated the collared peccary oocyte with the combination of ionomycin and 6D and obtained up to two-cell (40%) and 4-cell (10%) embryos. Thus, this is the first study that achieved the blastocyst stage of embryonic development in collared peccaries through artificial oocyte activation.

The low performance of CHX was observed in both artificial oocyte activation of collared peccaries and blastocyst quality. Although our results demonstrated a higher total number of cells in the 6D and CHX groups, CHX group exhibited a higher number of trophectoderm cells and a very low number of cells of the internal mass, which would later impair the embryonic development of the CHX group that had a low blastocyst rate compared to the other groups. The CHX + CB group exhibited higher ICM/total cell rates compared to the CHX group. This may be because CHX has no effect on blocking the extrusion of the second polar body. Thus, parthenotes treated with CHX were largely haploid and exhibited low blastocyst development, which does not improve blastocyst formation further [52]. The combination of 6D and CB was more effective in inhibiting the extrusion of the second polar body, which promotes the development of diploid embryos with higher developmental competence compared to haploid embryos [53].

The combination of ionomycin and 6D exhibited optimal performance as this was the only combination that produced embryos from GIII oocytes. As CB enhances the effect of CHX on GI and GII oocytes, its mechanism cannot be as efficient as 6D because the combination of CHX and CB could not promote the development of GIII oocytes to blastocysts. However, matured GIII oocytes were activated and developed into embryonic blastocyst only in the 6D group. Therefore, embryonic development depends on the efficiency of the activation protocol [53,54]. Thus, use of oocytes with heterogeneous cytoplasm and less than one layer of *cumulus* cells may be suitable when there is low availability of oocytes.

In conclusion, EGF can be used to supplement the maturation medium to obtain a greater quality of matured oocytes and to improve embryonic development. Moreover, the combination of ionomycin and 6D could produce collared peccary embryos from the activation of both GI/GII and GIII COCs. These optimized IVM conditions and artificial activation in collared peccaries represent the first steps for cloning this species through ARTs.

Acknowledgments

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ANEXO F – COMPROVANTE DO ARTIGO SUBMETIDO NA ANNALS OF ANIMAL SCIENCE

EFFECTS OF INCUBATION TIME AND METHOD OF CELL CYCLE SYNCHRONIZATION ON COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES

QUALIS: Quadriênio 2013-2016: Não classificado pela Medicina Veterinária/ Novo Qualis: A3

FATOR DE IMPACTO: 1,586

UFERSA mail

Alexsandra Fernandes Pereira <alexsandra.pereira@ufersa.edu.br>

Submission of manuscript

k.skupniewicz@izoo.krakow.pl <k.skupniewicz@izoo.krakow.pl> 22 de junho de 2020 09:39
Para: Alexsandra Fernandes Pereira <alexsandra.pereira@ufersa.edu.br>

Dear Author,
Thank you for your submission. Your manuscript entitled "Effects of incubation time and method of cell cycle synchronization on collared peccary skin-derived fibroblast cell lines" has been assigned the following number: 317/20 and has been sent for evaluation to the reviewers.

Sincerely,
Editorial Board of Annals of Animal Science
Katarzyna Skupniewicz

W dniu 2020-06-17 23:21, Alexsandra Fernandes Pereira napisał(a):

> Dear Dr. Skupniewicz,
>
> I would like to submit for evaluation in ANNALS OF ANIMAL SCIENCE the manuscript titled “EFFECTS OF INCUBATION TIME AND METHOD OF CELL CYCLE SYNCHRONIZATION ON COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES”.
>
> Briefly, our manuscript proposes to develop another stage of cloning by somatic cell nuclear transfer (SCNT) in collared peccary, a wild mammal of ecological, economic and scientific importance and that has suffered population reduction. In the recent years, our group has been working to establish the use of the karyoplast (donor nuclei), which forms a database of somatic resources for the collared peccary (Borges et al., 2017; Borges et al., 2018; Queiroz Neta et al., 2018), in techniques such as cloning by SCNT. We aimed to achieve this goal by defining appropriate methodologies for the synchronization of these cells.
>
> In the manuscript, we evaluated three different approaches for cell cycle synchronization in terms of their effects on synchronization in G0/G1 phases and on the viability of collared peccary skin fibroblasts:
>
> (I) Serum starvation (SS) for 1 to 4 days,
>
> (II) Contact inhibition (CI) for 1 to 3 days,
>
> (III) Using cell cycle regulatory inhibitors (dimethyl sulfoxide, cycloheximide, cytochalasin B, or 6-dimethylaminopurine) for 1 and 2 days.
>
> Several cell cycle synchronization protocols in G0/G1 stages have been proposed. However, the cellular response to these protocols has been variable in domestic and wild mammals, necessitating the evaluation of each protocol for a given cell type and species. Thus, we showed that CI for 3 days was the most efficient method for cell cycle synchronization in collared peccary fibroblasts. To our knowledge, this is the first work to elucidate nuclear reprogramming in cells of this species. Thus, we established the last step of the preparation for the use of these fibroblast as karyoplast for application in somatic cell nuclear transfer of collared peccary, with potential application to conservation of the species.
>
> Moreover, all authors of manuscript agree with the content of the manuscript and submission in this journal. Additionally, none of the authors have conflict of interest. Finally, we sent to an English review company for conference to correct grammatical language, according to Certificate of Editing English. All material submitted

