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INCORPORAÇÃO DE ANTIMICROBIANOS, DETERGENTES E ANTIOXIDANTES AO DILUENTE PARA CRIOPRESERVAÇÃO DO SÊMEN DE CATETOS (*Pecari tajacu* LINNAEUS, 1758)

MOSSORÓ, RN
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Linha de Pesquisa: Morfofisiologia da Reprodução e Biotecnologia Animal

Orientador: Alexandre Rodrigues Silva, Prof. Dr.

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SAMARA SANDY JERÔNIMO MOREIRA – Nascida no Município de Russas– Ce, no dia 01/04/1994, filha de Fátima Coêlho Jerônimo e José Evamberto Moreira, concluiu o Ensino Médio na escola Unidade Educacional Coração Imaculado de Maria - UNECIM, Ce. Graduou-se em Biotecnologia pela Universidade Federal Rural do Semi-Árido (UFERSA) em 2016.1, onde foi bolsista no programa permanência acadêmica (2013.2 – 2014.1) por um ano, PIVIC (Novembro/2014 a Outubro/2015) por um ano e PIBIC (Agosto/2015 a Fevereiro/2016) por um semestre. Durante a graduação, desenvolveu trabalhos sobre a conservação de material genético de espécies silvestres. Em 2019 concluiu o mestrado em Ciência Animal pelo Programa de Pós-Graduação em Ciência Animal (PPGCA/UFERSA). Durante o período do mestrado foi colaboradora das disciplinas Fisiopatologia da Reprodução, Ginecologia e obstetrícia veterinária do curso de Medicina Veterinária na Universidade Federal Rural do Semi-Árido (UFERSA). Em Fevereiro de 2019 foi selecionada pelo Programa de Pós-Graduação em Ciência Animal (PPGCA/UFERSA). Durante o período do doutorado, foi colaboradora na disciplina de Fisiopatologia da Reprodução, Ginecologia e obstetrícia veterinária, Biotecnologia da reprodução e Reprodução animal e inseminação artificial do curso de Medicina Veterinária, assim como da disciplina de Biotecnologia animal do curso de Biotecnologia da mesma instituição.

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“A vida não é sobre metas e conquistas. É sobre quem você se torna durante a caminhada”.

Phablo Ricardo

RESUMO

Haja vista a importância ecológica dos catetos (*Pecari tajacu*) como dispersores de sementes e membros da cadeia alimentar em diferentes biomas, o objetivo desta tese foi aprimorar o diluente para a criopreservação de sêmen nesta espécie por meio da incorporação de diferentes agentes, com o intuito de contribuir para os programas de conservação de seu germoplasma. A tese foi configurada em quatro capítulos. No primeiro, foram investigados os efeitos da suplementação do diluente com os antibióticos gentamicina 70µg/mL ou estreptomicina-penicilina 1 mg/mL/1000 UI/mL. Observou-se que o processo de criopreservação resultou em uma diminuição significativa ($P < 0,05$) da carga bacteriana, independentemente de antibióticos. Os parâmetros cinéticos pós-descongelação não foram afetados pela ausência ou presença de diferentes antibióticos, exceto pela frequência de batimento cruzado que foi significativamente ($P < 0,05$) prejudicada pela suplementação de estreptomicina-penicilina (31,7 ± 1,0) em comparação com o grupo sem antibióticos (35,6 ± 0,9). Após a descongelação, a morfologia espermática, a funcionalidade e integridade da membrana, a atividade mitocondrial e a capacidade de ligação espermática também não foram afetadas pela presença ou ausência de antibióticos. Conclui-se que embora o sêmen de cateto possa ser eficientemente criopreservado na ausência de antibióticos no diluidor, o uso de gentamicina ou a combinação estreptomicina-penicilina é recomendado como suplementação antibiótica eficaz para um maior controle das cargas bacterianas sem afetar os parâmetros espermáticos. No segundo capítulo, verificou-se o efeito da suplementação do diluente com detergentes como o Equex STM® a 0,5% ou o dodecil sulfato de sódio (SDS) a 0,1%, 0,3% ou 0,5%. Verificou-se que os tratamentos sem o SDS (41,8 ± 3,5%) e aqueles contendo Equex (41,8 ± 4,4%) ou SDS a 0,1% (41,2 ± 5,5%) proporcionaram maior motilidade espermática ($P < 0,05$) comparados àqueles contendo SDS 0,3% (30,5 ± 4,7%) e 0,5% (31,2 ± 6,3%). Imediatamente após a descongelação, apenas o grupo contendo 0,1% de SDS preservou efetivamente o índice de progressão espermática (STR) quando comparado ao grupo sem detergente. Ainda, o SDS a 0,5% prejudicou a funcionalidade da membrana e a atividade mitocondrial após a descongelação ($P < 0,05$). Desse modo, sugere-se que a adição de SDS 0,1% ao diluente otimiza a criopreservação do sêmen de catetos. No terceiro capítulo, o diluente foi suplementado com agentes antioxidantes como a catalase (CAT) a 200 ou 400 IU/mL ou superóxido dismutase (SOD) a 150 ou 300 IU/mL), ou sua combinação (CAT-SOD: 200 IU/mL + 150 IU/mL). No entanto, não foram observados efeitos significativos da suplementação com antioxidantes em nenhum dos parâmetros espermáticos avaliados, e nem mesmo em relação ao estresse oxidativo intracelular. Desse modo, a adição de SOD ou CAT, nas concentrações testadas, não promove benefícios à criopreservação do sêmen de cateto. Finalmente, o último capítulo mostra uma análise retrospectiva dos parâmetros espermáticos nos ejaculados de catetos que apresentam congelabilidade variada. Inicialmente, os animais foram classificados como bons (> 40% de espermatozoides móveis), moderados (de 30 a 40% de espermatozoides móveis) e maus (< 30% de espermatozoides móveis) congeladores, com base na motilidade espermática pós-descongelação determinada por um sistema de análise computadorizada. No sêmen fresco destes animais, apenas a análise da integridade de membrana mostrou algum valor preditivo quanto a congelabilidade do sêmen, uma vez que os maus congeladores (77,3 ± 1,7%) apresentaram uma significativa menor proporção ($P < 0,05$) de membranas intactas que os animais classificados como moderados (86,4 ± 2,1%) e bons (84,7 ± 3,1%) congeladores.

Palavras-chave: Biobanco; Vida Selvagem; Germoplasma; Tayassuídeo.

ABSTRACT

In view of the ecological importance of collared peccaries (*Pecari tajacu*) as seed dispersers and members of the food chain in different biomes, the objective of this thesis was to improve the extender for cryopreservation of semen in this species through the incorporation of different agents, with the aim of to contribute to programs for the conservation of their germplasm. The thesis was set up in four chapters. In the first, the effects of diluent supplementation with the antibiotics gentamicin 70µg/mL or streptomycin-penicillin 1 mg/mL/1000 IU/mL were investigated. It was observed that the cryopreservation process resulted in a significant ($P < 0.05$) decrease in bacterial load, regardless of antibiotics. Post-thaw kinetic parameters were not affected by the absence or presence of different antibiotics, except for the cross-beating frequency which was significantly ($P < 0.05$) impaired by streptomycin-penicillin supplementation (31.7 ± 1.0) in comparison with the group without antibiotics (35.6 ± 0.9). After thawing, sperm morphology, membrane functionality and integrity, mitochondrial activity and sperm binding capacity were also unaffected by the presence or absence of antibiotics. It is concluded that although collared peccary semen can be efficiently cryopreserved in the absence of antibiotics in the extender, the use of gentamicin or the streptomycin-penicillin combination is recommended as an effective antibiotic supplementation for greater control of bacterial loads without affecting sperm parameters. In the second chapter, the effect of supplementing the diluent with detergents such as 0.5% Equex STM® or 0.1%, 0.3% or 0.5% sodium dodecyl sulfate (SDS) was verified. It was found that treatments without SDS (41.8 ± 3.5%) and those containing Equex (41.8 ± 4.4%) or 0.1% SDS (41.2 ± 5.5%) provided greater sperm motility ($P < 0.05$) compared to those containing SDS 0.3% (30.5 ± 4.7%) and 0.5% (31.2 ± 6.3%). Immediately after thawing, only the group containing 0.1% SDS effectively preserved the sperm progression index (STR) when compared to the group without detergent. Furthermore, 0.5% SDS impaired membrane functionality and mitochondrial activity after thawing ($P < 0.05$). Thus, it is suggested that the addition of 0.1% SDS to the extender optimizes the cryopreservation of collared peccary semen. In the third chapter, the diluent was supplemented with antioxidant agents such as catalase (CAT) at 200 or 400 IU/mL or superoxide dismutase (SOD) at 150 or 300 IU/mL), or their combination (CAT-SOD: 200 IU/mL + 150 IU/mL). However, no significant effects of supplementation with antioxidants were observed on any of the evaluated sperm parameters, and not even in relation to intracellular oxidative stress. Thus, the addition of SOD or CAT, at the concentrations tested, does not promote benefits to the cryopreservation of collared peccary semen. Finally, the last chapter shows a retrospective analysis of sperm parameters in collared collared peccary ejaculates with varied freezability. Initially, animals were classified as good (> 40% motile sperm), moderate (30 to 40% motile sperm) and poor (< 30% motile sperm) freezers, based on post-thaw sperm motility determined by a computerized analysis system. In the fresh semen of these animals, only the membrane integrity analysis showed some predictive value regarding the semen freezability, since the bad freezers (77.3 ± 1.7%) had a significantly lower proportion ($P < 0.05$) of intact membranes than animals classified as moderate (86.4 ± 2.1%) and good (84.7 ± 3.1%) freezers.

Keywords: Biobank; Wild life; Germplasm; Tayassuid.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

%	: Porcentagem
=	: Igual
±	: Mais ou menos
°C	: Graus Celsius
µL	: Microlitro
ACP	: água de coco em pó
ALH	: *amplitude of lateral head*
BCF	: *beat cross frequency*
CAT	: catalase
dL	: Decilitro
g	: Grama
GPx	: glutationa peroxidase
L	: Litro
LDL	: Lipoproteínas de baixa densidade
LIN	: *linearity*
LVC	: Low velocity average pathway cutoff
Mg	: Miligrama
µg	: Micrograma
mL	: Mililitro
mmol	: Milimol
MVC	: Medium velocity average pathway cutoff
SDS	: Dodecil sulfato de sódio
SOD	: Superóxido dismutase
STR	: *Straightness*
UFC	: Unidades formadoras de colônias
UI	: Unidade Internacional
VAP	: *Velocity average pathway*
VCL	: *Curvilinear velocity*
VSL	: *Velocity straight line*

SUMÁRIO

1. INTRODUÇÃO

O cateto é um taiassuídeo silvestre, presente nos mais diversos habitats e caracterizado por sua importância ecológica e seu valioso potencial produtivo (Garcia et al., 2015). Sua população encontra-se atualmente estável quanto ao risco de extinção, em termos mundiais. No entanto, em virtude da fragmentação de seu habitat e da caça predatória, sua população vem se extinguindo em alguns territórios como nas regiões leste e sul da Argentina (Gongora et al., 2011). Dada a importância da espécie e sua fácil adaptação ao ambiente de cativeiro, estas vêm sendo criadas com o propósito científico em pesquisas conservacionistas, visando o seu uso sustentável (Garcia et al., 2015). Assim, tecnologias desenvolvidas para os catetos poderiam servir como protótipos para espécies filogeneticamente próximas como o *Tayassu pecari* (Keuroghlian et al., 2013) e *Catagonus wagneri* (Altrichter et al., 2015), que têm suas populações drasticamente reduzidas.

Nesse sentido, fica evidente o impacto positivo que o desenvolvimento de biotecnologias reprodutivas poderia acarretar à conservação e multiplicação da espécie. Para este propósito, a criopreservação de germoplasma masculino, em especial de sêmen, representa uma ferramenta importante para a formação de bancos genéticos de espécies silvestres (Castelo et al., 2010a), podendo o material manter-se conservado ao longo de anos a baixas temperaturas (Pegg, 2007). Dessa forma, desde 2010 quando foi relatado o primeiro trabalho com criopreservação de sêmen em catetos (Castelo et al., 2010a), diversos estudos têm sido conduzidos no intuito de aprimorar a composição do atual diluente de congelação. Até hoje, já foram definidas a fonte energética do meio diluente (Castelo et al., 2010a), as curvas de congelação/descongelação (Castelo et al., 2010a; Silva et al., 2013), os crioprotetores (Alves et al., 2013; Souza et al., 2015; Souza et al., 2016) e até mesmo os antimicrobianos necessários durante a refrigeração (Santos et al., 2021).

Portanto, estando definido um meio base capaz de promover a conservação espermática após a descongelação, o próximo passo para a otimização do meio diluente seria a busca por aditivos que promovam o acréscimo à viabilidade espermática pós-descongelação. Neste sentido, em razão dos riscos de contaminação obtidos durante o processamento do sêmen, e da possibilidade de danos morfológicos (Diemer et al., 2000)

e funcionais às células (Villegas et al., 2005), a incorporação de agentes antimicrobianos torna-se uma possibilidade de controle da população bacteriana no sêmen, impedindo possíveis efeitos negativos às células espermáticas (Prieto-Martínez et al., 2014).

Vale destacar que um dos desafios para o aprimoramento do protocolo de congelação do sêmen de catetos está na baixa taxa de sobrevivência espermática após a descongelação. Embora já tenha sido relatado que a incorporação do detergente Equex STM (Nova Chemical Sales, Scituate Inc., MA, EUA) promoveria um leve aumento na longevidade espermática de catetos (30 minutos) (Bezerra et al., 2019), os efeitos do detergente sobre os demais parâmetros funcionais ainda precisam ser entendidos. Ressalta-se ainda, que por se tratar de um detergente comercial, sua formulação não é divulgada, sabendo apenas que seu princípio ativo é o dodecil sulfato de sódio (SDS), fazendo-se interessante que seja avaliado o efeito do SDS de forma isolada sobre os parâmetros seminais da espécie.

Por fim, o meio diluente contendo Tris-gema, associado ao processo de criopreservação, podem levar à formação de peróxido de hidrogênio (H_2O_2), que é altamente nocivo às células, ocasionando a redução na cinética espermática (Bilodeua et al., 2002). Em condições fisiológicas, os EROs são indispensáveis para diversos eventos reprodutivos (O'Flaherty et al., 2006; Aitken et al., 2017), no entanto, em níveis acima do necessário, ocasionam efeitos negativos à membrana celular, motilidade espermática (Griveau; Lelannou, 1997), ao DNA e mitocôndrias (Aitken; Marshall, 2002). Apesar de existirem mecanismos fisiológicos presentes no plasma seminal capazes de regular o excesso da produção de EROs, durante a criopreservação, tais mecanismos fisiológicos não costumam ser suficientemente eficientes para prevenir o dano espermático, sendo importante a suplementação do meio diluente com antioxidantes, permitindo a manutenção nos níveis de EROs no meio e mantendo a qualidade do sêmen após a descongelação (Halliwell; Gutteridge, 1999).

Embora a busca por aditivos que aprimorem o meio diluente de congelação seja uma perspectiva para aumentar a viabilidade das células espermáticas descongeladas de catetos, sabe-se que podem existir fatores ligados ao plasma seminal que contribuem para a congelabilidade do sêmen (Moreira et al., 2019), observados em espécies domésticas como como touros (Gomes et al., 2020), javalis (Uribe et al., 2016), cavalos (Bubenickova

et al., 2020), e búfalos (Ivanova et al., 2019). Além disso, existem animais com melhor congelabilidade em relação a outros, por razões ligadas à morfologia espermática (Esteso et al., 2006) ou envolvidas com a espermatogênese (Rajak et al., 2016), levando a classifica-los em bons ou maus congeladores. Em catetos, porém, não existem ainda informações sobre essa abordagem, sendo interessante a sua investigação.

Desta forma, este trabalho objetivou aprimorar o meio diluente de congelação do sêmen de catetos a partir da adição de antimicrobianos, detergentes e antioxidantes, bem como compreender os principais parâmetros espermáticos envolvidos com a congelabilidade do sêmen da espécie. Para melhor subsidiar a compreensão do assunto, segue-se uma revisão de literatura a respeito destas temáticas.

2. REVISÃO DE LITERATURA

2.1 Conservação do germoplasma masculino de catetos

A primeira tentativa de salvaguardar o material genético masculino da espécie foi efetuada por Castelo et al. (2010a). Estes mostraram ser possível a congelação do sêmen em diluente Tris-gema acrescido de glicose ou frutose, possibilitando a conservação em torno de 30% de células móveis e 40% de células viáveis e com membrana íntegra. Posteriormente, no mesmo ano, Castelo et al. (2010b) definiram a taxa de descongelação do sêmen da espécie para 37° C durante 1 min. Com essa curva, foi obtida uma média de 37,9 % de células móveis, com 47,4% de células viáveis e 41,7% de células com membrana íntegra, quando utilizado o diluente suplementado com frutose e 28,5% de células móveis, com 52,3% de células viáveis e 38,4% de células com membrana íntegra, quando utilizada suplementação com glicose.

Em 2012, Silva et al. investigaram diferentes concentrações de gema de ovo (10% e 20%) e glicerol (1,5% e 3%) ao diluente água de coco em pó (ACP-116c; ACP Biotecnologia, Fortaleza, Brasil). Foram constatadas as concentrações 20% de gema e 3% de glicerol como as mais eficientes para conservar os parâmetros espermáticos de catetos, obtendo-se uma média de 48,3 % de células móveis, com vigor de 2,8, 45,3 % de células viáveis, 59,9 % com membranas funcionais, 25,8 % com membranas íntegras e 64,8 % de células morfologicamente normais. No ano seguinte, Alves et al. (2013) investigaram diferentes concentrações de gema de ovo (5%, 10% e 20%) e glicerol (3% e 6%), desta vez, adicionados ao diluente Tris. Assim, Tris adicionado de 20% de gema e 3% de glicerol foi capaz de manter 50,5% de espermatozoides móveis, com vigor 3, dos quais 67,8% eram morfologicamente normais, 98,5% com membranas intactas, 47,8% viáveis e 44,2% com membranas funcionais, após a descongelação.

No mesmo ano, Silva et al. (2013) avaliaram os efeitos das curvas de congelação (lenta: -10° C/min; rápida: -40° C/min) e descongelação (37° C/1 min e 70° C/8 s), assim como dos volumes das palhetas (0,25 mL e 0,5 mL) sobre a eficiência da congelabilidade das amostras de sêmen. A curva de congelação rápida (-40° C/min) associada à taxa de descongelação 37° C/1 min, obteve 42,1% de espermatozoides móveis, com 35,6% de células viáveis, 29,8% com membranas funcionais e 58,1% de células morfologicamente normais, independente do volume de palheta utilizado para o armazenamento.

Apesar de todos os trabalhos já desenvolvidos até aquele ano, ainda havia uma preocupação pela busca de crioprotetores que pudessem ser utilizados em substituição a gema de ovo, visto os possíveis riscos com contaminação bacteriana que os crioprotetores de origem animal poderiam ocasionar às amostras de sêmen (Iaffaldano et al.,2014). Desta forma, em 2015, Souza et al. estudaram os efeitos da adição de diferentes concentrações de lipoproteínas de baixa densidade (LDL; 5%, 10% e 20%) ao diluente Tris, em substituição a gema de ovo (20%), uma vez que essa lipoproteína consiste no principal componente da gema (Cook; Martin, 1969). Desta forma, as LDL a 20%, mostraram-se superiores à gema na mesma concentração (20%), sendo capazes de manter 36,4% de espermatozoides móveis e 27,4% com membranas intactas após a descongelação, enquanto a gema de ovo (20%) manteve apenas 21,8% de células móveis e 8,4% de células com membranas íntegras.

No ano seguinte, ainda se buscava um crioprotetor alternativo à gema de ovo. Desta vez, foi testado um crioprotetor de origem vegetal. Souza et al. (2016) analisaram os efeitos do extrato de *Aloe vera* (5%, 10% e 20%) junto ao Tris, em comparação à gema de ovo (20%), durante a congelação do sêmen. Desse modo, a adição do extrato de *Aloe vera* a 20% resultou em 46,4% de espermatozoides móveis, dos quais 66,9% eram morfologicamente normais, 23,3% viáveis, 31,4% com membranas funcionais e 27% com membranas intactas.

Apesar de estabelecido o meio base para a congelação de sêmen de catetos, a criopreservação ainda se deparava com um grande obstáculo, a baixa sobrevivência espermática (Campos et al., 2014). Campos et al. (2014) observaram que aos 15 minutos após a descongelação, as células espermáticas já apresentavam uma redução considerável em sua motilidade espermática, tanto na presença do diluente Tris (T0: 43,8 ± 8%; T15: 25 ± 6,5%) quanto do ACP (T0: 43,8 ± 7,7%; T15: 29,4 ± 8,5%). O Tris manteve as células viáveis até os 45 minutos após a descongelação (T0: 29,4 ± 8,5%; T45: 18,8 ± 5,4%) enquanto o ACP já apresentou uma queda significativa aos 30 minutos (T0: 30,6 ± 7,6%; T30: 26,6 ± 4,9%). Além disso, o diluente Tris manteve as células íntegras até os 30 minutos de descongelação (T0: 38,8 ± 6,9%; T30: 21,4 ± 5,1%), diferente do ACP que aos 15 minutos já apresentavam danos consideráveis em sua estrutura (T0: 36 ± 4,8%; T15: 24,6 ± 4,3).

Desta forma, com o propósito de suprimir essa carência, Bezerra et al. (2019) investigaram os efeitos da adição do detergente Equex STM® (0,5% e 1,0%) ao diluente Tris-gema (20%), sobre a longevidade espermática. Desta forma, a incorporação do detergente a 0,5% ao meio diluente possibilitou 25,5% de espermatozoides móveis aos 30 minutos após a descongelação, mantendo a membrana plasmática intacta até os 15 minutos (29,4%), assim como os parâmetros cinéticos VAP (37,9 µm/s), VSL (26,1 µm/s), VCL (88,7 µm/s) e STR (64,7%) até 30 min após a descongelação. No entanto, por se tratar de um detergente comercial, sua formulação não é conhecida, embora saiba que sua base consista no princípio ativo dodecil sulfato de sódio (SDS) e que sua concentração presente também não é conhecida. Além disso, por ser um gel extremamente viscoso, sua manipulação torna-se difícil, não sendo possível garantir com precisão o volume que é incorporado ao diluente. Por essas razões, é interessante que haja uma investigação sobre os efeitos do SDS, isolado, sobre os parâmetros espermáticos descongelados de catetos.

Recentemente, Santos et al. (2021) preocupados com os riscos que a contaminação bacteriana poderia acarretar às amostras de sêmen, suplementaram o meio diluente Tris com os antimicrobianos: estreptomicina/penicilina (1 mg/mL - 1.000 UI/mL ou 2 mg/mL -2.000 UI/mL) e gentamicina (30 µg/mL ou 70 µg/mL) durante a refrigeração a 15 º C, ao longo de 36 horas. A incorporação dos antimicrobianos permitiu o controle da carga bacteriana no sêmen, bem como, manter a integridade da membrana e a atividade mitocondrial por até 36 h. A associação de estreptomicina/penicilina a 2 mg/mL - 2.000 UI/mL prejudicou a integridade da membrana espermática (51,1%) às 36 h de armazenamento, quando comparada à gentamicina tanto a 30 µg/mL (60%) como a 70 µg/mL (58,3%). Quanto aos parâmetros cinéticos, a estreptomicina/penicilina (1 mg/mL - 1.000 UI/mL) e a gentamicina (70 µg/mL) mantiveram a motilidade progressiva durante a refrigeração, declinando em 36 h. O parâmetro VCL foi mantido até 36 h nos grupos contendo estreptomicina-penicilina a 2 mg/mL - 2.000 UI/mL (61,7 µm/s) e gentamicina a 30 ou 70 µg/mL (80,6 µm/s; 76,7 µm/s). Além disso, a gentamicina a 70 µg/mL foi capaz de manter o número de espermatozoides com motilidade rápida até 24 h (30%), enquanto os outros grupos diminuíam já às 12 h. Apesar dos efeitos positivos dos antimicrobianos ao meio diluente durante a refrigeração do sêmen de catetos, ainda não

se sabe se durante o armazenamento a longo prazo (congelação) esses efeitos permanecem.

2.2 Adição de antimicrobianos ao meio diluente de sêmen

Segundo Bianchi et al. (2006), as etapas críticas para a manutenção da qualidade do sêmen, em termos bacteriológicos, compreendem o modo de obtenção dos ejaculados, a manipulação e a sua conservação. À vista disso, a contaminação microbiana pode acarretar perdas econômicas significativas aos centros de inseminação artificial, bem como aos animais, através da propagação de doenças e infecções por patógenos, levando à perda da qualidade dos ejaculados e podendo contribuir para a infertilidade dos animais (Maes et al., 2008).

Dentre as origens da contaminação microbiológica no sêmen, destaca-se a de origem animal, que provêm de infecções que acometem o animal, e a de origem não animal, consistindo nas etapas que envolvem a coleta e o processamento do sêmen (Maes et al., 2008). Adicionalmente, o processo de congelação envolve etapas que podem acarretar a propagação de patógenos às amostras, como a adição de substâncias de origem animal ao meio diluente, a exemplo do crioprotetor externo gema de ovo, e o armazenamento em nitrogênio líquido, podendo, esse último, atuar como um potencial contaminante às amostras mediante a conservação dos patógenos a baixas temperaturas (Mazurova; Krpatova, 1990; Fountain et al., 1997).

Em suínos, espécie filogeneticamente próxima aos catetos, os ejaculados podem conter de 10^3 a 10^5 unidades formadoras de colônias (UFC) por mL (Althouse; Lu, 2005; Schulze et al., 2015), dentre as bactérias, *Escherichia coli* (79%), *Proteus* sp. e *Serratia* spp. (36%), *Enterobacter* spp. (29%), *Klebsiella* spp. (14%), *Staphylococcus* spp. (12%), *Streptococcus* spp. (9%), *Pseudomonas* spp. (8%) e bactérias anaeróbias (1%), em sua maioria gram-negativas (Maroto Martin et al., 2010).

Nesse aspecto, segundo Gall et al. (1998), há indícios de que os microrganismos presentes nos ejaculados suínos não provém de seus órgãos reprodutivos. Assim, são apontados como principais veículos de contaminação do sêmen suíno os procedimentos de coleta e processamento do sêmen, além da presença de longos pelos na região prepucial

(Goldeberg et al., 2013; Schulze et al., 2015; Kuster; Althouse, 2015), que podem resultar em perdas significativas da qualidade seminal.

