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CARACTERIZAÇÃO E CRIOPRESERVAÇÃO DE SÊMEN DE ONÇA-PINTADA (*Panthera onca*)

FORTALEZA-CEARÁ
2019

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do grau de Doutor em Ciências Veterinárias. Área de Concentração: Reprodução e Sanidade Animal.

Orientadora: Prof.ª Drª. Lúcia Daniel Machado da Silva.
Coorientador: Prof. Dr. Alexandre Rodrigues Silva.

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RESUMO

Um dos principais pontos da biotecnologia reprodutiva é a criopreservação de sêmen, que possibilita a criação de um banco genético de fácil acesso, bem como permite maximizar a utilização dos gametas masculinos. Portanto, objetivou-se primeiramente descrever as características morfológicas e ultraestruturais do sêmen fresco de onça-pintada e, posteriormente, comparar a qualidade do sêmen pós-descongelação, utilizando-se os diluidores à base de TRIS ou água de coco em pó (ACP-117c). Para tanto, foram utilizados cinco machos de onça-pintada que foram submetidos a duas coletas seminais por eletroejaculação. O sêmen foi avaliado quanto à motilidade total, vigor, funcionalidade da membrana espermática, atividade mitocondrial por meio do 3,3'-diaminobenzidina (DAB) e morfologia dos espermatozoides, além das avaliações ultraestruturais por microscopia eletrônica de varredura (MEV) e transmissão (MET), como também a análise morfométrica por meio do *software* Image J. Em seguida, foi realizado o processamento das amostras para criopreservação. Após o término das coletas, as amostras foram descongeladas e, realizadas as mesmas análises feitas nas amostras frescas, exceto a morfometria, além da avaliação computadorizada do sêmen (CASA), como também análise da integridade das membranas celulares por meio de análise de fluorescência e o teste de interação com membrana perivitelínea de ovo de galinha. Os resultados estão expressos na forma de média ± erro médio padrão. Os principais resultados do sêmen fresco foram: motilidade; vigor. As principais alterações morfológicas encontradas foram: na cabeça dos espermatozoides (9,0 ± 1,7%) e defeitos de cauda (12,5 ± 3,3%). A largura e o comprimento da cabeça foram 3,60 ± 0,03 μm e 4,9 ± 0,02 μm, respectivamente, peça intermediária 9,7 ± 0,3 μm, cauda 54,5 ± 4,4 μm, e o comprimento total do espermatozoide foi de 59,5 ± 0,1 μm. Regiões electronlucentes foram encontradas na região do núcleo e aproximadamente 54 espirais mitocondriais na peça intermediária foram identificadas usando microscopia eletrônica. As maiores porcentagens de células foram classificadas como DAB I (46,6 ± 4,9%) e DAB II (38,0 ± 4,4%). No tocante à criopreservação, as amostras preservadas no TRIS apresentaram melhor resultado do que as preservadas no ACP-117c: maior motilidade pós-descongelação (46,0 ± 7,7% *vs* 20,9 ± 5,4% de espermatozoides móveis; p=), melhor funcionalidade da membrana (60,5 ± 4,2% *vs* 47,1 ± 2,5% de membrana funcional; p=) e maior atividade mitocondrial (21,5 ± 3,7% *vs* 11,8 ± 1,7%; p=). Em relação às avaliações ultraestruturais, a MEV mostrou que ambos os diluidores foram capazes de preservar o plasmalema espermático, mas a MET revelou a ocorrência de pontos electronlucentes, especialmente em amostras preservadas em ACP-117c. Além disso, um maior número de

espermatozoides ligados à membrana perivitelina foi observado com as amostras diluídas em TRIS em comparação com o ACP-117c (29,5 ± 3,3% *vs* 18,6 ± 1,5%; p=). Em conclusão, o diluidor TRIS apresentou melhores resultados do que o diluidor ACP-117c, para a criopreservação de sêmen de onça-pintada.

Palavras-chaves: Avaliação seminal. Congelação. Felidae. Morfologia espermática. Morfometria espermática.

ABSTRACT

One of the main points of reproductive biotechnology is semen cryopreservation, which allows the creation of an easily accessible genetic bank, as well as maximizing the use of male gametes. Therefore, the objective was first to describe the morphological and ultrastructural characteristics of jaguar fresh semen, and then to compare the quality of the post-thawed semen using TRIS or powdered coconut water (ACP-117c) extender. For this, five male jaguars were submitted to two seminal collections by electroejaculation. Semen was evaluated for total motility, vigor, sperm membrane functionality, mitochondrial activity by 3,3'-diaminobenzidine (DAB) and sperm morphology, plus ultrastructural evaluation by scanning electron microscopy (SEM) and transmission (TEM), as well as morphometric analysis using a software (Image J). Then, the samples were cryopreserved. After the end of all collections, the samples were thawed and first performed the same analyzes made on fresh samples, except for morphometry, as well as computerized semen evaluation (CASA), as well as analysis of cell membrane integrity by fluorescence, and the interaction test with perivitelline chicken egg membrane. The main results of fresh semen were morphological abnormalities in sperm head (9 ± 1.7%) and tail defects (12.5 ± 3.3%). Head width and length were 3.6 ± 0.03 μm and 4.9 ± 0.02 μm, respectively, middle piece 9.7 ± 0.3 μm, tail 54.5 ± 4.4 μm, and the total sperm length was 59.5 ± 0.1 μm. Electronlucent regions were found in the nucleus region and approximately 54 mitochondrial spirals in the middle piece were identified using TEM. The highest cell percentages were classified as DAB I (46.6 ± 4.9%) and DAB II (38 ± 4.4%). Regarding cryopreservation, samples preserved in TRIS showed better post-thaw motility (46.0 ± 7.7%) and membrane functionality (60.5 ± 4.2%) and greater mitochondrial activity (21.5 ± 3.7, respectively) than those preserved in ACP-117c (20.9 ± 5.4% mobile sperm; 47.1 ± 2.5% functional membrane; 11.8 ± 1.7% mitochondrial activity). Regarding ultrastructural evaluations, SEM showed that both extenders were able to preserve sperm membrane, but MET revealed electronlucent points, especially in samples preserved in ACP-117c. In addition, a higher number of perivitelline membrane-bound sperm (29.5 ± 3.3%) was observed with samples diluted in TRIS compared with ACP-117c (18.6 ± 1.5%). In conclusion, we suggest the TRIS extender for jaguar semen cryopreservation.

Keywords: Seminal evaluation. Freezing. Felidae. Sperm morphology. Sperm morphometry.

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LISTA DE ABREVIATURAS E SIGLAS

cm	Centímetro
%	Porcentagem
/	Por
°C	Graus Celsius
µg	Micrograma
µl	Microlitro
µm	Micrômetro
ACP	Água de coco em pó
ALH	Amplitude do deslocamento lateral da cabeça (*Amplitude of lateral head displacement*)
ANOVA	Análise de Variância
AX	Axoneme
BCF	Frequência de batida cruzada *(Beat cross frequency*)
BSA	Albumina sérica bovina (*Bovine Serum Albumin*)
$CaCl_2 2H_2O$	Cloreto de cálcio dihidratado
CADI	Centro Avançado de Diagnóstico Por Imagem
CAP	*Capitulum*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAQ	Casa da Química
CASA	Análise de sêmen assistida por computador (*Computer-assisted sperm analysis*)
CE	Ceará
CENARGEN	Embrapa Recursos Genéticos e Biotecnologia
CFDA	Diacetato de 6-carboxifluoresceína (*6-carboxyfluorescein diacetate*)
CITES	Convenção sobre o Comércio Internacional de Espécies Ameaçadas de Fauna e Flora Selvagens (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico

DAB	3,3'-Diaminobenzidina
DMSO	Dimetil Sulfóxido
eCG	Gonadotropina coriônica equina (*Equine chorionic gonadotropin*)
EFA	Acessório de fluorescência episcópica (*Episcopic fluorescence attachment*)
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EY	Gema de ovo (*Egg yolk*)
FIV	Fertilização *in vitro*
FMVZ	Faculdade de Medicina Veterinária e Zootecnia
g	Grama
G	Força G para centrífuga
h	Hora
hCG	Gonadotropina coriônica humana (*Human chorionic gonadotropin*)
HEPES	Ácido 4-(2-hidroxietil)-1- piperazinaetanossulfônico
ICMBIO	Instituto Chico Mendes de Conservação da Biodiversidade
IF	Fossa de implantação (*Implantation fossa*)
IM	Intramuscular
IMV	*Instruments de Médecine Vétérinaire – IMV Technologies*
IU	Unidades internacionais (*International units*)
IUCN	União Internacional para Conservação da Natureza (*International Union for Conservation of Nature*)
KCl	Cloreto de potássio
Kg	Quilograma
L	Litro
LDL	Lipoproteínas de baixa densidade *(Low density lipoproteins*)
LH	Hormônio luteinizante
LIN	Índice de linearidade (*Linearity index*)
m	Metro
MET	Microscopia eletrônica de transmissão
MEV	Microscopia eletrônica de varredura
mg	Miligrama

MG	Minas Gerais
$MgCl_2$	Cloreto de magnésio
min	minuto
mL	Mililitro
MP	Peça intermediária (*Middle piece*)
MS	Bainha mitocondrial (*Mitochondrial spiral*)
n	Número
NA	Área do colo (*Neck área*)
NaH_2PO_4	Fosfato monossódico
$NaHCO_3$	Bicarbonato de sódio
NC	Carolina do Norte (*North Carolina*)
NCl	Cloreto de sódio
NM	Morfologia normal (*Normal morphology*)
ODF	Fibras densas externas (*Outer dense fibers*)
PB	Paraíba
PBS	Tampão salina fosfato (*Phosphate buffered saline*)
PC	Centríolo proximal (*Proximal centriole*)
PE	Pernambuco
pFSH	Hormônio folículo estimulante suíno (*porcine follicle-stimulating hormone*)
pH	Potencial Hidrogeniônico
PI	Iodeto de propídio (*Propidium iodide*)
PI	Piauí
PL	*Plasmalemma*
RN	Rio Grande do Norte
SAS	Software de Análise Estatística (*Statistical Analysis Software*)
SEM	Microcopia Eletrônica de Varredura (*Scanning electron microscope*)
SEM	Erro médio padrão (*Standard error*)
SISBio	Sistema de Autorização e Informação em Biodiversidade
SP	São Paulo
STR	Linearidade (*Straightness index*)

TEM	Microscopia eletrônica de transmissão (*Transmission electron microscopy*)
TM	Motilidade Total (*Total motility*)
Tris	Tris-hidroximetil-aminometano
UECE	Universidade Estadual do Ceará
UN	Não avaliado (*Unevaluated*)
USA	Estados Unidos da América (*United States of America*)
USP	Universidade de São Paulo
V	Volts
VAP	Velocidade média da trajetória (*Low-velocity average pathway*)
VCL	Velocidade curvilinear (*Curvilinear velocity*)
VSL	Velocidade linear progressiva (*Straight line velocity*)

SUMÁRIO

1 INTRODUÇÃO

A ordem Carnivora é uma das que mais apresenta espécies ameaçadas de extinção, totalizando um quarto de todas as espécies desta ordem (IUCN, 2019). Além disso, no tocante à família Felidae, de todas as 38 espécies de felídeos que já foram catalogados apenas o gato doméstico (*Felis silvestris catus*) não está em risco de extinção (HUNTER, 2015).

A onça-pintada é uma espécie pertencente à ordem dos carnívoros e da família dos felídeos, inicialmente esta espécie possuía uma distribuição que incluía quase todas as três Américas, indo desde os Estados Unidos à Argentina (EISENBERG; REDFORD, 1999). Entretanto, devido à ação antrópica, levando à perda ou fragmentação de seu habitat, hoje a espécie está extinta nos Estados Unidos, restringindo-se a áreas fragmentadas no México e em países da América Central. Entretanto, é na América do Sul que é encontrada sua maior distribuição (OLIVEIRA; CASSARO, 2005) (Figura 1). No Brasil, ocorre em todos os biomas, desde aqueles com grande cobertura florestal, como a Amazônia e a Mata Atlântica, ou regiões abertas, como o Cerrado, Caatinga e Pantanal OLIVEIRA et al., 2012).

Figura 1 – Distribuição original e atual da onça-pintada

Fonte: OLIVEIRA et al. (2012).

A onça-pintada é considerada um predador de topo de cadeia, sendo considerado o maior felídeo do continente americano, cujo peso varia de 61,0 a 158,0 kg. O comprimento total tem variação de 1,88 a 2,07m, com os machos maiores que as fêmeas

(EMMONS; FEER, 1997; EISENBERG; REDFORD, 1999). Nesta espécie podem acontecer grandes variações de tamanho devido ao habitat, onde indivíduos quem vivem em áreas abertas parecem ser maiores que aqueles que vivem em áreas de floresta (OLIVEIRA; CASSARO, 2005). Possui tradicionalmente a coloração amarelada desde a cabeça até a cauda, onde é possível encontrar marcações de rosetas com manchas negras, e possui o ventre e peito esbranquiçados (CHEBEZ, 1994; OLIVEIRA; CASSARO, 2005).

Em relação às características reprodutivas, por serem indivíduos solitários, macho e fêmea ficam juntos apenas durante o período de cio da fêmea, e a gestação varia entre 90 e 111 dias, com número médio de dois filhotes. As crias ficam sob cuidados maternos até cerca de dois anos (MARGARIDO; BRAGA, 2004).

A espécie está classificada como ameaçada, sendo listada no apêndice I da CITES (CITES, 2019) e na Lista Vermelha mundial da IUCN (IUCN, 2019), e sua população apresenta um acentuado declínio. A conservação da onça-pintada depende de ações capazes de reduzir sua vulnerabilidade, por meio de ações conservacionistas *in situ* (promovendo a proteção de seus habitats, reduzindo a retirada de indivíduos e combatendo a caça) e *ex situ* (promovendo programas de educação ambiental e desenvolvimento de estratégias voltadas para a reprodução natural ou assistida). Ações como estas estão definidas no Plano de Ação Nacional para a Conservação da Onça-pintada, elaborado pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, 2013), com o apoio de especialistas na área.

O estudo das características reprodutivas de uma espécie torna-se de fundamental importância para a aplicação de biotécnicas reprodutivas que visem à preservação de uma espécie ameaçada (SILVA et al., 2004). Uma das principais biotécnicas reprodutivas é a criopreservação de sêmen, que possibilita a criação de um banco genético de fácil acesso, bem como permite maximizar a utilização dos gametas masculinos (WATSON, 2000). No entanto, esse processo pode causar redução na qualidade da amostra, afetando a capacidade fecundante das células espermáticas devido a alterações criogênicas (HOLT, 2000). Essas alterações podem ser reduzidas dependendo do tipo de diluente utilizado (ROCA et al., 2004). Porém, há necessidade de estabelecer o melhor protocolo de criopreservação e descrever quais são as principais alterações que o congelamento pode causar aos espermatozoides.

2 REVISÃO DE LITERATURA

2.1 CAPÍTULO 1 - CRIOPRESERVAÇÃO DE SÊMEN PARA SALVAR A ESPÉCIE ONÇA-PINTADA

Criopreservação de semen para salvar a onça-pintada (*Panthera onca*)

(*Semen cryopreservation to save jaguar (Panthera onca)*)

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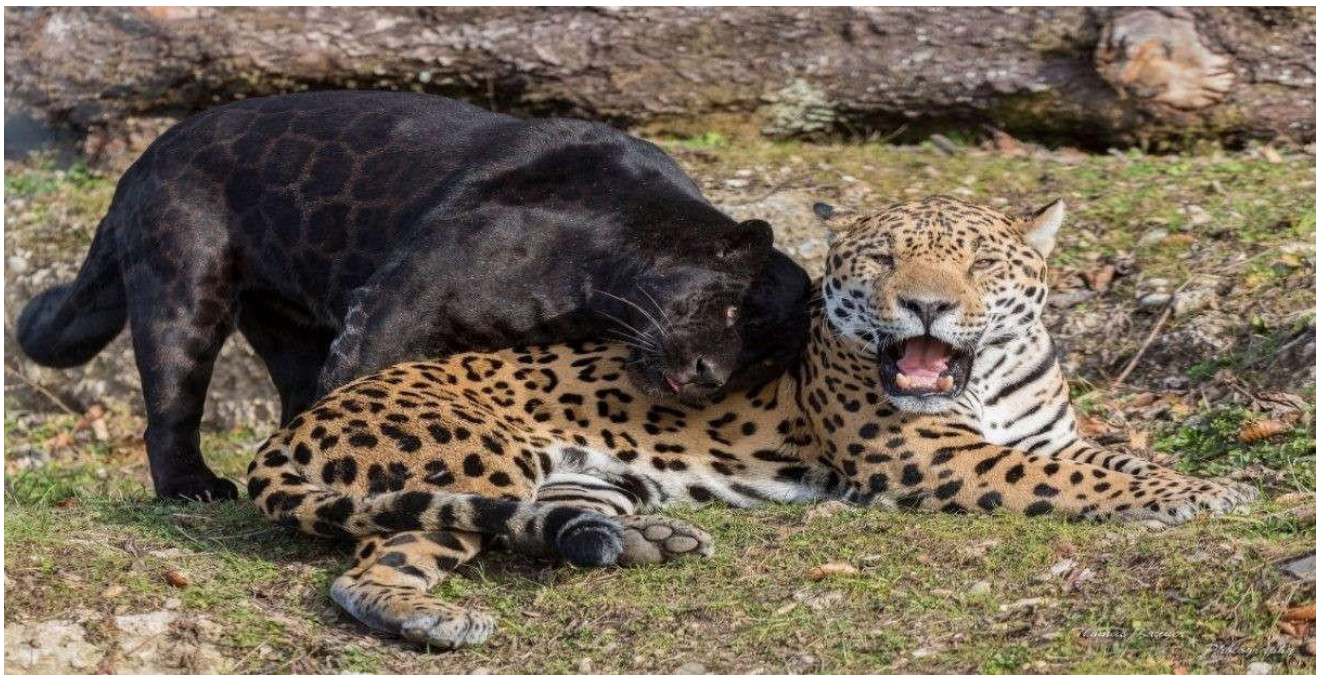
CRIOPRESERVAÇÃO DE SÊMEN PARA SALVAR A ESPÉCIE ONÇA-PINTADA

nanocell.org.br/criopreservacao-de-semen-para-salvar-a-especie-onca-pintada/

admin_cms

July 1, 2019



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A onça-pintada é um dos principais animais da fauna brasileira, sendo que além de ter sido utilizada como mascote do time brasileiro nas olimpíadas, ainda é representante do exército brasileiro e estampa a nossa cédula de cinquenta reais. Dentre todos os felinos conhecidos, a onça-pintada (Panthera onca) somente é menor que o leão (Panthera leo) e o tigre (Panthera tigris), e é classificada como o maior felino de todo o continente americano. Apesar de todo o reconhecimento e importância, a espécie vem sofrendo significativa redução no número de indivíduos. Seu habitat, que originalmente compreendia desde os Estados Unidos até o Sul da Argentina, hoje, basicamente se resume a áreas fragmentadas no México, América Central e do Sul (até o extremo norte da Argentina), no qual a maior parte da população é encontrada no Brasil (1) (Figura 1).

Figura 1: Onças-pintadas, variações pintada e melânica (preta).
Fonte: https://www.iguiecologia.com/onca-pintada/.

O status da população de onças-pintadas é de espécie vulnerável, segundo o Ministério do Meio Ambiente, e próximo de ameaçada, segundo classificação da Lista Vermelha da IUCN (1 e 2). As principais causas que levam à diminuição da população desta espécie estão ligadas à redução no número de suas presas naturais como cervos, queixadas e catetos, e principalmente devido à expansão de áreas urbanas, destruição dos habitats naturais, atropelamentos em rodovias e caça ilegal (1).