> for consideration is original and is being submitted exclusively to
> ANNALS OF ANIMAL SCIENCE.
>
> Yours sincerely,
>
> Alexsandra Fernandes Pereira
>
> --
>
> _Profa. Dra. Alexsandra Fernandes Pereira_
> Bolsista de Produtividade do CNPq - Nível 2
> Laboratório de Biotecnologia Animal - LBA
> _Programa de Pós-Graduação em Ciência Animal - PPGCA_
> [Texto das mensagens anteriores oculto]

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Katarzyna Skupniewicz

ANEXO G – RESUMOS PUBLICADOS NO RENORBIO 2017

I ENCONTRO DE BIOTECNOLOGIA NO NORDESTE

NA0007 - INFLUÊNCIA DE DIFERENTES CRIOPROTETORES SOBRE A VIABILIDADE DE CÉLULAS SOMÁTICAS DE CATETOS (PECARI TAJACU LINNAEUS, 1758)

Autores: Gabriela Pereira De Oliveira Lira - 1ºAutor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Alexandre Rodrigues Silva - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Moacir Franco De Oliveira - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Luiza Bento De Queiroz Neta - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Maria Valeria De Oliveira Santos - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Lucas Emanuel Nascimento - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Alana Azevedo Borges - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA)); Alexsandra Fernandes Pereira1 - Orientador (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO (UFERSA))

Área: Agropecuária **Tipo:**Pesquisa **Nível:**Graduação **STA2:**Não

Resumo: A criopreservação de células somáticas é uma técnica interessante para a conservação de material biológico visando o uso na investigação básica, transferência nuclear (clonagem) e formação de bancos genéticos. Para todas essas aplicações, a escolha dos crioprotetores a serem empregados na criopreservação dessas células consiste na primeira etapa a ser estabelecida. Portanto, o objetivo do presente trabalho foi comparar a influência de diferentes crioprotetores intracelulares (dimetilsulfóxido, DMSO, e etilenoglicol, EG) e extracelular (sacarose, SAC) sobre a viabilidade de células somáticas derivadas de catetos, mamíferos silvestres com importância cientifica, ecológica e econômica. Para tanto, fragmentos teciduais da região auricular periférica de cinco catetos, mantidos no Centro de Multiplicação de Animais Silvestres (CEMAS/UFERSA), foram cultivados in vitro por 40 dias. Posteriormente, células somáticas recuperadas desses fragmentos foram submetidas à congelação lenta usando a solução de criopreservação constituída por meio essencial mínimo modificado por Dulbecco (DMEM) acrescido de 2,2 g/L de bicarbonato de sódio, 2% de solução de antibiótico-antimicótico,10% de soro fetal bovino e seis combinações de crioprotetores a seguir: 10% de DMSO (DMSO), 10% de DMSO e 0,2 M de sacarose (DMSO-SUC), 10% de EG (EG), 10% EG e 0,2 M de sacarose (EG-SAC), 10% de DMSO e 10% de EG (DMSO-EG), 10% de DMSO, 10% de EG e 0,2 M de sacarose (DMSO-EG). Para todas as congelações, foi empregado o sistema de congelação Mr. Frosty (Mr. Frosty? Freezing Container) numa taxa de resfriamento de 1°C/min e concentração final de 2,0 x 106 células/mL. Além disso, células não criopreservadas foi considerada como grupo controle. Para a avaliação da viabilidade, células descongeladas e não criopreservadas foram submetidas ao ensaio de azul de tripan. Todas as análises foram realizadas em duplicatas. Os dados foram expressos como média ± desvio padrão e analisados usando ANOVA seguida de teste Tukey (P ? 0,05). Após as análises, apenas células somáticas derivadas da congelação lenta usando DMSO-SAC (69,8% ± 9,6) mantiveram a viabilidade similar ao grupo não criopreservado (85,7% ± 3,4; P > 0,05), evidenciando um efeito positivo da presença de sacarose sobre a viabilidade celular. Além disso, embora os demais grupos tenham sido diferentes do controle foram similares entre si (P > 0,05): EG (50,8% ± 19,5), EG-SAC (58,5% ± 7,1), DMSO (55,8% ± 10,1), DMSO-EG (58,7% ± 11,2) e DMSO-EG-SAC (47,9% ± 9,8). Adicionalmente, exceto para o grupo DMSO-SAC, a presença de sacarose não influenciou na manutenção da viabilidade de células somáticas após a congelação lenta. Em conclusão, 10% de DMSO acrescido de 0,2 M de sacarose manteve a viabilidade de células somáticas derivadas de catetos após a descongelação, podendo essa combinação de crioprotetores ser utilizada na conservação de material biológico desta espécie.