Dessa forma, Maroto Martin et al. (2010) observaram a incidência de aglutinação espermática em amostras de sêmen suíno contaminadas com *Escherichia coli* (acima de 2,09 x 10^3 UFC/mL), resultando negativamente no tamanho da ninhada (3,5 x 10^3 UFC/mL). Em amostras diluídas, a presença de *Escherichia coli* (10^8 UFC/mL^{-1}) mostrou efeito negativo sobre a motilidade e viabilidade celular a 37º C (até 96 h da inoculação) e 15º C (após 24- 48 h da inoculação), possivelmente em consequência da adesão das bactérias às membranas espermáticas (Bussaleu et al., 2011). Efeitos semelhantes foram encontrados em sêmen armazenado (15 – 17º C) na presença de *Pseudomonas aeruginosa* (2 x 10^7 e 2 x 10^8 UFC/mL), causando prejuízos à motilidade total e progressiva, a viabilidade espermática e a integridade acrossomal (Sepúlveda et al., 2014). Por outro lado, a contaminação por *Enterobacter cloacae* (1:5 e 1:10) afetou a viabilidade espermática, integridade do acrossoma, funcionalidade da membrana e a motilidade, causando aglutinação espermática e alteração do pH, durante armazenamento a 17º C (Prieto-Martínez et al., 2014).

Logo, existem fatores que podem contribuir para o grau de contaminação bacteriana no sêmen, entre eles a eficiência do antimicrobiano presente no meio diluente, o diluente utilizado, a temperatura de armazenamento, o tempo de exposição, os gêneros bacterianos, assim como a interação destes (Vyt et al., 2004; Althouse et al., 2008; Kuster; Althouse, 2015). Diante dessa problemática, os cuidados adequados durante os procedimentos de coleta e processamento do sêmen, através do uso de materiais e equipamentos previamente esterilizados, podem amenizar os riscos de contaminação dos ejaculados (Maes et al., 2008). Atrelado a esses cuidados, o uso de agentes antimicrobianos no meio diluente possibilita amenizar os prejuízos que os microrganismos poderiam acarretar à qualidade seminal (Maes et al., 2008).

No tocante aos animais silvestres, especialmente os catetos, os trabalhos ainda são insuficientes. Em Belém/PA a análise microbiológica dos ejaculados de catetos revelou a incidência de bactérias dos gêneros: *Streptococcus* sp. (35,6%), *Staphylococcus* sp. (32%), *Micrococcus* sp. (28,4%), *Corynebacterium* sp. (2,2%) e *Enterococcus* sp. (1,8%), no entanto, sem nenhum efeito sobre os aspectos reprodutivos (Bartha, 2009).

Em região semiárida, Santos et al. (2020) constataram a presença de *Corynebacterium* sp. e *Staphylococcus* sp. tanto na região prepucial (60,60%; 24,25%, respectivamente) quanto nos ejaculados (64,10%; 20,51%, respectivamente). Além disso, a presença de *Arcanobacterium* sp. foi identificada apenas em amostras de sêmen (5,13%) enquanto *Rhodococcus* sp. e *Microbacterium* sp. estiveram presentes apenas na região prepucial (3,03%). Embora não tenha sido observada correlações entre os parâmetros espermáticos e a carga bacteriana total, observou-se que a incidência de *Corynebacterium* sp. no sêmen estava envolvida negativamente com a integridade da membrana espermática e a velocidade curvilínea linear (VCL). Haja vista a presença desses microrganismos no sêmen da espécie, justifica-se a necessidade de incorporação de agentes antimicrobianos capazes de controlar a sua propagação, a qual poderia resultar em danos funcionais às células espermáticas, ou mesmo possibilitar a transmissão de doenças às fêmeas inseminadas.

De acordo com a definição, antibióticos são substâncias produzidas por um microrganismo que, em pequenas concentrações, podem inibir outros microrganismos, enquanto antimicrobianos são fármacos totalmente sintéticos (Tortora et al., 2017). Segundo as prerrogativas da Diretiva do Conselho das Comunidades Europeias (90/429/CEE), no anexo C2, que instaura as condições sanitárias do sêmen suíno e suas trocas intracomunitárias, após a diluição, devem ser adicionados antimicrobianos eficientes, em especial contra as leptospiras e os micoplasmas. Tal combinação deve apresentar efeito semelhante às diluições de 500 UI/ mL de estreptomicina, 500 UI/ mL de penicilina, 150 μg/ mL de lincomicina, 300 μg/ mL de espectinomicina.

À vista disso, a presença de antimicrobianos em diluente de sêmen devem ser capazes de controlar ou eliminar o crescimento bacteriano sem prejudicar a funcionalidade e a viabilidade dos espermatozoides armazenados, sendo indispensável a seleção adequada dessas substâncias, bem como de suas doses (Brinsko et al., 2011), uma vez que o uso indiscriminado dessas substâncias pode levar à resistência bacteriana (Morrell; Wallgren, 2011). Deste modo, destacam-se entre os antimicrobianos mais utilizados em diluentes de sêmen a penicilina, estreptomicina e gentamicina (Câmara et al., 2018), cujo uso já foi reportado em suínos (Toniolli et al., 2001), ovinos (Yániz et al., 2010), bovinos (Miraglia et al., 2003), e até mesmo em catetos (Santos et al., 2021).

2.2.1 Penicilina e estreptomicina

A penicilina foi o primeiro antibiótico a ser descoberto, constituída por um anel β-lactâmico central (Tortora et al., 2017). Seu mecanismo de ação está relacionado com a inibição da síntese da parede celular de bactérias gram-positivas. Uma vez que a parede celular das bactérias é constituída por peptidioglicanos (Tortora et al., 2017), a penicilina ao agir sobre sua síntese, torna a parede celular frágil e propícia à lise celular. Desta forma, a penicilina atua apenas sobre as células em crescimento, ou seja, nos estágios finais da formação da parede celular, impedindo a ligação cruzada de peptideoglicanos (Tortora et al., 2017). Em virtude de as células dos animais não apresentarem parede celular constituída por peptideoglicanos, sua toxicidade é reduzida nesses tipos celulares (Tortora et al., 2017).

Alguns trabalhos relatam o uso associado da penicilina à estreptomicina. Este antimicrobiano possui um núcleo de estreptamina (Shi et al., 2013), configurando-se como um aminoglicosídeo, com ação sobre as bactérias gram-negativas. A estreptomicina atua no interior da célula bacteriana a partir da ligação ao ribossomo, interferindo na síntese proteica (Oliveira et al., 2006) a partir de alterações na configuração da porção 30S do ribossomo, tornando a leitura do mRNA incorreta (Tortora et al., 2017). Desta forma, o uso combinando da penicilina a estreptomicina garante uma ação mais eficiente sobre a bactéria, ao passo que a lesão ocasionada, pela penicilina, à parede celular, favorece a introdução da estreptomicina na célula (Tortora et al., 2017).

Em suínos, a associação dos antimicrobianos (1.000 UI/L + 1 g/L), não controlou a população microbiana nos ejaculados (Toniolli et al., 2001). Além disso, a combinação ($8,4 \times 10^5$ UFC/mL), após 8 dias de armazenamento, resultou em uma contaminação 2,5 vezes maior que aquelas amostras contendo gentamicina ($3,3 \times 10^5$ UFC/ mL). Por sua vez, em touros, a combinação dos antimicrobianos foi eficiente para o controle das leptospiras (97,1%) (Miraglia et al., 2003). Em catetos, o uso associado dos antimicrobianos (1 mg/mL-1.000 UI/mL e 2 mg/mL-2.000 UI/mL) em meio diluente Tris-gema, possibilitou o controle da carga bacteriana ao longo de 36 h de armazenamento a 15º C, no entanto, a maior concentração prejudicou a integridade da membrana espermática (Santos et al., 2021).

Por outro lado, em ovinos, das bactérias presentes nos ejaculados (*Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Staphylococcus epidermis* e *Staphylococcus aureus*), 53% mostraram-se resistentes à penicilina (Yániz et al., 2010). Por outro lado, 15% das bactérias presentes (*Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Staphylococcus epidermis* e *Staphylococcus aureus*), mostraram-se resistentes à estreptomicina (Yániz et al., 2010), enquanto as bactérias *Staphylococcus* sp. e *Klebsiella* sp. se mostraram resistentes quando o antibiótico foi combinado com a penicilina (Madeira et al., 2014).

Por tanto, o uso de antimicrobianos no meio diluente de sêmen torna-se relevante por possibilitar o controle bacteriano, e consequentemente, permitir uma maior qualidade seminal, uma vez que diferentes estudos comprovarem efeitos negativos de diferentes bactérias sobre os parâmetros espermáticos (Maroto-Martin et al., 2010; Bussaleu et al., 2011; Prieto-Martínez et al., 2014; Sepúlveda et al., 2014). Além disso, de acordo com Ochsendorf (1999), infecções causadas por bactérias podem estar envolvidas com o aumento na produção de espécies reativas de oxigênio (EROs) no sêmen, que quando em níveis acima do necessário, tornam-se maléficas às células (Gavriliouk; Aitken, 2015; Aitken et al., 2016).

2.2.2 Gentamicina

A gentamicina é considerada o antimicrobiano mais comumente utilizado nos meios diluentes de sêmen suíno (Althouse; Lu, 2005). Assim como a estreptomicina, é um aminoglicosídeo, atuando inibindo a síntese proteica, com amplo espectro de ação. Seu mecanismo de ação ocorre após sua interação com a superfície celular, entrada no interior da célula bacteriana e ligação ao ribossomo (30S) (Althouse; Lu, 2005).

Desta forma, os aminoglicosídeos se ligam à parede celular em estruturas com cargas negativas, deslocando Ca^{2+} e Mg^{2+}, que são responsáveis por manter as células unidas. Consequentemente, são formados “buracos” na parede celular, seguida de alteração de sua permeabilidade. Quando os aminoglicosídeos atingem os ribossomos, estes agem sobre a síntese proteica, levando a erros de leitura do mRNA, causando o mau funcionamento da membrana celular e posterior morte, mediante saída de constituintes essenciais à sobrevivência da célula. Em virtude de sua entrada na célula, esse

antimicrobiano desenvolve toxidade à célula, no qual sua atividade antimicrobiana é melhor apresentada em meio aeróbio e pH alcalino, uma vez que é necessário oxigênio para que ocorra o transporte ativo nas células microbianas (Oliveira et al., 2006), agindo contra bactérias gram-negativas (Althouse; Lu, 2005).

Em suínos, a gentamicina mostrou excelentes resultados sob o controle da contaminação, durante armazenamento do sêmen a 17º C, reduzindo a população de 4,2 x 10^5 UFC/mL (dia 1) para 3,3 x 10^5 UFC/mL (dia 8) (Toniolli et al., 2001). Em ovinos, todas as bactérias encontradas nos ejaculados (*Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Staphylococcus epidermis* e *Staphylococcus aureus*) mostraram sensibilidade ao antimicrobiano gentamicina (Yániz et al., 2010). Entretanto, alguns gêneros bacterianos, comumente encontrados no sêmen suíno, demonstram resistência ao aminoglicosídeo gentamicina (Althouse et al., 2000). Além disso, alguns trabalhos relatam ação negativa da gentamicina sobre a motilidade e viabilidade espermática (Aurich; Spergser, 2007), bem como a ocorrência da liberação de endotoxinas tóxicas aos espermatozoides, como os lipopolissacarídeos (LPS), pelas bactérias gram-negativas durante a bacteriólise (Okazaki et al., 2010).

Em catetos, assim como a associação de estreptomicina e penicilina, a gentamicina (30 µg/mL ou 70 µg/mL) em meio diluente Tris-gema, também demonstrou efeito positivo sobre o controle da carga bacteriana, no decorrer de 36 h de armazenamento (15º C). Além disso, a gentamicina (70 µg/mL) permitiu uma preservação mais eficiente dos parâmetros cinéticos, integridade e atividade mitocondrial das amostras resfriadas (Santos et al., 2021). Embora seja evidente o impacto da adição de antimicrobianos ao meio diluente seminal de catetos durante a refrigeração, ainda não são conhecidos os impactos desses antimicrobianos durante a congelação.

2.3 Adição de detergentes ao meio diluente

Embora o protocolo de congelação em catetos esteja em constante desenvolvimento, com um meio base definido, a adaptação do protocolo de congelação na espécie apresenta como um dos principais desafios a sobrevivência dos espermatozoides após a descongelação (Campos et al., 2014).

Neste sentido, a incorporação de detergentes ao meio de congelação representa uma estratégia para aumentar a longevidade espermática após o processo de congelação-descongelação (Costa et al., 2013), uma vez que em meios diluentes contendo gema de ovo, a incorporação de detergentes possibilita a solubilização dos fosfolipídios e a permeabilidade da membrana, promovendo uma melhor conservação da integridade da membrana e motilidade das células submetidas ao processo (Maia et al., 2008).

Assim, pretendendo minimizar esse problema, Bezerra et al. (2019) suplementaram o diluente Tris com 0,5% do detergente Equex STM® (Nova Chemical, Scituate), obtendo células viáveis por até 30 minutos após a descongelação do sêmen de catetos. Por se tratar de um detergente comercial, sua fórmula não está ao alcance da sociedade, e por tanto, seus componentes não são conhecidos. Embora saiba-se que o principal responsável por sua atividade seja o dodecil sulfato de sódio (SDS), sua quantidade presente é desconhecida, havendo a necessidade que se estude a ação do SDS isoladamente sobre os parâmetros funcionais espermáticos da espécie.

Em termos funcionais, o SDS é um detergente aniônico, solúvel em água e capaz de solubilizar proteínas. Seu efeito protetor às membranas espermáticas deve-se a sua capacidade de interagir com a gema de ovo presente no diluente, liberando os seus fosfolipídios e facilitando a sua disponibilidade e interação com a membrana celular, protegendo assim as células de prováveis danos resultantes do processo de congelação (Rota et al., 1997).

Em suínos, o detergente Pasta Orvus Es (0,5%, 1% e 1,5%) proporcionou maiores porcentagens de espermatozoides móveis e com acrossoma normal (NAR), com efeitos dose-dependentes. Além disso, o número de oócitos fertilizados por espermatozoides submetidos à congelação com 0% (43 zigotos) ou 0,5% (81 zigotos) do detergente foi significativamente afetado (Pursel, Schulman e Johson, 1978). O detergente (0,5%) proporcionou ainda a conservação da integridade da membrana plasmática após a descongelação (Fraser et al., 2014). Ainda em suínos, o Equex STM® também mostrou bons resultados com a manutenção dos parâmetros espermáticos descongelados. O detergente (0,5% e 1,5%) incorporado ao diluente seminal possibilitou maior conservação da motilidade, viabilidade e acrossoma normal (NAR) após a descongelação do sêmen (Buranaamnuay1 et al., 2009; Wu et al., 2013).

Poucos trabalhos relatam o uso isolado do SDS em diluente seminal. Em cães, a adição de SDS (0,1 e 0,2%) ao diluente Tris-gema, não mostrou efeito sobre a qualidade seminal após a descongelação (Costa et al., 2013). Por outro lado, em camundongos, o SDS (0,0035% e 0,05%) em diluente contendo gema de ovo, foi capaz de conservar tanto a motilidade espermática como a sua capacidade fecundante após a congelação (Dewit et al., 2000).

Em ovinos, a incorporação de até 0,1% de SDS em meio diluente contendo Tris-gema promoveu maior conservação dos parâmetros cinéticos e integridade acrossomal. Acima dessa concentração, o SDS passou a exercer danos aos parâmetros espermáticos, possivelmente em razão das moléculas livres de SDS se ligarem diretamente às membranas espermáticas, ocasionando efeitos negativos (Aboagla; Terada, 2004).

Recentemente, também foi relatado em suínos, a incorporação de SDS isolado (0,025 %; 0,05 %; 0,1 %; 0,2 % e 0,4 %) em diluente contendo gema de ovo. O SDS aumentou significativamente a viabilidade e motilidade espermática. A viabilidade aumentou significativamente na presença de SDS a 0,1 (21,6% ± 0,75) e 0,2% (30,1% ± 2,15), após a descongelação. No entanto, a 0,4% houve uma redução (16,6% ± 1,0). A motilidade do sêmen seguiu o mesmo padrão, com as maiores motilidades adquiras com 0,1 % a 0,4 % de SDS (Silva et al., 2021). Tendo a espécie como a mais próxima filogeneticamente aos catetos e em razão dos excelentes resultados encontrados na espécie, este trabalho foi utilizado como suporte para a escolha das concentrações utilizadas em catetos, que será apresentado no capítulo III.

2.4 Adição de antioxidantes ao meio diluente

Durante o metabolismo espermático, é natural que sejam geradas espécies reativas de oxigênio (EROs) por meio do oxigênio molecular, dentre os quais, os mais conhecidos são: o ânion superóxido (O_2^-), o radical hidroxila (OH) e o peróxido de hidrogênio (H_2O_2). As EROs podem ser categorizadas em radicais livres, quando as moléculas portam de um ou mais elétrons não pareados, e não radicais, quando dois radicais livres partilham seus elétrons desemparelhados (Birben et al., 2012).

Em condições fisiológicas, as EROs atuam nos processos de capacitação e sinalização celular, motilidade (Aitken et al., 2017), maturação espermática, por meio da

oxidação dos grupos tiol localizados na protamina, resultando na conservação da integridade da cromatina (O'Flaherty; Matsushita-Fournier, 2017) e no evento de fusão dos gametas feminino e masculino, por meio do aumento da fluidez da membrana, em especial os EROs O_2 • e H_2O_2 (Aitken, 1997; Griveau; Lannou, 1997).

Contudo, quando os níveis de EROs ultrapassam a capacidade da célula de detê-los, é desencadeado o estresse oxidativo, que ocasiona danos lipídicos, proteicos e ao DNA da célula espermática (Gavriliouk; Aitken, 2015; Aitken et al., 2016), afetando o potencial fecundante dos espermatozoides (Aitken et al., 2014).

Segundo Upreti et al. (1998), em espermatozoides mortos, a atividade de oxidase de aminoácidos aromáticos exibe disponibilidade ou atividade após a morte das células, no qual está intimamente ligada com a produção de EROs (H_2O_2) em espermatozoides de ovinos e touros. Portanto, o aumento na produção de EROS tem sido associado a danos na membrana celular, motilidade espermática (Griveau; Lelannou, 1997), degradação de bases e quebra no DNA (Birben et al., 2012), redução da atividade das mitocôndrias, mediante esgotamento da adenosina trifosfato (ATP) (Zhu et al., 2019) e degradação de proteases específicas que levam à proteólise de proteínas, por intermédio da fragmentação da cadeia peptídica, alteração da carga elétrica das proteínas e oxidação de aminoácidos, resultando em baixa capacidade fecundante das células espermáticas (Kelly; Mudway, 2003).

Durante a criopreservação, podem ocorrer alterações nos constituintes lipídicos de membrana. Visto os espermatozoides possuírem como principais constituintes de membrana os ácidos graxos poli-insaturados, estes se tornam mais suscetíveis à peroxidação lipídica, que pode estar envolvida negativamente com alterações bioquímicas e fisiológicas durante este processo (Cerolini et al., 2000; Watson, 2000). Em touros, Chatterjee e Gagnon (2001) confirmaram o aumento na produção do radical superóxido ($O_2^{\bullet-}$) durante a congelação e descongelação do sêmen, observando um aumento nos níveis de lipoperoxidação, que podem estar relacionados com redução da fluidez da membrana espermática. Acreditam-se que, a produção de EROs durante processo de congelação-descongelação poderia desencadear a capacitação prematura na célula, uma vez que a capacitação está relacionada com a produção de radicais superóxido e H_2O_2 (De Lamirande; Gagnon, 1993; Cormier et al., 1997).

Outro fator a ser considerado, quanto a geração de EROs, é a escolha do diluente seminal. Bilodeua et al. (2002) verificaram que em meio diluente contendo Tris - gema de ovo, o H_2O_2 é o principal encarregado na geração de EROs, refletindo diretamente na redução da motilidade espermática. Apesar das lipoproteínas presentes na gema de ovo possuírem atributos antioxidantes (Yamamoto; Omori, 1994), a presença de ferro e outros metais de transição na gema de ovo podem levar ao aumento nos níveis de H_2O_2, pela reação de Fenton (Abou-Ashour; Edwards, 1970; Bing, 1972; Halliwell; Gutteridge, 1984), uma vez que a presença de metais de transição pode induzir a peroxidação lipídica e a geração de altos níveis de EROs (Halliwell e Gutteridge, 1984; Bilodeua et al., 2002).

Endogenamente, podem existir diferentes antioxidantes presentes no sêmen. Em catetos, foram identificadas as presenças das enzimas superóxido dismutase (SOD) (Santos et al., 2018) e glutationa peroxidase (GPx) no plasma seminal (Santos et al., 2014). No entanto, diante dos inúmeros efeitos negativos que os EROs podem acarretar às células espermáticas, atrelado ao processo de criopreservação, a adição de antioxidantes ao meio diluente transfigura-se como uma estratégia para a manutenção da viabilidade espermática, mediante a prevenção da oxidação do substrato oxidável, convertendo as EROs em água, prevenindo assim, seus altos níveis que são danosos às células (Halliwell; Gutteridge, 1999).

O modo de atuação dos antioxidantes pode ocorrer mediante o impedimento da formação de radicais livres e não radicais, impedindo a ação desses ou ainda, favorecendo a reparação das estruturas biológicas lesionadas (Clarkson; Thompson, 2000; Koury; Donangelo, 2003). Em regra, os antioxidantes são classificados em não enzimáticos e enzimáticos. Dentre os antioxidantes não enzimáticos, compõem a vitamina C, vitamina E, α-tocoferol, β-caroteno, dentre outros. Já os antioxidantes enzimáticos são, a catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx) (Barbosa et al., 2010).

i. Superóxido Dismutase (SOD)

A enzima superóxido dismutase age nas células espermáticas na conversão do radical O_2^- em O_2 e H_2O_2, sendo este último o mais prejudicial às células (Trzcinska; Bryla, 2015). Em catetos, a enzima foi identificada no plasma seminal (0,033 ± 0,049

AU/mgP), no entanto, não mostrou ação sobre os parâmetros espermáticos (Santos et al., 2018).

Diferentes trabalhos relatam efeitos positivos da adição do antioxidante em meios de congelação de sêmen. Em sêmen descongelado de suínos, a enzima SOD (150 ou 300 UI/mL) demonstrou ação positiva sobre a motilidade total e viabilidade espermática, atenuando os níveis de EROs (Roca et al., 2005) e reduzindo a porcentagem de espermatozoides com alterações semelhantes a apoptose (Trzcinska; Bryla, 2015). Já em sêmen de suínos refrigerados (17º C), o SOD (200 UI/mL) proporcionou maior tempo de sobrevivência, integridade de membrana, integridade acrossomal, capacidade antioxidante total e menor conteúdo de malondialdeído (MDA) e H_2O_2 (Zhang et al., 2016).

ii. Catalase (CAT)

A catalase converte H_2O_2 em H_2O e O_2, eliminando a toxicidade potencial de EROs (Aitken, 1997). Em catetos, sua presença fisiológica no plasma seminal não foi identificada (Santos et al., 2018). No que diz respeito ao seu uso em meios de congelação, em suínos, o efeito do H_2O_2 sobre as células espermáticas foi atenuado pela adição da enzima CAT (400 UI/mL) ao meio diluente, proporcionando maior proteção às células espermáticas contra os danos causados pela redução de temperatura, além disso, reduziu a porcentagem de espermatozoides com alterações semelhantes a apoptose (Trzcinska; Bryla, 2015).

Ainda em suínos, o uso da catalase, independente da concentração (200 ou 400 IU/mL), implicou maiores taxas de motilidade e viabilidade espermática (Roca et al., 2005). Além do uso isolado dos antioxidantes, a CAT combinada à SOD independente das concentrações (200 + 150 UI/mL ou 400 + 300 UI/mL) influenciou positivamente os parâmetros cinéticos, integridade acrossomal, taxas de zigotos clivados e de blastocistos, além de refletirem em menores índices de EROs, após a descongelação do sêmen (Roca et al., 2005).

O uso combinado das enzimas SOD e CAT tem como vantagem o fato que elas se complementam quanto aos seus mecanismos de ação, uma vez que a SOD converte o radical O_2^- em O_2 e H_2O_2 que é nocivo às células (Trzcinska; Bryla, 2015), enquanto a CAT suprime a toxicidade do H_2O_2 reduzindo-o em H_2O e O_2 (Aitken, 1995).

2.5 Outros fatores que influenciam na criopreservação

A qualidade do sêmen descongelado, depende da capacidade das células de tolerarem o processo de congelação/descongelação, sem que haja danos em seus parâmetros cinéticos, funcionais e estruturais, uma vez que as células são expostas a condições rigorosas de temperatura (Sieme et al., 2008; Sieme et al., 2015).

Desta forma, além da incorporação de antimicrobianos, detergentes e antioxidantes ao meio diluente, outros fatores podem contribuir para o sucesso da criopreservação, como a escolha dos crioprotetores, da curva de congelação/descongelação, a existência de animais bons e maus congeladores, bem como, a composição bioquímica do plasma seminal, assim como outros fatores que contribuem para que as amostras tenham maior ou menor criotolerância (Hunter, 1982; Rizkallah et al., 2022).

i. Crioprotetores

Os agentes crioprotetores são utilizados com a finalidade de minimizar os danos causados pelo processo de criopreservação (Purdy, 2006). Esses agentes podem ser categorizados, de acordo com seu mecanismo de ação, em intracelulares, como o glicerol e extracelulares, como a gema de ovo (Kundu et al., 2000).

Portanto, a gema de ovo desempenha o papel de restaurar os fosfolipídios da membrana plasmática, que são perdidos ao longo da congelação, em razão do estresse térmico causado pela redução da temperatura (Holt, 2000), enquanto o glicerol, interage com as cabeças polares dos fosfolipídios e diminui o ponto de congelação (Kundu et al., 2000; Watson, 2000), no qual em concentrações elevadas, causa efeitos tóxicos às células (Alvarenga et al., 2000).

Em catetos, as diferentes concentrações de gema de ovo (5%, 10% e 20%) e glicerol (3% e 6%) adicionadas ao diluente Tris não repercutiram em efeitos significativos sobre os parâmetros de motilidade (40%), vigor (3) e viabilidade espermática (40%) após a descongelação. No entanto, a maior porcentagem de espermatozoides normais foi encontrada quando combinados 20% de gema e 3% de glicerol (67,8 %) assim como 10% de gema e 6% de glicerol (67,6 %). Além disso, a maior porcentagem de células íntegras foi encontrada quando utilizada a combinação de 20% de gema e 6% de glicerol (50,2 %)

(Alves et al., 2013). Por sua vez, em suínos, o glicerol a 3% resultou em melhores parâmetros de motilidade (43 ± 2%), viabilidade (45 ± 2%), integridade do acrossoma (44 ± 1%) e morfologia normal (89 ± 1 %), permanecendo móveis e viáveis por até 6 h após a descongelação (Yang et al., 2016).

ii. Curva de congelação/descongelação

Outro fator intimamente relacionado à eficiência do processo de congelação é a escolha da curva de congelação e descongelação. A curva ideal deve ser lenta o bastante para admitir a desidratação dos espermatozoides e rápida o suficiente para impedir a exposição prolongada das células espermáticas às altas concentrações do meio (Snoeck, 2003).