Para reverter este quadro, são desenvolvidas estratégias com o intuito de proteger a espécie, sendo que dentre elas ganha destaque o uso de biotécnicas reprodutivas, uma vez que tais técnicas são capazes de aumentar o número de indivíduos em um curto espaço de tempo. Nas biotécnicas reprodutivas, destaca-se a criopreservação (congelamento) de sêmen, técnica na qual se permite armazenar amostras sob temperaturas extremamente negativas, por um longo período de tempo e também deslocar o material para quaisquer localidades.

Para realizar a criopreservação é necessário primeiramente estipular a forma em que o sêmen será obtido. Existem dois principais protocolos de coleta na onça-pintada: a coleta farmacológica, em que a ejaculação do animal é induzida simplesmente pela combinação de medicamentos específicos; e a eletroejaculação, em que se utiliza um aparelho que emitirá pequenos impulsos elétricos suficientes para haver a liberação do sêmen. Para a realização deste método de coleta é necessário que o animal esteja anestesiado, a fim de facilitar a técnica e evitar acidentes (tanto com os profissionais, quanto com os animais) (3) (Figura 2).

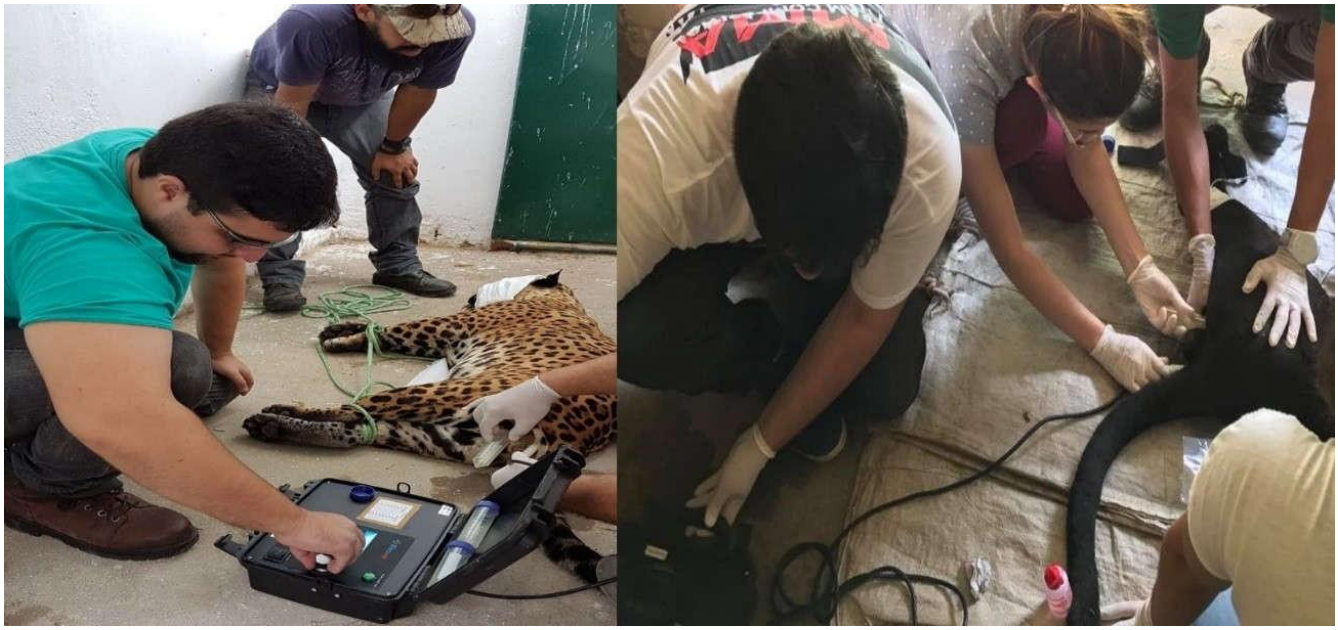

Figura 2: Coleta de sêmen em onça-pintada por eletroejaculação.

Após a coleta do sêmen, é necessário diluí-lo em substâncias capazes de nutrir os espermatozoides durante todo o processamento, bem como proteger as células das variações de temperatura. Alguns exemplos dos componentes dessas substâncias são: o Tris, que evita que o meio se torne excessivamente ácido ou básico, e também atua fornecendo açúcares para as células; a gema de ovo, que protege a superfície dos espermatozoides; e por fim oglicerol, que protege acélula contra possíveis rompimentos causados devido à formação de partículas de gelo (3).

Uma vez que o sêmen estiver diluído, as amostras estarão prontas para refrigeração, etapa em que serão submetidas a uma lenta redução de temperatura, que pode demorar até 3 horas. Na etapa final, as amostras são estocadas em pequenos tubos, e finalmente armazenadas até o dia do seu uso, em tanques de nitrogênio à temperatura de -196ºC. Nesta temperatura, os espermatozoides permanecem parados e congelados sem gasto de energia, até o momento de descongelamento. O descongelamento, diferentemente do processo de congelamento, é bastante rápido, sendo que as amostras ficam prontas para o uso em apenas 1 minuto, após serem submetidas à temperatura de 37ºC (3 e 4).

O armazenamento de amostras congeladas possibilita sua utilização em biotécnicas de reprodução assistida, assim como também pode contribuir para o aumento de variabilidade genética de espécies, visto que em felídeos, mais especificamente em guepardos (Acinonyx jubatus), foidemonstrado haver redução da capacidade reprodutiva em pequenas populações com alto grau de parentesco (5).

Quando comparada as espécies domésticas (como o cachorro, por exemplo), a pesquisa envolvendo criopreservação de sêmen nas onças ainda se encontra em seus primeiros passos. Embora os resultados publicados até o momento sobre a espécie sejam satisfatórios, é necessário realizar novos trabalhos a fim de determinar o protocolo ideal a ser utilizado. O próximo passo poderia ser então a inseminação artificial utilizando sêmen congelado, tendo em vista que recentemente (neste ano de 2019) foi reportado um trabalho conjunto entre a Associação Mata Ciliar, a Universidade de Mato Grosso (UFMT) e o zoológico de Cincinnati/EUA, sobre o

nascimento da primeira onça-pintada por meio de inseminação artificial utilizando sêmen fresco (6) (Figura 3).

Figura 3: Onça-pintada em cuidados com a cria. Fonte: Associação Mata Ciliar. **REFERÊNCIAS**

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2.2 CAPÍTULO 2 - BIOTÉCNICAS REPRODUTIVAS EM CARNÍVOROS NEOTROPICAIS

Biotécnicas reprodutivas em carnívoros neotropicais

(*Reproductive biotechniques in neotropical carnivores*)

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Biotécnicas reprodutivas em carnívoros neotropicais

Reproductive biotechniques in neotropical carnivores

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Resumo

Os carnívoros neotropicais incluem um grande número de espécies ameaçadas de extinção. É fundamental desenvolver esforços de conservação para garantir a sustentabilidade das populações *in situ* e *ex situ*. As principais prioridades são proteger os habitats naturais e entender melhor a biologia destas espécies. Os esforços de conservação também devem ser direcionados à implementação de programas de preservação da variabilidade genética e ao desenvolvimento de biotecnologias reprodutivas aplicáveis a realidade das instituições mantenedoras como zoológicos e bioparques. Também é fundamental criar bancos de germoplasma que contribuam para manter a diversidade genética em populações pequenas e ameaçadas. O presente artigo tem como objetivo revisar as biotécnicas reprodutivas aplicadas em carnívoros neotropicais e discutir sobre os resultados já obtidos na conservação dessas espécies.

Palavras-chave: conservação, animais selvagens, extinção.

Abstract

Neotropical carnivores include a large number of endangered species. Development of resources to guarantee sustainability of in situ and ex situ populations are fundamental. The main priorities are to protect natural habitats and understand about biology of these species. Conservation efforts should also be directed towards the implementation of breeding programs and development of reproductive biotechnologies applicable to reality of animal welfare institutions such as zoos and bioparks. It is also essential to establish genebanks that help maintain genetic diversity in small and endangered populations. Present article aims to review reproductive biotechniques on neotropical carnivores and to discuss results obtained in conservation of these species.

Keywords*: conservation, wildlife, extinction.*

Introdução

As espécies silvestres vêm sofrendo um declínio acentuado nas últimas décadas por causa de ações antrópicas, como desmatamento, queimadas e caça furtiva (Renctas, 2016). A interferência humana nos ecossistemas contribui para o desaparecimento de espécies em uma intensidade maior que as causas naturais (Ceballos et al., 2015). Especificamente, a extensa biodiversidade neotropical (área desde o México passando pela América Central, até a América do Sul) é altamente degradada pela atividade humana presente em países emergentes, e exige estratégias urgentes de conservação *in situ* e *ex situ* (Salvador et al., 2011).

Os carnívoros são as espécies mais afetadas pela perda do habitat natural. Por outro lado, a presença destes animais em seus habitats representa a estabilidade do ecossistema, uma vez que os carnívoros têm o potencial de controlar a população de espécies inferiores na cadeia alimentar (Salvador et al., 2011). Também devido à redução cada vez mais intensa do habitat, ocorre a reprodução entre pequenos grupos consanguíneos, assim reduzindo a variabilidade genética, refletindo em uma redução na performance reprodutiva e na baixa qualidade dos gametas (Wildt et al., 1983). Assim, é necessário o desenvolvimento de estratégias para o estudo e sustentabilidade de espécies carnívoras, favorecendo uma utilização mais dinâmica dos gametas e contribuindo para aumento da diversidade genética, consequentemente, influenciando de forma positiva na sua conservação.

Para o sucesso da conservação, é necessário o desenvolvimento de biotecnologias reprodutivas como: criopreservação de gametas, controle de ovulação, inseminação artificial, fertilização *in vitro* e transferência de embriões, possibilitando sua integração ao manejo genético de populações em centros de reprodução, zoológicos e parques (Wildt, 1992; Comizzoli e Holt, 2014; Comizzoli, 2017). Assim, o objetivo desta revisão foi descrever as biotécnicas reprodutivas já relatadas em algumas espécies de carnívoros neotropicais, discutindo seus principais resultados.

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Criopreservação de sêmen

As principais técnicas descritas para a criopreservação estão relacionadas ao uso de amostras do gameta masculino em vez do gameta feminino, com as metodologias variando amplamente entre as espécies. Cães e gatos geralmente, são os modelos experimentais ideais para estas biotecnologias, embora grandes adaptações sejam necessárias para o desenvolvimento da técnica em questão (Silva et al., 2004).

Primeiramente, é necessário se estabelecer o protocolo de obtenção das amostras para dar continuidade ao desenvolvimento da técnica. A eletroejacualação é o método mais utilizado para a coleta de sêmen em espécies carnívoras (Wildt et al., 1983). Este método permite a coleta segura tanto para o animal, quanto para a equipe executora, uma vez que é necessário a anestesia do animal para tal procedimento, como também, geralmente é o método de primeira escolha para obtenção de amostras em qualquer espécie selvagem. Entretanto, outros métodos de coleta alternativos já foram testados em carnívoros neotropicais.

A manipulação digital, que é o método mais comum de coleta de sêmen em cães, já foi realizada com sucesso em lobos-guará (*Chrysocyon brachyurus*) (Teodoro et al., 2012). Já a recuperação epididimária, um método capaz de recuperar espermatozoides de indivíduos que vieram a óbito, ou passaram por procedimento de esterilização, já foi realizado para coleta de amostras de várias espécies de carnívoros neotropicais como lobo-guará (*Chrysocyon brachyurus*), urso andino (*Tremarctos ornatos*), puma (*Puma concolor*) e onça-pintada (*Panthera onca*) (Jewgenow et al., 1997; Maksudov et al., 2008), porém, devido as condições de coleta realizada na maioria das vezes em animais senis ou mortos, ocorre o comprometimento da qualidade da amostra. Por fim, métodos mais atuais para obtenção de amostra de sêmen já vêm sendo desenvolvidos, se destacando a coleta farmacológica, onde são utilizadas substâncias anestésicas derivadas da medetomidina, que promovem a ejaculação sem a necessidade de estímulos elétricos, no qual já foram obtidos excelentes resultados em onças-pintadas (*Panthera onca*) preconizando a dose de 0,08 a 0,1 mg/kg (Araújo et al., 2017).

Após o sêmen coletado, previamente a criopreservação é importante que tenha uma avaliação criteriosa sobre a qualidade da amostra, como também a descrição das particularidades presentes na espécie estudada. Por exemplo, em procionídeos como o quati (*Nasua nasua*) e o guaxinim (*Procyon cancrivorus*) é verificada a presença de um capuz acrossomal diferenciado, que este não é encontrado nos outros carnívoros (Silva et al., 2014; Silva et al., 2015), já nos felídeos em geral, há um grande percentual de células apresentando defeitos morfológicos (Araújo et al., 2015; Araújo et al., 2017).

Após o sêmen coletado, é fundamental que este seja preparado para a criopreservação. Primeiramente há necessidade do uso de meios diluidores capazes de suprir as necessidades do espermatozoide durante a refrigeração até a criopreservação, como também evitar que a célula sofra alterações deletérias durante o procedimento (Silva et al., 2018).

Vários diluidores já foram testados, porém àqueles que possuem em sua composição o TRIS (Tris-hidroximetil-aminometano) obtiveram maior sucesso em espécies carnívoras como jaguatiricas (*Leopardus pardalis*), gato do mato (*Leopardus tigrinus*) (Araujo et al., 2015), onças-pintadas (*Panthera onca*) (Silva et al., 2017), pumas (*Puma concolor*) (Deco-Souza et al., 2013) e quatis de cauda anelada (*Nasua nasua*) (Paz e Ávila, 2015). Diluidores alternativos são uma opção para a composição do meio, dentre estes, temos o derivado de água de coco em pó (ACP-117c), que já apresentou resultados similares ao Tris até a etapa de refrigeração, em onças-pintadas (*Panthera onca*) (Silva et al., 2017).

Outros componentes também são fundamentais, para complementar o meio diluidor, dentre estes, a gema de ovo possui fundamental importância, pois funciona protegendo a membrana celular de alterações na bicamada fosfolipídica, sendo utilizada geralmente na proporção de até 20% (Amstislavsky et al., 2012).

A inclusão de um crioprotetor também se torna importante, pois este é responsável pela proteção contra a formação dos cristais de gelo, no qual geralmente é utilizado o glicerol em concentrações variáveis de 2 a 10%, porém outros crioprotetores como o DMSO já foram utilizados (Johnson et al., 2014a, Silva et al., 2018).

O sêmen pós-diluído, deve passar pelas etapas de refrigeração até a criopreservação, podendo então ser armazenadas em taques de nitrogênio por tempo indeterminado, entretanto, apenas poucas espécies já possuem protocolos descrevendo a criopreservação de sêmen com sucesso, como em jaguatiricas (*Leopardus pardalis*), gato do mato (*Leopardus tigrinus*) (Araujo et al., 2015), onças-pintadas (*Panthera onca*) (Paz et al, 2007), pumas (*Puma concolor*) (Deco-Souza et al., 2013) e quatis de cauda anelada (*Nasua nasua*) (Paz e Ávila, 2015), sendo necessário ainda o estudo em novas espécies, bem como o aprimoramento dos protocolos já descritos.

Indução da atividade ovariana

A indução da atividade ovariana com o uso de gonadotrofinas exógenas já é uma realidade bastante usual no controle da reprodução em diferentes espécies de felídeos visando a utilização de outras biotécnicas como a sincronização, a inseminação artificial, a fertilização *in vitro*, além da transferência e criopreservação de embriões. (Lima Neto et al., 2017).

Protocolos utilizando a gonadotrofina coriônica equina (eCG) e a gonadotrofina coriônica humana (hCG) são bastante usuais pois possuem uma prolongada meia vida na circulação (24 – 48h). Também podem ser utilizados métodos alternativos como o uso do folículo estimulante suíno (pFSH), ou o hormônio luteinizante suíno (pLH) e

seus derivados, estes possuem a biodisponibilidade inferior aos anteriormente citados, sendo de aproximadamente 2h (Paz, 2013).

Os protocolos estipulados com eCG e hCG para estimulação ovariana, visando inseminação artificial e FIV, já foram utilizados em vários felídeos como: jaguatirica (*Leopardus pardalis*), gato do mato pequeno (*Leopardus tigrinus*) (Swanson et al., 2002; Paz et al., 2006); puma (*Puma concolor*) (Barone et al., 1994); e onça-pintada (*Panthera onca*) (Jimenez et al., 1999; Morato et al., 2000). Em canídeos apenas em lobo guará (*Chrysocyon brachyurus*), foi realizado um estudo por Johnson et al., 2014b, realizando a indução ovariana pelo uso de LH, e foi percebido que dentre as espécies de canídeos já estudadas, apenas no lobo guará, há necessidade da presença do macho para que ocorra a ovulação.

Inseminação artificial

O sucesso da inseminação artificial em carnívoros está diretamente ligado ao local de deposição do sêmen no trato reprodutivo da fêmea. Os resultados relacionando os métodos de inseminação com deposição do sêmen na vagina e por meio cirúrgico diretamente no útero, demonstram que a deposição direta no útero apresenta resultados mais satisfatórios (Howard, 1993). Outro fator que pode interferir diretamente no sucesso da técnica é a qualidade do sêmen, no qual é visto uma resistência menor do sêmen descongelado quanto comparado a amostra fresca.

Em felídeos, já é possível realizar a técnica da inseminação artificial em algumas espécies com o resultado de gestação e filhotes ao complemento da técnica. As espécies que já foram descritas são: puma (*Puma concolor*) (Barone et al., 1994), no qual foi possível produzir um filhote saudável, utilizando a técnica da inseminação artificial por vídeo-laparoscopia; em jaguatirica (*Leopardus pardalis*) (Swanson et al., 1996) também da mesma forma, foi possível produzir um filhote saudável; em gato do mato pequeno (*Leopardus tigrinus*) (Moraes et al., 1997). Recentemente, uma parceria da Universidade Federal do Mato Grosso (UFMT), Mata Ciliar e o Zoológico de Cincinnati/EUA foi possível obter um filhote sadio através da técnica de inseminação artificial por vídeo- laparoscopia em onça-pintada (*Panthera onca*) (Cincinnati, 2019).

Em canídeos, apenas no lobo guará (*Chrysocyon brachyurus*), estão sendo realizados protocolos para a inseminação, onde o Smithsonian Conservation Biology Institute realiza estudos avançados para o desenvolvimento de biotécnicas reprodutivas que favorecem a conservação desta espécie, entretanto, estes ainda estão em fase inicial.

Fertilização *in vitro*

A fertilização *in vitro* (FIV) é uma técnica bastante útil para a preservação de espécies ameaçadas, uma vez que pode ser realizada previamente a recuperação de oócitos por meio de laparoscopia em animais que tiveram de passar por procedimento de ovariectomia, ou em indivíduos pós-mortem que ainda possuam tempo hábil para recuperação, não havendo comprometimento do material biológico (Paz, 2013).

Nos felídeos a maturação oocitária ocorre dentro de 24 a 32 horas no meio de cultivo, porém destes que atingem a maturação, 70% são capazes de serem fertilizados, e apenas 20 a 30% conseguem o desenvolvimento até blastocisto (Johnston et al, 1989). Nos canídeos como citado anteriormente, a taxa de sucesso é ainda menor devido principalmente, ao desenvolvimento do oócito ser mais tardio que em outras espécies (Santos et al., 2006).