Keywords: Palavras chaves: Mamíferos silvestres, criopreservação, cultivo in vitro, crioprotetores intracelulares, sacarose.

NA0005 - INFLUÊNCIA DO TEMPO DE MATURAÇÃO IN VITRO EM OÓCITOS DE CATETOS (PECARI TAJACU LINNEAUS, 1758)

Autores: Alexsandra Fernandes Pereira - 1ºAutor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Alexandre Rodrigues Silva - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Moacir Franco De Oliveira - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Luiza Bento De Queiroz Neta - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Lhara Ricarliany Medeiros De Oliveira - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Gabriela Pereira De Oliveira Lira - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Maria Valeria De Oliveira Santos - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO); Alana Azevedo Borges - Co-Autor (UNIVERSIDADE FEDERAL RURAL DO SEMI-ARIDO)

Área: Agropecuária **Tipo:**Pesquisa **Nível:** **STA2:**

Resumo: A maturação in vitro (MIV) de oócitos consiste numa etapa essencial para biotecnologias reprodutivas, como a fecundação in vitro, injeção intracitoplasmática de espermatozoides e transferência nuclear de células somáticas. Todas essas biotécnicas podem ser utilizadas como ferramentas para a conservação de mamíferos silvestres, como os catetos. Esses animais se destacam pelo seu potencial zootécnico e econômico, sendo considerados como fonte para pecuária alternativa de pequenos produtores rurais. Nesse sentido, para alcançar o sucesso da MIV é necessário o estabelecimento do tempo de incubação desses oócitos. Portanto, o objetivo do presente trabalho foi avaliar a influência do tempo de MIV (24 h vs. 48 h) sobre o percentual de oócitos maturados de catetos. Para tanto, ovários recuperados de nove fêmeas mantidas do Centro de Multiplicação de Animais Silvestres (CEMAS/UFERSA) foram aspirados e fatiados para a obtenção de oócitos imaturos. Posteriormente, todos os oócitos foram classificados morfologicamente de acordo com a homogeneidade do citoplasma, número e compactação das camadas de células do cumulus em: Grau 1 (3 ou mais camadas de células do cumulus e citoplasma homogêneo); Grau 2 (1?2 camadas de células do cumulus e citoplasma homogêneo); Grau 3 (oócito parcialmente desnudo e ligeiramente heterogêneo); Grau 4 (oócito desnudo e heterogêneo). Os oócitos 1 e 2 foram selecionados e submetidos à MIV em TCM199 com 2,2 g/L de bicarbonato de sódio, 0,2 mM de piruvato de sódio, 1% de solução de antibiótico-antimicótico e suplementado com 10 µg/mL de FSH, 10% de soro fetal bovino e 100 ?M de cisteamina a 38,5°C, 5% de CO2 e umidade máxima por 24 ou 48 h de incubação. Após cada período, as células do cumulus foram removidas por pipetagens e as estruturas desnudadas foram verificadas quanto à presença do primeiro corpúsculo polar (1CP) sob estereomicroscópio. Assim, os oócitos com 1CP foram considerados maturados. Os dados foram analisados pelo teste exato de Fisher ($P < 0,05$) e expressos em média percentual ± erro padrão. Um total de quatro repetições (2?3 fêmeas por repetição) foi realizado, resultando em 59 oócitos imaturos e com uma taxa de recuperação de 83,1% (59/71) e 3,3 oócitos por ovário. Destes, 42 estruturas foram classificadas como viáveis (2,3 oócitos viáveis por ovário). Após diferentes períodos de incubação, valores de maturação superiores a 50% foram observados para ambos os grupos. Além disso, diferenças ($P < 0,05$) foram observadas entre 24 e 48 h para a presença do 1CP (52,4 ± 2,2 (11/21) vs. 90,5 ± 2,0 (19/21). Em conclusão, o tempo de 48 h foi mais adequado para a MIV de oócitos derivados de catetos, quanto à presença do 1CP. Tais resultados evidenciam que a MIV nesta espécie ocorre em período similar aos oócitos de suínos domésticos, espécie filogeneticamente próxima a esta espécie silvestre.

Keywords: Mamíferos silvestres, biotécnicas reprodutivas, maturação nuclear.