Para tanto, em catetos, amostras diluídas em Tris-gema (20%) e expostas a uma curva de congelação lenta (-10° C/min), foram submetidas a duas curvas de descongelação. A primeira curva consistiu em 37° C/1 min e a segunda em 55° C por 7 s, seguido de mais 30 s a 37° C. No entanto, não foi observado diferenças entre as temperaturas empregadas, ambas as taxas de descongelação foram capazes de manter a funcionalidade das células espermáticas de catetos (Castelo et al., 2010b).

Por outro lado, em amostras diluídas em ACP-116c® acrescido de gema de ovo (20%) foram expostas a duas curvas de congelação, uma lenta (-10° C/min) e uma rápida (- 40 ° C/min), assim como a duas taxas de descongelação (37° C/1 min e 70° C/8 s seguida de mais 30 s a 37° C). As curvas de congelação não diferiram entre si. No entanto, as taxas de descongelação (37° C/1 min e 70° C/8 s) sinalizaram importantes diferenças na qualidade do sêmen descongelado de catetos. A descongelação a 37° C/1 min possibilitou uma melhor conservação dos parâmetros funcionais quando comparados a curva de 70° C/8 s, com o maior impacto sendo observado na cinética espermática, no qual a 37º C foram obtidos até 27,7 % de células móveis enquanto a 70° C esse número caiu drasticamente para até 0,4 % (Silva et al., 2013).

Em suínos a mudança nas condições de descongelação proporcionou melhor qualidade espermática em amostras de animais considerados maus congeladores. Neste caso, o ajuste combinado da concentração do crioprotetor (3% de glicerol) e das condições de congelação e taxa de descongelação (~1800° C/min) permitiram que

amostras consideradas de maus congeladores pudessem ter sua qualidade melhorada (Hernández et al., 2007). Desta forma, a partir de ajustes nas condições de congelação/descongelação, amostras consideradas inviáveis para a reprodução poderiam ter sua qualidade melhorada e assim serem utilizadas em biotécnicas reprodutivas (Hernández et al., 2007).

iii. Bons congeladores x maus congeladores

Atrelado a todos os fatores supracitados, podem existir diferenças intrínsecas entre os animais que contribuem para uma maior ou menor resistência ao processo de congelação. Em suínos, os bons congeladores apresentaram as maiores taxas de penetração oocitária, clivagem e formação de blastocistos em comparação aos maus congeladores (Gil et al., 2005), ao passo que em equinos, os espermatozoides dos maus congeladores se mostraram menos resistentes, após a descongelação (Bubeníčková et al., 2020).

As causas para a heterogeneidade entre as amostras de sêmen de uma mesma espécie ainda não estão totalmente elucidadas, sendo hipotetizado que se deve às características morfológicas dos espermatozoides (Esteso et al., 2006) ou ao processo de espermatogênese (Rajak et al., 2016). Essas informações são úteis para o ajuste no protocolo de congelação, uma vez que sabendo dessa heterogeneidade entre as amostras, é possível traçar estratégias para que essas diferenças sejam equiparadas.

iv. Plasma seminal

O plasma seminal parece ser mais que um simples veículo de transporte espermático, este possui efeito protetor às células, atuando sobre a sensibilidade espermática às alterações de temperatura (Hopkins; Evans, 1991). Esse efeito protetor se deve aos seus inúmeros constituintes bioquímicos (Juyena; Stelletta, 2012), que podem variar entre as diferentes espécies (Caballero et al., 2012; Samanta et al., 2018), raças (Ciereszko et al., 2000), idades (Fraser et al., 2016), indivíduos (Barranco et al., 2015), e estações (Moreira et al., 2019)

Em algumas espécies domésticas como touros (Gomes et al., 2020), suínos (Valência et al., 2020), cavalos (Bubenickova et al., 2020) e búfalos (Ivanova et al., 2019),

determinados componentes do plasma foram apontados como potenciais marcadores da congelabilidade espermática. Do mesmo modo, em catetos, foi observado que a maiores concentrações de frutose (849,2 mg/dL) no plasma seminal, observadas em decorrência do período climático (período chuvoso), está diretamente envolvida com melhores parâmetros cinéticos espermáticos após a descongelação do sêmen (Moreira et al., 2019).

Em suínos, o plasma seminal mostrou efeito protetor durante a criopreservação do sêmen em animais de baixa congelabilidade (Okazaki et al., 2009). Ainda, a congelação do sêmen de animais de baixa congelabilidade, na presença do plasma seminal de animais de boa congelabilidade resultou em melhor qualidade seminal e maior taxa de penetração (Hernández et al., 2007). Um dos motivos para tais efeitos, se deve aos antioxidantes presentes no plasma seminal dos suínos, conferirem às células maior tolerância ao processo de criopreservação (Li et al., 2018). Tais efeitos positivos do plasma seminal na criorresistência também foi verificado em outras espécies como bovinos (Garner et al., 2001), caprinos (Azerêdo et al., 2001), cavalos (Neuhauser et al., 2019) e veados (Martinez-Pastor et al., 2006).

Em razão da importância que a influência dos constituintes bioquímicos presentes no plasma seminal possui para a congelabilidade do sêmen, o assunto originou um artigo que será apresentado no primeiro capítulo desta tese.

3. JUSTIFICATIVA

A congelação de sêmen se configura como principal recurso utilizado para a conservação e multiplicação das espécies silvestres ameaçadas de extinção. Entretanto, durante as etapas de coleta, manipulação e congelação, pode haver contaminação do sêmen por patógenos, em especial bacterianos, favorecida pelo uso de materiais não esterilizados, crioprotetores de origem animal (gema de ovo) ou até mesmo em decorrência do armazenamento em nitrogênio líquido, podendo contribuir para a conservação dos patógenos a baixas temperaturas. Portanto, o uso de antimicrobianos ao meio diluente, durante a congelação, pode colaborar para o controle do crescimento bacteriano nas amostras de sêmen e consequentemente para a qualidade das amostras descongeladas, uma vez que a contaminação bacteriana pode contribuir com efeitos deletérios à funcionalidade da membrana e a cinética espermática.

Correlacionado ao processo de congelação, o sêmen pós- descongelado de catetos se depara com um grande obstáculo, que é a sobrevivência espermática. Anteriormente, ficou claro que a incorporação do detergente comercial Equex STM® (0,5%; Nova Chemical, Scituate) ao diluente, promove um sensível aumento na longevidade (30 min) espermática após a descongelação do sêmen. No entanto, o efeito do detergente em parâmetros relacionados à função espermática, ainda precisa ser esclarecido. Somado à falta de informações sobre a composição exata do detergente comercial, apesar de saber que o princípio ativo é o dodecil sulfato de sódio (SDS), a sua concentração não é divulgada. Logo, existe a necessidade de uma investigação em relação à incorporação do detergente SDS isolado em diluentes para conservação de sêmen de catetos, uma vez que a maior parte dos trabalhos descreve o uso de detergentes comerciais a base do composto.

Adicionalmente, em meio diluente contendo Tris-gema de ovo, são gerados peróxidos de hidrogênio (H_2O_2), que são altamente nocivos às células e considerados os principais responsáveis por causar a formação de espécies reativas de oxigênio (EROs) nos espermatozoides, resultando na redução da cinética espermática. No entanto, sabe-se que fisiologicamente, os espermatozoides produzem EROs durante o seu metabolismo e quando em condições adequadas, são indispensáveis para eventos de hiperativação, capacitação e maturação espermática. Apesar de endogenamente existirem mecanismos antioxidantes capazes de manter os níveis de EROs em condições adequadas às células,

durante o processo de congelação, pode haver mecanismos que propiciam o seu aumento além das condições ideias, podendo desencadear efeitos oxidativos às células. Desta forma, os antioxidantes possibilitariam o equilíbrio dos níveis de EROs em condições dentro da capacidade suportável pelas células, assegurando o armazenamento das amostras por longos períodos em temperatura sub zero, garantindo que as amostras tenham sua qualidade mantida após a descongelação.

Por fim, desde os primeiros trabalhados envolvendo a congelação de sêmen de catetos, foram observadas variações na congelabilidade do sêmen entre os animais. Tais variações são refletidas na qualidade das amostras descongeladas, no entanto esse fator nunca foi investigado. Em outros animais como suínos, bovinos e cervídeos, a existência de animais bons e maus congeladores pode estar ligada a fatores genéticos, metabólicos, espermatogênese, morfologia espermática ou aos componentes do plasma seminal. Neste sentido, a possibilidade de predizer a congelabilidade em catetos, a partir dos parâmetros espermáticos em ejaculados frescos, permitirá aperfeiçoar os bancos de germoplasma da espécie, a partir de coleções de germoplasmas de alta qualidade, com a perspectiva de selecionar os reprodutores de acordo com sua eficiência reprodutiva e consequentemente, contribuir para o sucesso das técnicas de reprodução assistida como a inseminação artificial e a fertilização in vitro.

4. HIPÓTESES

I. A adição de antimicrobianos ao meio diluente, durante a congelação, possibilita o controle da população bacteriana e contribui para a manutenção da qualidade seminal de catetos;

II. A adição de uma concentração adequada do detergente SDS ao meio diluente, contribui para a manutenção dos parâmetros funcionais espermáticos de catetos após a congelação;

III. A combinação dos antioxidantes SOD e CAT ao meio diluente possibilita maior conservação dos parâmetros espermáticos, em comparação ao seu uso isolado, uma vez que um complementa a ação do outro;

IV. A congelabilidade do sêmen de catetos pode ser prevista a partir dos parâmetros seminais em ejaculados frescos.

5. OBJETIVOS

5.1 Objetivo geral

✓ Aprimorar o meio diluente do sêmen de catetos, por meio da adição de antimicrobianos, detergentes e antioxidantes, investigando seus efeitos sobre os parâmetros funcionais espermáticos após a congelação- descongelação do sêmen.

5.2 Objetivos específicos

✓ Investigar a necessidade de suplementação de antibióticos ao diluente para criopreservação de sêmen de cateto, comparando o efeito de amostras sem antibióticos com aquelas suplementadas com penicilina e estreptomicina (1000 UI/mL e 1 mg/mL) e gentamicina (70 µg/mL) na carga bacteriana, nos parâmetros funcionais e morfológicos espermáticos e na capacidade de ligação espermática;

✓ Verificar o efeito da adição do detergente Equex STM® (0,5%) ou de diferentes concentrações de SDS (0%, 0,1%, 0,3% e 0,5%) em vários parâmetros funcionais do sêmen congelado-descongelado de catetos, como parâmetros cinéticos espermáticos, funcionalidade e integridade da membrana, atividade mitocondrial e capacidade de ligação espermática, além de avaliar a longevidade espermática por meio de teste de resistência térmica;

✓ Investigar o efeito da incorporação dos antioxidantes CAT (0, 200 ou 400 UI/mL) e SOD (0, 150 ou 300 UI/mL), em diferentes concentrações, isoladas ou combinadas (SOD/CAT: 200 mL/150 UI/mL), ao diluente para criopreservação de sêmen de cateto e avaliar os parâmetros morfológicos e funcionais espermáticos, sua capacidade de ligação e estresse oxidativo intracelular;

✓ Identificar se os animais classificados como bons (> 40% de espermatozoides móveis), moderados (de 30% a 40% de espermatozoides móveis) ou

congeladores ruins (< 30% de espermatozoides móveis), possuem diferentes características espermáticas que permitam predizer sua congelabilidade contrastante.

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CAPÍTULO I

Impact of Seminal Plasma Composition on Sperm Freezability in Wild Mammals:A Review

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REVIEW ARTICLE

Impact of Seminal Plasma Composition on Sperm Freezability in Wild Mammals: A Review

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This review was designed to summarize the most important information around seminal plasma composition and discuss its impact on the freezability of wild mammal semen samples. Seminal plasma is made up of various biochemical constituents, including ions, lipids, proteins, enzymes, and sugars, which vary between species in response to the presence and size of any relevant accessory glands. The biochemical constituents of seminal plasma may change as a result of age, individual variability, and seasonality. These constituents are responsible for supporting different functions in sperm cells, contributing to motility, acrosomal reaction, and fertilization events. A detailed understanding of seminal plasma biochemistry may help to optimize semen freezing protocols, enabling the dynamic alteration in diluents to allow for increased sperm viability rates after thawing.

Keywords: biochemistry, accessory glands, seminal plasma, biobanks

Introduction

To mitigate the growing risks of extinction, the establishment of biobanks has been the primary strategy used for the conservation and expansion of valuable genetic material for years.[1] The systematic collection and preservation of semen from wild species has advanced significantly over the past few decades.[2,3] For some individuals, such as the black-footed ferret (*Mustela nigripes*)[4] and the giant panda (*Ailuropoda melanoleuca*),[5] biobanks are now in active use to support species management and propagation.[1] However, this strategy is currently limited to a small group of individuals. Therefore, research into wildlife semen preservation needs to emphasize species-specific variations in sperm physiology as well as focus on the specific transformations experienced by gametes during chilling, freezing, and thawing.[6]

Recent evidence suggests that sperm freezability from both domestic[7] and wild animals[8,9] could be closely associated with seasonal variations in seminal plasma composition. Seminal plasma plays an important role in maintaining sperm function and helping sperm travel through the female reproductive tract.[10] The protective activities of this biological fluid seem to be attributed to its numerous biochemical constituents,[11] which may vary among different species,[12,13] breeds,[14] ages,[15] individuals,[16] and seasons.[9]

Most information regarding the functional attributes of seminal plasma come from studies conducted in humans[17] and domestic species,[18] and little is known about this subject in wildlife. Some studies have provided valuable descriptions of the seminal plasma proteome from certain wild species,[19–22] but few, if any, elucidate how seminal proteins affect sperm function and fertility in these species.

Knowing the biochemical constitution of seminal plasma, as well as the interactions between these constituents and sperm, could provide useful data for the improvement of extenders and protocols for semen preservation in wild species, thus contributing to the implementation of biobanks. This review aims at summarizing currently available information related to the composition of seminal plasma from wild animals and discussing its impact on sperm freezability and the formation of biobanks in these species.

Biochemical Composition and Seasonal Variation in the Seminal Plasma of Wild Animals

The composition of mammalian seminal plasma is quite diverse. Among the organic compounds, carbohydrates[23] and triglycerides[11] represent important energy sources for sperm, whereas cholesterol contributes to the fluidity of the sperm membrane.[24] Proteins, in turn, participate in numerous

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mechanisms such as sperm protection,[25–27] membrane stabilization,[28,29] and different stages of the fertilization process.[30,31] In addition, inorganic compounds such as calcium, magnesium, and iron contribute to the maintenance of sperm metabolism,[32] whereas zinc has antimicrobial and antioxidant activity.[33]

Given the huge array of diversity found in wild mammals, it makes sense that we also see a wide variety of compounds in the seminal plasma from different species, including significant changes in the amounts of both organic (Tables 1 and 2) and inorganic (Table 3) content. Despite the importance, relatively few studies have reported detailed seminal plasma composition for wild species. For instance, the seminal plasma of chimpanzees (*Pan troglodytes*) has been known to contain glycoproteins, galactose, and sialic acid since 1981, but the study did not describe their quantity in these samples.[40]

The biochemical constitution of seminal plasma can even have a significant impact on sperm parameters. This was clearly demonstrated in elephants, in which samples with motility of ≥65% and ≤10% varied significantly in terms of their average creatine phosphokinase (11.86 U/L; 3.97 U/L), alanine aminotransferase (3.46 U/L; 5.92 U/L), phosphorus (2.74 mg/dL; 7.44 mg/dL), sodium (109.64 mmol/L; 70.47 mmol/L), chloride (107.14 mmol/L; 80.50 mmol/L), magnesium (2.22 mg/dL; 6.66 mg/dL), and glucose (6.45 mg/dL; 1.42 mg/dL) levels, respectively.[22]

Seasonal variations in the biochemical content of seminal plasma were reported for the collared peccaries raised in the Caatinga biome, Northeast of Brazil.[9] There were higher levels of fructose and calcium in their seminal plasma during the rainy season than during the dry season, and these changes were also shown to be positively correlated with the kinetic parameters of their sperm.[9] Seasonal variation was also observed in the composition of seminal fluid from carnivores such as the Siberian tiger, in which the individual variability in some biochemical contents was evidenced, even when samples were collected within the same month of the year.[34]

In brown bears, higher levels of lactate dehydrogenase were obtained when testosterone was reduced; in contrast, when testosterone was elevated during the breeding season, both lactate and magnesium were secreted at higher concentrations in the seminal plasma.[19] In addition, seminal plasma proteins were identified as a binder of sperm protein 5 (BSP-5) and tissue inhibitor metalloproteinase-2 were more highly expressed during the breeding season (May) than during the non-breeding season (November) in the North American bison (*Bison bison*).[41]

It is important to consider the great diversity in mammalian species with regards to size and anatomy of accessory glands,[39,42,43] which may reflect differences in seminal plasma composition.[12] Further, variations in sperm quality may occur as a result of changes in accessory sex gland secretions by the use of different semen collection methods.[44] In wild animals, collection is mainly performed through electroejaculation,[45] which is known to overstimulate the accessory glands.[46] In addition, interactions between electroejaculation and anesthetic protocols can also influence the secretion of accessory sex glands, interfering with semen quality, as reported for armadillos (*Euphractus sexcinctus*).[47]

Proteomic and Metabolomic Evaluations of Seminal Plasma

The application of so-called ''omic'' technologies, especially proteomics and metabolomics, has become the backbone of studies designed to characterize the protein and metabolite composition of seminal plasma from a wide

Table 1. Seminal Plasma Protein and Enzymatic Contents in Different Wild Mammals

Seminal plasma contents	*Elephant (*Elephas maximus*), U/L*	*Collared peccaries (*Pecari tajacu*), g/dL*	*Siberian tiger (*Panthem tigris altaica*), IU/L*	*Brown bear (*Ursus arctos*), U/L*	*Red deer (*Cervus elaphus *L.), U/mL*
Total protein	4.67–8.23 mg/mL	7.0–8.0	0.162 g/dL	0.2 g/dL	—
Albumin	1.3–1.6 mg/mL	3.0–9.4	0.062 g/dL	0.1 g/dL	—
Creatine phosphokinase	3.97–11.86	—	—	—	—
Non-protein nitrogen	—	—	—	—	—
Total concentration of glutathione	—	—	—	—	194.1–678.10 μM
Creatine	—	—	—	3.3 mg/dL	—
Urea nitrogen	85.83–103.20 mg/dL	—	33.9 mg/dL	—	—
Fructosamine	—	125.6–403.6 mmol/L	—	—	—
Lactate dehydrogenase	6.20–3.48	—	—	5.0 mmol/L	—
Aspartate aminotransferase	9.21–12.33	—	—	—	—
Alanine aminotransferase	3.46–5.92	—	—	—	—
Alkaline phosphatase	462.08–500.78	—	855	1093.7	—
Acid phosphatase	—	—	5.6	—	—
Superoxide dismutase	—	0.033 AU/mgP	—	—	10.07–17.00
Alanine transaminase	—	—	9.8	—	—
Aspartate transaminase	—	—	7.0	—	—
Glutamic oxaloacetic transaminase	—	—	—	93.7	—
Lipase	—	—	—	6.1	—
Glutathione peroxidase	—	—	—	—	0.52–2.07
Reference	Kiso et al.[22]	Santos et al.,[20] Moreira et al.[9]	Schmehl et al.[34]	Anel-López et al.[19]	Koziorowska-Gilun et al.[35]

—, means absence or unidentified constituent.

Table 2. Seminal Plasma Lipids, Carbohydrates, and Other Constituents in Different Wild Mammals

Seminal plasma contents	*Elephant* (Elephas maximus), *mg/dL*	*Collared peccaries* (Pecari tajacu), *mg/dL*	*Alpaca* (Vicugna pacos), *mg/dL*	*Siberian tiger* (Panthem tigris altaica)	*Rhesus monkey* (Macaca mulata), *%*	*Brown bear* (Ursus arctos), *mg/dL*	*Kangaroos* (Macropus eugenii, Macropus giganteus, Megaleia rufa), *mg/dL*	*Agouti* (Dasyprocta leporina), mmol/L
Triglycerides	—	296.1–306.8	—	—	—	—	—	—
Cholesterol	10.50–13.73	152.3–332.0	—	—	—	0.1 mmol/L	—	—
Phospholipids	—	—	—	—	—	—	—	—
Phosphatidyl choline	—	—	—	—	21.8	—	—	—
Phosphatidyl ethanolamine	—	—	—	—	2.0	—	—	—
Ethanolamine plasmalogen	—	—	—	—	24.0	—	—	—
Sphingomyelin	—	—	—	—	16.9	—	—	—
Choline plasmalogen	—	—	—	—	3.5	—	—	—
Cardiolipin	—	—	—	—	1.3	—	—	—
N—acetylglucosamin	—	—	—	—	—	—	336.1–508.8 mg/100 g seminal plasma	—
Glucose	1.42–6.45	—	—	2.42	—	2.42	28.9–110.9	—
Fructose	—	119.4–849.2	5.0	—	—	—	—	14.3
Citric acid	—	140.8–169.7	4.3	—	—	—	—	97.1
Creatinine	3.93–5.94	—	—	2.05	—	—	—	—
Carbon dioxide	—	—	—	14.2 mEq/dL	—	—	—	—
Reference	Kiso et al.[22]	Moreira et al.[9]	Garnica et al.[36]	Schmehl et al.[34]	Darin-Bennett et al.[37]	Anel-López et al.[19]	Rodger and White[38]	Mollineau et al.[39]

—, means absence or unidentified constituent.

Table 3. Seminal Plasma Inorganic Contents in Different Wild Mammals

Seminal plasma contents	*Elephant (*Elephas maximus*), mg/dL*	*Collared peccary (*Pecari tajacu*), mg/dL*	*Siberian tiger (*Panthera tigris altaica*), mg/dL*	*Brown bear (*Ursus arctos*), mg/dL*	*Red deer (*Cervus elaphus *L.)*
Sodium	70.47–109.69 mmol/L	—	158.2 mEq/dL	—	—
Phosphorus	2.74–7.44	12.3–72.1	2.40	—	—
Potassium	18.95–24.18 mmol/L	—	13.6 mEq/dL	3.6	—
Calcium	8.81–17.45	15.6–32.3	4.5	0.4 mmol/L	—
Chloride	80.50–107.14 mmol/L	271.3–315.6 mEq/L	143.0 mEq/dL	—	—
Bicarbonate	5.48–6.60 mmol/L	—	—	—	—
Magnesium	2.22–6.66	5.7–5.9	—	2.0	—
Iron	—	210.9–423.2 μg/dL	—	—	—
Urea nitrogen	85.83–103.20	—	33.9	—	—
L-ascorbate	—	—	—	—	90.13–690.50 μM
References	Kiso et al.[22]	Moreira et al.[9]	Schmehl et al.[34]	Anel-López et al.[19]	Koziorowska-Gilun et al.[35]

—, means absence or unidentified constituent.

range of species.[13] As expected, most investigations have been carried out in humans[48] and domestic species[49,50] and the amount of information available for these samples is impressive.

In wild species, however, information related to this subject is scarce. An initial study described the composition of elephant seminal plasma and identified components that can affect sperm motility. Lactotransferrin was present in 85% of ejaculates with good motility (>65%) and absent in 90% of ejaculates with low motility (<10%), which clearly demonstrates its involvement in seminal quality.[22]

In 2014, a study conducted in our laboratory characterized the main proteome of collared peccary seminal plasma, using two-dimensional electrophoresis followed by analysis of polypeptide maps. Through tandem mass spectrometry, we identified 23 different proteins, including spermadhesin as the Porcine Seminal Plasma Proteins (PSP-1), clusterin, and bodesin 2, in addition to proteins with known antioxidant activity, such as glutathione peroxidase, albumin, and annexin 5.[20]

Recently, the proteome of the seminal plasma from some wild carnivores was investigated. These studies found that the seminal fluid from brown bears (*Ursus arctos*) was rich in serum albumins, nucleobinding, phospholipases, albumin protein, clusterins, binder of sperm protein (BSP), and spermadhesins, as well as two unidentified proteins. Interestingly, there was more BSP-1 in the seminal plasma of individuals with low testosterone levels. Moreover, the two unidentified proteins were found to be more highly expressed in high testosterone samples.[19] In another study describing the coatis (*Nasua nasua*), our research team identified 238 seminal proteins, most of which were structural (18%), extracellular (17%), and nuclear (14%) proteins. The protein composition in these samples included proteins involved in the conservation of sperm integrity (annexin-1, DJ-1 protein, nucleobindin-1), sperm motility (sperm-associated antigen 6, epididymal secreting protein E1, phosphoglycerate kinase), and sperm capacitation (L-lactate dehydrogenase, and a protein similar to calmodulin, acrosin-binding protein, and ezrin).[21]

It seems clear that the seminal plasma proteome is unique to each species and is probably linked to the structure of reproductive organs, animal physiology, evolution, and strategies for reproductive success. Therefore, it is necessary to carry out further studies using this technique to confirm this concept and understand how protein profiles interact with the function and freezability of sperm in wild species.

Seminal Plasma Composition and Sperm Freezability

There is little consistency regarding which aspects affect sperm freezability in mammals. Some evidence points to a variety of genetic factors[51] that could be related to the functionality of accessory sex glands and the seminal plasma composition.[52] Recently, hard data supporting the concept that specific seminal plasma components are reliable markers of sperm freezability in some domestic species, such as bulls,[49] boars,[53] horses,[54] and buffalos,[55] have been reported.

There is evidence that semen preservation procedures increase the production of reactive oxygen species, which can induce sperm DNA damage and cause rapid loss of sperm fertilizing potential through lipid peroxidation of the plasma membrane.[56] Therefore, some seminal plasma compounds such as enzymatic (catalase, superoxide demutase, and glutathione peroxidase) and non-enzymatic (albumin, taurine, hypotaurine, pyruvate, ascorbic acid, tocopherol, and ergothionine) antioxidant agents can provide protection to the sperm from cell damage caused by oxidative stress.[33]

During cryopreservation, cells are subjected to a series of harmful events that may change lipid composition, resulting in decreased fluidity and permeability of the plasma membrane, leading to premature capacitation-like and acrosome reaction-like changes in the sperm.[54] In domestic animals, it is postulated that some proteins present in the seminal plasma could interact with the sperm plasma membrane to prevent premature capacitation and acrosome reactions.[57] The absence of these proteins might lead to a disruption in sperm membrane function during cryopreservation procedures, thus decreasing the subsequent fertilization capacity of these samples.[58]

Recently, the specific activity of some seminal plasma proteins related to sperm protection during semen preservation procedures has been elucidated.[26,59] BSPs have been shown to bind to the membrane and protect sperm

from cold shock when added before freezing.[26] Moreover, it is suggested that heat shock proteins can provide resilience to sperm, providing them with resistance to stressors associated with the freezing procedure.[59]

All information related to how seminal plasma could contribute to sperm protection during freezing procedures is particularly important for wildlife, as it can directly interfere with the efficiency of germplasm banks. To address this subject, an initial study conducted by Santymire et al.[60] demonstrated that the seminal plasma osmolarity of ejaculates obtained via electroejaculation could influence the viability of black-footed ferret sperm when incubated in different media used as freezing extenders. In 2011, it was demonstrated that rhesus monkey (*Macaca mulatta*) semen (with seminal plasma) subjected to rapid cooling (4°C) and thawing (37°C) had better motility parameters and acrosomal integrity than semen samples stored without its seminal plasma,[61] suggesting that seminal plasma components contribute to sperm viability.