Nos carnívoros neotropicais, um dos primeiros trabalhos realizados foi em puma (*Puma concolor*), no qual foi possível a recuperação de 106 oócitos, com 43,8% maturados, e destes 40% sendo fertilizados com espermatozoide coespecífico e 26,5% com espermatozoide de gato doméstico (Miller et al., 1990). Já em 1998, Pope et al., produziu embriões de gato-mourisco (*Herpailurus yagouaroundi*) com taxa de sucesso de 55,6%.

Os primeiros trabalhos envolvendo a produção *in vitro* de embriões no Brasil foram realizados em onça-pintada (*Panthera onca*), onde por meio de estimulação ovariana utilizando pFSH e LH, foram obtidos 25 folículos viáveis/fêmea. Entretanto, em menos de 25% foi possível a realização da fertilização (Morato et al., 2000). Já em jaguatirica (*Leopardus pardalis*) e em gato do mato pequeno (*Leopardus tigrinus*), no qual as fêmeas foram tratadas com eCG/hCG, a obtenção média foi de 10 folículos viáveis/fêmea. Destes folículos 60% foram possíveis de realizar a FIV, sendo ao final obtidos 76 embriões de jaguatirica (*Leopardus pardalis*) e 52 de gato do mato pequeno (*Leopardus tigrinus*) (Swanson e Brown, 2004).

Transferência de embriões

As técnicas relacionadas à transferência de embriões realizadas nas espécies carnívoras neotropicais têm uma baixa taxa de sucesso. Este fato pode estar relacionado com a baixa porcentagem de clivagem embrionária pós descongelamento, como por exemplo em felinos, que possuem taxas inferiores a 70% (Pope, 2000). Tal fator pode estar associado devido uma baixa resistência dos embriões a criopreservação, ou até mesmo à uma sincronização inadequada da fêmea receptora a fim de manter a sobrevivência fetal (Swanson e Brown, 2004).

Uma tentativa de transferência de embriões de gato-mourisco (*Herpailurus yagouaroundi*) para gato doméstico foi realizada por Pope et al., (1998). Apesar de conseguir levar a técnica até a formação de embriões, não foi possível a obtenção final de filhotes.

Até o momento o único sucesso na transferência de embriões em carnívoros neotropicais foi através do trabalho em parceria do Zoológico de São Bernardo do Campo no Brasil e do Zoológico de Cincinnati nos Estados

Unidos, no qual foram produzidos filhotes de jaguatirica (*Leopardus pardalis*), através da transferência de embriões por meio de laparoscopia na tuba embrionária (Swanson, 2012).

Considerações finais

Há grande dificuldade em conservar espécies ameaçadas de extinção. Instituições como zoológicos e demais mantenedores de espécies selvagens, não estão conseguindo atingir os índices de manutenção demográfica e genética, que deveriam ter pelo menos 90% de armazenamento da diversidade genética.

Recentemente, houve um aumento no número de trabalhos destinados à conservação de espécies carnívoras, mas ainda há dados insuficientes para estabelecer protocolos ideais. Espécies carnívoras neotropicais, como cachorro-vinagre (*Speothos venaticus*), tara (*Eira barbara*), ariranha gigante (*Pteronura brasiliensis*), urso-de- óculos (*Tremarctos ornatus*), entre outras, não foram submetidas a pesquisas relacionadas à utilização de biotécnicas que visem a preservação destas espécies. Muitas destas não possui sequer uma descrição simples dos parâmetros de sêmen fresco, ou nem o protocolo de coleta de sêmen foi estabelecido.

Mesmo com a aplicação das biotécnicas reprodutivas, apenas em jaguatirica (*Leopardus pardalis*), foi possível o sucesso final com a produção de filhotes. Entretanto, muitas pesquisas relacionadas as espécies carnívoras neotropicais, como por exemplo na onça-pintada (*Panthera onca*) e no lobo guará (*Chrysocyon brachyurus*) estão em fase de desenvolvimento por Universidades brasileiras e instituições estrangeiras a fim de utilizar o recurso genético desses animais e de outras espécies ameaçadas como estratégias de conservação.

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2.3 CAPÍTULO 3 - SEMEN CRYOPRESERVATION AND BANKING FOR THE ONSERVATION OF NEOTROPICAL CARNIVORES

Semen Cryopreservation and Banking for the Conservation of Neotropical Carnivores

(Criopreservação e Bancos de Sêmen para a Conservação de Carnívoros Neotropicais)

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Semen Cryopreservation and Banking for the Conservation of Neotropical Carnivores

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Neotropical carnivores include a large number of threatened and endangered species. It is critical to develop conservation efforts to ensure the sustainability of populations *in situ* and *ex situ*. The highest priorities are to protect natural habitats and better understand the biology of rare species. Conservation efforts also are directed toward the implementation of breeding programs and the development of reproductive biotechnologies in which the cryopreservation of male gametes plays a major role. It also is fundamental to create semen banks that contribute to maintaining genetic diversity in small and endangered populations. The present article aims at reviewing the state of the art in cryopreservation of semen from neotropical carnivores and discuss the de- velopment of systematic banking for the conservation of these understudied species.

Keywords: Carnivora, conservation, America, semen cryopreservation

Introduction

WILD SPECIES POPULATIONS have suffered a sharp decline in the last decades because of anthropic actions such as deforestation, burning, and poaching.[1] Human inter- ference in ecosystems contributes to species disappearance at an intensity greater than natural causes.[2] Specifically, the extensive biodiversity in the Neotropics (from Mexico through the whole of the Caribbean to South America) is highly degraded by human activities and urgently required *in situ* and *ex situ* conservation strategies.[3]

As top predators, carnivores such as large felids, ursids, and canids are the species most affected by the loss of natural habitat. In contrast, the presence of theseindividuals in their habitats represents stability of the ecosystem, since carnivores have the potential to control the population of the lower species in the food chain.[3] Thus, it is necessary to develop strategies for the study and sustainability of carnivore species. One conservation effort involves the development of reproductive biotechnologies (artificial insemination, *in vitro* fertilization, and embryo transfer) that can be potentially integrated into the genetic management of small populations in breeding centers, zoos, and animal parks. In parallel, the creation of cryobanks also is critical as it allows storing of biological material with two major purposes: to advance biological knowledge and to sustain the genetic diversity of these rare and endangered species.[4–6]

Cryobiology is the science behind the creation of biobanks, allowing the conservation of embryos, somatic tissues, and germplasms.[7] So far, most efforts have been centered on banking of male gametes because (1) there is more experience in collecting semen in different species and(2) sperm cells are more resilient to freezing temperatures than other cells such as oocytes.[8] However, many steps are required during the delicate cryopreservation process, including seminal plasma separation, dilution, exposure to cryoprotectant, refrigeration, freezing, and thawing.[9,10] The objective of the article is to review the state of the art in cryopreservation of semen from neotropical carnivores and discuss the development of systematic banking for the conservation of these understudied species.

Sperm Collection and Handling

A good sperm collection is a prerequisite for a successful cryopreservation process. In carnivores, techniques used to obtain spermatozoa include diverse methods such as digital manipulation, electroejaculation, and urethral catheterization through pharmacological induction. Ejaculatory methods can be performed on more than one occasion. Digital manipulation is accomplished quite routinely in dogs; however, it is unusual in wild animals. This method is only possible in individuals conditioned to the technique; moreover, owing to the anatomy of the penis, it can only be

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performed in canids. This collection method has already been performed in maned wolf (*Chrysocyon brachyurus*), with samples having high total motility (76%) and vigor (3.9), but low sperm concentration (74 · 10^6 spermatozoa/mL) and normal morphology (36.5%).[11]

The most conventional method for collecting semen from wild carnivores is electroejaculation, because it is performed under anesthesia, which provides safety to the collection team and the animal.[12] For electroejaculation, it is necessary that the animal be anesthetized, and so, some anesthetic protocols are recommended, such as the combination of ti- letamine and zolazepam, which is most commonly used for many species, but the combination of ketamine and xylazine has also shown excellent results with respect to seminal quality.[13] The major impediment to electroejaculation is the ease of contamination with urine.[14] This issue has been re- ported in neotropical carnivores such as ring-tailed coatis (*Nasua nasua*),[13] crab-eating foxes (*Cerdocyon thous*),[15] and maned wolves (*C. brachyurus*).[16] To minimize this contamination, performing catheterization of the animal with a urethral catheter is recommended before electro- ejaculation, to remove all the contents of the bladder.[17] It is also recommended to perform a semen wash by centrifu- gation at 300 *g* for 10 minutes, using appropriate means to avoid reduction in seminal quality[18]; likewise, centrifuga- tion at 500 *g* for 5 minutes.[19]

Some precautions must be taken to avoid compromis- ing the technique and future use of the processed sample. Centrifugation with proper removal of seminal plasma contributes to the reduction of contaminants present in the sample, since this semen will be used for *in vitro* fertil- ization or insemination in the future. Studies have shown that without the removal of seminal plasma, it was pos- sible to verify the development of pyometra in 40% of the inseminated cats, regardless of whether the semen was diluted with antibiotics such as penicillin and streptomy- cin.[20] The main bacterium that causes infections in the reproductive tract is *Escherichia coli*; however, others act similarly. In neotropical felines, studies were conducted with the intention of describing the microbiota present in the reproductive tract of males and showed that these microorganisms have pathogenic potential to trigger in- fections in females. Paz et al.[21] described the most fre- quent presence of *Staphylococcus sp*, *Streptococcus sp*, *Escherichia coli*, and *Corynebacterium sp* in jaguars (*Panthera onca*). Guido et al.[22] described in oncilla (*Leopardus tigrinus*) the presence of *Escherichia coli*, *Proteus rettgeri*, and *Yersinia psedotuberculosis*; in ocelot (*Leopardus pardalis*): *Staphylococcus sp*, *Escherichia coli*, *Streptococcus sp*; and only *Staphylococcus sp* in Margay (*Leopardus wieddi*). There is also the possibility of viral transmission, such as feline immunodeficiency virus, which can be transmitted through semen.[23] Pre- cautions against these pathogens should be considered in order to be successful in biotechnology. For this reason, general and reproductive clinical exams should be used before reproductive works.

In the last decade, semen collection by urethral cathe- terization through pharmacological induction has been widely performed. This technique requires alpha-2 adren- ergic agonist drugs that act on seminal release, with me- detomidine being the most used and acting to stimulate erection and ejaculation in the animal. Lueders et al.[24] performed collection in maned wolves (*C. brachyurus*), with 40% sperm motility, 30% progressivity motile, 100 mL volume, and 10 million sperm per milliliter. Araujo et al.[25] developed this method of collection in jaguars (*P. onca*) with samples containing about 77% of motile spermatozoa with average vigor of 3.75.

There also is the possibility to recover epididymal spermatozoa, which involves flushing or slicing the epididymis.[26] Epididymal recovery is important for obtaining gametes from animals that have died, especially in zoos and parks, or those killed in road accidents, which is one of the main reasons for wild species casualties in America, as well as recovery of gametes from individuals dying unexpectedly in zoos and parks. Jewgenow et al.[26] reported the collection of spermatozoa from finely minced cauda epididymis of large felids such as leopards (*Panthera pardus*), cougars (*Puma concolor*), and jaguars (*P. onca*). Progressively motile spermatozoa varied between 60% and 85% after collection and between 25% and 65% after freezing and thawing. Maksudov et al.[27] described this form of postmortem collection in several wild species in a Russian zoo, including carnivores such as the maned wolf (*C. brachyurus*), but their best results were obtained with a spectacled bear (*Tremarctos ornatus*), in which motile (19%) and viable (62%) sperm were collected 3 days after the death of the animal. In general, semen quality of wild carnivores compared with domestic is considerably lower,[28] and greater care is required during handling of samples or during evaluations.

Extenders Composition

Methodologies described for semen cryopreservation in carnivores vary widely among species. Dogs and cats are ideal experimental models for biotechnologies, although large adaptations are necessary for developing the technique in question.[29] It is fundamental to understand the difference between extenders, cryoprotectants, and other components that will be added to the semen to obtain the best post-thawing results.

Egg yolk (EY) is one of the main components added to extender medium and mainly functions to protect the cell membrane, preventing the loss of phospholipids or replenishing them.[30] The likely way that the EY protects sperm is initially that low-density lipoprotein (LDL) present in EY can associate with sperm membrane and provide protection to sperm by stabilization of the membrane, and the second that phospholipids present in LDL protect the cell by forming a protective film on the surface of sperm or replacing phospholipids lost in the membrane during cryopreservation. Another hypothesis is that LDL interacts with seminal proteins performing this protection. Regardless of the protection mechanism, EY is still an essential component in extender preparation.[30] The combination of Tris and 20% EY is most commonly used for preparing carnivore semen extenders, and has demonstrated good results post-thawing in several canids, felids, ursids, and mustelids species[31]; however, EY concentration may vary depending on the extender. Because EY is a product of animal origin, it facilitates the propagation of bacteria and diseases, therefore, EY free extenders, which use soybean lectin as a substitute, could be used, such as the commercial extenders Bioxcell (IMV, L'Aigle, France) and AndroMed (Minitube,

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Tiefenbach, Germany). These have demonstrated excellent results in oncillas (*L. tigrinus*) and ocelots (*L. pardalis*).[30,31] As an alternative extender, it is also possible to use coconut water powder (ACP®), which has already shown good re- sults in maintaining the sperm quality of jaguar (*P. onca*) semen samples, similar to Tris.[32]

Extenders are one of the most important components that can influence the success of cryopreservation. In neotro- pical carnivores, some extenders have been tested, including Ham's F10 supplemented with bovine serum albumin in maned wolves (*C. brachyurus*)[16] and lactose-based solutions in jaguars (*P. onca*),[33] ocelots (*L. pardalis*), and oncillas (*L. ti- grinus*).[34] However, Tris (Tris-hydroxymethyl-aminomethane) is most commonly used in cryopreservation protocols for domestic carnivores and has been successfully tested in various species such as ocelots (*L. pardalis*), oncillas (*L. tigrinus*),[17] jaguars (*P. onca*), cougars (*P. concolor*),[35] and ring-tailed coatis (*N. nasua*).[36]

Combinations of Tris with other extenders such as TES (N-tris-methyl-2-sulfonic acid aminomethane) have been used successfully in carnivores such as ocelots (*L. pardalis*), oncillas (*L. tigrinus*),[31] and maned wolves (*C. brachyurus*).[16]

Detergents are commonly added to semen and can pro- vide greater longevity to spermatozoa, along with solubi- lizing phospholipids and increasing plasma membrane permeability, thus reducing the damage caused by osmotic stress. The most commonly used detergents are sodium dodecyl sulfate derivatives such as Equex STM paste, which has been successfully tested on canids and felids; in ocelot (*L. pardalis*) and oncilla (*L. tigrinus*), it was used at a concentration of 0.5% Equex added to the extender for cryopreservation.[17,18]

Glycerol is the most commonly employed cryoprotectant for freezing semen from different wild carnivore spe- cies.[16,36,37] The most commonly used concentrations in car- nivores vary between 2% and 10%.[36,37] Deco-Souza et al.[38] performed a comparative study of two glycerol concentra- tions (5% and 7.5%) in cougar (*P. concolor*) semen and verified that both are efficient for semen cryopreservation. Paz et al.[33] tested a final concentration of 4% glycerol in jaguar (*P. onca*) semen and obtained only 26.7% of mobile spermatozoa with 3.1 vigor.

In canids, the results obtained demonstrated that DMSO was able to surpass glycerol, when it was tested in the cryopreservation of semen from maned wolves (*C. bra- chyurus*).[16]

Although the ejaculate is a physiologically sterile fluid, inevitable contamination occurs as it passes through the urethra, penis, and prepuce.[21,22]Another important contam- ination factor is the EY used in the extender, which is a biological product capable of transporting microorgan- isms.[30] Environments such as zoos and habitats having free- living animals can also facilitate contamination of semen samples.[20] To avoid this problem, the use of bactericidal antibiotics in the extender is recommended. The most com- mon antibiotics combination used is penicillin and strepto- mycin. In carnivores, these antibiotics have already been tested together or alone for semen cryopreservation in jag- uars (*P. onca*) (1000 IU/mL penicillin, 1000 mg/mL strepto- mycin)[33] and cougars (*P. concolor*) (1 g/L streptomycin).[38] Amicacin is an alternative antibiotic in the protocol com- monly used for extender preparation, and has been tested in

TABLE 1. RESULTS OF FRESH AND POST-THAWING SEMEN METRICS IN NEOTROPICAL WILD CARNIVORES

	Fresh semen						*Frozen-thawed semen*		
Species	*Volume (mL)*	*pH*	*Concentration (10^6 spermatozoa/mL)*	*TM (%)*	*Vigor (0–5)*	*NM (%)*	*TM (%)*	*Vigor (0–5)*	*NM (%)*
Jaguar (*Panthera onca*)	7±0.6[33]	7.9±0.1[33]	1.6±0.6[33]	70±3.3[33]	3.7±0.2[33]	26.7±4.4[33]	26.7±4.4[33]	3.1±0.2[33]	UN
Cougar (*Puma concolor*)	0.4[38]	UN	165±151.9[38]	62±31.1[38]	3±1.2[38]	23.4±3.7[20]	42.5±6.5[38]	2.5±0.6[38]	UN
Ocelot (*Leopardus pardalis*)	0.3±0.1[20]	UN	28.0±17[20]	85±6.5[17]	4.3±0.3[17]	47.5±14[17]	25±12.6[17]	2.1±0.3[17]	23.1±6.9[17]
Oncilla (*Leopardus tigrinus*)	0.11±0.02[41]	UN	78.5±33.8[20]	80±0[17]	4.3±0.3[17]	81±2.6[17]	33.3±5.8[17]	2.5±0.5[17]	52.3±4.2[17]
Ring-tailed coati (*Nasua nasua*)	0.1±0.2[13]	7.8±0.5[13]	178.6±287.5[13]	84.3±11.6[36]	3.6±1.4[36]	81±16[13]	49.3±29.9[36]	2.2±1.1[36]	UN
Maned wolf (*Chrysocyon brachyurus*)	2±0.6[16]	7.4±0.2[16]	43.4±18.2[16]	59.8±4.9[16]	2.5±0.2[16]	28.1±4.4[16]	20.0±1.9[16]	UN	UN

Values are expressed as mean±standard deviation.
NM, normal morphology; TM, total motility; UN, unevaluated.

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oncillas (*L. tigrinus*) and ocelots (*L. pardalis*) in a concentration of 2 g/L.[17] However, in several studies, antibiotics are absent in extenders for freezing semen from other carnivore species.

Semen Processing

Semen dilution is directly linked to cooling steps that reduce thermal shock in the sample. The most commonly used cooling method is fractionation where the first part of the extender (without penetrating cryoprotectant) is added at 37°C,[33,36] followed by sample refrigeration with temperature maintained at 5°C for 3 hours, and a second dilution with the extender containing the penetrating cryoprotec- tant.[16,33,35] At this point, it is possible to determine the ideal refrigeration curve for carnivore semen. The speed at which temperature reduction occurs can influence the appearance of morphological anomalies, and consequently affect semi- nal quality.[39] Semen from carnivores, mainly canids, show resistance to temperature changes during the refrigeration curve, but in other carnivore species, it is possible to prevent semen damage using slower curves, such as those where the gradient of reduction ranges from -0.05°C/min to -0.4°C/min.[33,35] The most common methodologies have determined that semen after the first dilution should be re- frigerated to 5°C for 1 hour and after addition in the second part of extender, at this time containing cryoprotectant, to remain for another 30 minutes at 5°C. Then the samples are stored in straws and under nitrogen vapor for 20 minutes for later storage in nitrogen tanks.[33]

Several forms of packaging are used for semen storage, such as pellets and straws. However, in recent studies, the use of 0.25 mL straws for cryopreservation of wild carnivore semen has gained practically unanimous support.[16,17]

In the thawing process, it is important to consider the volume to be thawed; because the majority of studies use 0.25 mL straws, the methodologies are based mainly on this volume. A protocol widely used for several species that causes few sperm morphology alterations involves thawing at 37°C for 60 seconds. This protocol has been successfully used in ring-tailed coatis (*N. nasua*), cougars (*P. concolor*), and jaguars (*P. onca*).[35,36] It is also possible to perform thawing at higher temperature ranges with good results, as shown by Tebet[40] who used 46°C for 15 seconds for semen samples from ocelots (*L. pardalis*), oncillas (*L. tigrinus*), and domestic cats. Table 1 shows the results after thawing in the recently published articles using neotropical carnivore species. As shown in the table, the seminal values found for neotropical carnivores are within the range expected for seminal quality after thawing, similar to that observed in carnivores from other regions. Table 2 describes the composition of extenders.