ANEXO H – RESUMO PUBLICADO NO SEMIC 2018
XXIV SEMINÁRIO DE INICIAÇÃO CIENTÍFICA DA UFERSA

Área temática: Ciências Agrárias

Influência de crioprotetores extracelulares sobre a criopreservação de células somáticas de catetos (*Pecari tajacu* Linnaeus, 1758)

Gabriela Pereira de Oliveira Lira, Alexsandra Fernandes Pereira, Alana Azevedo Borges, Matheus Barbosa do Nascimento

Resumo

A criopreservação de células somáticas consiste numa interessante ferramenta na conservação de catetos, visando a formação de bancos de recursos genéticos. Esses bancos quando associados à clonagem por transferência nuclear de células somáticas resultam em estratégias eficientes na manutenção da população desta espécie. Contudo, o sucesso desses bancos depende da combinação de crioprotetores utilizados na criopreservação. Embora o crioprotetor intracelular mais empregado seja o dimetilsulfóxido (DMSO) na congelação lenta de células somáticas de catetos, os agentes extracelulares ainda necessitam ser estudados, visando aumentar as taxas de viabilidade após a descongelação. Portanto, o objetivo foi avaliar a influência dos crioprotetores extracelulares, sacarose (SAC) e soro fetal bovino (SFB), sobre a criopreservação de células somáticas de catetos. Para tanto, fragmentos teciduais da pele da região auricular periférica de cinco catetos, pertencentes ao Centro de Multiplicação de Animais Silvestres (CEMAS/UFERSA), foram coletados e transportados ao laboratório em meio essencial mínimo modificado por Dulbecco (DMEM) suplementado com 10% de SFB e 2% de solução de antibióticos e antimicóticos, a 37°C, por até 1 h. Todos os procedimentos foram aprovados pelo Comitê de Ética de Uso de Animais (CEUA/UFERSA, no. 23091.001072/2015-92) e Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, no. 48633-2). No laboratório, fragmentos (9,0 mm^3) foram cultivados *in vitro* sob condições e atmosfera controladas (5% de CO_2 e 38,5ºC). Posteriormente, células somáticas recuperadas desses fragmentos foram submetidas à congelação lenta utilizando DMEM acrescido 10% de DMSO suplementado com diferentes combinações de crioprotetores extracelulares: i) 0,2 M de SAC; ii) 10% de SFB; iii) 50% de SFB; iv) 0,2 M de SAC com 10% de SFB; v) 0,2 M de SAC com 50% de SFB. O sistema de congelação empregado foi Mr. Frosty numa taxa de resfriamento de 1°C/min e concentração final de 1,0 x 10^4 células/mL. Após a descongelação e remoção dos crioprotetores, as células foram submetidas ao ensaio de viabilidade por corante azul de tripan. Todos os dados foram expressos como média ± erro padrão e analisados por ANOVA e Tukey ($P < 0,05$). Após cinco repetições (1 animal/1 repetição), foi obtida uma taxa média de viabilidade antes da congelação de 78,0% ± 12,9. Após a descongelação, as taxas de viabilidade obtidas em cada grupo foram: 0,2 M de sacarose (66,0% ± 6,3); 10% de SFB (87,8% ± 7,5); 50% de SFB (73,8% ± 10,9); 0,2 M de sacarose com 10% de SFB (67,1% ± 14,5); 0,2 M de sacarose com 50% de SFB (66,6% ± 9,8). Nenhuma diferença foi observada nas taxas de viabilidade avaliada por azul de tripan para células congeladas e não congeladas ($P > 0,05$). Além disso, nenhuma diferença foi observada entre os grupos criopreservados contendo diferentes crioprotetores extracelulares ($P > 0,05$). Portanto, células somáticas derivadas de catetos podem ser criopreservadas com sucesso independente do tipo de crioprotetor extracelular empregado.

Palavras-chave: Congelação lenta. Cultivo *in* vitro. Mamíferos silvestres. Sacarose. Soro fetal bovino.

Agência financiadora: Bolsista IC-PIVIC (2017-2018).

ANEXO I – RESUMOS PUBLICADOS NO CONERA 2018
CONGRESSO NORTE-NORDESTE DE REPRODUÇÃO ANIMAL

Produção *in vitro* de embriões de cateto (*Pecari tajacu* Linnaeus 1758) usando diferentes ativadores químicos

[*In vitro* production of collared peccary (*Pecari tajacu* Linnaeus 1758) embryos using different chemical activators]

Alana Azevedo Borges[1*], Maria Valéria de Oliveira Santos[1], Lucas Emanuel Nascimento[1], Gabriela Pereira de Oliveira Lira[1], Érika Almeida Praxedes[1], Moacir Franco de Oliveira[2], Alexandre Rodrigues Silva[3], Alexsandra Fernandes Pereira[1]