In the arctic fox (*Vulpes lagopus*), semen quality indicators and biochemical markers were investigated at different stages of freezing and revealed a series of changes in the levels of the seminal biochemical constituents during this process (fresh, chilled, and frozen-thawed). Samples presented the highest alkaline phosphatase activity and the lowest levels of acrosine inhibitors before freezing. Then, during the refrigeration stage, acid phosphatase was shown to be upregulated and finally when the samples had completed their freeze/thaw cycle these same samples presented with an upregulation in their lactate dehydrogenase and aspartate aminotransferase concentrations. Moreover, the rate of increase in acrosin inhibitor activity in seminal plasma at different stages of cryopreservation can be used to evaluate the degree of damage in the sperm membranes in each sample, and thus a reduction in sperm fertilizing capacity. These findings indicate that biochemical constituents preserve semen quality by using a series of unknown molecular mechanisms.[62]

Pinyopummin et al.[63] elegantly demonstrated that the addition of stallion seminal plasma to elephant (*Elephas maximus*) sperm samples helped to preserve these cells at 4°C for 24 and 48 hours. These findings open up the possibilities for evaluating the constituents of heterologous seminal plasma as a source of exogenous supplementation when placing wild mammal samples in cryopreservation.

A study developed by our team showed that spermatozoa from collared peccaries were cryopreserved with greater efficiency during the rainy season in the semiarid region of northeastern Brazil, showing better kinetic parameters compared with cryopreservation in the dry season.[8] This observation was then validated by Moreira et al.,[9] who observed higher levels of fructose, an essential energy source for sperm metabolism, in the seminal plasma of peccaries during the rainy season when compared with the dry season. Based on this information, we were able to adjust the management of our scientific breeding center for peccaries and scheduled our semen collection and cryopreservation procedures for the middle of the rainy season. This simple change in procedure had a positive effect on the quality of the samples we maintain in our biobank, providing an important example of how these data regarding the composition of seminal plasma could be used in the future.

Conclusions

Wild species show differences in the size, morphology, and function of their accessory sex glands, which reflects the great degree of variation in their composition in seminal plasma. Moreover, seminal plasma composition can be affected by climate and season, influencing sperm function, freezability, and fertilizing capacity. A thorough understanding of the composition of the seminal plasma in different species is likely to allow for the development of improved protocols for semen cryopreservation. Although highly relevant to the field of wildlife conservation, these studies remain underrepresented in the literature.

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CAPÍTULO II

Investigating the need for antibiotic supplementation to the extender used forsemen cryopreservation in collared peccaries

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Investigating the need for antibiotic supplementation to the extender used for semen cryopreservation in collared peccaries

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The objective was to investigate the effects of semen freezing extender supplementation with antibiotics on bacterial load of semen samples, sperm functional and morphological metrics in the collared peccary. Fresh ejaculates from 10 males were extended in Tris-egg yolk-glycerol supplemented or not (control) with gentamicin (70 μg/mL) streptomycin-penicillin (SP; 1 mg/mL−1000 IU/mL) or and cryopreserved in liquid nitrogen. Bacterial load, sperm motility patterns, morphology, membrane functionality and integrity, mitochondrial activity, chromatin integrity and sperm-binding ability were evaluated in fresh and frozen-thawed samples. Regardless of the use of antibiotics, the sole cryopreservation provoked a significant decrease ($P < 0.05$) in bacterial load compared to fresh samples (from average values $> 1 \times 10^6$ CFU/mL to $<0.4 \times 10^6$ CFU/mL). Post-thawing sperm kinetic parameters were not affected by the absence or presence of different antibiotics, except for beat cross frequency that was significantly ($P < 0.05$) impaired by SP supplementation compared to the group without antibiotics. After thawing, sperm morphology, membrane functionality and integrity, and mitochondrial activity were also not affected by the presence or absence of antibiotics; however, a significant decrease was observed in the group without antibiotics ($P < 0.05$) in comparison to fresh samples. Regarding sperm-binding ability, there were no differences among the different groups. While collared peccary semen could be efficiently cryopreserved in the absence of antibiotics in the extender, the use of both gentamicin or the streptomycin-penicillin combination is recommended as effective antibiotic supplementation for a further control of bacterial loads without affecting sperm parameters.

KEYWORDS

wildlife, biobank, antibiotics, semen extender, bacterial load, collared peccary

Introduction

The collared peccary (*Pecari tajacu*) is a member of the Tayassuidae family that positively impacts Latin American ecosystems by acting as a seed disperser and prey for large carnivores. Despite being a species globally classified as stable, populations have been declining in various biomes like the Atlantic Forest (1). Thus, several efforts have been made to improve management in captivity and conservation, especially through the development of protocols for semen preservation and artificial insemination (2). Given the critical role of microbiomes on reproductive functions, the presence of *Staphylococcus spp*. and *Corynebacterium spp*. in the foreskin and semen of peccaries was recently investigated, with the proliferation of the latter bacterium significantly associated with damage to sperm membrane integrity and some kinetic parameters (3).

Negative effects of bacterial contamination in some sperm morphological (4) and functional (5) parameters have been reported. Thus, the use of antibiotics in semen extenders during freezing can contribute to the preservation of the quality and safety of male germplasm banks, especially because most of the microorganisms resulting from the semen processing can survive liquid nitrogen temperatures (−196°C) (6). On the other hand, the indiscriminate use of antibiotics can induce bacterial resistance (7). Besides, some antibiotics have been reported to negatively affect sperm quality in different species in a dose-dependent manner (8). Therefore, the possibility of avoiding antibiotic use in extenders for semen cryopreservation have been discussed, mainly because the cryopreservation process itself also causes a reduction in the bacterial load (9).

Thus, we aimed to investigate the need for antibiotic supplementation to the extender for collared peccary semen cryopreservation, by comparing the effect of samples without antibiotics with those supplemented with different antibiotics on the bacterial load, sperm functional and morphological metrics, and the sperm-binding ability.

Materials and methods

The ethics committee of the Federal Rural University of the Semi-Arid (UFERSA) approved the experimental protocols and procedures for the care of animals used in the experiment (n°. 05/2020). The study was authorized by the Chico Mendes Institute for Biodiversity (Opinion n° No. 37329/3). All chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Ten sexually mature collared peccaries of 40 months of age on average were used in the study. They were grouped in a maximum of three animals that were conditioned in paddocks (20 m × 3 m) containing a covered area (3 m × 3 m) under a natural photoperiod of 12 h, at the Center for Wild Animals Multiplication located on the UFERSA campus, Mossoró, Brazil (5°10'S-37°10'W; average temperature range, 27–29°C). During the study period, the animals were fed an isocaloric (3,300 kcal/kg) and isoprotein (14% protein) diet, supplemented with tropical fruits, in addition to water *ad libitum* (2).

The animals were fasted for 12 h before the procedures and first restrained with a net, followed by an anesthetic protocol with propofol (Propovan®, Cristália, Fortaleza, Brazil) in bolus (5 mg/kg) intravenously. Throughout the procedure, the animals' heart and respiratory rates were monitored (10). The animals were placed in lateral recumbency and submitted to semen collection using the electroejaculation protocol previously established for the species (11) consisting of a portable device (Autojac®, Neovet, Campinas, SP, Brazil) connected to a 12-V source. The stimulatory cycle comprised 10 stimuli in each voltage, starting from 5 V, followed by a voltage increase in steps from 1–12 V. Each electrical stimulus lasted 3 s, with intermittentbreaks of 2 s. The stimuli cycle was maintained for a 10-min duration from the beginning of the procedure. The electroejaculator probe was 15 1.3 cm, and it was inserted 12 cm into the rectum. Ejaculates were collected in plastic tubes and individually evaluated and processed.

For the microbiological analysis, an aliquot of 100 μL of each semen sample was inoculated in 900 μL of sterile 0.85% saline, obtaining a dilution of 10–1, followed by a serial dilution up to 10–5. Aliquots of 100 μL of each dilution were sown, with the aid of a Drigalski loop, on the surface of Petri dishes containing Plate Count Agar (Hi Media, Mumbai, India), all in duplicate and incubated in a bacteriological incubator (Fanem LTDA, São Paulo, Brazil) at 37°C for 24–48 h. Colonies were then counted on each plate and the number of microorganisms was expressed in Colony Forming Unit–CFU/mL multiplied by the inverse of each dilution (12).

Ejaculates were evaluated for color and appearance. The volume was measured using micropipettes, and the pH was determined using pH indicator strips (Neutralit®, Merck, Bucharest, Romania). Sperm concentration (millions of sperm/mL) was estimated in a Neubauer counting chamber (13).

Sperm kinetic patterns were analyzed using computerized semen analysis (IVOS 7.4 G; Hamilton-Thorne Research, Beverly, MA, USA), using settings previously established for peccaries (13). The settings of the instrument included temperature 37°C; 60 frames/s; minimum contrast, 45; straightness threshold, 30%; low-velocity average pathway (VAP) cutoff, 10 m/s; and medium VAPcutoff, 30 m/s. Five independent and non-consecutive microscopic fields were randomly selected and evaluated using scanning procedures. Values for the following parameters were analyzed: number of cells counted, total motility (%), velocity average pathway (VAP; mm/s), straight-line velocity (VSL: mm/s), curvilinear velocity (VCL: mm/s), amplitude lateral head (ALH; mm), beat cross frequency (BCF; Hz), straightness (STR; %) and linearity (LIN; %), as well as the sperm subpopulations: fast, medium, slow and

static. For a reliable assessment of sperm motility patterns, the Edit Tracks option of the IVOS 7.4 G system was used to exclude the debris derived from the extenders. A further dilution in salt solution (1:1) was conducted only if necessary (13).

Morphological analysis was performed using semen smears stained with Bengal Rose and evaluated under light microscopy (×1000; 200 cells/slide) (13). The functionality of the sperm membrane was analyzed through the osmotic response of sperm to the hypo-osmotic test with distilled water (0 mOsm/L), evaluated under light microscopy (x400; 200 cells/slide) (13). Chromatin integrity was assessed using a smear stained with toluidine blue dye [0.025% dye in McIlvaine buffer (sodium citrate: phosphate pH ¼ 4.0)] and evaluated under light microscopy (x1000; 500 cells; slide). Cells stained slightly blue were classified as normal (negative) and those stained from violet to dark blue were considered to have altered chromatin (positive) (14).

Sperm membrane integrity and mitochondrial activity were assessed using a combination of fluorescent solutions: 3 μL of Hoechst 342 (H342; Sigma-Aldrich, St Louis, MO, USA) (998.4 μL of DPBS + 1.6 μL of stock solution: 25 μg/mL), 5 μL of CMXRos (Mito Tracker red®, Molecular Probes, M-7512) (1 mL of TRIS + 0.1 μL of stock solution: 50 μg / 94 μL) and 2 μL of IP (Propidium Iodide, Sigma-Aldrich, Co., St Louis, MO, USA) (980 μl DPBS + 20 μl stock solution: 25 μg/ml). A total of 200 cells were evaluated under an epifluorescence microscope (Episcopic Fluorescent attachment EFA Halogen Lamp Set. Leica. Kista, Sweden), whose spermatozoa with a blue head (H-342) were judged to contain an intact membrane and those with a full head or partially red-labeled (PI) were considered to contain a non-intact membrane, and a red-labeled midpiece were considered having a mitochondrial function (12).

The binding ability of spermatozoa was investigated using the hen egg perivitelline membrane binding assay, as previously validated for peccaries (15). Briefly, egg yolk membranes from fresh and non-fertile chicken eggs were washed in saline solution at 37°C and submitted to 1 cm² cuts, with two membranes for each treatment. Together, the sperm samples (1: 1) were diluted in an incubation medium solution (114 mM NaCl; 3.1 mM KCl; 0.4 mM NaH_2PO_4; 10 mM calcium lactate; 25 mM $NaHCO_3$; 10 μg/mL phenol red; 1.4 mM caffeine; 2.0 mM $CaCl_2.2H_2O$; 0.5 mM $MgCl_2$; 10 mM Hepes; 6 mg/mL BSA; 5.5 mM glucose; 0.45 mM sodium pyruvate; 40 μg/mL gentamicin; pH 7.47.8), and subsequently centrifuged at 700 xg for 10 min. The pellet was resuspended to obtain 1 x 10^6 sperm/mL that was incubated in a 4-well plate with a membrane fragment at 38.5°C for 20 min in a water bath. After incubation, each membrane was washed in 100 μL drops of incubation medium for the removal of non-binding sperm, and subsequently kept in Hoechst 33250 for 15 min. Finally, the membranes were evaluated for the number of ligating sperm, evaluating six distinct and random fields using epifluorescence microscopy (Episcopic Fluorescent attachment EFA Halogen Lamp Set. Leica. Kista, Sweden).

For freezing, the ejaculates were diluted in Tris plus glycerol (3%) and egg yolk (20%), and separated into three aliquots that were kept without antibiotics (Control group) or supplemented with gentamicin (70 μg/mL) or streptomycin/penicillin (1 mg/mL/1000 IU/mL), as previously reported for peccary semen refrigeration (12). Final dilution resulted in a 100 x 10^6 sperm/mL concentration. The samples were refrigerated at 15°C for 40 min in isothermal boxes and stabilized at 5°C for another 30 min in a biological incubator (Quimis, Diadema, SP, Brazil). Then, they were filled in 0.25 mL plastic that were placed in contact with the nitrogen vapor (5 cm) for 5 min and finally stored in a cryobiological container at −196° C. After 1 week, the samples were thawed in a water bath at 37°C for 1 min (13) and evaluated for microbiological load and sperm metrics as described for fresh samples.

A total of 10 samples obtained from 10 animals (one sample per animal) were used in the experiment. Each individual sample was divided into three aliquots, which were allocated to each of the treatments tested. Data obtained were expressed as mean ± standard error of 10 replicates. Normality of residual was verified by the Shapiro-Wilk test and homogeneity of variance by Levene's test. The data of total motility, medium subpopulation, and membrane functionality and integrity were transformed into arcsine to attend to the parametric assumptions. Initially, fresh semen was considered as **one** of the treatments and Dunnett's test was applied to compare it with the other treatments. Subsequently, a one-way ANOVA, followed by Tukey's *post hoc* test to evaluate differences among treatments (thawed) were performed. For all analyses, Statistical Analysis Software, version 8.0 (SAS Institute Inc., Cary, NC, USA) was used and in all pairwise comparisons, a $P < 0.05$ was considered.

Results

Cryopreservation alone (values ranging from 0.04 to 1 x 10^6 to CFU/mL) already caused a significant decrease ($P < 0.05$) in bacterial load compared to fresh samples (values ranging from 0.4 to 21.3 x 10^6 CFU/mL). The most significant decrease ($P < 0.05$) of bacterial load was observed after the use of the streptomycin-penicillin combination (values ranging from 0 to 0.2 x 10^6 CFU/mL), while values from the gentamicin group (values ranging from 0.01 to 0.5 x 10^6 CFU/mL) did not differ from either the streptomycin-penicillin or the non-antibiotic control group (Figure 1).

Ejaculates presented a milky appearance, with a whitish color, and pH 7.5 ± 0.2. The average volume was 5.0 ± 1.2 mL, with average sperm concentration of 461.0 ± 59.2 ×10^6 sperm/mL. The mean number of total motile spermatozoa was 95.3 ± 0.8%, with 80.0 ± 4.3% morphologically normal cells, being 69.4 ± 8.6% with functional membrane, 80.9 ± 2.2% with intact membrane, 79.3 ± 2.8% with mitochondrial activity, and 99.4 ± 0.2% with normal condensed chromatin.

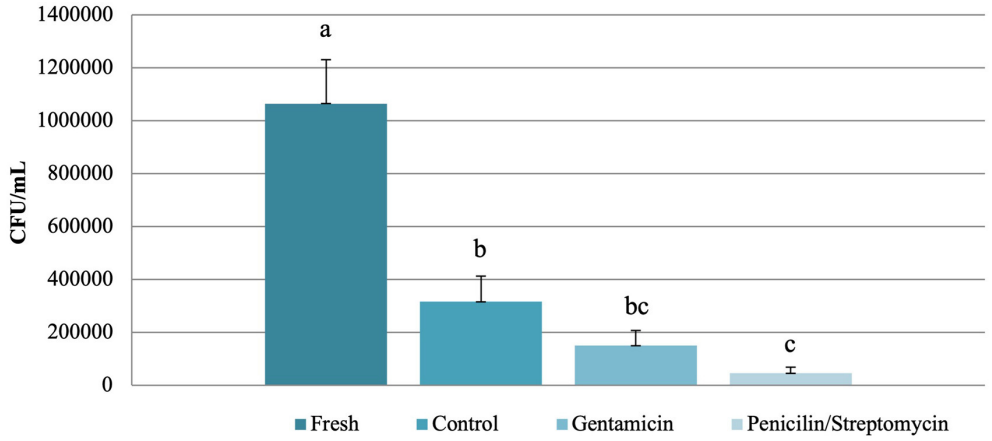

FIGURE 1
Mean (± SEM) values for bacterial load (CFU/mL) in fresh and frozen-thawed semen from collared peccaries ($n = 10$) supplemented of not with different antibiotics (gentamicin and streptomycin-penicillin combination). a–c Lowercase letters indicate significant differences for fresh and treatment groups (Dunnett's test; $P < 0.05$).

TABLE 1 Mean (± SEM) values for kinetic motility patterns in fresh and frozen-thawed semen from collared peccary ($n = 10$) supplemented or not with different antibiotics (gentamicin and streptomycin-penicillin combination).

	Fresh	Frozen-Thawed		
Sperm kinetic motility patterns		**Control**	**Gentamicin**	**Streptomycin-penicillin**
Total motility (%)	95.3 ± 0.8^{A}	34.1 ± 3.7^{Ba}	37.2 ± 6.1^{Ba}	32.6 ± 4.0^{Ba}
Progressive motility (%)	72.1 ± 3.5^{A}	20.2 ± 2.4^{Ba}	23.2 ± 4.6^{Ba}	20.0 ± 2.8^{Ba}
Velocity average pathway (mm/s)	69.2 ± 4.8^{A}	46.1 ± 2.7^{Ba}	47.7 ± 2.8^{Ba}	45.8 ± 2.5^{Ba}
Velocity straight line (mm/s)	56.8 ± 4.4^{A}	35.7 ± 3.1^{Ba}	36.0 ± 3.4^{Ba}	33.2 ± 3.0^{Ba}
Velocity curvilinear (mm/s)	119.4 ± 8.0^{A}	100.6 ± 5.5^{Aa}	100.2 ± 4.4^{Aa}	99.7 ± 5.0^{Aa}
Amplitude lateral head (mm)	5.3 ± 0.3^{A}	5.4 ± 0.2^{Aa}	5.6 ± 0.1^{Aa}	5.7 ± 0.2^{Aa}
Beat cross frequency (Hz)	37.2 ± 0.6^{A}	35.6 ± 0.9^{Aa}	34.1 ± 0.5^{Bab}	31.7 ± 1.0^{Bb}
Straightness (%)	77.5 ± 2.0^{A}	72.7 ± 2.4^{Aa}	70.9 ± 3.5^{Aa}	69.0 ± 2.2^{Aa}
Linearity (%)	47.0 ± 2.4^{A}	34.4 ± 1.5^{Ba}	34.8 ± 2.3^{Ba}	33.3 ± 1.5^{Ba}
Sperm subpopulations				
Rapid (%)	81.0 ± 3.6^{A}	23.5 ± 3.0^{Ba}	27.7 ± 4.9^{Ba}	23.4 ± 3.0^{Ba}
Medium (%)	14.4 ± 3.0^{A}	10.6 ± 1.8^{Aa}	9.4 ± 1.4^{Aa}	9.1 ± 1.6^{Aa}
Slow (%)	2.0 ± 0.2^{A}	2.7 ± 0.7^{Aa}	2.6 ± 0.4^{Aa}	2.4 ± 0.6^{Aa}
Static (%)	3.0 ± 0.6^{A}	63.1 ± 4.2^{Ba}	60.3 ± 6.4^{Ba}	65.0 ± 4.1^{Ba}

[AB]Superscript capital letters indicate a significant difference between fresh and thawed groups (Dunnett's test; $P < 0.05$).
[ab]Superscript lower case letters indicate significant difference between experimental groups (Tukey's test; $P < 0.05$).

After thawing, there was a significant decrease ($P < 0.05$) on most of the sperm kinetic parameters (Table 1) in comparison to fresh samples. However, post-thawing values for VCL, ALH, straightness, medium and slow populations in all the experimental groups were similar to those observed for fresh semen ($P < 0.05$). Furthermore, there were no differences among experimental groups for these kinetic parameters ($P > 0.05$). Regarding Beat Cross Frequency (Table 1), values obtained for the control group without antibiotics were the only similar to those observed for fresh samples ($P > 0.05$), while the group containing streptomycin-penicillin significantly impaired this parameter when compared to other treatments ($P < 0.05$).

Regarding sperm morphology (Table 2), similar values were observed among fresh samples and all the post-thawing experimental groups ($P > 0.05$). For the analysis of chromatin integrity (Table 2), groups containing antibiotics provided similar values as those observed for fresh samples ($P > 0.05$); however, a significant decrease on this parameter was observed

TABLE 2 Mean (± SE) values for sperm normal morphology, membrane functionality and integrity, mitochondrial activity and chromatin integrity in fresh and frozen-thawed semen from collared peccaries ($n = 10$) supplemented or not with different antibiotics (gentamicin and streptomycin-penicillin combination).

	Fresh	Frozen-Thawed		
Sperm Parameters		**Control**	**Gentamicin**	**Streptomycin-Penicillin**
Normal morphology (%)	80.0 ± 4.3^{A}	79.4 ± 2.2^{Aa}	75.1 ± 3.5^{Aa}	79.1 ± 3.3^{Aa}
Membrane functionality (%)	69.4 ± 8.62^{A}	50.0 ± 3.6^{Ba}	59.4 ± 6.1^{Ba}	50.5 ± 4.3^{Ba}
Chromatin integrity (%)	99.4 ± 0.2^{A}	97.5 ± 0.6^{Ba}	98.5 ± 0.5^{ABa}	98.4 ± 0.6^{ABa}
Membrane integrity (%)	80.9 ± 2.2^{A}	37.6 ± 5.4^{Ba}	40.1 ± 5.8^{Ba}	31.3 ± 2.5^{Ba}
Mitochondrial activity (%)	79.3 ± 2.8^{A}	31.3 ± 5.4^{Ba}	34.9 ± 5.9^{Ba}	30.6 ± 2.8^{Ba}

[AB]Superscript capital letters indicate a significant difference between fresh and thawed groups (Dunnett's test; $P < 0.05$).
[abc]Superscript lowercase letters indicate a significant difference between the experimental groups (Tukey's test; $P < 0.05$).

TABLE 3 Mean (± SE) values and range (Min—Max) for number of sperm bound to the perivitelline membrane of hen egg yolk in fresh and frozen-thawed semen from collared peccaries ($n = 10$) supplemented or not with different antibiotics (gentamicin and streptomycin-penicillin combination).

	Fresh	Frozen-Thawed*		
		Control	**Gentamicin**	**Penicillin/Streptomycin**
Number of bound sperm	212.0 ± 22.6^{A}	105.0 ± 16.1^{B}	100.7 ± 8.1^{B}	95.8 ± 13.4^{B}
Min	99.7	34.5	47.2	47
Max	351.2	185.5	148.3	196.7

[AB]Superscript capital letters indicate significant difference between fresh and frozen-thawed groups (Dunnett's test; $P < 0.05$).
*There were no differences among post-thawing experimental groups (Tukey's test; $P > 0.05$).

for the samples cryopreserved without antibiotics ($P < 0.05$). For membrane functionality and integrity, and mitochondrial activity (Table 2), there was a significant decrease ($P < 0.05$) after thawing in comparison to fresh semen ($P < 0.05$), but no significant differences were observed among treatment groups ($P > 0.05$).

Regarding sperm-binding assay (Table 3), values of 212.0 ± 22.6 sperm bound to perivitelline membranes were observed for fresh samples, but the cryopreservation process caused a significant decrease ($P < 0.05$) of ∼50% in the values found for all experimental groups, regardless of the use of antibiotics.

Discussion

While the sole cryopreservation process can cause a significant reduction in semen sample's bacterial loads, the study demonstrated the possibility of supplementing semen extender with different antibiotics for further control of bacterial load, without provoking extensive damage on sperm quality during the cryopreservation process.

Regarding bacterial contamination in peccary fresh ejaculates, we found values >1 x 10^6 CFU/mL, which are into the normal range previously described for the species as 0.04 to 2.2 x 10^6 CFU/mL (3). It is difficult to compare these findings with those reported for other species, as even in boars there is no consensus regarding the number of bacteria found in fresh ejaculates, which can vary from just 0.08 10^6 CFU/mL (16) to 370 x 10^6 CFU/mL (17). In fact, semen collection in farm animals is not a sterile procedure, with some bacterial flora contaminating the semen (16), but the adoption of adequate sanitary practices can help to reduce sample contamination.

In general, the combination of streptomycin-penicillin, as well as gentamicin, are among the antibiotics most commonly used in the composition of extenders for the semen of farm animals (8), which is why these were the antibiotics chosen to be used in the present study. The use of antibiotics during peccary semen freezing allowed an effective control of bacterial load. Both streptomycin and gentamicin are aminoglycosides that acts in the protein synthesis of gram-negative bacterial cells (18, 19). In the other hand, the penicillin is a β lactam that acts by inhibiting the synthesis of cell wall of gram-positive bacteria (19). Even if this is the first time that these antibiotics were used of the cryopreservation of peccary sperm, they were previously proven to provide efficient control of bacterial load during peccary semen chilling for 36 h. At this point, the streptomycin-penicillin combination was able to reduce the bacterial load to zero in various samples (12).

As demonstrated by the present study, freezing conditions alone could be maintain contamination at acceptable levels in peccary semen as previously observed for ram (9). It has already been shown that bacteriostasis can be achieved during storage of swine semen even at a temperature of 5°C in the absence of antibiotics (20). Furthermore, in domestic swine, it is assumed that a complete elimination of microorganisms in semen samples cannot be justified, given the high exposure of the female genital tract to commensal bacteria in ejaculates during natural mating, which could play a role in important physiological immunogenic role (20). Therefore, the adoption of strict hygiene measures should be focused on avoiding the exposure of animals to pathogenic microorganisms that could then be transmitted *via* semen samples.