Creation of Semen Banks and Applications

There is great difficulty in practicing endangered species preservation. Institutions such as zoos are failing to achieve the optimal indices for demographic and genetic maintenance, which should be at least 90% storage of gene diversity.[42]

The use of stored semen samples can reduce the number of individuals in parks and zoos by up to 50%, where many solitary species such as leopards, jaguars (*P. onca*), bears, and others are housed in pairs, which is not common in free living, thus increasing the stress levels and reducing the reproductive performance of these individuals.[6,43]

In the past decade, there has been an increase in the number of works aimed at cryopreservation of carnivore species, but there are still insufficient data to establish ideal protocols. Neotropical carnivore species such as the crab-eating raccoon (*Procyon cancrivorus*), tayra (*Eira barbara*), giant river otter (*Pteronura brasiliensis*), spectacled bear (*T. ornatus*), and others have not undergone research related to seminal cryopreservation, and many species do not even have semen collection protocols. These animals are included in the fauna present in South America, where, despite great degradation of biomes, new species conservation policies are being implemented.

Currently, applications of reproductive biotechnologiesin neotropical carnivores are restricted to a few species with cryopreservation protocols still in development. Studies about reproductive peculiarities in each species are in initial phases. Use of frozen-thawed semen samples for artificial inseminations are not routinely used in species such as the giant panda, and large amounts of samples still have to be stored.[44] Other programs such as those for the black-footed ferret (*Mustela nigripes*) have integrated semen cryopres- ervation into the management of the populations.[45] Among felids, an extensive range of species have been subjected to artificial insemination to gestate females, including the leopard cat (*Prionailurus bengalensis*), ocelot (*L. pardalis*), and cheetah (*Acinonyx jubatus*).[46] In canids, few reports of artificial insemination using frozen semen are available, among which the most significant result was described by Seager et al.,[47] who obtained seven gray wolf (*Canis lupus*) pups. Unfortunately, there are few records about successful insemination in neotropical carnivores using frozen-thawed semen. One of the most celebrated is the production of ocelot pups (*L. pardalis*),[48] but using fresh semen was possible to produce puppies in cougars (*P. concolor*) and oncillas (*L. tigrinus*).[49,50]

TABLE 2. SEMEN EXTENDER COMPOSITION AND CRYOPROTECTANTS USED IN NEOTROPICAL CARNIVORES

Species	*Medium*	*Presence of egg yolk*	*Cryoprotectants*	*Antibiotics*
Jaguar[33] (*P. onca*)	Lactose-based	Yes	Glycerol	Penicillin + streptomycin
Cougar[38] (*P. concolor*)	Tris	Yes	Glycerol	Streptomycin
Ocelot[17] (*L. pardalis*)	Tris	Yes	Glycerol	Amicacine
Oncilla[17] (*L. tigrinus*)	Tris	Yes	Glycerol	Amicacine
Ring-tailed coati[36] (*N. nasua*)	Dilutris® Minitube	Yes	Glycerol	—
Maned wolf[16] (*C. brachyurus*)	TES-T	Yes	DMSO	—

Final Considerations

One way to ensure the perpetuation of these species is the inclusion of breeding programs where the seminal cryopreservation technique is highlighted, as it is able to store male gametes for long periods and facilitate genetic vari- ability. Many studies aim to improve cellular preservation protocols to obtain the highest percentage of viable sper- matozoa that can still maintain fertilization capacity. Sper- matic cryobiology is still a field that needs to be explored. This is especially important in carnivore species to improve existing techniques as well as propose new methodologies to ensure minimal loss of sperm cells after thawing.

Recently, the jaguar (*P. onca*) has become more promi- nent in the development of protocols for seminal cryopres- ervation, of free-living and captive individuals, developed by researchers in Brazil, with the purpose of creating cryobanks for the genetic storage of gametes and possibly the ex- change of samples, with the purpose of increasing the genetic variability of the species. In Brazil, for example, some uni- versities and genetic resources organizations (EMBRAPA/ CENARGEN) are responsible for maintaining the genetic material of some native species.

Author Disclosure Statement

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3 JUSTIFICATIVA

A ordem Carnivora é uma das que mais apresenta maior número de espécies ameaçadas de extinção (IUCN, 2019). Dentro desta ordem está inserida a família dos felídeos, na qual apenas o gato doméstico não apresenta risco de extinção (HUNTER, 2015). Algumas espécies selvagens podem apresentar maior ou menor potencial de extinção, e entre os carnívoros, uma das espécies com maior risco é a onça-pintada (*Panthera onca*), uma vez que devido à destruição de seu habitat, caça, redução de presas naturais, conflitos com produtores de gado, atropelamentos, corroboram para a queda no número de indivíduos (IUCN, 2019).

Mesmo diante do aumento do interesse em pesquisas voltadas para a conservação desta espécie, ainda existe uma grande carência de informações relacionadas a seus aspectos reprodutivos (ARAÚJO, 2016). Logo é de suma importância um conhecimento apurado sobre as características ligadas à reprodução, a fim de propor técnicas que possam auxiliar na conservação da espécie.

A criopreservação de germoplasma é uma das biotécnicas mais difundidas mundialmente, através dela é possível armazenar gametas por um longo período, bem como contribuir para uma maior variabilidade genética (FERNANDEZ-GONZALEZ et al., 2015). Entretanto, esta técnica pode causar ações deletérias sobre os gametas capazes de reduzir a capacidade fecundante, sendo necessário um melhor entendimento sobre estas alterações.

Assim, devido à importância da criopreservação para a formação de bancos de germoplasma e, consequentemente, para a conservação das espécies, tornam-se válidos os testes sobre essa biotécnica reprodutiva em espécies ameaçadas como a onça-pintada, devido ao fato desta estar em alto risco de extinção, como também pelo fato de possuir um número de exemplares em cativeiro possível de ser utilizado a fim de estudo dos parâmetros reprodutivos, não havendo necessidade de retirada de indivíduos de seu habitat para uso experimental.

4 HIPÓTESES

O sêmen da onça-pintada (*Panthera onca*) é criopreservado, de forma eficiente, utilizando-se o diluidor ACP-117c®.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Descrever detalhadamente a morfologia dos espermatozoides e avaliar o sêmen criopreservado em Tris e ACP-117c® de onças-pintadas (*Panthera onca*).

5.2 OBJETIVOS ESPECÍFICOS

- Descrever as características ultraestruturais de espermatozoides provenientes de sêmen *in natura* e descongelado de onças-pintadas;
- Comparar o efeito de diferentes diluidores (Tris e ACP-117c®) sobre os parâmetros de qualidade do sêmen;
- Verificar a capacidade fecundante pós-descongelação dos espermatozoides de onças-pintadas através do teste de ligação à membrana perivitelínea de ovo de galinha.

6 CAPÍTULO 4 - MORPHOLOGY, MORPHOMETRY, ULTRASTRUCTURE, AND MITOCHONDRIAL ACTIVITY OF JAGUAR (*Panthera onca*) SPERM

Morphology, morphometry, ultrastructure, and mitochondrial activity of jaguar (Panthera onca) sperm

(Morfologia, morfometria, ultraestrutura e atividade mitocondrial de espermatozoides de onça-pintada (*Panthera onca*))

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Morphology, morphometry, ultrastructure, and mitochondrial activity of jaguar (*PANTHERA ONCA*) sperm

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ABSTRACT

The jaguar is categorized as "Near Threatened". Conservation strategies, therefore, are needed which include use of reproductive biotechniques. For implementation of biotechnique use, the reproductive characteristics of the species must be understood, which is currently not the case. This study, therefore, aimed to describe the detailed morphology of jaguar sperm, and to evaluate the sperm mitochondrial activity. Five male adults were used. Slides stained with Rose Bengal were used for morphometric and morphological analyses. The length and the width of the sperm head were measured, as well as the length of the middle piece, the tail, and the total length. Scanning and transmission electron microscopy were used for ultrastructural analysis. Mitochondrial function was assessed using the marker 3,3′-diaminobenzidine (DAB). The results are expressed as means ± SEM. The most significant morphological abnormalities observed were head (9 ± 1.7%) and tail defects (12.5 ± 3.3%). The width and length of the head were 3.6 ± 0.03 μm and 4.9 ± 0.02 μm, respectively. The middle piece measured 9.7 ± 0.3 μm, the tail measured 54.5 ± 4.4 μm, and the total length of the sperm was 59.5 ± 0.1 μm. Electron- lucent regions and approximately 54 mitochondrial spirals in the middle piece were identified in the nucleus using electron microscopy. The greatest percentages of cells were classified as DAB I (46.6 ± 4.9%) and DAB II (38 ± 4.4%). The data provide detailed information on the sperm characteristics of jaguars and can support research on germplasm conservation for the species.

1. Introduction

The jaguar (*PANTHERA ONCA*) is monotypic within the genus *PANTHERA*, which is native to the American continent. The geographical distribution initially extended from the southern United States to northern Argentina, but at present its natural habitat is confined to small fragmented areas of the Central America, Mexico, and South America (Quigley et al., 2017). Habitat destruction, combined with predatory hunting, has caused populations to markedly decrease and the species is now considered "Near Threatened" according

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to the IUCN Red List (Quigley et al., 2017).

Consequently, conservation strategies which include use of the reproductive biotechniques are needed to preserve the species. To identify the most suitable biotechniques, however, the reproductive physiology of each species must be understood (Silva et al., 2004). Characterization of sperm is essential for the improvement of assisted reproduction techniques, contributing to the creation of biobanks that enable the preservation of the genetic material of the species (Silva et al., 2015).

One of the main methods for evaluating sperm is morphological analysis, in which the physiological structure of the cell can be determined, as well as possible anomalies that might affect fertilization (Chemes and Sedo, 2012). One such method is ultrastructural evaluation, which can also predict the disposition and integrity of the cell organelles, and can allow the observation of possible abnormalities that may go unnoticed with conventional light microscopy (Chemes and Rawe, 2003). In the Felidae, this technique has already been used to ascertain the potential abnormalities in the sperm of domestic cats and tigers (*PANTHERA tigris ALTAICA*) (Schmehl and Graham, 1989); however, the ultrastructure of jaguar sperm has not been previously studied.

Morphometric analysis is another method used to evaluate morphologyand there can be use of this approach to determine sperm defects occurring mainly in head, including macro or microcephaly (Maree et al., 2010). Another important application for sperm morphometry in wild animals is to provide reference values for species that can be used in computer-assisted sperm analysis software (CASA), because most of these analyses do not include references for all the species (Soler et al., 2017).

Evaluation of mitochondrial function can predict the quality of sperm motility, given that the potential for movement of the cell is dependent on metabolic energy resulting from mitochondrial functions (Marchetti et al., 2004). The most common method used to accomplish this analysis is through use of fluorescent probes and a microscopes suitable for this type of analysis; however, for free- living wild animals or those in zoos a feasible method of sample preparation is needed at the site of collection for subsequent assessment. Accordingly, cytochemistry using 3,3′-diaminobenzidine (DAB) dye is an alternative technique to assess mitochondrial functions (Brito et al., 2018).

The present study, therefore, was conducted to describe for the first time the sperm structure of the jaguar, and provide information on the sperm morphology, morphometry, and mitochondrial functions.

2. Materials and methods

2.1. Animals

The study was approved by the Ethics Committee of the State University of Ceará (Nº 5098414/2016) and by the System of Authorization and Information of Biodiversity (Sistema de Autorização e Informação da Biodiversidade – SISBIO) (no. 54741–1). Five male jaguar weighing 70.8 ± 5.9 kg and ranging from 4 to 17 years of age were used. All the animals were housed in a zoo. There was one jaguar from each of the following that was used in the study: Ecological Park – EcoPoint, Fortaleza, CE, Brazil (3° 43′ S, 38° 30′ W); São Francisco Zoo, Canindé, CE, Brazil (4° 21′ S, 39° 18′ W); Teresina Zoobotanical Park, Teresina, PI, Brazil (5° 05′ S, 42° 48′ W); Arruda Câmara Zoobotanical Park, João Pessoa, PB, Brazil (7° 06′ S, 34° 51′ W); and Dois Irmãos Park, Recife, PE, Brazil (8° 03′ S, 34° 52′ W). All the zoos were located in the intertropical zone in the Brazilian northeast and, therefore, the animals were not subject to a possible reproductive seasonality due to differences in photoperiod at the various zoos. Each animal was subject to the usual management practices of each institution, with the regular provision of red meat or slaughtered chicken, as well as vitamin supplementation. Water was provided *AD libitum*. There is listing of further information regarding each individual animal in Table 1. All animals were captured in their natural habitat before being housed in the zoos that were previously described. Males 1, 2 and 5 were captured in locations of the Amazon Forest and Males 3 and 4 from the region of the Atlantic Forest. All animals were classified as being healthy and free of disease. None of the males had produced offspring because of matings before the time that the present study was conducted. All males had penile spicules, which characterize male hormonal stimulations of these tissues had occurred (Fig. 1 A–E).

2.2. Anesthesia

With each procedure, the animals were chemically restrained using blowgun darts containing dexmedetomidine (Dexdomitor ®, Zoetis, Campinas – SP, Brazil) at a dose of 0.04 mg/kg, IM, combined with ketamine hydrochloride (Ketalar®, Pfizer, São Paulo-SP, Brazil) at a dose of 5 mg/kg, IM. Fifteen minutes after administration of the combined drug the animals were in a decubitus position and, when necessary, a third of the initial dose was administered to maintain anesthesia. The total time to perform the anesthetic safety procedure was approximately 1 h. To reverse the anesthesia after semen collection, yohimbine was administered at a dose of

Table 1
All data about jaguars (*PANTHERA ONCA*) used.

Animals	City located	Age (Years)	Weight (Kg)	Housed with female	Origin
Male 1	Fortaleza/CE	17	66	Yes	Captive
Male 2	Canindé/CE	6	81	Yes	Captive
Male 3	Recife/PE	8	70	No	Captive
Male 4	João Pessoa/PB	9	69	Yes	Captive
Male 5	Teresina/PI	4	68	Yes	Captive

Fig. 1. Genitals of males, testicles and penis. (A) Male 1; (B) Male 2; (C) Male 3; (D) Male 4; (E) Male 5; Note penis in conical for mat, with approximate size of 5 cm and spicules present in penile base.

0.4 mg/kg, IM, and after a few minutes the animals were conscious (Lueders et al., 2012).

2.3. Semen collection

Semen was collected from the five animals between March and September 2017. Two collections were performed per animal (n = 10), with a minimum interval of 2 months between collections for each animal.

The preparation for semen collection began as soon as the animal was under the effects of anesthesia. The jaguar was placed in a lateral recumbency position (Fig. 2A) and the penile region was cleaned with saline solution (0.9%). Urine was completely expelled using a urethral probe, and three washes were performed by injecting and removing 10 mL of saline solution (0.9%), using the probe. The washing was performed slowly to avoid traumato the urethral canal, as well as reduce the possibility of contamination of the semen with urine (Fig. 2B; Curren et al., 2013).

Feces were then removed from the rectum of the animal and lubricating gel was used to facilitate the penetration of the electroejaculation probe which was inserted until reaching the prostate close to the rectal area (Fig. 2C), with the electrodes positioned towards the prostatic surface. Electroejaculation was performed using an electro-mechanical device (Autojac V2®, Neovet, Uberaba, MG, Brazil). The electrode probe had three 3-cm-long metal electrodes (positive, neutral, and negative). The procedure consisted of three sets of stimuli, with ten stimuli for each voltage, the first series starting with 5 V, 6 V, and 7 V; the second with 6 V, 7 V, and 8 V; and the third with 8 V and 9 V (Wildt et al., 1983). Each stimulus lasted for 2 s, with 5-minute intervals between each series. Samples were collected in 50-mL graduated tubes in which it was possible to completely insert the penis of the animal. The semen was evaluated immediately after collection (Fig. 2D).

2.4. Semen Analysis

The semen was initially evaluated for the macroscopic parameters of volume and color, as well as the microscopic parameters of sperm motility (%) and vigor (scale 0–5; 0: all sperm without movement; 1: slowly lateral-lateral movements, without progression; 2: fast lateral- lateral movements, without progression; 3: rapid lateral-lateral movements, with occasional progression; 4: slow and continuous progression; 5: fast and continuous progression -Christiansen, 1984). The sperm concentration was determined from a 5 µL aliquot of semen diluted in a 1% formol saline solution, and subsequently evaluated using a light microscope at 400× magnification and a Neubauer chamber was used for cell counting. To evaluate sperm morphology, samples were fixed using 10 µL of the samples diluted in 90 µL Rose Bengal (distilled water 20 mL; sodium citrate 0.58 g; formaldehyde 0.8 mL; Rose Bengal 0.3 g; CAQ – Casa da Química, São Paulo-SP, Brazil) and observed using a light microscope at 1000x magnification (Cardoso et al., 2005). For each sample, 200 sperm were used to determine the proportion of normal to defective cells and to characterize the defects at different

Fig. 2. (A) Lateral recumbency position, before semen collect; In male 3; (B) Removal of urine before semen collect; in Male 1; (C) Feces removal, before to insertion of electroejaculator probe; in Male 5; (D) Electrobejaculator probe, inserted into the rectus of animal, to start the electrical stimuli; Male 5.

locations (acrosome, head, middle piece, or tail) of the sperm cell (Wildt et al., 1986). The functionality of the sperm membrane was determined using a hypoosmotic test in which 10 μL of semen were diluted in 90 μL distilled water. The sample was subsequently stored at 37 °C for 40 min, followed by evaluation of the cell membrane of the cells that reacted positively (tail winding), or ne- gatively (tail completely straight) (Silva et al., 2015; Lima et al., 2016).

2.5. Analysis of mitochondrial function

A 20 μL volume of semen was added to a 50 μL solution containing 15 mg/mL 3,3′-diaminobenzidine (DAB) diluted in phosphate buffered saline (PBS) and incubated in a water bath at 37 °C for 40 min, protected from light. Two 10 μL smears of each sample were prepared on microscope slides and dried atambient temperature, fixed in 10% formaldehyde for 10 min, rinsed in distilled water, and
then dried at ambient temperature (20–25 °C) (Hrudka, 1987). A total of 100 sperm were counted using a phase-contrast microscope (400×), and classified as class I DAB (100% of the middle piece was stained, indicating relatively greater mitochondrial function); class II DAB (> 50% of the middle piece was stained, indicating average mitochondrial function); class III DAB (< 50% of the middle piece was stained, indicating relatively lesser mitochondrial function); and class IV DAB (absence of staining in the middle piece, indicating the absence of mitochondrialfunction). The values obtained in each class were expressed as a percentage (Flores et al., 2016).