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A ativação artificial consiste numa etapa crucial para o sucesso da produção de embriões clones por transferência nuclear de células somáticas (TNCS). Nesse sentido, o estabelecimento de protocolos de ativação oocitária em catetos, mamíferos silvestres de importância ecológica, econômica e científica, representa um dos pontos fundamentais para o desenvolvimento da TNCS nesta espécie. Em mamíferos domésticos, o uso de ativadores químicos, como os ativadores primários de liberação inicial de cálcio [ionomicina] em combinação com ativadores secundários de inibição de proteína quinase [6-dimetilaminopurina (6-DMAP)] ou de síntese proteica [ciclohexamida (CHX)] tem sido empregado para a ativação oocitária com respostas variáveis entre essas associações. Portanto, o objetivo foi produzir embriões *in vitro* de catetos por ativação oocitária artificial usando a ionomicina em associação com diferentes ativadores secundários. Para tanto, oócitos imaturos derivados de doze fêmeas adultas estimuladas com 600 UI de PG600® (MSD Saúde Animal, São Paulo, Brasil) foram recuperados após eutanásia dos animais. Em seguida, apenas oócitos com mais de uma camada de células do *cumulus* e citoplasma homogêneo foram maturados *in vitro* por 44 h (38,5°C e 5% de CO_2) em meio TCM199 modificado. Decorrido o período de maturação, oócitos com o primeiro corpúsculo polar foram ativados artificialmente usando 5,0 µM de ionocimina por 4 min seguida de incubação por 3 h com 1,9 mM de 6-DMAP (grupo 6D) ou 10 µg/mL de CHX (grupo CHX). Os presumíveis zigotos foram cultivados *in vitro* por sete dias e avaliações foram realizadas em D3 e D7 quanto às taxas de clivagem e blastocisto, respectivamente. Além disso, para avaliação da qualidade embrionária, blastocistos foram marcados com Hoechst 33342 para contagem do número de blastômeros. Os dados foram descritos como média ± erro padrão, sendo as taxas de produção embrionária e número de blastômeros analisados pelo teste do chi-quadrado e ANOVA seguido de teste de Tukey, respectivamente ($P < 0,05$). Após três repetições (4 fêmeas por repetição), nenhuma diferença foi observada para as taxas de clivagem entre os grupos, sendo evidenciado valores superiores a 78% de embriões clivados (6D: 82,9% ± 2,4 e CHX: 78,4% ± 2,2). Além disso, nenhuma diferença foi observada para as taxas de blastocistos, tanto quando foram calculadas pelo número de total de estruturas ativadas (6D: 20,0% ± 0,3 e CHX: 5,4% ± 0,2), quanto pelo número total de embriões clivados (6D: 24,1% ± 0,3 e CHX: 6,9% ± 0,3). Finalmente, também nenhuma diferença foi observada quanto ao número total de blastômeros entre os grupos (6D: 172,0 ± 76,9 e CHX: 195,0 ± 0,0). Esses resultados indicam que ativadores químicos podem ser empregados com sucesso na produção de blastocistos de catetos, independente do mecanismo de ação do ativador secundário (6DMAP ou CHX). Em conclusão, a associação de ionomicina independente do tipo de ativador secundário pode ser empregada na ativação artificial de oócitos de catetos, visando seu uso na clonagem desta espécie.

Palavras-chave: ativação artificial, clonagem, mamíferos silvestres.
Keywords: artificial activation, cloning, wild mammals.

ANEXO J – RESUMO PUBLICADO NO ISABR 2018

VII INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION

Evaluation of epidermal growth factor on *in vitro* maturation of collared peccaries' (*Pecari tajacu* Linnaeus, 1758) oocytes