Besides controlling bacterial load, the treatment containing no antibiotics was able to provide an effective preservation of all sperm metrics, except the chromatin integrity of frozen-thawed peccary sperm. As reported for human sperm, bacterial contamination can directly alter the sperm function, increasing the phosphatidylserine translocation and the apoptosis activation, which could be related to DNA condensation and fragmentation (5). In collared peccaries, however, the aforementioned deleterious effect on chromatin integrity does not seem to be reflected on fertility, since a sperm binding capacity similar to the groups containing antibiotics was observed. Besides it, no differences were observed between the groups containing or not containing antibiotics in relation to the rates obtained in the sperm binding test, despite differences relative to BCF especially with the inclusion of SP to the extender. Furthermore, there were no differences between groups with or without antibiotics regarding post-thawing sperm morphology, membrane functionality and integrity, and mitochondrial activity.

The discussion concerning the need to control microorganisms present in semen is still quite controversial, especially for wild animal species. If, on one hand, the microbiome of wild animals is still unknown, and its impact on the physiology of these species remains to be elucidated (21), on the other hand, there is a possibility that biological samples collected in the field may transmit unknown microorganisms to captive populations or even for men (22). In this context, our results provide novel information related to the possibility of freezing collared peccary semen samples without the presence of antibiotics. It is worth mentioning that the animals used in the present study have been bred in captivity for several generations, and the description of their reproductive microbiome had already been previously performed (3). Besides, semen collection procedures were carried out taking all necessary sanitary precautions to reduce sample contamination. Under field conditions, however, it is not always possible to carry out the collection in an aseptic way, and the use of antibiotics may be advised for this purpose, especially if samples are destined to *in vitro* fertilization trials (23).

In conclusion, it is noteworthy that the cryopreservation process alone can control bacterial load, without promoting effective damage to collared peccary semen samples. However, if necessary, the antibiotic supplementation, both gentamicin and the streptomycin-penicillin combination are indicated for the extender used in the cryopreservation of collared peccary semen. These are important findings to be considered when using germplasm from populations kept in captivity or from those living in the wild to create safe biobanks and also assisted reproductive technologies (ART) or artificial insemination (AI).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of the Federal Rural University of the Semi-Arid (UFERSA) approved the experimental protocols and procedures for the care of animals used in the experiment (n°. 05/2020). The study was authorized by the Chico Mendes Institute for Biodiversity (Opinion n° No. 37329/3).

Author contributions

All authors equally contributed for manuscript conceptualization, methodology, data analysis, and writing and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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CAPÍTULO III

Effect of Detergents Based on Sodium Dodecyl Sulfate on Functional Metrics ofFrozen–Thawed Collared Peccary (*Pecari tajacu*) Semen

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Effect of Detergents Based on Sodium Dodecyl Sulfate on Functional Metrics of Frozen–Thawed Collared Peccary (*Pecari tajacu*) Semen

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Simple Summary: Constant threats to wildlife have led countless researchers to develop or improve conservation strategies, among which the formation of biobanks stands out. The systematic storage of male gametes in freezing temperatures is useful for future use in assisted reproduction and for improving the reproductive management of rare species. In this study, the effect of including Equex STM® paste or different concentrations of sodium dodecyl sulfate (SDS) on various functional metrics of frozen and thawed peccary semen, such as sperm kinetic parameters, membrane functionality and integrity, mitochondrial activity and binding capacity, was verified. In parallel, sperm longevity was evaluated through a thermal resistance test. In general, it was demonstrated that the addition of 0.1% SDS to the Tris–egg yolk–glycerol diluent favored the maintenance of the sperm kinetic parameters of peccaries during freezing and thawing procedures.

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Abstract: We evaluated the effects of detergents based on sodium dodecyl sulfoxide (SDS) on the functional parameters of collared peccary frozen–thawed sperm. Semen aliquots from ten individuals were diluted in a Tris–egg yolk–glycerol extender alone or with 0.5% Equex STM® paste or SDS (at 0.1%, 0.3% or 0.5% (*v/v*) concentration). Samples were fast frozen in liquid nitrogen with a post-thaw evaluation of motility, membrane functionality and integrity, mitochondrial activity, sperm binding ability and thermal resistance. The treatments without SDS (41.8 ± 3.5%) and those containing Equex (41.8 ± 4.4%) or 0.1% SDS (41.2 ± 5.5%) provided greater sperm motility ($p < 0.05$) than those containing SDS 0.3% (30.5 ± 4.7%) and 0.5% (31.2 ± 6.3%). Immediately after thawing, only treatments containing 0.1% SDS effectively preserved sperm straightness (STR) when compared to the negative control. All treatments preserved the amplitude of lateral head (ALH) and straightness (STR) during a thermal resistance test ($p > 0.05$), but SDS 0.5% impaired the membrane functionality and mitochondrial activity after thawing ($p < 0.05$). All treatments provided a similar recovery of sperm binding ability after thawing ($p < 0.05$). Our results showed that the addition of 0.1% SDS to the Tris–yolk–glycerol extender optimized the freeze–thaw recovery of peccary semen.

Keywords: *Tayassuids*; biobanking; sperm; membrane integrity; SDS

1. Introduction

In view of constant threats to the survival of various wild species, the need for efficient conservation strategies has been imminent. Among such strategies, biobanks emerge as important repositories of valuable genetic material from endangered animals [1]. Important studies have focused on the cryopreservation of the male gametes of ecologically important individuals, such as collared peccaries (*Pecari tajacu* Linnaeus, 1758). These peculiar ungulates that originally lived from North to South America have today suffered a

decline in their population, having even disappeared in some regions such as eastern and southern Argentina [2].

Since 2010, the protocol for the cryopreservation of collared peccary semen was developed [3] from the incorporation of egg yolk and glycerol with diluents based on Tris [4] or coconut water [5]. One of the great challenges to improving the protocol lies in the short-term peccary sperm survival after thawing [6]. To overcome this obstacle, a preliminary study demonstrated that the addition of Equex STM paste (Nova Chemical Sales, Scituate, MA, USA) would promote a slight extension of just 30 min in the maintenance of sperm longevity after thawing [7]. However, the effect of the paste on other sperm parameters, mainly related to sperm function, still needs to be elucidated. Furthermore, the exact composition of the paste in terms of excipients and other compounds is not known, even if manufacturers highlight sodium dodecyl sulfate (SDS) as the main active component, albeit at an unknown concentration. In general, there is a lack of information regarding the incorporation of isolated SDS with diluents for semen conservation, since most studies have reported the use of commercial pastes containing this substance.

The addition of commercial SDS-based supplements to extenders for semen cryopreservation has contributed not only to increased sperm longevity in different species, but also stands out for maintaining the post-thawing sperm kinetic rate, as recently reported for buffaloes [8]. In addition, the incorporation of SDS-based pastes with diluents has also promoted a positive effect on sperm binding and fertilizing ability in dogs [9], cats [10] and boars [11]. Despite the extensive use of SDS-based supplements in semen freezing in different species, their effects on the mitochondrial activity of sperm from any species remain to be elucidated. This would be important information to show, as Bezerra et al. [7] demonstrated through an ultrastructural analysis using transmission electron microscopy, that cryopreservation promotes mitochondrial vacuolation, thus, possibly contributing to a reduction in post-thawing sperm survival in peccaries.

Therefore, the aim of the present study was to verify the effect of including Equex STM® paste or different concentrations of sodium dodecyl sulfate (SDS) on various functional metrics of frozen–thawed peccary semen such as sperm kinetic parameters, membrane functionality and integrity, mitochondrial activity and sperm binding ability, in addition to evaluating sperm longevity through a thermal resistance test.

2. Materials and Methods

2.1. Animal Ethics and Husbandry

All experimental procedures were authorized by the ethics committee of the Universidade Federal Rural do Semi-Árido (UFERSA) (nº 05/2020). The protocols were authorized by the Chico Mendes Institute for Biodiversity (opinion nº 37329/3). Sigma Chemical Co. (St. Louis, MO, USA) provided all reagents used in the experiment, unless otherwise specified.

Ten sexually mature collared peccaries, with a mean age of 40 months, were used. The animals were exposed to a natural photoperiod of 12 h and up to three animals were allocated into paddocks (20 m × 3 m), presenting a covered area (3 m × 3 m) at the Wild Animal Multiplication Center located on the UFERSA campus, Mossoró, Brazil (5°10′ S–37°10′ W; medium temperature range, 27–29 °C). For the study, an isocaloric (3300 kcal/kg) and isoprotein (14% protein) diet, supplemented with tropical fruits and water ad libitum, was offered to the animals.

2.2. Experimental Design

After obtaining ejaculate from ten individuals, one aliquot of fresh semen was immediately evaluated, and five other aliquots were diluted in Tris–egg yolk–glycerol extenders and used for cryopreservation. Regarding the dilution, the first aliquot received no detergent supplementation (negative control group); the second aliquot was supplemented with 0.5% Equex STM paste (Nova Chemical Sales, Inc., Scituate, MA, USA), constituting a positive control group [7]; the other three semen aliquots were supplemented with SDS only in increasing concentrations of 0.1%, 0.3% or 0.5% *v/v*, as previously tested for boars [12],

the domestic species most closely related to peccaries. Fresh and frozen–thawed samples were evaluated for sperm kinetic parameters, membrane functionality and integrity, mitochondrial activity, sperm binding ability and longevity.

2.3. Semen Collection and Initial Evaluation

After a 12 h fast, the peccaries were restrained with a net and anesthetized with intravenous propofol (Propovan®, Cristália, Fortaleza, Brazil) in bolus (5 mg/kg). During the anesthesia, the cardiac and respiratory parameters of the animals were evaluated [13]. The animals were placed in lateral recumbency, and the semen was obtained through the electroejaculation protocol, as previously established for the species [3]. For this purpose, a portable device (Autojac®, Neovet, Campinas, SP, Brazil) connected to a 12 V source provided a stimulatory cycle comprised of 10 stimuli in each voltage, starting from 5 V, followed by a voltage increase in steps from 1 V to 12 V. Each electrical stimulus lasted 3 s, with intermittent breaks of 2 s. The stimulus cycle lasted 10 min from the commencement of the procedure. The electroejaculator probe measured 15 × 1.3 cm, of which 12 cm was introduced into the rectum. Ejaculates were stored in plastic recipients and immediately analyzed for semen volume, which was measured with repeated pipetting. The pH was defined using pH indicator strips (Neutralit®, Merck, Bucharest, Romania) [13] and the sperm concentration (in millions of sperm/mL) was determined by counting cells in a Neubauer chamber after dilution (1:2) in a formalized buffered solution (10%) [14].

2.4. Semen Freezing–Thawing Procedures

For freezing, the samples were diluted in Tris containing egg yolk (20%), glycerol (3%) and gentamicin (70 µg/mL) [15], and separated according to the following treatments: (A) negative control (no detergent), (B) positive control (0.5% Equex STM® paste (Equex–Nova Chemical Sales, Inc., MA, USA), (C) 0.1% SDS, (D) 0.3% SDS and (E) 0.5% SDS. Semen was frozen according to a fast-freezing curve previously described by Silva et al. [16], in which diluted semen cooled at 15 °C for 40 min in isothermal containers and stabilized at 5 °C for another 30 min in an incubator (Quimis, Diadema, SP, Brazil). Then, samples were packed in 0.25 mL plastic straws that were exposed to nitrogen vapor (5 cm) for 5 min and, finally, stored in a cryobiological cylinder at −196 °C. After 1 week, the samples were thawed in a water bath at 37 °C for 1 min [17], and the functional sperm parameters were immediately evaluated. For a thermal resistance test, samples were incubated in a water bath at 37 °C and re-evaluated at 30 and 60 min.

2.5. Computer-Aided Semen Analysis

Information on fresh and frozen–thawed sperm motility characteristics and kinetic parameters was obtained with a computerized semen analysis (IVOS 7.4 G; Hamilton-Thorne Research, Beverly, MA, USA) with pre-established settings for the species, such as a temperature of 37 °C; 60 frames/s; minimum contrast 45; straightness threshold 30%; low-velocity average pathway (VAP) cutoff, 10 m/s; and a medium VAP cutoff of 30 m/s [17]. The parameters evaluated were the total and progressive motility (%), velocity average pathway (VAP; µm/s), velocity straight line (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head (ALH; µm), beat cross frequency (BCF; Hz), straightness (STR; %) and linearity (LIN; %). When there was a low VAP cutoff (LVC) and medium VAP cutoff (MVC), the overall sperm population was subdivided into four categories: rapid, with VAP > MVC; medium, with LVC < VAP < MVC; slow, with VAP < LVC; and static for the absence of cell motility. For a reliable assessment of sperm motility patterns, the Edit IVOS 7.4 G System Tracks option was used to exclude debris derived from the diluents. There was further dilution in a saline solution (1:2) only when necessary [17].

2.6. Sperm Membrane Functionality

A hypoosmotic test using distilled water as the hypoosmotic solution (0 mOsm/L) was used to assess the sperm membrane functionality. Fresh and frozen–thawed semen

aliquots were diluted following the proportion of 1:9 and incubated at 37 °C for 45 min. Then, a total of 200 cells were counted, and those with curled tails were judged as having a functional membrane under phase contrast microscopy (Alttion®, Wuzhou, China) [18].

2.7. Plasma Membrane Integrity and Mitochondrial Activity

The plasma membrane integrity and mitochondrial activity in fresh and frozen–thawed samples were examined simultaneously through the combined use of fluorophores Hoechst 342 (H342; Sigma-Aldrich, St. Louis, MO, USA), Mito Tracker red® (CMXRos, Molecular Probes, M-7512) and propidium iodide (IP, Sigma-Aldrich, Co., St. Louis, MO, USA). A total of 200 cells were counted under epifluorescence microscopy (EFA fluorescent accessory Halogen Lamp Set. Leica. Kista, Sweden). Cells that had a head marked in blue (H-342) were classified as having an intact membrane, while those that had a head marked in red (PI) were identified as having a nonintact membrane. In addition, those with the intermediate piece marked in red were considered to have an active mitochondrial function [17,19].

2.8. Sperm Binding Ability Assay

For fresh and frozen–thawed samples, an interaction test with a hen's egg perivitelline membrane was used to evaluate the sperm binding capacity, as previously established for collared peccaries [20]. Briefly, fresh and infertile eggs, whose membranes were obtained by separating the yolk and white, were used, standardizing cuts of 1 cm^2, with two membranes per treatment. The semen samples were diluted (1:1) in an incubation solution (114 mM NaCl; 3.1 mM KCl; 0.4 mM NaH2PO4; 10 mM calcium lactate; 25 mM NaHCO3; 10 µg/mL phenol; 1.4 mM caffeine; 2.0 mM $CaCl_2.2H_2O$; 0.5 mM $MgCl_2$; 10 mM Hepes; 6 mg/mL BSA; 5.5 mM glucose; 0.45 mM sodium pyruvate; 40 µg/ mL gentamicin; pH 7, 47, 8) and further centrifuged at 700× *g* for 10 min to establish a sperm concentration of 1×10^6 sperm/mL. The membranes, together with the sperm solution, were incubated in a water bath at 38.5 °C for 20 min, washed in 100 µL drops of the incubation solution and kept in Hoechst 33,250 for 15 min. Then, the membranes were stretched over a slide and coverslip and six random fields were analyzed under epifluorescence microscopy (Episcopic Fluorescent attachment EFA Halogen Lamp Set. Leica. Kista, Sweden) for the number of binding spermatozoa.

2.9. Statistical Analysis

Data obtained were expressed as mean ± standard error and all analyses were performed using the Statistical Analysis Software, version 8.0 (SAS Institute Inc., Cary, NC, USA). The Shapiro–Wilk test verified the normality of residual and the Levene test evaluated the homogeneity of variance. A two-way ANOVA, which considered the effects of the treatment (SDS-based extenders), incubation time and its interaction, was performed. Then, to assess the potential differences among the means, Tukey's post hoc test was performed. All pairwise comparisons were considered significant when a $p < 0.05$.

3. Results

3.1. Fresh Semen Parameters

The peccary ejaculates showed a milky aspect, with a whitish color and pH 7.2 ± 0.1. The mean volume obtained was 3.9 ± 0.4 mL, with a sperm concentration of $470 \pm 33.7 \times 10^6$ sperm/mL, among which 87.8 ± 4.4% showed motility with kinetic parameters, shown in Table 1. Regarding sperm subpopulations, fresh samples presented 75.7 ± 4.6% rapid, 12.1 ± 2.0% medium, 5.6 ± 2.5% low and 6.7 ± 2.9 static sperm.

Table 1. Kinetic parameter values (mean ± SE) of the collared peccaries (n = 10) fresh ejaculates used in the experiment.

		Sperm Kinetic Motility Patterns								
Treatments	**Time**	**Total Motility (%)**	**Progressive Motility (%)**	**VAP (μm/s)**	**VSL (μm/s)**	**VCL (μm/s)**	**ALH (μm/s)**	**BCF (Hz)**	**STR (%)**	**LIN (%)**
Fresh		87.8 ± 4.4	71.2 ± 4.6	57.4 ± 5.1	45.0 ± 4.4	110.0 ± 8.8	5.6 ± 0.3	33.4 ± 1.0	76.1 ± 1.1	41.3 ± 1.7

Additionally, fresh samples presented an 85.9 ± 3.3% functional membrane, 87.8 ± 2.2% viable sperm and 87.4 ± 2.1% with mitochondrial activity. An average of 204.2 ± 11.6 peccary sperm was bound to the hen eggs' perivitelline membrane.

3.2. Sperm Motility Characteristics, Kinetic Parameters and Subpopulations

Immediately after thawing, the highest concentrations of SDS, 0.3% (30.5 ± 4.7%) and 0.5% (31.2 ± 6.3%), impaired the preservation of sperm motility ($p < 0.05$) when compared to the control treatment without SDS (41.8 ± 3.5%) or to the treatments containing Equex (41.8 ± 4.4%) or 0.1% SDS (41.2 ± 5.5%), as observed in Figure 1. Additionally, the treatment containing 0.1% SDS optimized the preservation of STR immediately after thawing in comparison to the negative control ($p < 0.05$). During the thermal resistance test, all treatments maintained the ALH and STR for 30 min after thawing ($p < 0.05$), while the other kinetic parameters suffered a significant drop at each evaluation at 30 and 60 min.

For sperm subpopulations (Figure 2), the 0.5% SDS increased ($p < 0.05$) the proportion of static sperm in comparison to the negative control and groups containing Equex or SDS 0.1%. This was even more evident during the thermal resistance test, since a significant increase in the number of static spermatozoa was seen in all treatments at each evaluation, with a consequent reduction ($p < 0.05$) in motile spermatozoa in the rapid, medium or slow subpopulations.

3.3. Sperm Membrane Functionality and Integrity and Mitochondrial Activity

Immediately after thawing, the highest concentration of SDS (0.5%) impaired the membrane functionality and mitochondrial activity (Figure 3) when compared to other treatments ($p < 0.05$). As for the thermal resistance test, all treatments maintained membrane functionality for 30 min, only suffering a significant drop at 60 min ($p < 0.05$). The membrane integrity and mitochondrial activity parameters declined after 30 min in all treatments ($p < 0.05$). The group containing 0.5% SDS caused a significantly more pronounced decline in membrane functionality and mitochondrial activity over time ($p < 0.5$) compared to the control group.

3.4. Sperm-Binding Ability

There were no differences among treatments after thawing with regard to sperm binding ability (Table 2).

Table 2. Mean (±SE) values and range (min–max) for number of sperm bound to the hen egg yolk perivitelline membrane in collared peccary frozen–thawed semen diluted in Tris–egg yolk, with or without detergent (Equex STM® paste 0.5%; or sodium dodecyl sulfate–SDS: 0.1%, 0.3% or 0.5%) (n = 10).

Number of Bound Sperm	**Frozen–Thawed Semen**				
	Negative Control	**Equex**	**SDS 0.1%**	**SDS 0.3%**	**SDS 0.5%**
Mean ± SE	138.1 ± 10.8 [A]	126.1 ± 9.0 [A]	131.8 ± 8.0 [A]	144.3 ± 13.6 [A]	131.6 ± 9.3 [A]
Min	84.4	74.3	110.0	103.4	81.0
Max	188.7	174.7	193.6	147.0	180.2

[A] Uppercase superscript letters indicate a significant difference between the experimental groups ($p < 0.05$).

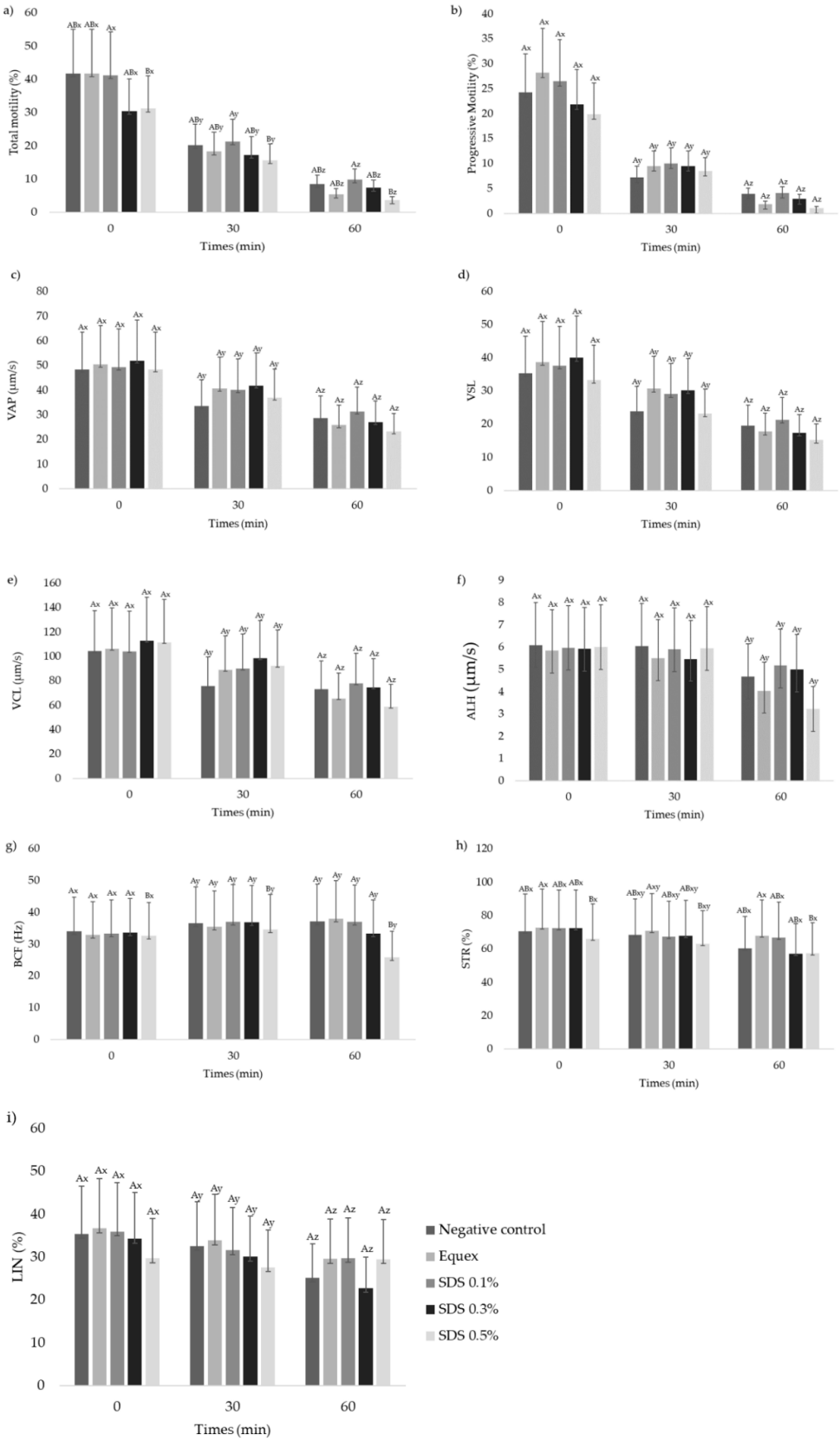

Figure 1. Values (mean ± SE) for the kinetic parameters (**a**–**i**) of the frozen–thawed semen of collared peccaries diluted in Tris–egg yolk, with or without detergent (Equex STM® paste 0.5%; or sodium dodecyl sulfate—SDS: 0.1%, 0.3% or 0.5%) (n = 10). ^{AB} Uppercase superscript letters indicate a significant difference between experimental groups within the same time ($p < 0.05$); ^{xyz} lowercase superscript letters indicate a significant difference within the experimental group over time ($p < 0.05$).

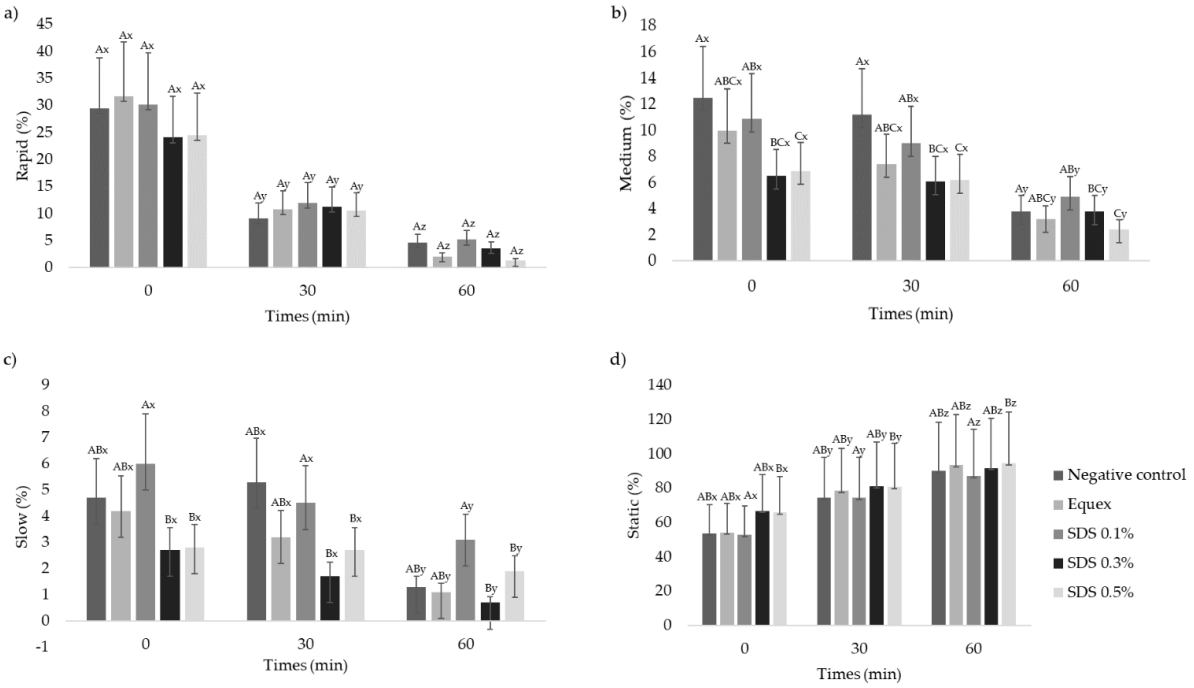

Figure 2. Values (mean ± SE) for sperm subpopulations rapid% (**a**), medium% (**b**), slow% (**c**) and static% (**d**) of the frozen–thawed semen of collared peccaries diluted in Tris–egg yolk, with or without detergent (Equex STM® paste 0.5%; or sodium dodecyl sulfate—SDS: 0.1%, 0.3% or 0.5%) (n = 10). ^{ABC} Uppercase superscript letters indicate a significant difference between experimental groups within the same time ($p < 0.05$); ^{xyz} lowercase superscript letters indicate a significant difference within the experimental group over time ($p < 0.05$).