2.6. Morphometric analysis

The slides prepared previously for the morphological evaluation, stained with Rose Bengal, were used for morphometric analysis. Images of 200 normal sperm cells were recorded at random fields in each semen sample, and evaluated using a light microscope (1000x) connected to a computer. Cells were measured using image analysis software (ImageJ Software, Wayne Rasband–National Institute of Health, Maryland, United States).

The following sperm variables were measured: sperm head (width and length), middle piece (length), tail (length), and sperm (total length). The length of the sperm head was measured from the apex of the acrosome to the base of the head, and the width was measured along the transverse axis of its largest diameter. The length of the middle piece was measured from its insertion site at the base of the head to the region of the annulus (Jensen's ring). The length of the tail was measured from the beginning of the middle piece to the end of the caudal portion. The total length of the sperm was measured from the apex of the acrosome to the end of the caudal portion (Silva et al., 2015).

2.7. Analysis by scanning electron microscopy (SEM)

Scanning electron microscopy was performed using 5 μL aliquots of pooled semen samples from each animal. The samples were fixed in a 2.5% glutaraldehyde solution buffered in 0.1 M phosphate buffer (pH 7.4) and subsequently fixed in osmium tetroxide 1%. The samples were stored in a refrigerator at 4 °C until all the samples had been collected, after which the samples were processed for SEM evaluation at the Laboratory of Applied Animal Morphophysiology at the Federal Rural University of Semi-Árido. The samples were washed three times in 0.1 M phosphate buffer at pH 7.4 and twice with distilled water, followed by treatment with 1% tannic acid and dehydration with an alcohol series (50%, 70%, 90%, and 100%). After dehydration, drying was performed using a critical point apparatus with carbon dioxide as the atmosphere. The material was subsequently assembled in the sample holder (stub) and finely coated with gold through cathode pulverization, and then observed using a scanning electron microscope (LEO VP® 435 – Carl- Zeiss, Oberkochen, Germany; Silva et al., 2015).

2.8. Analysis by transmission electron microscopy (TEM) data

After each collection, a 5 μL sample of semen from each animal was fixed using a modified Karnovsky solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 (PB-Co® Sigma, St. Louis, United States) and kept in a refrigerator at 4 °C. The samples were submitted for processing at the Electron Microscopy Laboratory of the University of São Paulo (Faculty of Medicine – Campus of Ribeirão Preto, SP, Brazil). The fixed samples were washed with phosphate buffer and then post-fixed with 1% osmium tetroxide, dehydrated in propylene oxide, and embedded in Epon resin (Embed 812, Electron Microscopy Sciences, Hatfield, United States). Ultrathin sections (60–70 nm) were stained manually with uranyl acetate and contrasts were detected by using lead citrate. The samples were subsequently evaluated with a transmission electron microscope at the *Centro Avançado em Diagnóstico por Imagem (CADI)* of the School of Veterinary Medicine and Animal Science (FMVZ) of the University of São Paulo (USP), São Paulo, SP, Brazil. Different fields were selected randomly and evaluated using TEM (JEOL 1010, Japan), and images were recorded for later analysis and description (Silva et al., 2015).

2.9. Statistical analysis

The data were analyzed using the statistical software R-project© version 3.3.2 (The R Foundation, Vienna, Austria), being submitted to the Cramer-Von Mises normality test and the Box-Cox Homoscedasticity test. For comparison of means, the data were submitted to the Kruskal Wallis test followed by Dunn's test, with a confidence interval of 95%. The results are expressed as mean ± standard error.

Table 2
General seminal parameters for jaguars (*PANTHERA ONCA*). Values (Mean ± SEM), (n = 10; 2 ejaculates/male).

Male	Total motility (%)	Vigor (0-5)	Volume (mL)	Concentration (spz/mL) x 10^6	Functional membrane (%)
1	95 ± 0.0	5 ± 0.0	7.5 ± 2.5	120 ± 40.0	84 ± 2.0
2	92.5 ± 2.5	4.75 ± 0.3	5.5 ± 4.5	150 ± 50.0	70 ± 0.0
3	95 ± 0.0	4.75 ± 0.3	5.5 ± 1.5	75 ± 5.0	85 ± 2.0
4	95 ± 0.0	5 ± 0.0	6.3 ± 1.3	220 ± 120.0	84 ± 2.0
5	87.5 ± 7.5	4.5 ± 0.5	6.5 ± 0.5	145 ± 5.0	87.5 ± 2.5
Total	93 ± 1.5	4.8 ± 0.1	6.25 ± 0.86	142 ± 25.68	82.5 ± 2.21

* There was no statistical difference among the animals (P > 0.05).

3. Results

3.1. Semen evaluation

All semen samples were slightly cloudy, with translucent staining, and no urine contamination was observed in any of the ten assessed samples. The largest seminal volume was obtained during the period of use of the second cycle of electrical stimuli. The total volume of the ejaculate was 6.25 ± 2.7 mL, but there was wide variation in the volumes, varying from a minimum of 1 mL to a maximum of 10 mL, with both these samples at the range extremes being from Animal 2. Animal 5 had a greater variation in sperm motility and vigor, while the remaining animals had more homogeneous values. Animal 5, along with Animal 4, however, had the least variation in sperm concentration. All the samples were homogeneous with regard to membrane functionality, with an average of
82.5 ± 2.21%. The semen variable values for all individuals are presented in Table 2.

Normal sperm morphology was observed in 76 ± 3.5% of the samples. Animal 1 had the greatest percentage of morphologically normal cells among all the animals assessed (82.5 ± 1.5%), and Animal 5 had the least (66 ± 11%; Table 3). Even though there was considerable variation in values, there were no significant differences in sperm morphology among the individual animals. Fig. 3 depicts a normal sperm and some of the primary abnormalities that were detected.

3.2. Evaluation of mitochondrial activity

To evaluate mitochondrial activity, the four DAB categories were compared using the data from the five animals. The greatest percentages observed were for DAB I and II sperm meaning of these cells there was at least 50% of the intermediate pieces that were stained, with values of 46.6 ± 4.9% for DAB I and 38 ± 4.4% for DAB II, followed by DAB III with 9.5 ± 1.4%, and the least values for DAB IV with 5.9 ± 1%. There, however, were individual differences that were similar to the same general comparison pattern, with DAB I and II being in the greatest percentages compared to the other classifications. The other values for each animal are presented in Table 4.

Table 3
Morphological evaluation in jaguars (*PANTHERA ONCA*) sperm. Values (Mean ± SEM), (n = 10; 2 ejaculates/male).

Characteristics	Male 1	Male 2	Male 3	Male 4	Male 5	Total
Normal sperm	82.5 ± 1.5	77.5 ± 4.5	73.0 ± 10.0	81.0 ± 11.0	66.0 ± 11.0	76.0 ± 3.5
Head defects	5.5 ± 0.5	14.5 ± 5.5	6.5 ± 3.5	6.5 ± 4.5	12.0 ± 1.0	9.0 ± 1.7
Detached	0.0 ± 0.0	0.5 ± 0.5	4.5 ± 4.5	0.0 ± 0.0	2.0 ± 2.0	1.4 ± 0.9
Micro	1.5 ± 1.5	10.5 ± 8.5	0.5 ± 0.5	3.0 ± 1.0	3.0 ± 2.0	3.7 ± 1.8
Macro	1.0 ± 1.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.5	0.4 ± 0.2
Degenerate	2.5 ± 2.5	3.0 ± 3.0	0.0 ± 0.0	3.5 ± 3.0	6.5 ± 1.5	3.1 ± 1.1
Piriform	0.5 ± 0.5	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3
Middle piece (MP) defects	2.5 ± 2.5	2.0 ± 2.0	0.5 ± 0.5	1.0 ± 0.0	1.0 ± 0.0	1.4 ± 0.5
Abaxial	1.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
Incomplete	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
Thick	0.5 ± 0.5	0.0 ± 0.0	0.5 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	0.3 ± 0.2
Reverse	0.5 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
Bent	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.5	1.0 ± 0.0	0.3 ± 0.2
Tail defects	8.0 ± 0.0	3.5 ± 1.5	20.0 ± 7.0	10.0 ± 8.0	21.0 ± 12.0	12.5 ± 3.3
Very coiled	3.0 ± 3.0	0.5 ± 0.5	7.0 ± 2.0	2.0 ± 1.0	0.5 ± 0.5	2.6 ± 1.0
Coiled	5.0 ± 3.0	3.0 ± 2.0	13.0 ± 9.0	8.0 ± 7.0	20.5 ± 12.5	9.9 ± 3.3
Acrossomal defects	1.5 ± 1.5	2.0 ± 1.0	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0	1.0 ± 0.5
Broken	1.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
Withdrawn	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0	0.6 ± 0.4
Fragmented	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Teratogenics	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Total defects	17.5 ± 1.5	22.5 ± 4.5	27.0 ± 10.0	19.0 ± 11.0	34.0 ± 11.0	24.0 ± 3.5

* There was no statistical difference among the animals (P > 0.05).

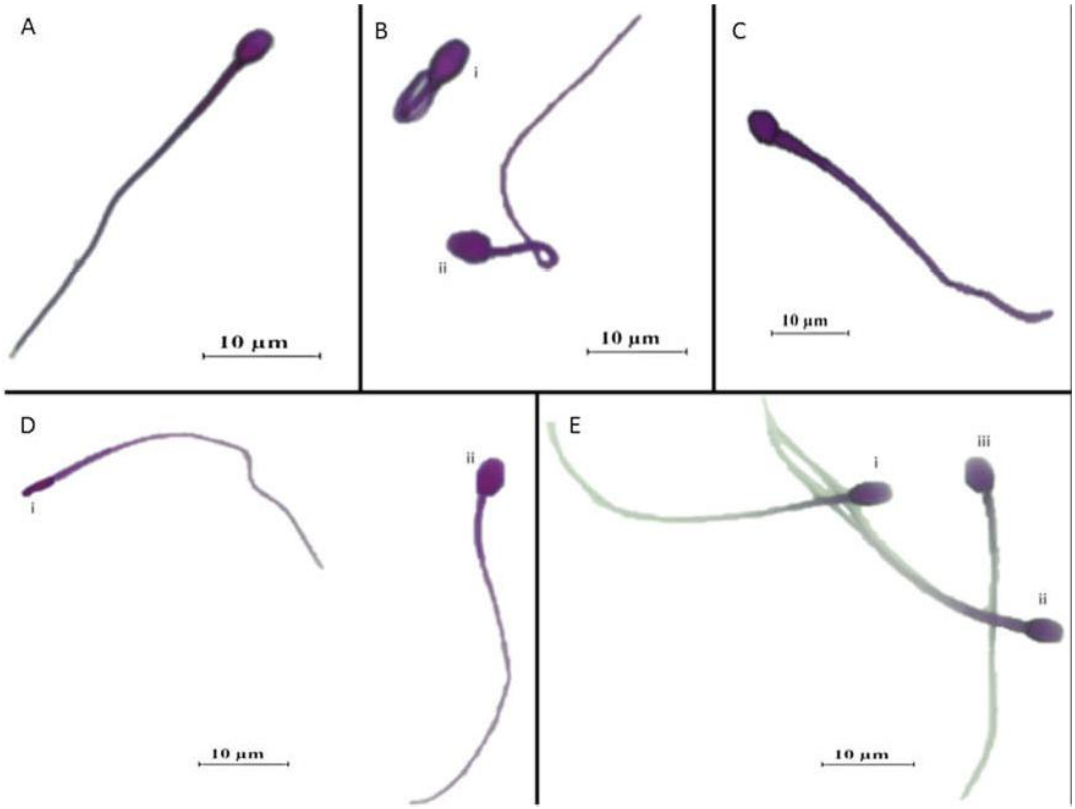

Fig. 3. Normal sperm morphology and sperm defects in jaguar (*PANTHERA ONCA*) - 1000x magnification. (A) Normal sperm (B) i - Very coiled tail; ii - Coiled tail (C) Thick middle piece (D) i - Micro head; ii - Normal sperm (E) i - Normal sperm; ii - Two tails; iii - Abaxial middle piece.

Table 4
Evaluation of mitochondrial activities in jaguar (*PANTHERA ONCA*) sperm through the use of 3,3′-diaminobenzidine (DAB). Values (Mean ± SEM), ($n = 10$; 2 ejaculates/male).

Male	DAB 1 (%)	DAB 2 (%)	DAB 3 (%)	DAB 4 (%)
1	51 ± 1.0^{a}	39 ± 1.0^{b}	6.5 ± 1.5^{c}	3.5 ± 1.5^{c}
2	58 ± 12.0^{a}	29 ± 5.0^{b}	7 ± 3.0^{b}	6 ± 4.0^{b}
3	36 ± 4.0^{a}	51 ± 5.0^{b}	9 ± 1.0^{c}	4 ± 2.0^{c}
4	44 ± 22.0^{a}	40 ± 22.0^{a}	9 ± 1.0^{a}	7 ± 1.0^{a}
5	44 ± 12.0^{a}	31 ± 7.0^{a}	16 ± 4.0^{a}	9 ± 1.0^{a}
Total	46.6 ± 4.9^{A}	38 ± 4.4^{A}	9.5 ± 1.4^{B}	5.9 ± 1.0^{C}

Lowercase letters comparing classes in each animal. Different superscript lowercase letters in the same row means that there was a statistical difference among DAB's classes ($P < 0.05$).
Uppercase letters comparing classes in total. Different superscript uppercase letters in the same row means that there was a statistical difference among DAB's classes ($P < 0.05$).

3.3. Sperm morphometric evaluation

The sperm of the jaguar had a head with a slightly oval shape, 3.6 ± 0.03 µm wide and 4.9 ± 0.02 µm long. The length of the intermediate piece was 9.7 ± 0.3 µm, while the total length of the tail was of 54.5 ± 0.1 µm. The total length of the sperm cell was 59.5 ± 0.1 µm, with homogeneity in individual values. The remaining results are shown in Table 5.

3.4. Ultrastructural evaluation of sperm by scanning and transmission electron microscopy

The images obtained using scanning electron microscopy were used to characterize the sperm of Jaguar. These sperm had a slightly oval head (Fig. 4A), nearly round, but with an easily distinguishable equatorial region dividing the acrosome hood (Fig. 4B). In the images obtained using transmission electron microscopy, there was characterization of a very electron-dense nucleus that was

Table 5
Values (Mean ± SEM) for sperm morphometry in in jaguars (*PANTHERA ONCA*) sperm, (n = 1000; 200 sperm cells/male).

Male	Head width (μm)	Head length (μm)	MP length (μm)	Tail length (μm)	Total length (μm)
1	3.1 ± 0.02[a]	4.6 ± 0.03[a]	9.2 ± 0.05 [a]	55.4 ± 0.3 [a]	60.1 ± 0.3 [a]
2	3.5 ± 0.03[b]	5.2 ± 0.03[b]	9.7 ± 0.07 [b]	54.3 ± 0.3 [a]	59.5 ± 0.3 [a]
3	4.1 ± 0.07[b]	4.9 ± 0.03[c]	9.3 ± 0.06 [a]	51.0 ± 0.2 [b]	56.0 ± 0.3 [b]
4	4.2 ± 0.08[b]	5.1 ± 0.04b[c]	10 ± 0.08[c]	54.9 ± 0.3 [a]	60.0 ± 0.3 [a]
5	3.1 ± 0.02[a]	4.7 ± 0.03 [a]	10.2 ± 0.06[d]	57.1 ± 0.3[c]	61.7 ± 0.3[c]
Total	3.6 ± 0.03	4.9 ± 0.02	9.7 ± 0.3	54.5 ± 0.1	59.5 ± 0.1

Different superscript lowercase letters in the same column means that there was a statistical difference ($P < 0.05$).

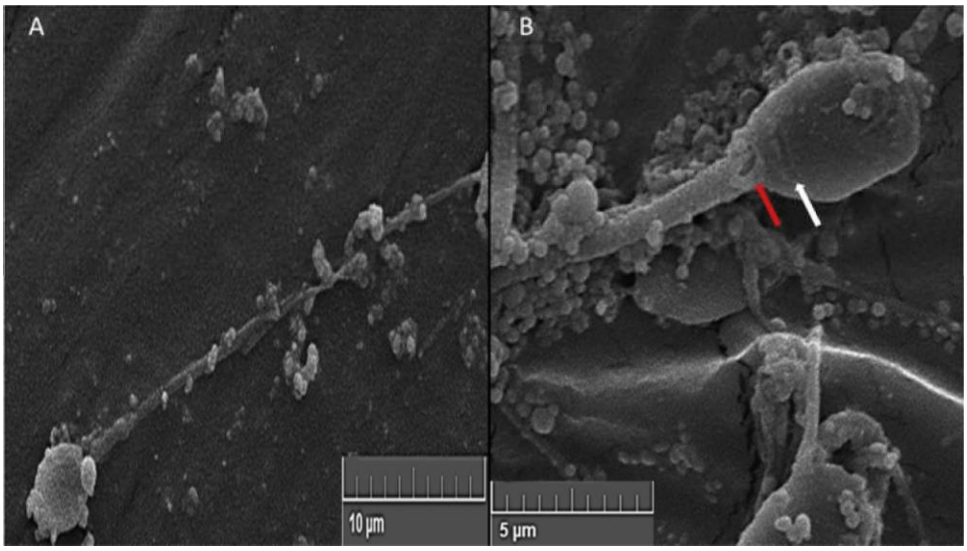

Fig. 4. Electron microphotograph of sperm morphology in jaguar (*PANTHERA ONCA*); (A) Sperm in a total visualization by microscopy scanning; (B) Head of the sperm - equatorial segment (white arrow); Neck area (red arrow) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

almost 100% homogeneous, as well as diffuse small electronic spots, in a longitudinal cut in the head of the sperm (Fig. 5A). In this region, besides a separated nucleus, the separation of the nuclear, and internal and external acrosomal membranes could be discerned, as well as the plasmalemma. In the basal end of the head, there was observation of a deployment pit of the middle piece and, in this region, the capitulum was detected below the proximal centriole. The first spiral of the mitochondria in the neck region could also be visualized. As shown in Fig. 5B, there was observation of a cross-section of the tail, containing the axoneme with a set of nine dense external fibers, surrounded by a mitochondrial ring. In Fig. 5C, there is observation of the section of the sperm from the head to the middle piece. In this section, there are approximately 54 mitochondrial spirals around the dense outer fiber along the length of the piece and extending to the region of the annulus, forming the mitochondrial sheath.