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Oocyte *in vitro* maturation (IVM) is the first step of the *in vitro* reproductive technologies that enables matured oocytes to be used for embryo production. In this sense, the establishment of culture environment, as medium composition, is essential for the success of the IVM. In some domestic mammals, the supplementation of IVM medium with 10 ng/mL of epidermal growth factor (EGF) increased the IVM rates, and we hypothesized that this effect could also be observed for collared peccary oocytes. Therefore, the aim was to evaluate the EGF on the IVM rates of collared peccary oocytes, wild mammals of great commercial and ecological interest. Thus, eight adult collared peccaries (two females per session) were ovarian stimulated with 600 IU of PG600® (400 IU eCG and 200 IU hCG, MSD Saúde Animal, São Paulo, Brazil). Four days after hormone administration, ovaries were recovered, and all visible follicles with a 3–6 mm diameter were aspirated for the oocyte recovery using a 21 G needle attached to a 5.0 mL syringe containing oocyte recovery medium. Oocytes were classified with a stereomicroscope and only oocytes with more than one layer of *cumulus* cells and homogeneous cytoplasm were used for IVM. Thus, oocytes were matured in TCM199 contained 20 µg/mL of FSH-LH, 10% of fetal bovine serum, 100 µM of cysteamine, 1% of antibiotic-antimycotic solution and in the absence (group without EGF) or presence of 10 ng/mL of EGF (group with EGF). Oocytes were divided randomly in both groups and matured *in vitro* for 44 h at 38.5°C in a humidified atmosphere with 5% CO_2. Immediately after the IVM, oocytes were evaluated for expansion of *cumulus* cells using a stereomicroscope. After, oocytes were denuded and assessed for the presence of first polar body using a stereomicroscope. Finally, to evaluate the nuclear stage, denuded oocytes were fixed in 4% paraformaldehyde in PBS for 30 min. Then, oocytes were stained with Hoechst 33342 (10 µg/mL) for 15 min and visualized with a fluorescent microscope for identification of nuclear status in second metaphase. All data were expressed as mean ± standard error and analysed by the chi-square test ($P < 0.05$). A total of 172 follicles were aspirated after four sessions of ovarian stimulation. The oocyte recovery rate was 69.8% ± 8.3 (120/172) with an average of 7.5 ± 2.4 oocytes per ovary, and 5.3 ± 1.7 viable oocytes per ovary. After the IVM, no difference ($P > 0.05$) was observed between oocytes matured in absence and presence of EGF for expansion of the *cumulus* cells (97.6% ± 1.2 *vs.* 100% ± 0.0), presence of first polar body (65.9% ± 1.2 *vs.* 70.5% ± 1.8) and nuclear status in second metaphase (62.5% ± 11.6 *vs.* 68.4% ± 4.9), respectively. Probably, as occurred in swine oocytes, domestic mammals phylogenetically closely to the collared peccaries, the presence of EGF during the IVM can act in the protein synthesis and this effect could be observed during the embryonic development. In conclusion, according to the meiotic potential observed in collared peccary oocytes, EGF has not improved the IVM rates in this species.

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ANEXO L – RESUMO PUBLICADO NO CBRA 2019

CONGRESSO BRASILEIRO DE REPRODUÇÃO ANIMAL

Efeito da privação de soro sobre a sincronização do ciclo celular de fibroblastos derivados da pele de catetos, *Pecari tajacu* (Linnaeus, 1758)

Effect of serum starvation on cell cycle synchronization fibroblast derived from skin collared peccaries, Pecari tajacu (Linnaeus, 1758)

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A clonagem por transferência nuclear de células somáticas consiste numa alternativa para a conservação de catetos, mamíferos silvestres de grande relevância por seu potencial econômico, científico e ecológico. Nesse contexto, a otimização das etapas envolvidas nessa biotécnica, como a sincronização dos carioplastos em G0/G1 do ciclo, são cruciais para o seu sucesso na espécie de interesse. Portanto, o objetivo foi avaliar o efeito da privação de soro fetal bovino (SFB) sobre a sincronização na fase G0/G1 de fibroblastos derivados de catetos. Para tanto, foram utilizados fibroblastos criopreservados da terceira passagem obtidos de fragmentos de pele de quatro catetos adultos. Após a descongelação, fibroblastos cultivados e com 70% de confluência tiveram o meio de cultivo com 10% de SFB substituído por 0,5% de SFB. Em seguida, células foram avaliadas de 24 h a 96 h de cultivo em privação de soro. Células em crescimento com 70% de confluência e não submetidas à supressão de SFB foram consideradas como grupo controle. Para análise do ciclo celular, células ao final do seu período de tratamento foram tripsinizadas, centrifugadas, fixadas em etanol e armazenadas a -4°C. Posteriormente, as células foram incubadas em solução composta por iodeto de propídio (20 μg/mL) e RNAse (50 μg/mL) por 50 min. Subsequentemente, todas as células foram analisadas por citômetro de fluxo Guava Easycyte Desktop (Guava Technologies). Para cada amostra, 15.000 eventos foram registrados, e histogramas gerados para avaliar o percentual de células em cada fase do ciclo celular (G0/G1, S, G2/M) usando o software MODFIT versão 5.0. Os dados foram expressos como média ± erro padrão e analisados pelo software GraphPad ($P < 0,05$). Assim, após cinco repetições (um animal/repetição), fibroblastos submetidos à privação de SFB por 96 h apresentaram um maior percentual de G0/G1 (79,0% ± 1,6), quando comparados aos fibroblastos não submetidos à sincronização (68,1% ± 8,5, $P < 0,05$). Além disso, nenhuma diferença foi observada entre os fibroblastos não sincronizados e sincronizados por 24 h (78,7% ± 2,3), 48 h (78,1% ± 1,7) e 72 h (75,8% ± 2,9). Adicionalmente, nenhuma diferença foi observada entre as demais fases (S, G2/M) em nenhum dos períodos avaliados ($P > 0,05$). Portanto, a privação de SFB por 96 h promoveu a sincronização de fibroblastos de catetos na fase G0/G1. Estes resultados são relevantes para o desenvolvimento da técnica de clonagem em catetos.