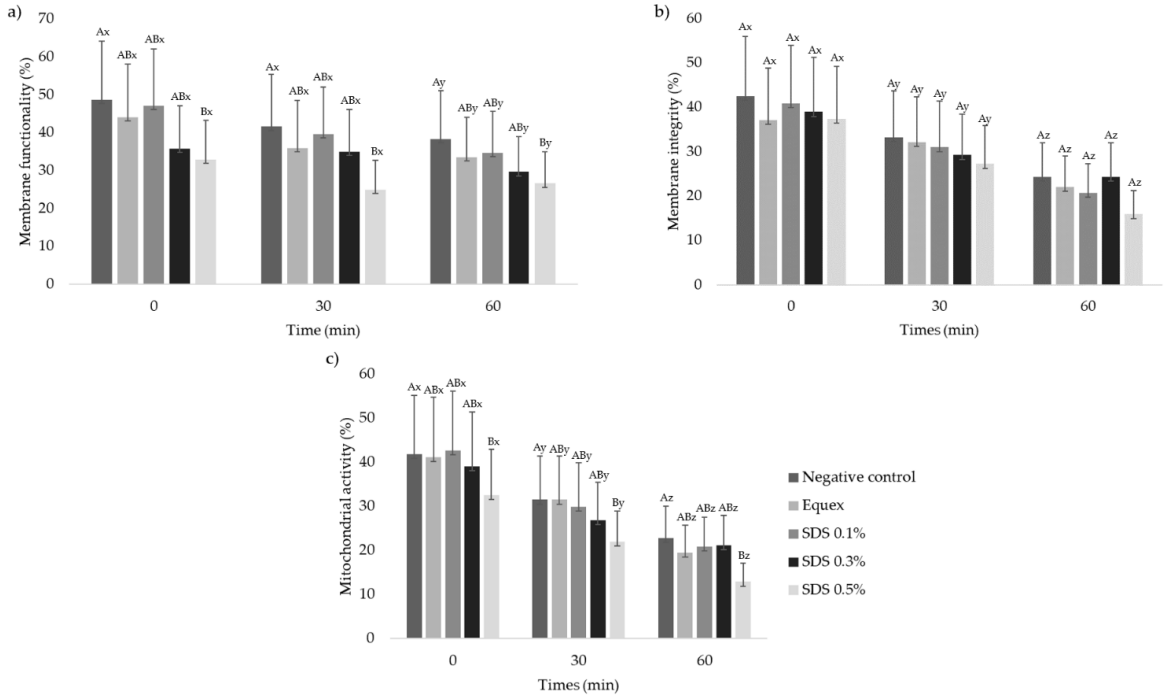

Figure 3. Values (mean ± SE) for membrane functionality (**a**), integrity (**b**) and mitochondrial activity (**c**) of collared peccary frozen–thawed semen diluted in Tris–egg yolk, with or without detergent (Equex STM® paste 0.5%; or sodium dodecyl sulfate—SDS: 0.1%, 0.3% or 0.5%) (n = 10). ^{AB} Uppercase superscript letters indicate a significant difference between experimental groups within the same time ($p < 0.05$); ^{xyz} lowercase superscript letters indicate a significant difference within the experimental group over time ($p < 0.05$).

4. Discussion

In an attempt to contribute to the formation of biobanks, we provided effective information about the actions of SDS-based detergents on some functional parameters of collared peccary sperm submitted to freezing and thawing processes. In general, we found that SDS alone had a positive effect on sperm kinetic parameters, especially regarding motility and straightness (STR). However, an increase in its concentration could cause significant damage to the functional parameters of collared peccary sperm, especially the plasmatic membrane functionality and mitochondrial activity. From an experimental point of view, the possibility of using isolated SDS in the formulation of extenders for semen would be an advantage over the commercial detergent Equex STM paste, since its composition is not exactly known.

SDS is an anionic detergent that seems to potentialize the cryoprotective effect of the egg yolk, allowing for a greater distribution of phospholipid molecules in the yolk and, consequently, providing greater protection to the sperm membrane [21], thus, enhancing other sperm functional parameters, such as the motility. As observed in domestic pigs, in which 0.1% SDS showed a positive effect on sperm motility and a negative effect at concentrations above 0.2% [12], the best results in the present study were achieved when 0.1% SDS was incorporated into the extender for the cryopreservation of collared peccary semen. This SDS concentration promoted not only obtaining adequate values of peccary sperm motility immediately after thawing, but also helped contribute to the maintenance of some kinetic parameters, such as the STR. It is known that some kinetic parameters are jointly considered as indicators of sperm vigor, and their evaluation is used as a criterion for the occurrence of hyperactivation, since such parameters are usually intensified in the capacitation event [22,23]. In this sense, SDS seemed to influence the prevention of the early capacitation of collared peccary spermatozoa from being able to maintain such parameters during a short-time incubation after thawing.

Generally, the sperm plasma membrane is the main target to be damaged by cryopreservation and thawing procedures. This probably occurs due to thermal stress that usually promotes changes in the structure of the phospholipid–protein bilayer [24]. In parallel to thermal and osmotic stress, sperm cells are very sensitive to oxidative stress resulting from freezing and thawing procedures, especially because they have a high concentration of polyunsaturated fatty acids in the membrane and a low percentage of antioxidant agents [25]. Protecting the integrity of the sperm plasma membrane is a crucial point in the development of any sperm cryopreservation protocol. If, on the one hand, the addition of SDS in adequate concentrations can promote the protection of the plasma membrane by increasing the solubility of most egg yolk particles, making them more accessible to spermatozoa, and, thus, improving their freezability, when SDS is used in high concentration in the diluent, free SDS molecules increase and can bind directly to the sperm, with devastating results on the sperm membrane [26]. These negative effects are generally related to the excessive fluidity of the plasmatic membrane of spermatozoa exposed to high SDS concentrations [27], as verified for peccary semen exposed to an extender containing 5% SDS. Interestingly, the harmful effect of the high concentration of SDS (0.5%) on collared peccary spermatozoa was more evident on membrane functionality than on its integrity. In fact, a similar event had previously been described in elephants, in which it was suggested that detergents such as Equex may protect the structural integrity of the membrane, but were not able to maintain its capacity for osmotic regulation [28].

In parallel, the highest concentration of SDS (0.5%) promoted a negative effect on the mitochondrial activity of collared peccary sperm, emphasizing that the detergent may perhaps act on the function of this organelle. In fact, the influence of detergent incorporation on this parameter has been little studied and remains to be better elucidated. In a single study found in the consulted literature, the effect of different diluents on the mitochondrial activity of spermatozoa from Iberian pigs was evaluated, but a direct action of detergents on this parameter was not identified [29].

The action of SDS-based detergents on sperm longevity seems to vary according to the species, since the addition of Equex paste to the freezing extender negatively affected the post-thawing quality of Andalusian donkey spermatozoa [30] and seems not necessarily essential for the cryopreservation of dromedary camel semen [31]. Unlike what is observed for canine spermatozoa, which have a post-thawing longevity of approximately seven hours when exposed to diluents containing the SDS detergent [9], collared peccary spermatozoa began a significant decline in most of their functional parameters as early as 30 min after thawing. Despite this fact being previously known [6], the present study showed that not even the incorporation of detergents based on SDS had the capacity to prolong the sperm longevity of collared peccaries after thawing. This emphasizes the importance of using semen immediately after thawing, associated with an efficient method of monitoring the estrous cycle of females [32], when implementing artificial insemination programs in collared peccaries [33].

In the present study, the incorporation of Equex paste or SDS in different concentrations with the Tris–yolk–glycerol diluent did not influence the binding capacity of collared peccary spermatozoa, since similar results were obtained when these supplements were not present in the diluent. Based on the data obtained of our results, SDS had no toxic effect of on sperm binding phenomena. Moreover, we believe that the use of SDS alone would be a more standardized process than the addition of Equex paste, as its composition is not completely known. However, the assay's limitations were evident, and only in vivo insemination tests could reveal the real fertilizing capacity of the frozen/thawed semen samples in the absence or presence of SDS, as recently postulated for canine semen [25].

When establishing an efficient diluent for freezing the semen of a given species, it is essential to consider the practical factors for its use. Extenders containing surfactants such as SDS are known to reduce the occurrence of sperm agglutination in thawed brown bear samples [34]. Likewise, less evidence of sperm agglutination was seen in collared peccary spermatozoa in those groups containing SDS-derived detergents, compared to the group that did not. More than that, the solubilization effect promoted by the detergents also seemed to allow for a better visualization of the collared peccary semen samples when they were evaluated with CASA, presumably by dispersing egg yolk–lipid conglomerates formed after the dilution of the semen [35].

5. Conclusions

In summary, this study revealed important interactions between the SDS-based detergent and the functional parameters of collared peccary spermatozoa, evidencing a relationship with the proportion of this substance in the diluent, since the use of the highest concentration (0.5%) mainly affected its sperm motility, membrane functionality and mitochondrial activity. In this sense, the incorporation of SDS 0.1% in the Tris–yolk–glycerol diluent optimized the sperm functional parameters of peccaries during freezing and thawing procedures.

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Institutional Review Board Statement: All procedures were approved by the UFERSA Animal Ethics Committee, opinion no. 05/2020.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO IV

Supplementation of extender with catalase and superoxide dismutase does notimprove frozen/thawed semen parameters of collared peccary (*Pecari tajacu*)

Periódico a ser submetido:
Reproduction in Domestic Animals
NOVO QUALIS: A3
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Supplementation of extender with catalase and superoxide dismutase does not improve frozen/thawed semen parameters of collared peccary (*Pecari tajacu*)

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Abstract

We evaluated the effects of the addition of catalase (CAT) and superoxide dismutase (SOD) antioxidants, alone or combined, on frozen/thawed collared peccary semen parameters, as well as on intracellular oxidative levels. Semen aliquots from ten individuals were diluted in Tris-yolk with or without the presence of antioxidants CAT (200 IU/mL or 400 IU/mL) and SOD (150 IU/mL or 300 IU/mL) alone or in combination

(CAT: 200 IU/mL + SOD: 150 IU/mL) and then cryopreserved in liquid nitrogen (-196º C). Samples were evaluated for kinetic parameters, membrane functionality and integrity, mitochondrial activity, normal chromatin, sperm morphology, sperm cell binding capacity and intracellular oxidative levels. Fresh seminal parameters were within the normal range for the species. Soon after thawing, there were no significant differences in any of the evaluated parameters. The samples showed total motility ranging from 28.4 ± 4.1% (SOD; 300 IU/mL) to 43.7 ± 3.6% (without antioxidant). All samples were able to maintain 50% of functional cells, 40% of intact cells with mitochondrial activity, over 99% of cells with condensed chromatin, as well as over 70% of morphologically normal cells. With regard to binding capacity, the minimum and maximum values found ranged from 33.8 to 194 spermatozoa bound to the perivitelline membrane. Finally, regarding intracellular oxidative levels, all samples showed similar levels, with values ranging from 0-9. In summary, the addition of SOD or CAT, at the concentrations tested, alone or in combination, does not promote benefits to the cryopreservation of collared collared peccary semen.

Keywords: Cryopreservation; antioxidants, intracellular oxidative stress.

1. Introduction

The formation of biobanks appears as an important alternative to safeguard the genetic material of vulnerable or ecologically important species, such as collared peccaries (*Pecari tajacu* Linnaeus, 1758). Although this ungulate species is distributed throughout the Americas, its population is already considered extinct in northern Argentina, and is in decline in the Atlantic Forest biome (Gongora et al., 2011). At this sense, efforts to establish efficient protocols for cryopreservation of peccary semen determined that the use of Tris extender (Castelo et al., 2010) added with egg yolk (20%),

glycerol (3%) (Alves et al., 2013) and gentamicin (70 μg/mL) (Moreira et al., 2023) is capable of providing post-thawing sperm motility around of 40%. Bearing in mind the idea that the fluid from the accessory glands of the genital tract may play an important role in semen freezability (Moreira et al., 2022), different aspects of collared peccary seminal plasma were deciphered, such as its proteomic profile that highlights the presence of the natural antioxidant glutathione peroxidase (GPX) (Santos et al., 2014), and the identification of a very low antioxidant activity of superoxide dismutase (SOD), in addition to an undetermined action of catalase (CAT) (Santos et al., 2018).

Although this cannot be clearly stated for collared peccaries, the triad formed by the GPX, SOD and CAT enzymes is highlighted as the main antioxidant system generally present in the seminal plasma of most of the mammals, acting as endogenous regulators responsible for maintaining the balance in the physiological reactions of reduction and oxidation (Kowalczyc, 2022). Physiologically, sperm cells produce reactive oxygen species (ROS) through their metabolism and, when under appropriate conditions, are indispensable for sperm maturation (O'Flaherty and Matsushita-Fournier, 2017), and capacitation events (Aitken et al., 2017). At levels above necessary, however, ROS can trigger oxidative effects on lipids, proteins and DNA (Gavriliouk and Aitken, 2015; Aitken et al., 2016), affecting sperm quality (Aitken et al., 2014). Under stressful conditions, the antioxidant mechanisms intrinsic to seminal plasma are not efficient enough to maintain ROS in adequate levels for cell survival (Chatterjee and Gagnon 2001).

The choice of extender, along with the freeze-thaw process, can be a determining factor for semen ROS levels. For example, the use of the Tris-egg yolk diluent can lead to the generation of hydrogen peroxide (H_2O_2), an ROS that is highly harmful to cells, capable of causing a reduction in the sperm kinetic parameters (Bilodeua et al., 2002). In

this regard, the incorporation of antioxidant agents to the semen extender could contribute to the oxidative balance and maintenance of the quality of semen parameters during the freezing and thawing procedures. Among the antioxidants, SOD and CAT are among the agents commonly added to the semen of human (Rossi et al., 2001) and domestic species (Prete et al., 2016; Arslan et al., 2019; Schafer-Somi et al., 2021;), given their positive effects on the conservation of sperm parameters during the freezing/thawing processes. In pigs, the domestic species most phylogenetically close to collared peccary, the use of antioxidant enzymes SOD and CAT, isolated or combined, in the diluent for semen cryopreservation, resulted in positive effects on kinetic parameters and sperm viability, providing adequate ROS rates (Roca et al., 2005), in addition to reducing the percentage of sperm with changes similar to apoptosis (Trzcinska and Bryla, 2015).

Thus, this study aimed to investigate the effect of incorporating the antioxidants CAT and SOD in different concentrations, isolated or combined, to the extender for the cryopreservation of collared peccary semen, evaluating the morphological and functional parameters of the sperm, its binding ability, and intracellular oxidative stress.

2. Materials and methods

2.1 Animal Ethics and management

All experimental procedures were authorized by the ethics committee of the Universidade Federal Rural of Semi-Árido (UFERSA) (nº 05/2020). The experimental protocols were approved by the Instituto Chico Mendes de Biodiversidade (Opinion nº 37329/3). Reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Ten sexually mature collared peccary, with an average age of 40 months, were used. These were grouped in paddocks (20 m × 3 m) with up to three animals, under a

natural photoperiod of 12 h, at the Wild Animal Multiplication Center located on the UFERSA campus, Mossoró, Brazil (5°10'S-37°10' W; medium temperature range, 27 – 29 °C). The study was conducted at the end of the rainy season (May to June 2022). Throughout the experimental period, the animals were fed an isocaloric (3300 kcal/kg) and isoproteic (14% protein) diet, supplemented with tropical fruits and water *ad libitum*.

2.2 Experimental Design

After obtaining ejaculates through electroejaculation, an aliquot of fresh semen was immediately evaluated, and another six aliquots were diluted in Tris-yolk-glycerol and then cryopreserved in liquid nitrogen. Regarding the dilution, the first aliquot did not receive antioxidant (control group), while the second and third aliquots were supplemented with catalase (200 IU/mL and 400 IU/mL; Sigma-Aldrich, St Louis, MO, USA), the fourth and fifth aliquots were supplemented with superoxide dismutase (150 IU/mL and 300 IU/mL; Sigma-Aldrich, St Louis, MO, USA) and the sixth and last aliquot was supplemented with the combination of 200 IU/mL catalase and 150 IU/mL of superoxide dismutase, as previously tested in swine (Roca et al., 2005). Frozen/thawed samples were evaluated for sperm kinetic parameters, membrane functionality and integrity, mitochondrial activity, chromatin condensation, sperm morphology, sperm binding ability and level of intracellular reactive oxygen species.

2.3 Semen collection and evaluation

The animals were fasted for 12 hours and then restrained with a handnet, accompanied by anesthetic protocol with propofol (Propovan®, Cristália, Fortaleza, Brazil) in bolus (5 mg/kg) intravenously. During the experiment, the animals were monitored for their cardiac and respiratory parameters (Souza et al., 2009). The animals

were positioned in lateral decubitus and submitted to semen collection using the electroejaculation protocol previously established for the species (Castelo et al., 2010), using of a portable device (Autojac®, Neovet, Campinas, SP, Brazil) connected to a 12-V source. The stimulatory cycle consisted of 10 stimuli at each voltage, starting at 5 V, followed by an increase in voltage from 1 V to 12 V, lasting 3 seconds of the electrical stimulus, with intermittent intervals of 2 seconds, over 10 minutes. The electroejaculator probe had the dimensions of 15 x 1.3 cm, in which 12 cm was inserted into the rectum.

Ejaculates were collected in plastic tubes and immediately evaluated for appearance and color. Semen volume was measured by repeated pipetting. The pH was defined with pH indicator strips (Neutralit®, Merck, Bucharest, Romania) (Souza et al., 2009) and the sperm concentration (in millions of sperm/mL) was determined by counting cells in a Neubauer chamber, after dilution (1:2) in formalized buffered solution (10%) (Silva et al., 2014). Fresh semen was subjectively evaluated, still in the field, for motility and vigor (0 – 5), using light microscopy (100×) (Castelo et al., 2010).

2.4 Semen Freezing-Thawing

For freezing, the samples were diluted and cryopreserved in Tris containing egg yolk (20%), glycerol (3%) and gentamicin (70 µg/mL) (Moreira et al., 2022) and destined for the following treatments: (A) control (without antioxidant), (B) 200 IU/mL of catalase (CAT; Sigma-Aldrich, St Louis, MO, USA) C) 400 IU/mL of CAT (Sigma-Aldrich, St Louis, MO, USA) , (D) 150 IU/mL superoxide dismutase (SOD; Sigma-Aldrich, St Louis, MO, USA), (E) 300 IU/mL SOD (Sigma-Aldrich, St Louis, MO, USA) and (F) combination of 200 IU/mL CAT and 150 IU/mL SOD, as previously tested for domestic swine (Roca et al., 2005). The samples were frozen following a rapid freezing curve previously described by Silva et al., 2013. Briefly, the curve consisted of refrigerating the

samples at 15° C for 40 min in isothermal boxes and stabilization at 5° C for another 30 min in a biological incubator (Quimis, Diadema, SP, Brazil). Then, the samples were placed in 0.25 mL plastic straws, which were exposed to nitrogen vapor (5 cm) for 5 min and stored in a cryobiological cylinder at -196º C. After 1 week, the samples were thawed in a water bath at 37°C for 1 min (Souza et al., 2016) and their sperm parameters were immediately evaluated.

2.5 Computer Aided Semen Analysis

Information on frozen-thawed sperm motility characteristics and kinetic parameters was obtained by computerized semen analysis (IVOS 7.4 G; Hamilton-Thorne Research, Beverly, MA, USA), with pre-established settings for the species, as temperature 37 °C; 60 frames/s; minimum contrast, 45; straightness threshold, 30%; low-velocity average pathway (VAP) cutoff, 10 m/s; and medium VAP cutoff, 30 m/s (Souza et al., 2016). The parameters evaluated were total and progressive motility (%), velocity average pathway (VAP; µm/s), velocity straight line (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head (ALH; µm), beat cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %). When there was a low VAP cutoff (LVC) and medium VAP cutoff (MVC), the overall sperm population was subdivided into four categories: rapid, with VAP > MVC; medium, with LVC < VAP < MVC; slow, with VAP < LVC; and static for the absence of cell motility. For a reliable assessment of sperm motility patterns, the Edit IVOS 7.4G System Tracks option was used to exclude debris derived from the diluents. There was further dilution in saline solution (1:2) only when necessary (Souza et al., 2016).

2.6 Sperm Membrane Functionality

Sperm membrane functionality was observed through osmotic response to the hyposmotic test using distilled water as hyposmotic solution (0 mOsm/L). 200 cells were counted in which those with curly tail were judged as having a functional membrane under light phase contrast microscopy (Alttion®, Wuzhou City, Guangxi Province, China) (Santos et al., 2013).

2.7 Plasma Membrane Integrity and Mitochondrial Activity

Plasma membrane integrity and mitochondrial activity in fresh and thawed samples were concomitantly observed through the association of fluorophores Hoechst 342 (H342; Sigma-Aldrich, St Louis, MO, USA), Mito Tracker red® (CMXRos, Molecular Probes, M -7512) and Propidium Iodide (IP, Sigma-Aldrich, Co., St Louis, MO, USA). A total of 200 cells were counted under epifluorescence microscopy (EFA halogen fluorescent lamp set. Leica. Kista, Sweden), in which those with the head marked in blue (H-342) were considered to have an intact membrane and those with the head marked in red (PI) as with non-intact membrane. Also, cells with the midpiece marked in red were considered to have active mitochondrial function (Sousa et al., 2013).

2.8 Chromatin condensation status

The state of chromatin condensation in fresh and thawed samples was evaluated using a seminal smear fixed in ethanol: 3:1 acetic acid (1 min), 70% ethanol (3 min) and in 4M HCl solution (25 min) and stained with toluidine blue dye. A total of 500 cells were counted under light microscopy (100×), in which those slightly stained blue were considered to have normal chromatin (negative) and those stained from violet to dark blue were considered to have altered chromatin (positive) (Beletti et al., 2004).

2.9 Sperm morphology

Sperm morphology in fresh and thawed samples was verified through semen smears stained with rose bengal dye, under light microscopy (100×). 200 cells were counted per slide, whose morphology was classified according to head, middle piece and tail defects (Sousa et al., 2013).

2.10 Sperm binding ability assay

The binding capacity of fresh and frozen/thawed spermatozoa was evaluated using the hen egg perivitelline membrane binding test, as previously established for peccaries (Campos et al., 2017). Briefly, fresh, infertile eggs were used. The membranes were obtained by separating the yolk and the white, standardizing cuts of 1 cm^2, with two membranes per treatment. Semen samples were diluted (1:1) in an incubation solution (114 mM NaCl; 3.1 mM KCl; 0.4 mM NaH2PO4; 10 mM calcium lactate; 25 mM NaHCO3; 10 µg/mL phenol; 1 .4 mM caffeine; 2.0 mM CaCl2.2H2O; 0.5 mM MgCl2; 10 mM Hepes; 6 mg/mL BSA; 5.5 mM glucose; 0.45 mM sodium pyruvate; 40 µg/mL gentamicin; pH 7,47,8) and then centrifuged at 700 xg for 10 min in order to establish a sperm concentration of 1 x 106 sperm/mL. The membranes accompanied by the sperm solution were incubated at 38.5°C for 20 min in a water bath and then washed in 100 µL drops of the incubation solution and kept in Hoechst 33250 for 15 min. Finally, the membranes were placed on a slide and coverslip and evaluated for the number of attached spermatozoa in six random fields under epifluorescence microscopy (400x; Episcopic Fluorescent Attachment EFA Halogen Lamp Set. Leica. Kista, Sweden).

2.11 Intracellular oxidative stress

Thawed samples were evaluated for intracellular oxidative stress by means of epifluorescence microscopy (400x; Episcopic Fluorescent Attachment EFA Halogen Lamp Set. Leica. Kista, Sweden), using the marker 2', 7'-dichlorofluorescein diacetate (H2DCFDA) (Thermo Fisher Scientific, USA).

For the evaluation, a sperm concentration of 50 x 10^6 sperm/mL was established. Initially, an aliquot of 50 µL of a solution of PBS and H2DCFDA [2.5 mL of PBS and 0.5 µL (0.1 M) of 2', 7'-dichlorofluorescein diacetate] was homogenized in an aliquot of 50 µL of sperm suspension (1:1) and incubated at 37°C for 30 minutes in the dark. Then, the sperm suspension was subjected to two washes in PBS (50 µL and 20 µL) after centrifugation at 500 G for 5 min (Santos et al., 2023).

Finally, an aliquot of 5 µL of the sample was added to the slide, covered with a coverslip, and sperm images obtained in an epifluorescence microscope (Episcopic Fluorescent Attachment EFA Halogen Lamp Set. Leica. Kista, Sweden) in order to determine the percentage of viable spermatozoa with intracellular ROS, where the intensity The fluorescence of the images were quantified using the ImageJ 1.45s software (National Institutes of Health, Bethesda, Maryland, USA) (Santos et al., 2023). The background signal intensity was subtracted from the values obtained for the treatment images. From each sample, up to 100 sperm were selected for the quantification of fluorescence intensity. The group without antioxidant supplementation (control) were used to calibrate the measurements, and the measured value of each sperm was divided by the mean of the calibrator to generate relative expression levels [arbitrary fluorescence units (AFU)] (Santos et al., 2023).

2.12 Statistical analysis

Results were expressed as mean and standard error. Data were verified for

normality by the Shapiro-Wilk test and homoscedasticity by the Levene test. The percentage data were transformed into arcosen, when necessary, to meet the parametric assumptions. An ANOVA was performed, followed by Tukey's post hoc test to assess the differences between treatments, using the Jamovi 2.1 software. Data regarding viability, morphology, binding capacity, ROS, VAP, VSL, VCL, ALH, STR and slow sperm subpopulations were analyzed using the non-parametric Kruskal-Wallis test. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Fresh Semen Parameters

The ejaculates had a milky, whitish appearance. An average volume of 3.04 ± 0.5 mL was obtained, whit a pH of 7.07 ± 0.07 and sperm concentration of 429 ± 40.4 $x10^6$ spermatozoa/mL, among which 96.1 ± 1.1% were motile and with vigor of 4.78 ± 0.1, 92.1 ± 1.7% with functional membrane, 80.1 ± 3.0% membrane integrity, 79.0 ± 4.8% with mitochondrial activity, 99.6 ± 0.1% with normal chromatin and 70.1 ± 4.0% morphologically normal. An average of 245 ± 20.4 sperm was found to be bound to the perivitelline membrane of hen eggs in peccary fresh semen samples.