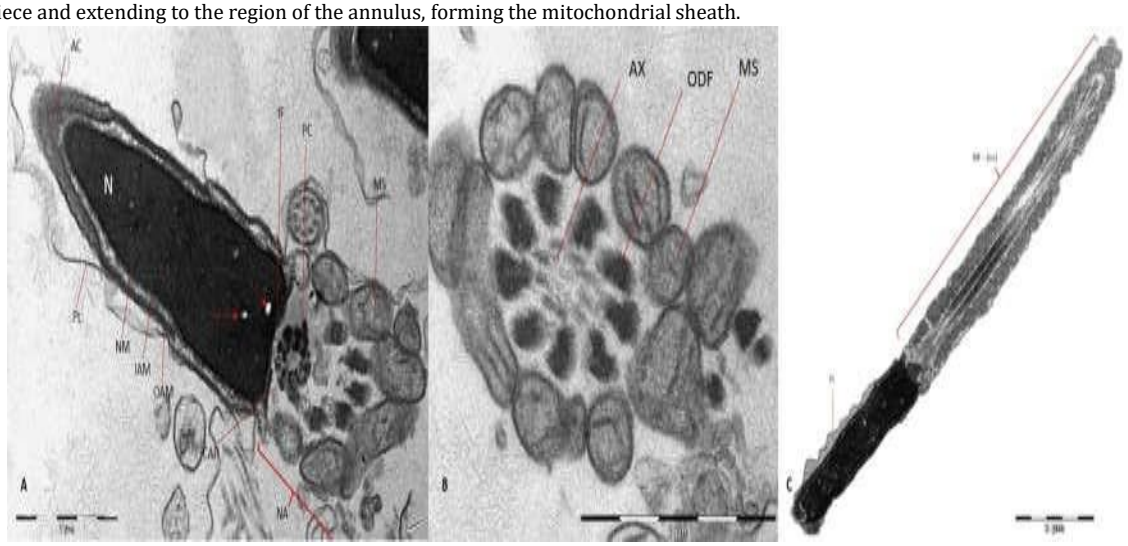

Fig. 5. Electron microphotograph of sperm morphology in jaguar (*PANTHERA ONCA*); (A) AC: acrosome; OAM: outer acrosome membrane; IAM: inner acrosome membrane; PL: plasmalemma; NM: nuclear membrane; N: nucleus; electron lucent nuclear dots (red arrows); NA: neck area; IF: implantation fossa; CAP: *CAPITULUM*; PC: proximal centriole; ODF: outer dense fibers; MS: mitochondrial spiral; (B) AX: axoneme; MS: mitochondrial sheath; ODF: outer dense fibers; (C) Head and middle piece PL: plasmalemma; MP: middle piece (ms: mitochondrial spiral) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

The semen collections in the present study were successful, with samples being obtained using a combination of dexmedetomidine and ketamine. In relation to the variables assessed, the percentage of the average total motility was high (93 ± 1.5%), which was also previously reported in results from other studies with jaguars (Paz et al., 2007; Araujo et al., 2017; Jimenez Gonzalez et al., 2017). Furthermore, the sperm vigor exceeded 3 on a scale of 0–5 (4.8 ± 0.1) indicating the samples collected were of excellent quality.

The volume of ejaculate was similar to that reported from results in previous studies using electroejaculation in jaguars (Paz et al., 2007), and the average sperm concentration was very high compared to values reported in previous studies where there was use of electroejaculation. This can be explained by the use of dexmedetomidine which increases the potential for erection, and consequently for ejaculation, due to its pharmacokinetic actions of this drug.; With use of the electroejaculation approach conducted in the present study, sperm count in the ejaculate, and cells with a functional membrane were both greater than 70% (Swanson et al., 2017).

A high percentage of sperm cells in the Felidae have morphological defects (Wildt et al., 1986; Axnér and Linde Forsberg, 2007), although this value may vary widely in some species, and can also be affected by the seasonal reproduction period (Swanson et al., 1996). There, however, was observation of a relatively greater percentage of normal cells (76.0 ± 3.5%) in the present study, in which there were the greatest percentages of defects in the tail (12.5 ± 3.3%) and head (9 ± 1.7%) of sperm cells. The percentages of normal cells vary substantially in studies with jaguars; Paz et al. (2007) observed only 27.6%, while Araujo et al. (2017) observed 60.7% and 51% in captive and free-living animals, respectively. Araujo et al. (2017) also reported that the greatest percentage of defects was related to malformations of the sperm tail.

Morphometry is a morphological method to analyze sperm that can be used to determine changes in the size of a cellular structure, and assist in determination of a standard for sperm size of a species. In jaguar, the morphology of sperm cells was not big compared with that of other carnivores such as coatis (*Nasua nasua*; Silva et al., 2015), American black bear (*Ursus americanus*; Brito et al., 2010), and artic fox (*Alopex lagopus*; Soler et al., 2017). The sperm size of the jaguar is similar to that previously reported for the puma (*Puma concolor*; Cucho et al., 2016) with the length and width of the head being nearly the same, however, for the jaguar being slightly longer than it is wide. The size and shape of the sperm can greatly affect the function of the cell, including the capacity to undergo the acrosome reaction and bind to the zona pellucida; therefore, these characteristics may also affect the capacity to predict male fertility potential (Maree et al., 2010).

The main objective of assessing mitochondrial function is to ascertain the percentage of cells with the greatest motility potential (Hrudka, 1987). In this regard, the ejaculate *in natura* of jaguars indicated there was a high percentage of cells with mitochondrial activity, with most of the cells classified as DAB I and II, indicating there was mitochondrial function in greater than 50% of the intermediate piece. This finding may be associated with the high values obtained for the general variables evaluated in the seminal sample. This indicates there may be a positive correlation between the high mitochondrial activity, and the high percentage of motile cells as well as those with a high progressive motility index.

In the SEM analysis, the sperm of the jaguar had a slightly oval shape, tending toward a more rounded morphology. There have been similar results reported by Schmehl and Graham (1989) for tiger sperm (*Panthera tigris altaica*) which also had a rounded morphology, unlike the sperm of the domestic cat that has a more elongated head. This finding suggests that this sperm head shape is the most common in species belonging to the subfamily Pantherinae, because Wildt et al. (1986) described a more elongated sperm head in cheetahs (*Acinonyx jubatus*) which, together with the cat, belongs to the subfamily Felinae. The differences in head morphology suggest a way of preventing the penetration of the oocyte by sperm that are compromised from a morphological perspective (Schmehl and Graham, 1989).

The characteristics of the jaguar sperm cell that can be described from using TEM initially includes the nucleus, which had an almost completely uniform size, from the region closest to the neck up to the most apical zone. This characteristic is similar to that of other Felidae such as cats and tigers (Schmehl and Graham, 1989), but not that of other carnivores such as the coati (*Nasua nasua*; Silva et al., 2015), artic fox (*Vulpes lagopus*; Hofmo and Berg, 1989), mustelids (Van der Horst et al., 1991), dog (Silva et al., 2009), and American black bear (*Ursus americanus*; Brito et al., 2010). In these species the sperm has a thin end near the acrosome with enlargement along the structure resulting a triangular shape. This similarity in sperm structure among these species may be related to the phylogenetic proximity of these species in which there is greater morphological similarities among sperm cells of the different species due to greater phylogenetic proximity. Several electron-lucent regions were observed in the cell nucleus, similar to what has been reported in other carnivores such as the Americans black bear (Brito et al., 2010) and coatis (Silva et al., 2015). The presence of these regions is related to changes that may occur during spermatogenesis with development of areas of condensed chromatin that, together with chromatin stabilization, can serve as markers of sperm maturity (Lazaros et al., 2011). Mitochondria were observed to be located in the sperm middle piece with these organelles having a spiral formation around a longitudinal axis, where these organelles function to provide energy for the movement of the flagella, providing motility to the cell (Amaral et al., 2013). The orientation of the mitochondria in the jaguar is very similar to that already described for the tiger and cheetah (Wildt et al., 1986; Schmehl and Graham, 1989), where a circular orientation of the mitochondria from the neck area was described. The number of mitochondrial spirals in the jaguar sperm (54 spirals) is very similar to that previously described for coatis (55 spiral; Silva et al., 2015) and black bears (59 spirals; Brito et al., 2010). In general, the jaguar has values for sperm variables that are indicative that sperm should have a very acceptable fertilizing capacity compared to other wild carnivores, especially other Felidae. Although the jaguar sperm is relatively small, it has almost the same amount of mitochondrial spirals as other carnivores with larger sperm. Together with a high potential for sperm mitochondrial functionality, this suggests that the sperm architecture may be associated with sperm that have a relatively longer motility period in

this species because larger sperm require more energy, and consequently mitochondria, for the motility functions (Levitan, 2000).

There were few significant differences in values for variables evaluated in individual animals, indicating a homogeneity of sperm in the samples from the different animals. The values for variables evaluated also provide a reliable estimate for the jaguar indicating the expected values that should be expected when collecting semen from this species. Furthermore, the typical ultrastructural sperm cell morphological characteristics ascertained in the present study for the jaguar provide important background information for future studies with this species. The morphometry results from the present study can serve as a basis for computer-aided semen assessment in the *P. ONCA* species, as well as a model for other species of the Pantherinae subfamily because of the similarities in cellular structure with phylogenetic proximity. Nevertheless, in the present study, it was not possible to obtain semen with use of pharmacological collection procedures from all the animals; therefore, electroejaculation was the method selected for semen col- lection for standardization purposes. The jaguar usually has excellent semen quality *in NATURA*, and has sperm cells with relatively greater mitochondrial function than some other species. The values for other variables assessed in the present study provide in- formation about the ideal characteristics and possible sperm pathologies in this species. This information can be used to develop cryopreservation protocols, serving as a database, and consequently contributing to the conservation of the species.

Conflict of interest statement

The authors have stated that there are no competing interests. None of the authors has financial or personal relationships that may influence or distort the content of the article.

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7 CAPÍTULO 5 - INFLUENCE OF DIFFERENT EXTENDERS ON MORPHOLOGICAL AND FUNCTIONAL PARAMETERS OF FROZEN-THAWED SPERMATOZOA OF JAGUAR (*Panthera onca*)

Influence of different extenders on morphological and functional parameters of frozen-thawed spermatozoa of jaguar (*Panthera onca*)

(Influência de diferentes diluentes nos parâmetros morfológicos e funcionais de espermatozoides congelados e descongelados de onça-pintada (*Panthera onca*))

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Influence of different extenders on morphological and functional parameters of frozen-thawed spermatozoa of jaguar (*Panthera onca*)

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ABSTRACT

Due to the global decrease in jaguar population, conservation strategies are essential and the development of effective semen cryopreservation protocols would contribute to the formation of germplasm banks. Therefore, the objectives were to (1) evaluate the use of TRIS and ACP-117c extenders for jaguar semen freezing, (2) describe the ultrastructural changes in sperm after cryopreservation, and (3) evaluate the binding capacity of the thawed sperm. Eight ejaculates from five mature individuals were collected by electroejaculation, extended in TRIS or a coconut based-extender (ACP-117c), and frozen in liquid nitrogen. Samples were evaluated for sperm motility, vigor, membrane functionality, mitochondrial activity, morphology (using light microscopy, scanning electron microscopy - SEM and transmission electron microscopy - TEM), sperm kinetic parameters (by computerized analysis - CASA), and sperm binding capability using an egg yolk perivitelline membrane assay. Samples preserved in TRIS presented better post-thaw motility (46.0 ± 7.7%) and membrane functionality (60.5 ± 4.2%) and higher mitochondrial activity (21.5 ± 3.7%) than those preserved in ACP-117c (20.9 ± 5.4% motile sperm; 47.1 ± 2.5% functional membrane; 11.8 ± 1.7% mitochondrial activity). Regarding ultrastructural evaluations, SEM showed that both extenders were able to preserve the superficial membrane of the sperm, but TEM revealed the occurrence of nuclear electron lucent points, especially in samples extended in ACP-117c. Additionally, TRIS also provided a higher number of sperm bound to the perivitelline membrane (29.5 ± 3.3%) in comparison to samples diluted in ACP-117c (18.6 ± 1.5%). Overall, we suggest the use of a TRIS-based extender for cryopreservation of jaguar semen.

1. Introduction

As a top predator of the food chain, the jaguar (*Panthera onca*) can be considered essential for the conservation of the biological diversity and the integrity of the ecosystems in which they live [17]. Jaguar popula- tion has significantly decreased owing to the destruction of habitats, predatory hunting of the species and its prey, and lack of interest in preserving the species. As a result, jaguar is considered close to being endangered according to the International Union for Conservation of Nature Red List [26]. Therefore, it is necessary to develop conservation and sustainability strategies for the species. Assisted reproductive techniques associated with genome resource banking can be potentially integrated into the genetic management of small populations in breeding centers, zoos, and parks [33]. Creation of germplasm cry- obanks is fundamental, since it allows the storage of biological material with two main purposes: to promote biological knowledge and to sup- port the genetic diversity of these rare and endangered species [12]. For semen cryopreservation, however, many steps are required during this delicate process, including seminal plasma separation, dilution, cryo- protectant exposure, refrigeration, freezing, and thawing [9,33].

Semen cryopreservation methods have been successfully described in several species of felids such as lions (*Panthera leo*) [15,20], cougar (*Puma concolor*) [13], ocelot (*Leopardus pardalis*), and oncilla (*Leopardus tigrinus*) [3]. In jaguar, according to Paz et al. [24], the use of an extender based on 20% egg yolk, 11% lactose, 1000 IU/mL penicillin, and 1000 mg/mL streptomycin, in PDV-62, provides only 26.7%

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post-thawing motility. Although the TRIS-based extender, or its variation in TES-TRIS, is widely recognized and used for semen cryopreservation in various felids [1,15,19,20] its effect on the morphofunctional parameters of jaguar sperm remains unknown. In parallel, it is important to propose alternative extenders that are capable of providing excellent post-thawing quality, at low cost and that are easy to prepare. Among these, extenders based on powdered coconut water (ACP) have been proposed for semen cryopreservation in several species [6,22,23,35], including the domestic cat (ACP-117c®) [36].

Cryopreservation can induce damage to the sperm cells that is not always easily detectable by classical evaluation methods. Some sperm ultrastructural cryodamage can only be observed at a nanometric scale using scanning or transmission electron microscopy [11], as reported for domestic cats and tigers (*Panthera tigris altaica*) [28]. In the jaguar, the first ultrastructural description of fresh sperm was recently reported by Silva et al. [34], but the effect of the freezing-thawing protocols on the ultrastructural integrity of its sperm remains unknown.

Because of the difficulties in accessing females for artificial insemination trials or oocyte collection, alternative methods to evaluate the functional parameters of the sperm have been proposed [10]. The first attempt at evaluating potential fertility of jaguar sperm was by Paz et al. [24], who demonstrated a 15.4% penetration rate in hamster denuded oocytes. For ocelot and oncillas, Araujo et al. [3] proposed the use of the perivitelline membrane of egg yolk as a heterologous substrate for testing the sperm-binding capability. The composition of such membranes presents similarities to the glycoproteins present in the zona pellucida [10], and the assay is relatively simple, requiring some equipment, and has a lower cost[3].

Thus, combining the results of the post-thaw semen quality analysis with the evaluation of the ultrastructural damage and the perivitelline membrane binding test, it is possible to determine an adequate extender to preserve the jaguar semen samples. Therefore, the objectives of the present study were to (1) evaluate the jaguar sperm cells frozen with TRIS or ACP-117c extenders, (2) describe the ultrastructural changes in the sperm during the freezing process, and (3) evaluate the binding capacity of the post-thawing spermatozoa using the perivitelline membrane of chicken eggs to estimate the fertilizing capacity.

2. Materials and methods

2.1. Animals, anesthesia and semen collection

The study was approved by the Ethics Committee of the State University of Ceará (no. 5098414/2016) and by the System of Authorization and Information of Biodiversity (Sistema de Autorização e Informação da Biodiversidade – SISBIO) (no. 54741-1). Five male jaguars weighing 70.8 ± 5.9 kg and ranging from 4 to 17 years of age were used. Each animal belonged to a zoo of different cities in north-eastern Brazil. One was from an Ecological Park – EcoPoint, Fortaleza, CE, Brazil (3° 43' S, 38° 30' W), weight: 66 kg, age: 17 years; one from São Francisco Zoo, Canindé, CE, Brazil (4° 21' S, 39° 18' W), weight: 81 kg, age: 6 years; one from the Teresina Zoobotanical Park, Teresina, PI, Brazil (5° 05' S, 42° 48' W), weight: 70 kg, age: 8 years; one from the Arruda Câmara Zoobotanical Park, João Pessoa, PB, Brazil (7° 06' S, 34° 51' W), weight: 69 kg, age: 9 years; and one from the Dois Irmãos Park, Recife, PE, Brazil (8° 03' S, 34° 52' W), weight: 68 kg, age: 4 years. Each animal was subject to the usual management practices of each institution, with the regular provision of red meat or slaughtered chicken, as well as vitamin supplementation. Water was provided ad libitum.

The animals were chemically restrained using blowgun darts containing dexmedetomidine (Dexdomitor ®, Zoetis, Campinas – SP, Brazil) at a dose of 0.04 mg/kg, IM, combined with ketamine hydrochloride (Ketalar®, Pfizer, S~ao Paulo-SP, Brazil) at a dose of 5 mg/kg, IM, when necessary, a third of the initial dose was administered to maintain anesthesia, to reverse the anesthesia, yohimbine was used at a dose of 0.4 mg/kg, IM [34].

Two collections at a two-month interval were performed on three animals, while collection was performed only once on the other two individuals, totaling the collection of eight ejaculates. The electroejaculation was performed for semen collection and using an electromechanical device (Autojac V2®, Neovet, Uberaba, MG, Brazil) as previously described [34].

2.2. Initial analysis of semen and sperm mitochondrial activity analysis

Samples of each animal were collected and evaluated in the zoos. Samples were evaluated macroscopically for color and volume, and microscopically for motility, vigor, morphology, sperm concentration and membrane functionality. These evaluations were carried out as previously described [34].

The functionality of the sperm membrane was determined using a hypoosmotic test in which 10 µL of semen were diluted in 90 µL distilled water. The sample was then stored at 37 °C for 40 min before evaluation of the cell membrane and classification either as positive reaction (tail winding) or negative reaction (tail completely straight) [34].

Regarding the assessment of the mitochondrial activity, 20 µL of semen was added to 50 µL of a solution containing 15 mg/mL 3,3'-diaminobenzidine (DAB) diluted in phosphate buffered saline (PBS). After incubation (protected from natural light) in a water bath at 37 °C for 40 min, 10 µL of cell suspension were smears on a microscope slide and dried at ambient temperature (20–25 °C). Slides were fixed in 10% formaldehyde for 10 min, rinsed in distilled water, and then dried at ambient temperature [34]. A total of 100 spermatozoa were observed under a phase-contrast microscope (400 magnification) and classified as: Class I DAB (100% of the middle piece was stained, indicating high mitochondrial activity); Class II DAB (>50% of the middle piece was stained, indicating attenuated mitochondrial activity); Class III DAB (<50% of the middle piece was stained, indicating low mitochondrial activity); and Class IV DAB (absence of staining in the middle piece, indicating the absence of mitochondrial activity). Values obtained in each class were expressed as a percentage [34].

Scanning electron microscopy (SEM)

SEM was performed using 5 µL aliquots of pooled semen fixed in 2.5% buffered glutaraldehyde solution and subsequently fixed in osmium tetroxide 1%. The samples were stored in a refrigerator at 4 °C until all the samples had been collected. Then, the samples were processed for SEM evaluation at the Laboratory of Applied Animal Morphophysiology at the Federal Rural University of Semi-Árido as previously described [34], and then evaluated using a scanning electron microscope (LEO VP® 435 – Carl-Zeiss, Oberkochen, Germany).

2.3. Transmission electron microscopy (TEM)

TEM was performed using 5 µL aliquots of pooled semen fixed in modified Karnovsky solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 (PB-Co® Sigma, St. Louis, USA) and kept in a refrigerator at 4 °C. The samples were sent for processing at the Electron Microscopy Laboratory of the University of Sa~o Paulo (Faculty of Medicine – Campus of Ribeirão Preto, SP, Brazil). The samples were processed as described by Silva et al. [34]. The samples were subsequently evaluated with a transmission electron microscope at the Laboratory of Transmission Electron Microscopy of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo – FMVZ – USP, Sa~o Paulo, SP, Brazil). Different fields were selected randomly and evaluated by TEM (JEOL 1010, Japan), and imaged for later analysis and description [34]. Acrosomal loss was evaluated using electron transmission microphotographs in image analysis software (ImageJ, Wayne Rasband–National Institute of

Health, Maryland, USA).