Palavras-chave: carioplasto, transferência nuclear de células somáticas, mamíferos silvestres.
Keywords*: karyoplast, somatic cell nuclear transfer, wild mammals.*

ANEXO M – RESUMOS PUBLICADOS NO RENORBIO 2019

II ENCONTRO DE BIOTECNOLOGIA NO NORDESTE

RENORBIO 2019 - II Encontro de Biotecnologia do Nordeste

Avaliação dos aspectos morfométricos de ovários oriundos de catetos após estimulação hormonal

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Palavras-chave: mamíferos silvestres, desempenho reprodutivo, protocolos hormonais,

A produção *in vitro* de embriões está diretamente ligada à função ovariana, uma vez que os ovários possuem funções essenciais na produção de oócitos a ser empregados nas biotecnologias reprodutivas, como a fecundação *in vitro*. Nesse sentido, protocolos de estimulação ovariana são essenciais na recuperação de um maior quantitativo de oócitos. Contudo, a identificação dos aspectos morfométricos dos ovários é etapa importante para o aprimoramento desses protocolos. Portanto, o objetivo foi avaliar os aspectos morfométricos de ovários direitos e esquerdos de catetos, mamíferos silvestres de significativa importância econômica em virtude da qualidade de seus produtos (carne e couro). Para tanto, vinte fêmeas com idade de 25,1 ± 2,5 meses foram estimuladas com 600 UI de PG600® por via intramuscular (400 UI eCG e 200 UI hCG). Após quatro dias da administração hormonal, ovários foram recuperados e transportados ao laboratório em NaCl a 0,9% (37°C por 30 min). No laboratório, com o auxílio de um paquímetro digital, ovários direitos e esquerdos foram mensurados, quanto ao comprimento (mm), largura (mm) e espessura (mm). Além disso, usando uma balança digital, ovários foram mensurados quanto ao volume (mL) e peso (g). Todos os dados foram expressos como média ± erro padrão e analisados por ANOVA seguida por teste de Tukey ($P < 0,05$). Nenhuma diferença foi observada entre os ovários direitos e esquerdos, respectivamente, para comprimento (21,2 ± 0,9 mm vs. 22,4 ± 0,9 mm), largura (16,1 ± 0,8 mm vs. 16,9 ± 0,8 mm), espessura (3,0 ± 0,3 mm vs. 3,8 ± 0,4 mm) e peso (3,0 ± 0,3 g vs. 3,3 ± 0,3 g). Portanto, a estimulação hormonal ovariana com PG600® promoveu padrão similar morfométrico entre os ovários direitos e esquerdos de catetos.

RENORBIO 2019 - II Encontro de Biotecnologia do Nordeste

Efeito do fator de crescimento epidermal sobre a morfometria de oócitos maturados de catetos

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Palavras-chave: mamíferos silvestres, maturação *in vitro*, produção *in vitro* de embriões.

A produção *in vitro* de embriões (PIVE) é o foco central da agropecuária, principalmente em virtude do interesse no aumento da produtividade por meio da multiplicação de indivíduos interessantes economicamente e de alta adaptação a cativeiros. Nesse cenário, incluem-se os catetos, os quais possuem uma interessante capacidade produtiva e potencial de mercado para seus produtos, especialmente em países da América Latina. Contudo, a eficiência da PIVE depende da bem-sucedida maturação *in vitro* (MIV) de oócitos. Portanto, o objetivo foi avaliar o efeito do fator de crescimento epidermal (EGF) sobre a morfometria de oócitos maturados de catetos. Para tanto, oócitos viáveis morfologicamente e derivados de oito fêmeas adultas e previamente submetidas à estimulação hormonal ovariana por quatro dias com 600 UI de PG600® (400 UI eCG e 200 UI hCG) foram maturados *in vitro*. Para a MIV, oócitos foram incubados por 44 h (38,5°C e 5% de CO_2) de acordo com os grupos: ausência ou presença de 10 ng/mL de EGF. Após a MIV, oócitos foram desnudos e avaliados morfometricamente, quanto diâmetro externo do oócito (DEO); espessura da zona pelúcida (EZP); diâmetro do ooplasma (DO); área do espaço perivitelino (AEP) e comprimento do espaço perivitelino (CEP). Tais parâmetros foram analisados por meio do software ImageJ, utilizando-se a barra de escala como escala arbitrária para análise de pixels. Os dados foram expressos como média ± erro padrão de quatro repetições (dois animais por repetição) e analisados a uma significância de $P < 0,05$. Nenhuma diferença foi observada entre os grupos maturados na ausência e presença de EGF, respectivamente, para DEO (382,2 ± 5,3 µm vs. 373,9 ± 4,4 µm), DO (285,6 ± 3,0 vs. 284,3 ± 4,2 µm), AEP (6284,0 ± 390,9 vs. 7312,6 ± 823,1 μm^2) e CEP (13,8 ± 0,8 vs. 16,2 ± 2,1 µm). Contudo, quanto à EZP, oócitos maturados na presença de EGF apresentaram uma redução de sua espessura (33,3 ± 1,2 µm), quando comparados aos oócitos maturados na ausência de EGF (38,6 ± 1,5 µm). Esta diferença implica em uma característica positiva uma vez que a espessura da zona pelúcida consiste em um indicativo relevante para a eclosão e implantação de blastocistos derivados desses oócitos. Portanto, de acordo com os parâmetros morfométricos, sugere-se o uso do EGF como suplementação no meio de MIV de oócitos de catetos.