3.2 *Post-thawing sperm motility and kinematic parameters*

Immediately after thawing, the samples showed total motility ranging from 28.4 ± 4.1 % (SOD; 300 IU/mL) to 43.7 ± 3.6 % (without antioxidant), with a progressive motility ranging from 17.0 ± 3.0 % (SOD; 300 IU/mL) to 25.8 ± 3.4 % (without antioxidant). However, these parameters, as well as the other kinematic parameters and their subpopulations, did not differ among treatments (Table 1).

Table 1. Values (mean ± SE) for kinetic parameters of collared peccary (n=10) frozen-thawed semen diluted in tris-yolk-glycerol, without or with antioxidants [Catalase (CAT) 200 or 400 UI/mL; Superoxide Dismutase (SOD) 150 or 300 UI/mL and CAT/SOD 200/150 UI/mL].

	Frozen-Thawed Semen*					
Sperm kinetic motility patterns	Control	CAT (200 UI/mL)	CAT (400 UI/mL)	SOD (150 UI/mL)	SOD (300 UI/mL)	CAT/SOD (200 UI/mL/ 150 UI/mL)
Total Motility (%)	43.7 ± 3.6	36.5 ± 4.2	31.4 ± 3.7	34.7 ± 5.9	28.4 ± 4.1	31.9 ± 4.1
Progressive Motility (%)	25.8 ± 3.4	20.7 ± 2.6	17.3 ± 2.7	20.6 ± 4.2	17.0 ± 3.0	19.6 ± 3.0
Velocity average pathway (µm /s)	30.1 ± 2.5	30.2 ± 2.7	27.4 ± 1.4	29.3 ± 1.3	33.6 ± 3.5	30.8 ± 2.9
Velocity straight line (µm /s)	27.9 ± 2.3	28.7 ± 2.1	25.3 ± 1.1	28.1 ± 1.1	32.2 ± 3.1	29.5 ± 2.4
Velocity curvilinear (µm /s)	50.3 ± 3.1	50.4 ± 3.7	48.6 ± 2.1	48.8 ± 4.1	54.1 ± 4.8	51.0 ± 4.3
Amplitude lateral head (µm/s)	2.4 ± 0.3	2.4 ± 0.2	2.1 ± 0.2	2.2 ± 0.3	2.5 ± 0.3	2.4 ± 0.2
Beat cross frequency (Hz)	16.3 ± 1.1	17.4 ± 0.8	16.0 ± 0.8	16.1 ± 1.2	17.0 ± 1.4	16.6 ± 1.1
Straightness (%)	20.2 ± 1.4	21.4 ± 1.5	20.0 ± 1.3	20.3 ± 1.3	21.7 ± 0.8	21.3 ± 0.9
Linearity (%)	17.4 ± 0.9	17.7 ± 0.8	17.3 ± 0.2	17.5 ± 0.4	18.4 ± 0.7	18.9 ± 0.6
Sperm subpopulations						
Rapid (%)	31.6 ± 3.7	26.3 ± 3.9	21.3 ± 3.0	24.2 ± 4.4	20.5 ± 3.2	23.8 ± 3.4
Medium (%)	12.2 ± 1.4	10.4 ± 1.0	10.1 ± 1.6	10.3 ± 2.3	7.8 ± 1.2	8.1 ± 1.2

Slow (%)	5.0 ± 1.5	5.8 ± 2.1	3.7 ± 0.8	3.1 ± 0.7	2.5 ± 0.4	2.9 ± 0.4
Static (%)	51.6 ± 3.3	57.6 ± 4.4	64.9 ± 4.2	62.2 ± 6.3	69.1 ± 4.4	65.2 ± 4.3

*There were no significant differences among the different treatments ($P > 0.05$).

3.3 *Post-thawing sperm functional and morphologic parameters*

After thawing, all treatments were similarly capable to maintain 50% sperm membrane functionality, regardless the use of antioxidants (Table 2). Furthermore, membrane integrity and mitochondrial activity were maintained in 40% of cells after thawing regardless the treatment ($P > 0.05$) (Table 2). Regarding chromatin condensation, there were no differences among treatments that similarly provided more than 99% cells with normal status (Table 2). For sperm morphology, all treatments provided the preservation of >70% normal cells, regardless the presence of antioxidants in the extender (Table 2).

Table 2. Values (mean ± SE) for membrane functionality, viability, mitochondrial activity and normal morphology of collared peccary (n= = 10) frozen-thawed semen diluted in tris-yolk-glycerol, without or with antioxidants [Catalase (CAT) 200 or 400 UI/mL; Superoxide Dismutase (SOD) 150 or 300; UI/mL and CAT/SOD 150/200 UI/mL].

	Frozen-Thawed Semen*					
Sperm Parameters*	Control	CAT (200 UI/mL)	CAT (400 UI/mL)	SOD (150 UI/mL)	SOD (300 UI/mL)	CAT/SOD (200 UI/mL/150 UI/mL)
Membrane functionality (%)	55.2 ± 5.3	53.6 ± 7.0	50.4 ± 5.7	55.9 ± 7.3	51.1 ± 5.1	54.1 ± 5.6
Membrane Integrity (%)	46.3 ± 2.8	39.1 ± 1.7	33.5 ± 3.7	33.8 ± 4.6	39.6 ± 5.0	38.0 ± 2.0
Mitochondrial activity (%)	43.3 ± 3.4	35.4 ± 2.7	34.7 ± 3.5	33.2 ± 4.8	31.9 ± 3.8	38.5 ± 2.4
Normal chromatin (%)	99.3 ± 0.1	99.5 ± 0.1	99.4 ± 0.1	99.5 ± 0.1	99.6 ± 0.1	99.6 ± 0.1
Normal morphology (%)	74.8 ± 4.6	73.5 ± 6.5	79.2 ± 4.7	77.9 ± 4.3	70.5 ± 6.4	76.3 ± 5.3

* There were no significant differences among the different treatments ($P > 0.05$).

3.4 Post-thawing sperm-binding ability

There were no differences among treatments after thawing with regards to the sperm binding ability (Table 3). Based on all samples, the minimum and maximum values found ranged from 33.8 to 194 spermatozoa bound to the perivitelline membrane of chicken egg yolks.

Table 3. Values (mean ± SE) for the number of collared peccary (n = 10) spermatozoa cryopreserved in tris-yolk-glycerol, without ow with antioxidants (Catalase (CAT) 200 or 400 UI/mL; Superoxide Dismutase (SOD) 150 or 300 UI/mL and CAT/SOD 200/150 UI/mL) bound to the perivitelline membrane (n = 10).

Number of Bound Sperm	Frozen-Thawed Semen*					
	Control	CAT (200 UI/mL)	CAT (400 UI/mL)	SOD (150 UI/mL)	SOD (300 UI/mL)	CAT/SOD (200 UI/mL/150 UI/mL)
mean ± SE	106 ± 14.0	120 ± 12.6	139 ± 11.0	121 ± 11.0	136 ± 16.0	126 ± 8.3
Min	33.8	54.9	86.9	43.5	36.3	89.7
Max	162	178	171	150	194	177

* There were no significant differences among the different treatments (P > 0.05).

3.5 Post-thawing intracellular oxidative stress

Regardless the use of antioxidants, all treatments provided similar ROS levels. The minimum and maximum ROS values found in the thawed samples were 0-9, respectively (Figure 1).

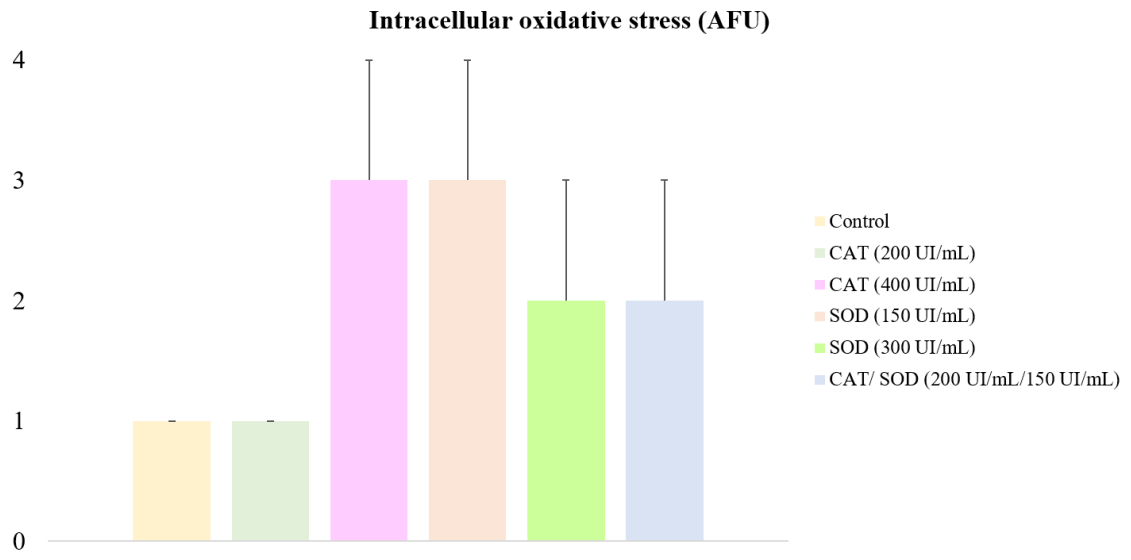

Figure 1. Intracellular oxidative stress (AFU) in collared peccary semen cryopreserved in Tris-yolk-glycerol without or with antioxidants (Cat 200 or 400 IU/mL; Sod 150 or 300 IU/mL and CAT/SOD 200/150 IU/mL).

4. Discussion

As an attempt not only to better preserve collared peccary semen, but also in order to better understand their sperm physiology, we investigated the effect of antioxidants added to the extender for semen cryopreservation. Thus, we verified that the addition of SOD or CAT, in the tested concentrations, alone or in combination, do not promote benefits to the cryopreservation of collared collared peccary semen. In fact, this work denotes that the improvement of the collared peccary freezing protocol is closely linked to biochemical investigations of their seminal plasma or sperm cells, which seem to be more complex than we believe to be.

In this way, the reason for using CAT and SOD antioxidants in the seminal extender is due to the confirmed presence of the SOD antioxidant in the enzymatic system of the seminal plasma of collared peccaries (Santos et al., 2018), whose role is to protect sperm cells against oxidative damage resulting from the generation of ROS, converting the O^{2-} radical into O_2 and H_2O_2 (Trzcinska and Bryla, 2015), in which CAT would promote the reduction of H_2O_2, which is highly harmful to cells, into H_2O and O_2 (Aitken, 1997). Thus, the concentrations and combinations of antioxidants used were based on previous work in pigs, a species phylogenetically close to peccaries (Roca et al., 2005; Trzcinska and Bryla, 2015), in which excellent results were obtained regarding sperm survival and reduction of oxidative levels after semen thawing (Roca et al., 2005; Trzcinska and Bryla, 2015). Despite the similarity between the species, possibly the concentrations of antioxidants used in our work were not enough to promote significant effects on the seminal parameters of collared peccaries.

In species such as sheep, the presence of low concentrations of antioxidants (5, 10, 20 or 100 U/mL) showed no effect on refrigerated or thawed seminal parameters (Graaf et al., 2007, Câmara et al., 2011). In turn, at higher concentrations (CAT: 200 U/mL-1, SOD: 800 U/mL-1), antioxidants resulted in greater sperm survival and higher rates of fertilized oocytes after semen refrigeration (5º C or 25º C) (Maxwell and Stojanov, 1996). Furthermore, the combination of enzymes, during storage at 5º C, provided better sperm survival than that observed when the enzymes were used alone. However, particularly CAT, at concentrations above 200 U/mL^{-1}, revealed toxic effects on sperm cells, causing a reduction in motility and integrity of the acrosome (Maxwell and Stojanov, 1996).

In addition to the doses of antioxidants used, another factor that may have contributed to our results was the presence of egg yolk in Tris diluent medium. This is

because the egg yolk has lipoproteins with antioxidant properties in its composition (Yamamoto and Omori, 1994). Thus, this may have contributed to the control of ROS levels in thawed samples (Yamamoto and Omori, 1994), preventing the oxidation of polyunsaturated fatty acids (Sakanaka et al., 2003).

Endogenously, seminal plasma is characterized by different biochemical constituents responsible for the regulation of sperm physiological mechanisms (Guasti et al., 2012). Among these components, there are some enzymatic (catalase, superoxide demutase and glutathione peroxidase) and non-enzymatic (albumin, taurine, hypotaurine, pyruvate, ascorbic acid, tocopherol and ergothionine) antioxidant agents that can act to protect sperm cells against damage by oxidative stress (Guasti et al., 2012).

In collared peccaries, seminal plasma collected during the dry period of the northeastern semi-arid region has SOD enzymes (0.033 AU/mgP) (Santos et al., 2018) and glutathione peroxidase (GPx; 6.2%) as an enzymatic system (Santos et al., 2014), while the presence of CAT was not identified during this period (Santos et al., 2018).Therefore, we presume that the levels of antioxidant components in the seminal plasma of collared peccaries may vary due to the seasonal period, as already verified in other biochemical components of the seminal plasma of the species (Moreira et al., 2019). This is because our work was conducted during the rainy season in the semi-arid region, characterized by offering better conditions for freezing collared peccary semen (Maia et al., 2018). However, the hypothesis of influence of the seasonal period on the antioxidant system of the seminal plasma of collared peccaries can only be confirmed through a comparative study between the antioxidant components present in the seminal plasma during the dry and rainy seasons of the region.

5. Conclusions

In summary, the addition of SOD or CAT, at the concentrations tested, alone or in combination, does not promote benefits to the cryopreservation of collared collared peccary semen. This is valuable information to be considered, as it proves the existing complexity in the seminal plasma of these animals, revealing the need for further investigation into its antioxidant constituents, as well as the existence of possible changes due to the seasonal period.

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Conflict of interest

None of the authors have conflict of interest.

Data Availability Statement

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

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CAPÍTULO V

Retrospective analysis of sperm metrics in fresh ejaculates of collared peccaries presenting contrasting semen freezability

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Retrospective analysis of sperm metrics in fresh ejaculates of collared peccaries presenting contrasting semen freezability

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Abstract

Based on a retrospective study of sperm parameters in fresh ejaculates of collared peccaries classified as good, moderate and bad freezers, this work aimed to identify whether these animals have different sperm characteristics that allow predicting their contrasting freezability. For this purpose, ejaculates of twenty-six animals were obtained, from which an aliquot of fresh semen was immediately evaluated, while another aliquot was diluted in Tris-yolk-glycerol diluent and cryopreserved. Fresh and frozen/thawed samples were evaluated for sperm kinetic parameters, membrane integrity, mitochondrial activity, membrane functionality, sperm morphology and morphometry, and sperm binding capacity. Based on post-thaw motility, the animals were classified as good (>40% motile sperm), moderate (30% to 40% motile sperm), and frozen poor (<30% motile sperm). Based on the information obtained, a retrospective study of fresh ejaculates was carried out, in which the samples were

reclassified according to the groups obtained after thawing. Data were checked for normality using the Shapiro-Wilk test and homoscedasticity using the Levene test. Percentage data were arc-transformed whenever necessary to meet parametric assumptions. An ANOVA was performed, followed by Tukey's post hoc test to assess differences between treatments, using Jamovi 2.1 software. Differences were considered significant when $P < 0.05$. Based on the post-thaw motility determined by CASA (Table 1), seven individuals were classified as good freezers, ten as moderate and nine as poor. Certainly, the highest ($P < 0.05$) mean sperm motility values were found for good freezers (50.1 ± 3.3%), to the detriment of moderate (35.0 ± 1.0%) and poor freezers (18 .3 ± 1.6%). The good freezers also had the highest mean values ($P < 0.05$) for progressive sperm motility (30.6 ± 3.7%) and fast sperm ratio (36.0 ± 4.0%), while the bad ones freezers had the highest amount ($P < 0.05$) of static spermatozoa (80.0 ± 1.6%). As for fresh semen, no significant difference was verified regarding the semen characteristics of the three groups, however, the bad freezers provided the lowest ($P < 0.05$) percentage of spermatozoa with intact membrane (77.3 ± 1.7%) . With regard to sperm morphometry, there was no statistical difference between the groups in terms of head and midpiece length ($P > 0.05$). However, the longest tail length was found in moderate freezers (38.4 ± 0.5 µm), while the shortest tail length was found in poor freezers 26.2 ± 0.3 µm. It can be concluded that sperm membrane integrity in fresh ejaculates can predict freezability in good, moderate and poor freezers.

Keywords: *Collared peccary*; cryresistance; sperm motility.

1. Introduction

The Collared peccary is a wild tayassuid, widely distributed in different biomes, being predominantly concentrated in the Amazon and Pantanal [1]. Its presence is an important indicator of environmental quality [2], and it is currently considered stable in terms of extinction risk, despite being almost threatened in the Atlantic Forest, due to predatory hunting in the region and fragmentation of its habitats [3]. As a strategy for the conservation and multiplication of the species, cryopreservation of male germplasm has been the main biotechnique used, providing the conservation of samples at -196 º C for an indefinite period [4,5,6].

It is well known that the freezing/thawing process causes a significant reduction in the quality of sperm parameters, when compared to fresh semen, compromising the fertility of the samples [7]. However, such reduction in post-thawing sperm quality is

largely influenced by an individual variation. In this aspect, there is a clear difference between the animals, even a heterogeneity between the ejaculates themselves, which may or may not favor greater resistance to osmotic stress resulting from the freezing process [7]. These differences may be favored by the existence of factors linked to the intrinsic characteristics of the animals, such as genetic aspects [8], nutritional status or the presence of antioxidants in seminal plasma [9,10,11,12], as well as extrinsic factors, such as environmental conditions [13]. Such differences in freezability make it possible to differentiate among animals in good and bad freezers, already observed in species such as dogs [14], bulls [15], red deer (*Cervus elaphus hispanicus*) [16] and pigs [17,18,19], a species phylogenetically close to collared peccaries. In pigs, moreover, some authors also support the classification of a third category of animals with moderate freezability, in parallel with good and bad freezers, based on post-thawing sperm motility results [17].

In the abovementioned studies that emphasize such differences between groups of animals of the same species, the existence of characteristics inherent to the sperm parameters in the fresh ejaculate that can predict this contrasting freezability was demonstrated. Among such parameters, the sperm morphology patterns [17,18], the sperm head morphometry [16], the sperm membrane integrity and functionality [20], the binding ability [21], and the sperm motility and kinematic patterns determined by computerized semen analysis systems - CASA [14] have been highlighted for presenting differences in the fresh ejaculate of animals with varied freezability.

From the initial experiments [4] to the most recent ones [6], variability in the freezability of collared peccary semen has been empirically observed; however, this factor has never been explored before. In this sense, the present work shows a retrospective analysis of sperm parameters in fresh ejaculates of peccaries classified as

good, moderate and bad freezers. The objective was to identify whether such animals have different sperm characteristics that allow predicting their contrasting freezability.

2. Materials and methods

2.1 Bioethics and animals

The experimental procedures involving the use of animals were approved by the ethics committee of the Federal Rural University of the Semi-Arid (UFERSA) (nº 05/2020). Twenty-six adult males with a mean age of 40 months were used. These animals were donated by the Center for the Multiplication of Wild Animals (CEMAS-UFERSA), which were allocated in groups of three animals in paddocks (20 m × 3 m) containing a covered area (3 m × 3 m) under a natural photoperiod of 12 pm. The animals received an isocaloric (3300 kcal/kg) and isoproteic (14% protein) diet, supplemented with tropical fruits and water ad libitum.

2.2 Experimental design

After obtaining ejaculates from twenty-six animals, an aliquot of fresh semen was immediately evaluated, while another aliquot was diluted in Tris-yolk-glycerol diluent and cryopreserved. Fresh and frozen/thawed samples were evaluated for sperm kinematic parameters, membrane integrity, mitochondrial activity, membrane functionality, sperm morphology and morphometry sperm and binding ability. Based on post-thawing motility, animals were classified as good (> 40% motile sperm), moderate (from 30% to 40% motile sperm) and poor freezers (≤<30% motile sperm). Based on the information obtained, a retrospective study was carried out regarding the fresh ejaculates,

in which a reclassification of the samples was performed according to the groups obtained after thawing, and subsequently a statistical analysis was performed comparing them.

2.3 Semen collection and evaluation

Prior to the collections, the animals were submitted to a 12-hour fasting. The animals were restrained with a net followed by an anesthetic protocol using propofol (Propovan®, Cristália, Fortaleza, Brazil) bolus at 5 mg/kg intravenously. During the experimental procedure, the animals were submitted to fluid therapy (sterile 0.9% saline solution) and monitored for heart and respiratory rate [22]. The collection took place through electroejaculation using a protocol previously established for the species. For this, a portable device (Autojac®, Neovet, Campinas, SP, Brazil) connected to a 12 V source was used, in which a stimulus cycle consisting of ten stimuli at each voltage was applied, starting at 5 V, followed by a voltage increase in steps from 1 V to 12 V, over 10 minutes, using an electroejaculatory probe measuring 15 × 1.3 cm [4].

Ejaculates were collected in graduated plastic tubes and immediately evaluated for appearance, color, volume using micropipettes and pH using pH indicator strips (Neutralit®, Merck, Bucharest, Romania) [22]. Then, the sperm concentration (in millions of sperm/mL) was counted in a Neubauer chamber by dilution (1:2) in formalized saline solution (10%) [23].

2.4 Computer Aided Semen Analysis

Information on sperm motility characteristics and kinetic parameters was obtained by computerized semen analysis (IVOS 7.4 G; Hamilton-Thorne Research, Beverly, MA, USA), with pre-established settings for the species, as temperature 37 °C;

60 frames/s; minimum contrast, 45; straightness threshold, 30%; low-velocity average pathway (VAP) cutoff, 10 m/s; and medium VAP cutoff, 30 m/s [5]. The parameters evaluated were total and progressive motility (%), velocity average pathway (VAP; µm/s), velocity straight line (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head (ALH; µm), beat cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %). When there was a low VAP cutoff (LVC) and medium VAP cutoff (MVC), the overall sperm population was subdivided into four categories: rapid, with VAP > MVC; medium, with LVC < VAP < MVC; slow, with VAP < LVC; and static for the absence of cell motility. For a reliable assessment of sperm motility patterns, the Edit IVOS 7.4G System Tracks option was used to exclude debris derived from the diluents. There was further dilution in saline solution (1:2) only when necessary [5].

2.5 Sperm functional parameters

Membrane integrity and mitochondrial activity were evaluated using a combination of fluorescent probes containing Hoechst 342 (Sigma-Aldrich, St Louis, MO, USA), Mito Tracker red® (CMXRos, Molecular Probes) and Propidium Iodide (IP, Sigma-Aldrich, Co., St Louis, MO, USA). For this, 200 cells were evaluated in epifluorescence microscopy (episcopic fluorescent accessory EFA Halogen Lamp Set. Leica. Kista, Sweden), whose cells with the head labeled in blue (H) were considered to have an intact membrane and those with the head labeled in red (IP) as having non-intact membrane, in addition, cells with the midpiece marked in red were considered to have mitochondrial activity [24].

Membrane functionality was investigated through the hyposmotic test using distilled water (0 mOsm/L) as hyposmotic solution. An aliquot of semen was diluted (1:9)

and incubated at 37ºC for 45 min. Then, a total of 200 cells were counted in phase contrast microscopy (400x; Alttion®, Wuzhou City, Guangxi Province, China) and analyzed for their osmotic response, whose coiled tail cells were considered to have a functional membrane [25].

The binding capacity of the cells was verified through the test of binding to the perivitelline membrane of the hen egg. For this, fresh and infertile chicken eggs were used, whose membranes were acquired by separating the yolk and the white. After obtaining, the membranes were washed in saline solution at 37º C and submitted to 1 cm² cuts, assigning two membranes to each animal. Then, the samples were diluted (1:1) in a solution of incubation medium (114 mM NaCl; 3.1 mM KCl; 0.4 mM NaH2PO4; 10 mM 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; 5.5 mM glucose; 0.45 mM sodium pyruvate; 40 µg / mL gentamicin sulfate; pH 7.47.8) [26], and subsequently centrifuged at 700 x g for 10 minutes. After centrifugation, the supernatant was discarded and the pellet re-diluted in incubation medium (100 µL) to obtain a final concentration of 1 x 10^6 sperm / mL [26]. Each membrane was incubated with 1 x 10^6 spermatozoa/mL in a water bath at 38.5º C for 20 minutes. Then, they were washed in 100 µL drops of incubation medium and incubated in Hoechst 33250 for 15 min. Finally, the membranes were placed on coverslipped slides and evaluated for the number of bound sperm in six random fields using epifluorescence microscopy (EFA halogen set accessory fluorescent episcopic lamp. Leica. Kista, Sweden).

2.6 Sperm morphology and morphometry

The morphological evaluation was performed using a semen smear stained with Rose Bengal. A total of 200 cells were counted per slide, which were classified according to the presence or absence of morphological defects in light microscopy (1000×), with the aid of immersion oil [24].

The same slides were used for the evaluation of sperm morphometry. In this case, head, midpiece, and tail measurements were performed separately on 100 sperm cells from random fields using the ImageJ 1.45s software (National Institutes of Health, Bethesda, Maryland, USA) [24].

2.7 Sperm freezing/thawing

The samples were diluted in Tris/yolk (20%) and subsequently refrigerated at 15° C for 40 minutes in isothermal boxes and then stabilized at 5°C for 30 minutes in a biological incubator (Quimis, Diadema, SP, Brazil). Then, the samples were glycerolized (3%) and packed in 0.25 mL straws that were exposed to nitrogen vapor (5 cm) for 5 minutes and then stored in a cryobiological cylinder at -196º C [6]. After at least 1 week, the samples were thawed in a water bath at 37°C for 1 minute and subjected to the analysis of post-thawing sperm motility and kinematic parameters as described for fresh sperm.

2.8 Statistical analysis

Based on post-thawing sperm total motility, the animals were selected and classified as good freezer (> 40% motile sperm), moderate freezer (motility varying from 30% to 40%) and poor freezer (< 30% motile sperm). Then, a retrospective analysis of sperm parameters in fresh semen was performed, separating animal data according to the respective classification groups for comparison purposes.