2.4. Semen processing

Two extenders were used for semen dilution. The first, based on powdered coconut water (ACP-117c, from ACP Biotecnologia, Fortaleza-Ceará, Brazil) was prepared according to the manufacturer's instructions, including 2.63 g of the powder in 50 mL of distilled water, with pH and final osmolarity of approximately 8.2 and 310 mOsm/L, respectively. The second was based on TRIS (tris-hydroxymethyl-ami- nomethane - Sigma, Darmstadt, Germany), containing 3.028 g TRIS hydroxymethyl aminomethane, 1.78 g citric acid monohydrate and 1.25 g, D-fructose (Dynamic® Química Contempora^nea Ltda., Indaia- tuba, S~ao Paulo, Brazil) diluted in 100 mL of distilled water, with pH 7.0 and osmolarity 316 mOsm/L.

After initial analysis, ejaculates were divided in two aliquots that were immediately diluted in ACP-117c or TRIS (1:1). Samples were centrifuged at 300 g for 10 min to remove excessive seminal plasma [24]. The supernatant was removed, and the samples were resuspended in the same extender (TRIS or ACP-117c), with the addition of 20% egg yolk. The dilution method used was by v:v (1:1) [30].

After the dilution, samples were stored in a thermal box with ice and equilibrated for 40 min at 15 °C (cooling rate: 0.4 °C/min). After this period, the samples were added to TRIS-egg yolk or ACP-egg yolk plus 12% glycerol (at 5 °C), resulting in a final concentration of 6% glycerol in the extenders and equilibrated for 30 min at 5 °C (cooling rate: 0.2 °C/min). At each refrigeration step, the samples were evaluated for total motility, vigor, membrane functionality, morphology, and mitochondrial activity [30,42].

The samples were packed into 0.25 mL plastic straws and placed horizontally in an insulated box for 5 min, 5 cm above the liquid nitro- gen level. Finally, the straws were plunged into liquid nitrogen for storage [30,42].

Thawing was performed in a water bath at 37 °C for 1 min and the contents of the straws were emptied into a glass tube pre-warmed to 37 °C. After warming (37 °C for 10 min), samples were evaluated as described for fresh semen and rewarmed using a microscope attached to a warmed plate (37 °C) and samples evaluated in 1 min, including acrosomal defects in morphology, as well as for sperm kinetic parameters using CASA sperm membrane integrity using fluorescent probes, and for sperm binding capability using an egg yolk perivitelline mem- brane assay, as described in sections 2.10 and 2.11, respectively.

2.5. Computer assisted semen analysis

Frozen-thawed samples were also evaluated using a computer- assisted semen analyzer (IVOS 7.4G; Hamilton-Thorne Research, Bev- erly, MA, USA). The evaluations were based on the configurations pre- viously determined for the cat, the species closest related to the jaguar, but adjusted according to the sperm morphometric settings previously described for the species [34]. The settings of the instrument were temperature 37 °C; 60 frames/s; minimum contrast, 30; straightness threshold, 80%; low-velocity average pathway (VAP) cutoff, 30.0 μm/s; and programming minimum VAP, 70.0 μm/s; straight line velocity (VSL) cutoff, 20.0 μm/s; and cell size, 8 pixels. The following spermatic pa- rameters were analyzed by CASA: total and progressive motility (%); subpopulations of fast, medium, slow, and static sperm (%); average path velocity (VAP - μm/s), curvilinear velocity (VCL - μm/s), straight line velocity (VSL - μm/s); linearity index (LIN - %); straightness index (STR - %); amplitude of lateral head displacement (ALH - μm), and beat cross frequency (BCF - Hz) [40].

2.6. Sperm membrane integrity

A fluorescent solution containing fluorophores 6-carboxyfluorescein diacetate (0.46 mg CFDA/1 mL dimethylsulfoxide) and propidium iodide (0.5 mg PI/1 mL 0.9% saline solution) was prepared to evaluate the integrity of the frozen-thawed sperm membrane. An aliquot of 10 μL of thawed semen was added to 40 μL of fluorescent solution. After 10 min, the microscope slides from the stained samples were evaluated under epifluorescence microscopy (Episcopic Fluorescent attachment "EFA" Halogen Lamp Set, Leica, Kista, Sweden). A total of 200 sperm were counted for each sample stained with CFDA/PI and classified as having an intact plasmalemma or not. Cell membranes stained in green (CFDA) were considered intact, while those stained in red (PI) or partially stained were considered non-intact [40].

2.7. Sperm binding capability assay

To verify the binding capability of the thawed sperm of jaguars using TRIS or ACP-117c extenders, the perivitelline membrane binding assay was used as previously reported for ocillas and ocelots [3] with slight modifications. The perivitelline membranes were obtained from fresh and unfertilized chicken eggs and prepared by separating yolk from albumen. The intact yolks were placed in parafilm and membrane separation followed by saline washes (NaCl, 0.9%). Then, the membranes were placed in a Petri dish and cut into squares (1 cm2). Subsequently each piece was inserted into four plates containing the incubation me- dium. This medium comprised 125 mM NaCl; 3.2 mM KCl; 0.4 mM NaH2PO4; 10 mM calcium lactate; 25 mM NaHCO3; 10 μg/mL phenol red; 1.4 mM caffeine; 2.0 mM CaCl2·2H2O; 0.5 mM MgCl2; 10 mM HEPES; 6 mg/mL BSA; 0.45 mM sodium pyruvate; 5.5 mM glucose; and 50 μg/mL gentamicin sulfate at pH 7.4–7.5.

After semen thawing, the samples were washed in the incubation medium (1:1), and centrifuged (700 g/10 min) at room temperature (approximately 23 °C). The supernatant was discarded, and the pellet diluted in incubation medium, adjusting the concentration to 1 x 10^6 sperm/mL for 1 perivitelline membrane. The samples were incubated for 20 min at 38.5 °C in a water bath. After incubation, the perivitelline membranes were washed to remove unbound sperm; then, the peri- vitelline membranes were stained with Hoechst 33342 (10 mg/mL) and attached sperm were counted using epifluorescence microscopy (Episcopic Fluorescent attachment EFA Halogen Lamp Set, Leica, Kista, Sweden) (400, magnification). For each sample, all sperm attached to the membrane in five different fields and the mean was determined [4].

Experimental design and statistical analysis

Samples of each animal were collected and evaluated in the zoos. Samples were evaluated macroscopically for color and volume, and microscopically for motility, vigor, membrane functionality, and sperm concentration. For the evaluation of mitochondrial activity, morphology and for electron microscopy, the fresh, freezing and thawed samples were fixed for further processing and laboratory analysis. Additionally, in the zoos the samples were processed for cryopreservation (from cooling using a refrigerator to cryopreservation in the nitrogen tanks). Samples were thawed at the Laboratory of Animal Germplasm Conservation (Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid - Mossoró/RN - Brazil), to perform the same evaluations as done for the fresh semen, computer-assisted sperm analysis (CASA), the membrane integrity test, and the perivitelline membrane binding test. Owing to the small volume of the samples and the number of assays, only one-time point after thawing was considered. Statistical analysis was performed using the R-project © statistical software version 3.3.2 (The R Foundation, Vienna, Austria). Experi- ments were performed in designs randomized by Stat view software for Windows (SAS Institute Inc. Copyright 1992–1998, version 5.0, Cary, NC, USA). The results were expressed as mean standard error of mean (SEM) and data were checked for normal distribution (Shapiro Wilk test) and homoscedasticity (Levene's test). To evaluate the effect of the diluent at each step, data were analyzed with Test T ($P < 0.05$) when normally distributed or Kruskal Wallis test followed by Wilcoxon Mann

Whitney test (P < 0.05) when not normally distributed (VAP, VSL, BCF, STR, fast, middle; morphology and DAB 1/5 °C; motility and vigor/ 15 °C). To evaluate the effect of steps on sperm characteristics in each diluent, data were analyzed by ANOVA followed by Duncan Test (P < 0.05) when normally distributed or Kruskal Wallis test followed by Wilcoxon Mann Whitney test (P < 0.05) when not normally distributed (Motility, vigor and DAB 4/TRIS). Arcsine transformation of the data was performed prior to evaluate the effect of the diluents on motility at 5 °C and the effect of temperatures on the TRIS diluent on the percentage of normal spermatozoa.

3. Results

3.1. Semen initial analysis

The semen volume was obtained from the second cycle of electrical stimuli, and semen color was translucent, owing to electrical stimulation on the prostate, that causes it to increase the volume of the ejaculate, releasing more prostatic fluid. Total volume of the ejaculate was 6.8 ± 2.3 mL, ranging from 4 to 10 mL. The sperm concentration was 132.3 ± 89.9 106 cells/mL, ranging from 70 to 340 106 cells/mL.

As expected, both extenders elicited significant reduction in sperm motility at 15 °C (P < 0.05), which was further increased after thawing (P < 0.05). However, TRIS (48.8 ± 4.8%) provided a better post-thaw motility in comparison to ACP-117c (25.0 ± 4.6%, P < 0.05) as evaluated by light microscopy (Fig. 1A). Similar effects were observed for vigor, which also presented decreasing values after cooling or thawing (P < 0.05), but also with TRIS (3.1 ± 0.2) providing better post-thawing values in comparison to ACP-117c (2.2 ± 0.3, P < 0.05) (Fig. 1B). Regarding membrane functionality, only TRIS samples demonstrated reduced values after thawing, while ACP samples were affected by cooling at 5 °C (P < 0.05); after thawing, samples frozen in TRIS (60.5 ± 4.2%) presented better values for functional membranes in comparison to ACP-117c (47.1 ± 2.5%, P < 0.05) (Fig. 1C). For normal morphology, only those samples frozen-thawed in ACP-117c (64.0 ± 3.9%) presented values lower than those observed for fresh samples (80.0 ± 3.5%, P < 0.05), but there were no differences between extenders (Fig. 1D) (P > 0.05). Moreover, a similar increase in acrosomal defects was observed for both extenders, TRIS (5.9 ± 1.3%) and ACP-117c (8.4 ± 1.8%), after thawing when compared to fresh samples (0.8 ± 0.5%, P < 0.05).

For mitochondrial activity evaluation, it is very important that cells are classified in the more than 50% stained category (observed in DAB I and II classifications) after thawing. In the DAB I category, both ex- tenders had reduced percentages compared with that of the fresh samples (42.8 ± 5.0%, P < 0.05), but in TRIS, this reduction started at 5 °C and in ACP-117c started at 15 °C. Between extenders, TRIS was better than ACP-117c, at 15 °C and in thawed samples (Fig. 2A, P < 0.05). In the DAB II classification, there was no difference between temperatures and extenders evaluated (Fig. 2B). DAB III and IV are described in Fig. 2C and D, respectively.

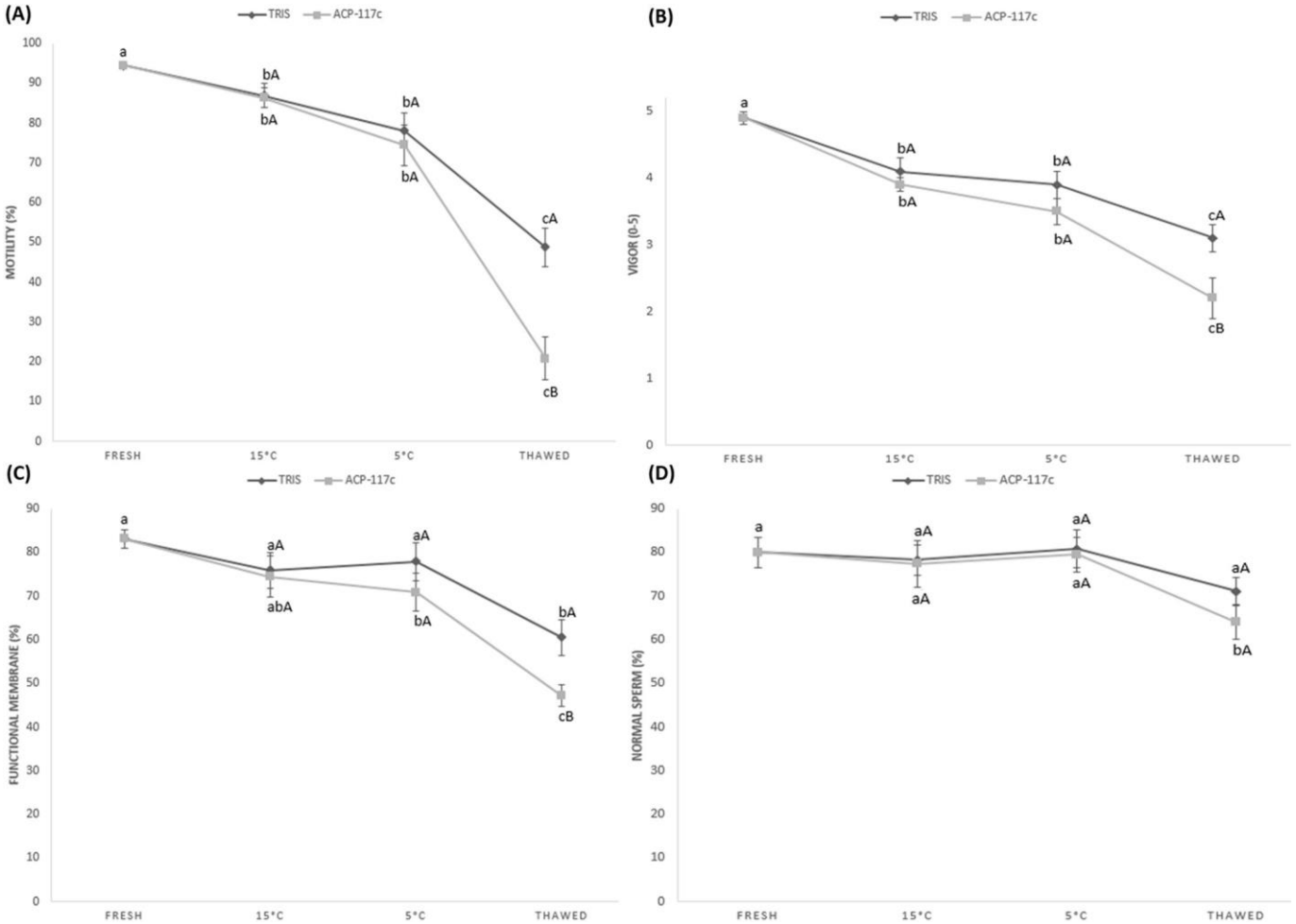

Fig. 1. (A) Total motility; (B) vigor; (C) functional sperm membrane, and (D) normal sperm in jaguars, evaluated in semen diluted in TRIS and ACP-117c, cooled at 15 °C, 5 °C, and thawed (mean ± SEM). Different letters, abc indicate a statistical difference between cryopreservation steps and AB the difference between extenders, (*) means ACP-117c.

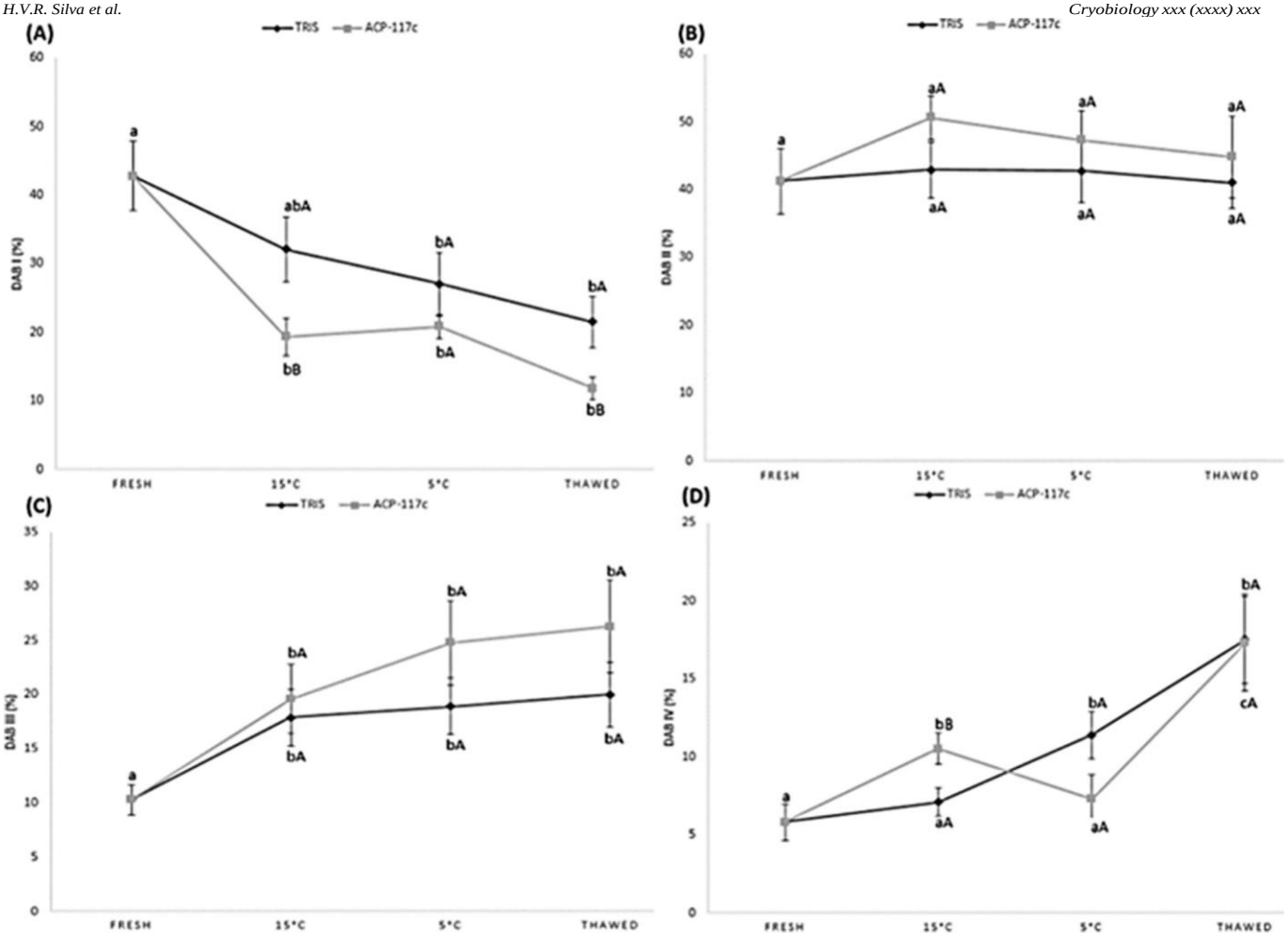

Fig. 2. (A) DAB I, (B) DAB II, (C) DAB III, and (D) DAB IV in jaguars, evaluated in semen diluted in TRIS and ACP-117c, cooled at 15 °C, 5 °C, and thawed (mean ± SEM). Different letters, abc indicate a statistical difference between cryopreservation steps, and AB the difference between extenders, (*) means ACP-117c.

3.2. Computer assisted semen analysis

Regarding post-thawing sperm kinetic parameters evaluated by CASA (Table 1), it was notable that TRIS provided significantly better values for total motility than ACP-117c ($P < 0.05$). This finding was also reflected in the sperm subpopulation, in which TRIS provided higher values for the medium and slow sperm while ACP-117c provided higher values for only the static sperm ($P < 0.05$).

Table 1
CASA parameters in jaguar (Panthera onca), frozen and thawed semen comparing TRIS and ACP-117c extenders. Values (mean ± SEM) (n 8 ejaculates).