ANEXO N – RESUMO PUBLICADO NO SEMIC 2019
XXV SEMINÁRIO DE INICIAÇÃO CIENTÍFICA DA UFERSA

Área do conhecimento: Ciências Biológicas

EFEITO DO FATOR DE CRESCIMENTO EPIDERMAL SOBRE A MATURAÇÃO *in vitro* DE OÓCITOS DE CATETOS, *Pecari tajacu* LINNAEUS, 1758 (ARTIODACTYLA: TAYASSUIDAE)

Luanna Lorenna Vieira Rodrigues; Alexsandra Fernandes Pereira; Lucas Emanuel Nascimento; Alana Azevedo Borges; Maria Valéria de Oliveira Santos

A produção *in vitro* de embriões (PIVE) tem se estabelecido como uma ferramenta interessante para a conservação de mamíferos silvestres, como os catetos. Nesse contexto, o desenvolvimento de meios adequados para a maturação *in vitro* (MIV) consiste numa etapa fundamental para a eficiência da PIVE. Portanto, o objetivo foi avaliar o efeito do fator de crescimento epidermal (EGF) durante a MIV de complexos *cumulus*-oócito (CCOs) derivados de catetos. Inicialmente, todos os procedimentos foram aprovados pelo Comitê de Ética e Uso de Animais (CEUA/UFERSA, no. 23091.001072/2015-92) e Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, no. 48633-2). Para tanto, oito fêmeas foram estimuladas hormonalmente com 600 UI de PG600® e, após a eutanásia dos animais no quarto dia de aplicação, os ovários foram removidos e transportados até o laboratório. Os folículos ovarianos com 2–6 mm de diâmetro foram aspirados e os CCOs apresentando mais de uma camada de células do *cumulus* e citoplasma homogêneo foram direcionados à MIV. Assim, CCOs foram maturados por 44 h (38,5°C e 5% de CO_2) em meio TCM199 modificado e na ausência e presença de 10 ng/mL de EGF. Após a MIV, oócitos foram avaliados quanto à expansão e viabilidade das células do *cumulus* por azul de tripan, maturação nuclear pela presença do primeiro corpúsculo polar (1CP) e níveis de espécies reativas de oxigênio (EROs) usando sonda fluorescente. Todos os dados foram expressos como média ± erro padrão e comparações foram realizadas usando o teste ANOVA seguido de teste de Tukey ($P < 0,05$). Após quatro repetições (dois animais/repetição), foram obtidos 16 ovários, resultando em 231 folículos aspirados e 120 CCOs recuperados, perfazendo uma taxa de recuperação de 49,1%. Assim, de acordo com a avaliação e classificação morfológica, 85 (70,8%) CCOs viáveis e 35 (29,2%) CCOs não viáveis foram recuperados, sendo empregado para a etapa de MIV apenas os CCOs viáveis. Após a MIV, os grupos avaliados mostraram altas taxas de expansão das células do *cumulus* (ausência de EGF: 98,8% ± 1,2 e presença de EGF: 100% ± 0,0) com apenas expansão nível III e IV identificadas ($P > 0,05$). Quanto à viabilidade das células do *cumulus*, nenhuma diferença foi observada entre os grupos na ausência (79,4% ± 2,7) e presença de EGF (79,4% ± 2,7). Além disso, nenhuma diferença foi observada entres os grupos de CCOs maturados na ausência e presença de EGF para as taxas de maturação nuclear (73,1% ± 9,0 *vs.* 76,0% ± 8,1) e níveis de EROs (0,3 ± 0,1 *vs.* 0,5 ± 0,2 unidades de fluorescência arbitrária). Em conclusão, o EGF não influencia as taxas de maturação nuclear, expansão e viabilidade das células do *cumulus* e níveis de EROs em CCOs de catetos. Provavelmente, o efeito dessa suplementação poderia ser melhor visualizado durante o desenvolvimento embrionário.

Palavras-chave: Mamíferos silvestres. Produção de embriões. Complexos *cumulus*-oócito.
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