	TRIS	ACP-117c
Total motility (%)	46.0 ± 7.7[a]	20.9 ± 5.4[b]
Velocity average pathway (μm/sec)	56.0 ± 5.0[a]	74.2 ± 7.7[a]
Straight line velocity (μm/sec)	44.1 ± 6.3[a]	65.7 ± 7.9[a]
Curvilinear velocity (μm/sec)	102.3 ± 6.9[a]	101.0 ± 8.5[a]
Amplitude of lateral head displacement (μm)	8.4 ±0.4[a]	7.6 ± 0.4[a]
Beat cross frequency (Hz)	15.9 ± 2.1[a]	14.1 ±1.9[a]
Straightness index (%)	72.1 ± 5.9[a]	84.8 ± 1.3[a]
Linearity index (%)	48.1 ±7.5[a]	63.4 ±2.8[a]
Sperm subpopulations		
Fast (%)	0.9 ±0.4[a]	0.5 ± 0.2[a]
Middle (%)	3.1 ±0.9[a]	1.1 ±0.2[b]
Slow (%)	42.0 ± 7.0[a]	16.5 ±5.2[b]
Static (%)	54.0 ± 7.7[b]	79.1 ±5.4[a]

*Different lowercase letters in the same line mean that there was a statistical difference between extenders.

3.3. Sperm ultrastructural evaluation

Scanning electron micrographs showed that jaguar fresh sperm presents a homogeneous surface denoting its external membrane integrity through the regions of the head, midpiece, or tail (Fig. 3A). After thawing, no evident damage on the ultrastructure of the sperm membrane surface was observed either for samples diluted in TRIS (Fig. 3B) or for those diluted in ACP-117c (Fig. 3C).

Through TEM, we verified the presence of electron lucent points in the fresh sperm nucleus (Fig. 4A), similar to that observed for samples frozen in TRIS (Fig. 4B); however, an increase in electron lucent spots (white points inside nucleus) was observed in samples frozen in ACP- 117c (Fig. 4C). For all the samples, the outer membrane was less adhered to the cell (Fig. 4A, B, and C). In contrast to fresh samples (Fig. 4A), the acrosomal area lost in both extenders in frozen sperm (Fig. 4B and C).

The most striking differences between the extenders were observed over the mid-piece. In fresh samples the mitochondria were well preserved showing several internal cristae immersed in a dense matrix (Fig. 4D, G). The frozen-thawed sperm mid-piece showed the same structure as observed for the fresh sperm, however, the mitochondrial sheath showed signs of damage, since mitochondria were vacuolated as if the matrix had been washed out and few cristae were visible. Such damage was more evident in samples frozen in ACP-117c (Fig. 4F, I) than in those diluted in TRIS (Fig. 4E, H).

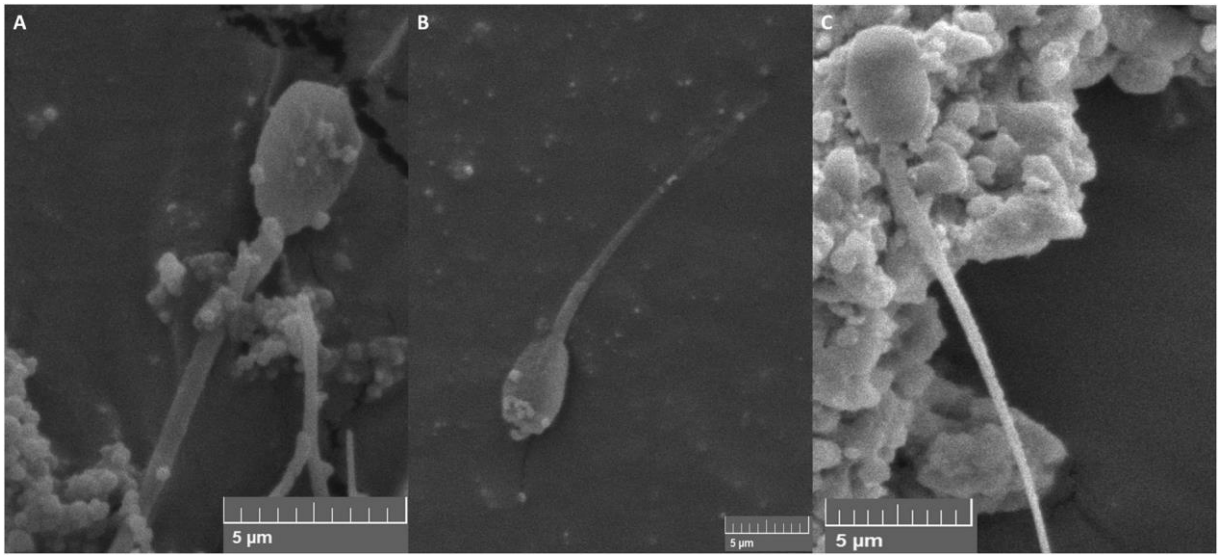

Fig. 3. Electron microphotograph in SEM of sperm morphology in jaguar (*Panthera onca*). (A) fresh semen, (B) semen diluted in TRIS, and (C) semen diluted in ACP-117c.

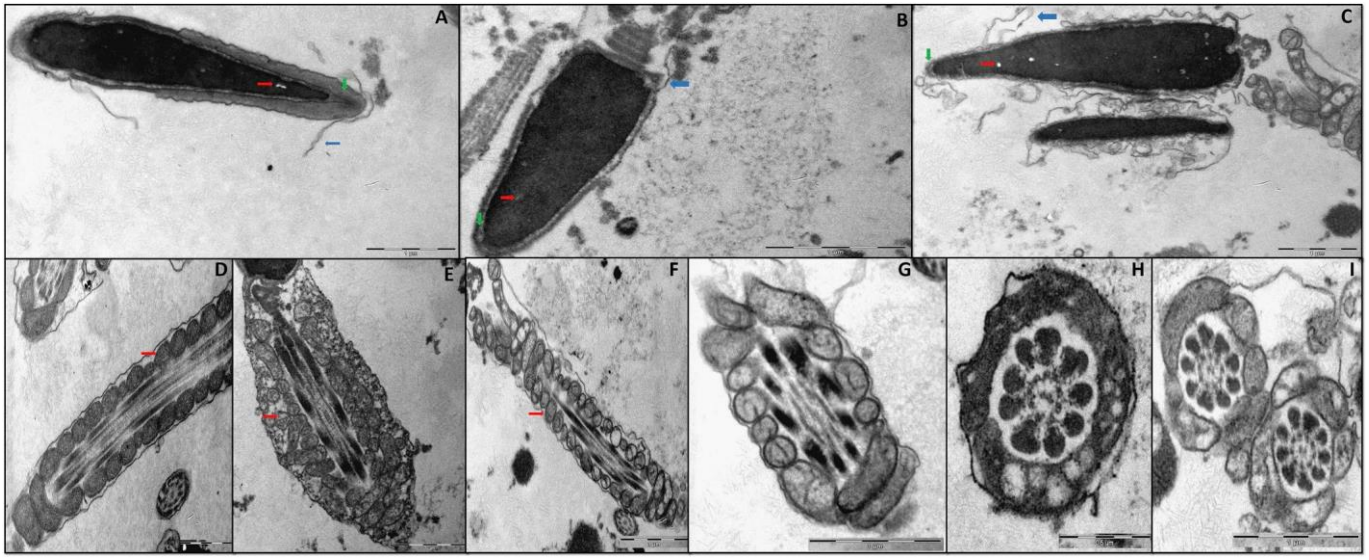

Fig. 4. Electron microphotograph in TEM of sperm morphology in jaguar (*Panthera onca*). (A) fresh semen, (B) semen diluted in TRIS, and (C) semen diluted in ACP-117c. Heads in longitudinal plane. Red arrow, electron lucent point; blue arrow, outer membrane; green arrow, acrosomal area. (D) fresh semen, (E) semen diluted in TRIS, and (F) semen diluted in ACP-117c; (DEF, red arrow – mitochondrial area). Description of mitochondria changes in the middle piece area in longitudinal plane. (G) fresh semen, (H) semen diluted in TRIS, and (I) semen diluted in ACP-117c. Description of mitochondria changes in the middle piece area in transversal plane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Evaluation of membrane integrity and perivitelline membrane binding test

Regarding the evaluation of membrane integrity by epifluorescence (Fig.5A, there were no significant differences between the samples frozen-thawed in TRIS (46.1 ± 4.5%) and those diluted in ACP-117c (35.1 ± 3.5%). In contrast, the TRIS extender provided an amount of sperm (29.5 3.3) bound to the egg yolk perivitelline membrane Fig. 5B) significantly higher ($P < 0.05$) than that observed with the use of ACP-117c (18.6 ± 1.5).

4. Discussion

Despite the immense ecological importance of the jaguar, strategies for both ex situ as well as in situ conservation are lacking. In this regard, we provide important information for the formation of male germplasm banks related to the effective use of a TRIS extender for cryopreservation of jaguar semen.

Concerning sperm motility, which is generally the main parameter evaluated for determining the quality of a semen sample [39], TRIS provided significantly better post-thawing results than those observed for the ACP-117c, both under subjective and objective (CASA) analysis. Moreover, our results with TRIS (46%) were better than those previously reported (26.7% motile sperm) in a study that described cryopreserva- tion of jaguar semen, but with the use of a PDV-62 extender (20% egg yolk, 11% lactose, 1000 IU/ml penicillin, 1000 mg/ml streptomycin)
[24].

These results confirm that the extender selected can significantly influence post-thawing parameters of a semen sample. The buffer components serve to control pH and reactive oxygen species (ROS). In

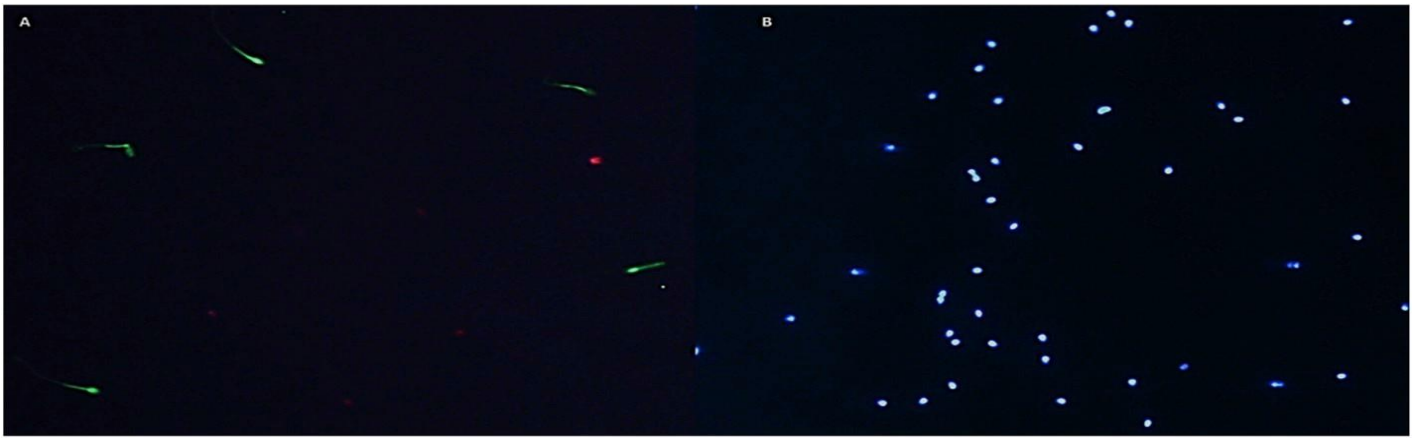

Fig. 5. (A) Jaguar sperm cells with green colored membranes (CFDA) classified as intact membranes and red (PI) colored or partially-colored cells classified as damaged membranes. (B) Sperm colored in Hoechst 33342 (blue) bound to the perivitelline membrane (1 cm^2). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the TRIS extender, citric acid performs this function; ACP-117c has only ascorbic acid (Vitamin C), which despite having a similar function does not have the same effectiveness as citric acid, and in some cases may increase the damage caused to sperm by ROS [21,33]. In fact, TRIS not only has a buffering activity [31], but also slows the consumption of the fructose present in the medium. It has been shown in felids such as domestic cats and cheetahs (Acinonyx jubatus), having spermatozoa with a high metabolic rate, that the sperm quickly consume the energetic substrate which can lead to rapid loss of motility [37].

For mitochondria activity evaluation after thawing, no significant differences were determined between the extenders tested for jaguar sperm. We however confirmed the occurrence of a gradual decrease in the jaguar sperm mitochondrial activity as the freezing stages passed, which is an expected side-effect of the cryopreservation process [41]. In fact, TEM revealed various midpiece alterations in jaguar sperm, specially related to mitochondria vacuolization and deforming. Such mitochondrial membrane injury occurs because of thermal shock, a phenomenon caused by abrupt cooling [18], and mainly because of the ROS originating from the respiratory cycle of cells [29]. Moreover, the addition of glycerol to the extender during the freezing process is re- ported to affect the reactions that require ATP and therefore may cause an ATP deficit in mitochondrial membrane potential [16].

It is necessary to highlight that DAB evaluation for mitochondrial activity analysis is not a commonly conducted analysis even for the domestic cat, in which values of 50% epididymal sperm with high mitochondrial activity (DAB I plus II) were verified in samples extended in phosphate buffer saline and cooled at 5 °C for 72 h [2]. In this sense, we believe that the values related to DAB (I and II) for jaguar sperm frozen in TRIS (62.5%) and ACP-117c (56.6%) are adequate, especially because the freezing technique for this species is still under develop- ment. Interestingly, percentages of cells with mitochondrial activity (DABs I, II and III) were high as we analyzed, for example, post-thawing motility values. This might be due to the fact that although cells are immotile, they still retain intact physiological functions including par- tial mitochondrial activity without the possibility to move the cell [27]. We assume that mitochondria activity close to 100% to trigger motility. Using light microscopy, we verified a large percentage of morpho-logically normal cells preserved with the use of both diluents, which was confirmed by SEM. However, cryopreservation induced an increase in acrosomal damage, regardless of the extender used, and the TEM evi-denced the occurrence of acrosomal content loss in cryopreserved samples. In cats, the occurrence of this kind of alteration in thawed samples is generally related to an early acrosomal reaction induced by cryopreservation [42], and such phenomena could also have occurred in some jaguar sperm.

It is worth noting that morphological evaluation by light microscopy is limited, while electron microscopy allows analysis under a nanometric scale [25]. In this sense, TEM revealed the occurrence of plasmalemma detachment in cryopreserved samples, especially those diluted in the ACP-117c extender. Such results corroborate those found by the hypo- osmotic test that revealed the TRIS extender is more effective in pre- serving membrane functionality of jaguar frozen sperm than ACP-117c. The glycerol included in the extenders, while protecting the cell against the formation of ice crystals, has the negative side of toxicity which may cause protein denaturation, actin interaction and alterations, and direct disruption of the plasma membrane [32]. It is plausible that the TRIS-glycerol interaction was better than that of the ACP-117c-glycerol, because membrane damage was more evident in the latter extender.

TEM also showed an increase in the amount of electron lucent points in the nucleus of jaguar sperm subjected to cryopreservation. The presence of these points was previously described for fresh jaguar sperm as a failure of chromatin integrity [8]. However, the increase in the number of nuclear electron lucent points after sperm cryopreservation, which was also previously described by Bezerra et al. [7] in peccaries (Pecari tajacu), may be related to the failure in sulfide bridges or histone loss, generally derived from the ROS action, thus causing DNA damage [5]. Some spermatozoa with chromatin defects can fertilize oocytes, but DNA damage may follow to the embryonic stage, leading to apoptosis and embryonic fragmentation [14,38].

In the present study, the use of a perivitelline membrane binding test provided us unprecedent and promising results for cryopreservation of jaguar sperm. It highlighted the most efficient action of the TRIS extender at preserving some functional aspects of the jaguar sperm in comparison to that of ACP-117c. Results found for jaguar (29.5 ± 3.3 bound sperm) were better than those found for the oncilla (Leopardus tigrinus - 15.6 ± 10.7) but lower than those reported for the ocelot (Leopardus pardalis - 38.5 ± 19.3) [3]. Such results may also suggest a different (species-specific) binding affinity to the chicken egg mem- brane. Moreover, the present results are better than those previously reported by Paz et al. [24], who incubated the jaguar thawed sperm with heterologous denuded oocytes and observed a 15.4% penetration rate. The perivitelline membrane binding assay is therefore highlighted as a simple, practical, and low-cost test that was proved as effective for testing different diluents for jaguar sperm freezing. To complement this evaluation method, it was necessary to use the appropriate medium composition enabling the sperm to bind to the perivitelline membrane. In conclusion, we suggest the use of a TRIS-based extender for the cryopreservation of jaguar semen. Of course, we cannot neglect the possibility of using the ACP-117c extender for the same purpose as an alternative. Additionally, we provided data for the first time regarding the ultrastructural damage to the jaguar sperm caused by cryopreser- vation, and demonstrated the possibility of testing its sperm binding capability using a perivitelline membrane assay. These results are initial steps for the creation of germplasm banks for this endangered species, thus contributing to the development of assisted reproductive programs for its conservation.

Declaration of competing interest

The authors have stated that there are no competing interests. None of the authors has financial or personal relationships that may influence or distort the content of the article.

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8 CONCLUSÕES

As onças-pintadas mantidas em cativeiro no nordeste brasileiro apresentam uma boa qualidade da amostra seminal. Nesta espécie não foram determinadas particularidades da estrutura morfológica do espermatozoide, logo apresenta morfologia similar à grande maioria dos carnívoros.

O sêmen da onça-pintada pode ser criopreservado com sucesso, utilizando e um protocolo rápido, eficiente, e o Tris possui potencial maior de proteção das células contra crioinjúrias.

9 PERSPECTIVAS

A definição de um protocolo de criopreservação pode contribuir para futuras pesquisas no tocante à reprodução das onças-pintadas, principalmente relacionadas à criação de banco de germoplasma para a espécie, uma vez que o Tris é sugerido como base para o melhor diluidor. Como também é possível propor futuramente o uso de amostras congeladas para inseminações, com o objetivo de aumentar a variabilidade genética nas populações de onças.

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ANEXOS

ANEXO A: COMPOSIÇÃO DOS MEIOS DE CONSERVAÇÃO E AVALIAÇÃO ESPERMÁTICA

Água de coco em pó (ACP®).

- 1 Sachê de ACP-117c® (4,25 g)
- 50 mL Água destilada autoclavada

Preparo do Tris

- 3,028g de TRIS hydroximetil-aminometano
- 1,78 g Ácido cítrico monohidratado
- 1,25 g D-frutose

OBS_1: Diluir em água destilada (100 mL).

OBS_2: Verificar pH 7,0 osmolaridade 316 mOsm/L (Valores aproximados).

Formulação do Rosa de Bengala

- 20 mL de água destilada
- 0,58g de citrato de sódio
- 0,8 mL de formaldeído
- 0,3g de Rosa de Bengala

Obs: Após a mistura dos três primeiros componentes, a solução é homogeneizada e acrescenta-se a Rosa de Bengala

Solução de formaldeído

- Solução 1:80 de formalina 40%, em solução fisiológica

Solução 3,3-diaminobenzidina

- 3,75 mg de 3,3-diaminobenzidina
- 250µL solução PBS

Obs[1]: Com a solução pronta, manter protegida da luz, à 4°C. Usar dentro de 1 semana.
Obs[2]: Para avaliações, 50 µL de solução DAB + 20 µL amostra sêmen. Incubar à 37°C/40 min. Realizar esfregaços com 10 µL da mistura DAB + amostra. Incubar A lâmina em formaldeído 10% por 10 min.

ANEXO B: ETAPAS DA CONGELAÇÃO

Diluição a temperatura ambiente com parte A do diluidor; Etapa 2: Resfriamento em caixa térmica por 40 minutos; Etapa 3: Resfriamento em refrigerador por 30 minutos; Etapa 4: Diluição com parte B do dluidor; Congelação em vapores de nitrogênio por 5 minutos; Etapa 5: Armazenamento em nitrogênio líquido (CARDOSO, 2005).