Results were expressed as mean ± standard error. Data were checked for normality using the Shapiro-Wilk test and homoscedasticity using the Levene test. Percentage data were transformed into arcsen, when necessary, to meet parametric assumptions. An ANOVA was performed, followed by Tukey's post hoc test to assess differences between treatments, using Jamovi 2.1 software. Data relating to volume, pH, mitochondrial activity (fresh), sperm morphology (thawed), total motility (fresh and thawed), progressive motility (fresh), BCF (thawed), STR (thawed), LIN (thawed) and subpopulations sperm (fresh) were analyzed using the non-parametric Kruskal-Wallis test. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Post-thawing sperm motility and kinematic parameters

With basis on post-thawing motility as determined by CASA (Table 1), seven individuals were classified as good freezers, ten as moderate freezers and nine as bad freezers. Certainly, the highest ($P < 0.05$) average values of post-thawing sperm motility were verified for the good freezers (50.1 ± 3.3%), to the detriment of the moderate (35.0 ± 1.0%) and bad ones (18.3 ± 1.6%). Good freezers also presented the highest ($P < 0.05$) average values for post-thawing sperm progressive motility (30.6 ± 3.7%) and proportion of rapid sperm (36.0 ± 4.0%), while bad freezers presented the largest amount ($P < 0.05$) of static sperm (80.0 ± 1.6%). Furthermore, there were no significant differences among the groups regarding the VAP, VSL, VCL, ALH, BCF and slow sperm subpopulation parameters ($P > 0.05$).

Table 1. Kinetic motility patterns (mean ± SE) in thawed semen of collared peccary peccary good freezers (> 40% motile sperm), moderate freezers (from 30 to 40% motile sperm) and bad freezers (< 30% motile sperm) through semen computerized analysis - CASA.

	Thawed		
Sperm kinetic motility patterns	Good Freezer	Moderate Freezer	Bad Freezer
Nº	N=7	N=10	N=9
Total Motility (%)	50.1 ± 3.3[a]	35.0 ± 1.0[b]	18.3 ± 1.6[b]
Progressive Motility (%)	30.6 ± 3.7[a]	17.7 ± 1.7[b]	11.1 ± 2.0[b]
Velocity average pathway (µm/s)	48.0 ± 3.4	43.8 ± 3.5	46.8 ± 3.8
Velocity straight line (µm/s)	36.6 ± 3.1	30.7 ± 2.5	34.0 ± 3.8
Velocity curvilinear (µm/s)	101.0 ± 7.8	98.8 ± 7.3	103.0 ± 7.4
Amplitude lateral head (µm/s)	5.8 ± 0.1	6.0 ± 0.2	5.6 ± 0.2
Beat cross frequency (Hz)	33.7 ± 0.4	35.0 ± 0.6	36.8 ± 1.1
Straightness (%)	73.6 ± 2.3[a]	67.2 ± 1.4[b]	68.6 ± 4.1[ab]
Linearity (%)	37.1 ± 2.2[a]	32.0 ± 0.7[b]	32.6 ± 2.2[ab]
Sperm subpopulations			
Rapid (%)	36.0 ± 4.0[a]	22.5 ± 2.2[b]	13.2 ± 1.8[c]
Medium (%)	15.0 ± 2.4[a]	12.2 ± 1.4[a]	5.0 ± 1.3[b]
Slow (%)	4.6 ± 1.1	5.2 ± 1.4	1.7 ± 0.3
Static (%)	45.0 ± 3.6[a]	60.1 ± 1.6[b]	80.0 ± 1.6[c]

[ab]Small superscript letters indicate a significant difference within the experimental group ($p < 0.05$).

3.2 General fresh semen characteristics

All ejaculates had a milky appearance and whitish color. No significant difference was verified regarding the characteristics of the fresh semen of the three groups, as can be seen in Table 2. The seminal volume ranged from 3.9 ± 0.7 (bad freezers) to 4.7 ± 0.8 mL (good freezers), pH ranged from 7.33 ± 0.2 (bad freezers) to 7.5 ± 0.3 (moderate freezers) and sperm concentration from 413 ± 28.9 (good freezers) to 506 ± 46.2 million sperm/mL (poor freezers).

Table 2. Mean values (±SE) for volume, pH and sperm concentration in fresh semen of collared peccary good freezers (> 40% motile sperm), moderate freezers (from 30% to 40% motile sperm) and bad freezers (< 30% motile sperm).

Sperm Parameters Nº	Good Freezers N=7	Moderate Freezers N=10	Bad Freezers N=9
Volume (mL)	4.7 ± 0.8	4.6 ± 1.1	3.9 ± 0.7
pH	7.33 ± 0.3	7.5 ± 0.3	7.33 ± 0.2
Sperm concentration (sperm/mL)	413 ± 28.9	505 ± 53.8	506 ± 46.2

*No significant difference between the different treatments, P>0.05

3.3 Sperm motility and kinematic parameters in fresh semen

Regarding fresh semen motility, the good freezers showed 95.0 ± 1.2% of motile cells, of which 73.3 ± 2.4% had progressive motility, the moderate ones had 87.6 ± 4.3% of motile cells and 69.4 ± 5.0% with progressive motility and the bad freezers 93.4 ± 2.0% mobile and 67.0 ± 5.8% progressive motility. There were no statistical difference among groups with regards to sperm motility or any of the kinematic parameters assessed by CASA ($P<0.05$), with the exception of head lateral amplitude parameters (ALH; µm/s), in which the good freezers (5.0 ± 0.2 µm/s) differed from the moderate freezers (6.1 ± 0.2 µm/s), while poor freezers (5.7 ± 0.4 µm/s) was similar to the others (Table 3).

Table 3. Kinetic motility patterns (mean ± SE) in fresh semen of collared peccary good freezers (> 40% motile sperm), moderate freezers (from 30% to 40% motile sperm) and bad freezers (< 30% motile sperm) as determined through semen computerized analysis - CASA.

	Fresh		
Sperm kinetic motility patterns	Good Freezers	Moderate Freezers	Bad Freezers
Nº	N=7	N=10	N=9
Total Motility (%)	95.0 ± 1.2	87.6 ± 4.3	93.4 ± 2.0
Progressive Motility (%)	73.3 ± 2.4	69.4 ± 5.0	67.0 ± 5.8
Velocity average pathway (µm /s)	57.0 ± 5.7	66.0 ± 5.1	69.5 ± 6.6
Velocity straight line (µm /s)	46.5 ± 5.5	51.1 ± 4.8	54.3 ± 6.0
Velocity curvilinear (µm /s)	100.0 ± 7.1	127.0 ± 7.6	126.0 ± 11.0
Amplitude lateral head (µm/s)	5.0 ± 0.2[a]	6.1 ± 0.2[b]	5.7 ± 0.4[ab]
Beat cross frequency (Hz)	34.8 ± 1.6	35.5 ± 0.8	36.7 ± 0.7
Straightness (%)	78.1 ± 2.0	74.5 ± 1.5	73.7 ± 2.4
Linearity (%)	45.7 ± 2.6	40.3 ± 2.0	43.0 ± 2.8
Sperm subpopulations			
Rapid (%)	79.1 ± 2.6	77.1 ± 5.0	78.7 ± 6.0
Medium (%)	15.7 ± 2.0	10.5 ± 2.0	14.8 ± 4.3
Slow (%)	1.9 ± 0.2	5.7 ± 2.5	2.4 ± 0.5
Static (%)	3.4 ± 1.1	6.5 ± 2.8	4.2 ± 1.5

[ab]Small superscript letters indicate a significant difference within the experimental group ($p < 0.05$).

3.4 Sperm functional parameters in fresh semen

Poor freezers provided the lower (P < 0.05) percentage of sperm presenting intact membranes (77.3 ± 1.7%), in comparison to good and moderate freezers that were similar to each other. Concerning other functional sperm parameters as membrane integrity and functionality, mitochondrial activity and sperm-binding ability, there were no significant differences among fresh ejaculates from individuals of contrasting freezability (Table 4).

Table 4. Mean (±SE) values for membrane integrity, mitochondrial activity, chromatin condensation, membrane functionality, normal morphology and number of spermatozoa attached to the hen egg perivitelline membrane in thawed semen from collared peccary good freezers (> 40% motile sperm), moderate freezers (from 30% to 40% motile sperm) and bad freezers (< 30% motile sperm).

	Fresh		
Sperm Parameters	Good Freezer	Moderate Freezer	Bad Freezer
Nº	N=7	N=10	N=9
Membrane Integrity (%)	84.7 ± 3.1[a]	86.4 ± 2.1[a]	77.3 ± 1.7[b]
Mitochondrial activity (%)	87.3 ± 3.0	74. 9 ± 9.2	79.8 ± 1.7
Membrane functionality (%)	74.9 ± 7.2	83.1 ± 5.4	75.4 ± 8.6
Normal morphology (%)	85.0 ± 4.4	70.1 ± 4.6	73.2 ± 7.4
Number of connected sperm	203.0 ± 9.5	232.0 ± 21.5	232.0 ± 24.6

[ab]Small superscript letters indicate a significant difference within the experimental group (p < 0.05).

3.5 Sperm morphology and morphometry in fresh semen

Ejaculates from individuals classified as good (85.0 ± 4.4%), moderate (70.1 ± 4.6%) and bad (73.2 ± 7.4%) freezers did not differ in terms of normal sperm morphology. Regarding sperm morphometry, head length ranged from 5.6 ± 0.1 µm (Moderate Freezer) to 5.7 ± 0.1 µm (Good Freezer and Bad Freezer), head width ranged from 3.6 ±

0.0 µm (Moderate Freezer) to 3.9 ± 0.1 µm (Good Freezer), midpiece length ranged from 11.6 ± 0.3 µm (Moderate Freezer) to 12.0 ± 0.2 µm (Good Freezer), tail length ranged from 26.2 ± 0.3 µm (Bad Freezer) to 38.4 ± 0.5 µm (Moderate Freezer) and the total length ranged from 42.4 ± 0.5 µm (Good Freezer) to 44.4 ± 0.5 µm (Moderate Freezer). There was no statistical difference between groups regarding head and midpiece length (P > 0.05). Good and bad freezers differed significantly in head width (3.9 ± 0.1 µm and 3.6 ± 0.0 µm, respectively) and total sperm length (42.4 ± 0.5 µm and 44 µm). .4 ± 0.5 µm, respectively). Regarding tail length, moderate freezers had the longest tail length (38.4 ± 0.5 µm), while bad freezers had the shortest tail length (26.2 ± 0.3 µm).

Table 5. Mean (±SE) values sperm morphometry of good freezer collared peccary (> 40% motile spermatozoa), moderate freezers (30% to 40% motile spermatozoa) and bad freezers (< 30% motile spermatozoa).

	Fresh		
	Good Freezer	Moderate Freezer	Bad Freezer
Nº	N=7	N=10	N=9
Head length (µm)	5.7 ± 0.1[a]	5.6 ± 0.1[a]	5.7 ± 0.1[a]
Head width (µm)	3.9 ± 0.1[a]	3.6 ± 0.0[b]	3.7 ± 0.0[ab]
Midpiece length (µm)	12.0 ± 0.2[a]	11.6 ± 0.3[a]	11.7 ± 0.1[a]
Tail length (µm)	36.2 ± 0.8[a]	38.4 ± 0.5[b]	26.2 ± 0.3[c]
Total length (µm)	42.4 ± 0.5[a]	44.4 ± 0.5[b]	43.4 ± 0.4[ab]

[abc]Small superscript letters indicate a significant difference within the experimental group (p < 0.05).

3. Discussion

Since the first studies involving the freezing of collared peccary semen [4], marked differences in freezability between individuals have been verified, reflected in their post-thawed sperm parameters. Such differences led us to classify collared peccary into three groups, good (> 40% motile spermatozoa), moderate (from 30 to 40% motile spermatozoa) and bad freezers (< 30% motile spermatozoa), based on sperm motility after thawing, considered the most important parameter in semen analysis [27]. The motility ranges used to consider the animals as good, moderate and bad freezers were based on the classification proposed for pigs [8], however, studies on collared peccary are more recent and, therefore, the results are still unclear. lower, for this reason lower motility ranges were chosen.

In search of factors that may indicate semen freezability in fresh collared peccary ejaculates, we found that bad freezers seem to be more prone to damage to their spermatic membrane, reflected in a higher percentage of cells with damaged membranes, compared to the other groups ($P < 0.05$). Therefore, the spermatic membrane integrity parameter in fresh ejaculates could be used to predict semen freezability in collared peccaries, since the spermatic membrane is closely related to reproductive events, therefore, damage to its structure would compromise sperm fertility [28].

Despite this, the factors that predispose to greater protection of the spermatic membrane in fresh collared collared peccary ejaculates have not yet been elucidated. However, it is estimated that factors inherent to animals can determine certain characteristics in the structure of the membrane that can contribute to sperm survival after cryopreservation [7], as verified in spermatozoa from pigs, which in addition to the presence of genetic markers involved with membrane integrity, markers linked to sperm kinetics were also verified in good and bad freezer animals [8], in addition to the presence of metabolites involved in energy metabolism [29].

In our results, in addition to alterations in the structure of the spermatic membrane, we identified significant differences in the morphometry of the cells of individuals of different freezability, also observed in other species such as pigs [17,30] and deer (*Cervus elaphus hispanicus*) [16]. In general, the values found in our work are a little lower than the reference values previously determined by Sousa et al. [24], however, the good freezers were the ones that came closest to these standard values.

Among the significant differences between individuals in different freezing conditions, the tail length stood out the most among the morphometric parameters, in which the longest tail length was observed in moderate freezers, and the shortest tail length observed in bad freezers ($P < 0.05$). We can attribute such differences in tail length to sperm kinetics, since its length is involved with the quality of sperm movement, whose cells with greater tail length are capable of presenting better kinetic performance [31]. However, the energy expenditure in cells with greater tail length becomes greater, which may result in a short period of sperm survival [31]. This information corroborates the results found in our study, since the best post-thawed kinetic parameters were observed in good freezers.

Taking into account the numerous components present in seminal plasma, this may be another important factor capable of differently influencing animals in terms of semen freezeability, as already verified in pigs [32] and horses [33, 34]. Thus, in pigs, the presence of seminal plasma from good freezer animals resulted in better seminal quality and a higher penetration rate in samples of low seminal quality [32]. In horses, the addition of autologous seminal plasma to frozen-thawed semen improved the sperm quality of moderate and poor freezers [33], which is mainly due to differences in the protein profile of the seminal plasma of these animals, especially the good and bad freezers, with a total of 33 proteins differing significantly [35].

On the other hand, in collared peccaries, most animals are still in the poor to moderate freezer range. In this sense, it is necessary to better understand the aspects that involve the sperm physiology of the species so that it is possible to improve the freezing protocol and, consequently, provide ideal conditions for the freezers that are in lower motility ranges, to reach the condition good freezers. Previously in the species, the susceptibility of collared peccary semen to environmental factors was verified, with better freezeability during the rainy season compared to the dry season [13], confirmed through changes in some organic and inorganic components of the seminal plasma [36]. Therefore, the environmental parameters could also contribute to the freezability of the ejaculates of individuals with contrasting freezability, from alterations in the components present in the seminal plasma, involved with the protection of the plasmatic membrane. However, this can only be confirmed through future research, comparing the seminal plasma of these individuals in different seasonal periods.

In light of the above information, we believe that conventional methods of assessment, with the exception of plasma membrane integrity, are unlikely to have predictive value for the freezability of collared peccary semen. In addition to membrane integrity, we also found that sperm morphometry can also be directly linked to semen freezability in these animals. Despite the relevance of the findings in our work, it is also suggested that other factors be explored such as the investigation of proteomics [35], membrane lipid composition [37], seminal plasma [32] and sperm DNA integrity [38], the in order to offer greater precision to the investigations.

4. Conclusions

In summary, plasma membrane integrity can be considered a predictive parameter of semen freezability from different freezers. In addition, we confirmed the existence of changes in sperm morphometry among individuals of different freezing

conditions, which may be linked to seminal quality. This information will serve as a basis for the establishment of high quality germplasm banks in the species, in addition to being able to contribute to the knowledge of sperm physiology of collared peccaries.

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Conflict of interest

None of the authors have conflict of interest.

Data Availability Statement

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

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CONCLUSÕES GERAIS

- O processo de criopreservação sozinho pode controlar a carga bacteriana, sem promover dano efetivo às amostras de sêmen de cateto. Porém, se necessário, a suplementação com antibióticos, tanto a gentamicina quanto a combinação estreptomicina-penicilina são indicadas para o diluente utilizado na criopreservação do sêmen de cateto;

- A incorporação de SDS 0,1% no diluente Tris-gema-glicerol otimizou os parâmetros funcionais espermáticos de catetos durante a congelação e descongelação;

- A adição de SOD ou CAT, nas concentrações testadas, isoladamente ou em combinação, não promoveu benefícios à criopreservação do sêmen de cateto;

- A integridade da membrana espermática em ejaculados frescos pode ser utilizada como um parâmetro preditivo para a congelabilidade contrastante observada em catetos classificados como bons, moderados e maus congeladores.

PERSPECTIVAS

Este trabalho representa um passo importante para o entendimento a respeito da fisiologia espermática e das particularidades do sêmen de catetos, mostrando que a incorporação de antibióticos e detergentes ao meio diluente de congelação podem ser úteis para melhorar a qualidade do sêmen descongelado de catetos. Apesar da adição de SOD ou CAT, nas concentrações testadas, isoladamente ou em combinação, não terem promovido benefícios à criopreservação do sêmen de cateto, é sugerido que novas concentrações sejam investigadas, para melhor compreender seus efeitos sobre o sêmen pós-descongelado de catetos.

Adicionalmente, parece que as diferenças existentes na congelabilidade entre os catetos podem estar ligadas a fatores relacionados principalmente à integridade da membrana dos espermatozoides em ejaculados frescos. Desta forma, o próximo passo será identificar a existência de diferenças entre os componentes bioquímicos do plasma seminal de catetos bons congeladores e congeladores ruins e verificar quais deles podem estar ligados à congelabilidade. Por tanto, essas informações possibilitarão o aperfeiçoamento dos bancos de germoplasma da espécie, permitindo o uso eficiente em técnicas de reprodução assistida como a inseminação artificial e a fertilização in vitro.

ANEXOS

RESUMOS PUBLICDOS EM EVENTOS REGIONAIS

CATEGORIZAÇÃO DA CAPACIDADE DE CRIOPRESERVAÇÃO DE SÊMEN EM CATETOS (*Pecari tajacu*) - RESUMO CIENTÍFICO

CATEGORIZATION OF SEMEN CRYOPRESERVATION CAPACITY IN COLLARED PECCARIES *(Pecari tajacu)*

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Os catetos vêm sendo classificados como estáveis quanto ao risco de extinção. No entanto, a espécie apresenta-se em declínio populacional no bioma Mata Atlântica (1). Neste sentido, biotécnicas reprodutivas estão sendo desenvolvidas visando à conservação e multiplicação desta espécie. Dentre as biotécnicas, a criopreservação pode ser aplicada com o intuito de conservar o material genético por longos períodos. No entanto, este procedimento pode causar efeitos deletérios aos espermatozoides, podendo ser mais evidentes em determinados indivíduos, conforme já descrito para bovinos, nos quais os animais têm sido categorizados como “bons ou maus congeladores” de sêmen (2). A identificação possibilita individualizar os protocolos de congelação espermática. Nesse sentido, objetivou-se identificar a existência de indivíduos com boa e má congelabilidade de sêmen na espécie *Pecari tajacu*. Os protocolos experimentais foram aprovados pelo Comitê de Ética em Experimentação Animal da UFERSA (Parecer n. 05/2020). Foram utilizados 14 machos adultos provenientes do Centro de Multiplicação de Animais Silvestres (CEMAS). Os animais foram contidos com propofol (Propovan®, Cristália, Fortaleza, Brasil) a 5mg/kg em bolus, via endovenosa. A coleta se deu pela aplicação de um protocolo de eletroejaculação anteriormente estabelecido para a espécie (3). Após a coleta, as amostras foram avaliadas macroscopicamente quanto ao aspecto, coloração e volume por meio de pipetagens. Em seguida, microscopicamente quanto à motilidade (%) por análise computadorizada do sêmen (CASA). As amostras foram diluídas em Tris-gema (20%) acrescidos de glicerol (6%), e congeladas de acordo com o protocolo anteriormente definido para catetos (4). Decorrido 1 semana, as amostras foram descongeladas e submetidas às mesmas avaliações das amostras frescas. Após a descongelação, os animais foram classificados quanto à motilidade espermática em bons congeladores (7 animais; ≥ 30%) e maus congeladores (7 animais; ≤ 30). Os resultados foram expressos em média e erro padrão e avaliadas pelo software Statview 5.0 utilizando ANOVA seguida do teste t não pareado ($P < 0,05$). As amostras frescas apresentaram aspecto

leitoso e coloração esbranquiçada. As amostras frescas dos bons congeladores 94,5±0,9ª (≥ 30%) não diferiram dos maus congeladores 92,1±2,3ª (≤ 30%). Após a descongelação, as amostras sofreram uma redução significativa em seus parâmetros espermáticos (P < 0,05). Quanto às amostras descongeladas, verificou-se uma nítida diferença no que se refere à capacidade de criopreservação das amostras, uma vez que aqueles categorizados como bons congeladores (≤ 30%) apresentaram 40,4 ± 2.9% dos espermatozoides móveis enquanto os maus congeladores (≤ 30%) apresentaram uma motilidade significativamente menor de 15,0 ± 6.0% dos espermatozoides móveis. Note-se a baixa variação dentro de cada uma das categorias, refletida em baixos erros padrão da média em cada grupo. Identificou-se, pela primeira vez, a existência de diferentes populações de catetos quanto a capacidade de criopreservação do sêmen, observando-se que a motilidade é, de fato, um parâmetro bastante afetado por essa variação individual. Provavelmente, a congelabilidade das amostras de sêmen depende de fatores intrínsecos aos animais e, a partir dessas informações é possível selecionar os bons congeladores a serem utilizados como doadores de sêmen para a formação de biobancos, quando do uso de espécimes mantidos em cativeiro.

Palavras-Chave: Animais silvestres; Banco de germoplasma; Criopreservação.

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PARÂMETROS MORFOFUNCIONAIS DOS ESPERMATOZOIDES DE CATETOS CRIOPRESERVADOS COM DIFERENTES ANTIMICROBIANOS

(Morphofunctional parameters of collared peccary sperm frozen with different antimicrobials)

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ABSTRACT

The aim was to evaluate the effects of the addition of antimicrobials to the diluent for the cryopreservation of the semen of collectors, especially on the morphofunctional parameters. Ten ejaculates from adult males were obtained by electroejaculation. The samples were evaluated for volume, concentration, motility, morphology, membrane functionality, sperm viability, mitochondrial activity and binding capacity. Subsequently, they were cryopreserved in Tris with egg yolk (20%) and glycerol (3%) added or not (control) with gentamicin (70µg/mL), or with the penicillin (1000 IU/mL) + streptomycin (1mgE/mL). After one week, the samples were thawed and evaluated according to the fresh semen. As for the results, no significant differences were observed between the control treatment and those added with antimicrobials, emphasizing that these do not damage the sperm morphofunctional parameters during cryopreservation. In this sense, it is suggested that both gentamicin and the penicillin/streptomycin combination could be added to the extender for the cryopreservation of the collared peccary semen.

Key words: Biobank, Semen, Cryopreservation, Antimicrobials.

INTRODUÇÃO

O cateto (*Pecari tajacu* Linnaeus, 1758) é um taiassuídeo silvestre cuja população vem diminuindo em biomas como a Mata Atlântica. Diante disso, seu desaparecimento nos ecossistemas ocasionaria significativas perdas ecológicas, visto sua importância para a manutenção da fauna e flora (Gongora *et al*., 2011). Assim, o desenvolvimento de biotecnologias reprodutivas, como a criopreservação de germoplasma masculino, poderia repercutir na conservação e multiplicação da espécie (Domingues *et al*., 2011). No entanto, durante as etapas que envolvem a congelação de sêmen, pode haver contaminação por agentes bacterianos, os quais podem sobreviver à baixas temperaturas (-196 ºC) (BIELANSKI e VAJTA, 2009) e causar danos às células espermáticas (Prieto-Martínez *et al*., 2014).

Nesse contexto, o uso de antimicrobianos contribuiria para o controle da população bacteriana, bem como minimizaria possíveis danos que a contaminação poderia acarretar. Porém, a depender das concentrações, eles podem acarretar danos aos parâmetros espermáticos. Dentre os antimicrobianos mais usados em diluentes para sêmen de diferentes espécies, destacam-se a associação penicilina/estreptomicina e a gentamicina (Câmara *et al*., 2018). Assim, o presente trabalho objetivou investigar a ação dos antimicrobianos:

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gentamicina (70 µg) e penicilina/estreptomicina (1000 UI/mL/ 1mgE/mL) sobre a qualidade espermática de catetos no processo de congelação/descongelação do sêmen.

MATERIAL E MÉTODOS

Os protocolos experimentais foram aprovados pelo Comitê de Ética em Experimentação Animal da UFERSA (nº. 05/2020). Foram utilizados dez ejaculados oriundos de machos sexualmente maduros, mantidos sob fotoperíodo natural de 12h. Os animais receberam alimentação comercial para suínos e frutas da estação, e água à vontade. Antecedendo ao período de coleta, os animais foram mantidos em jejum alimentar de 12h e contidos com auxílio de propofol (Propovan®, Cristália, Fortaleza, Brasil) a 5mg/kg em bolus, via endovenosa. Posteriormente, foi realizada a coleta mediante aplicação do protocolo de eletroejaculação estabelecido para a espécie (Castelo *et al*., 2010).

Após a coleta, as amostras foram avaliadas quanto ao volume, concentração espermática (em milhões de espermatozoides/mL) por contagem em câmara de Neubauer (400x), morfologia espermática em esfregaços corados com Rosa Bengala, contando-se 200 células (1000×), funcionalidade da membrana pelo teste hipo-osmótico com água destilada (0 mOsm/L) (400x). Em adição, verificou-se a viabilidade espermática e a atividade mitocondrial por marcação com Hoechst 342, Mito Tracker red® e Iodeto de Propídio, contando-se 200 células em microscópio de epifluorescência (Episcopic Fluorescent attachment EFA Halogen Lamp Set. Leica. Kista, Sweden). Os espermatozoides com cabeça marcada em azul foram considerados com membrana intacta e aqueles com cabeça marcada em vermelho com membrana não intacta e com peça intermediária marcada em vermelho foram considerados com função mitocondrial (SOUSA *et al*., 2013). A análise da capacidade ligante das células espermáticas também foi investigada, utilizando-se o teste de ligação à membrana perivitelina do ovo de galinha conforme descrito por Campos *et al*. (2017).

Alíquotas do sêmen foram criopreservadas em diluente Tris-gema (20%) e glicerol (6%) seguida da adição dos antimicrobianos, consistindo nos seguintes grupos: (A) Controle (sem antimicrobiano/C), (B) 70µg/mL de gentamicina (G) e (C) 1000 UI/mL de penicilina + 1 mgE/mL de estreptomicina (P+E). Após uma semana, as amostras foram descongeladas e submetidas às mesmas avaliações do sêmen fresco.

Os dados obtidos foram expressos em média±erro padrão (média±SEM). O teste de Dunnett comparou o sêmen fresco com os demais tratamentos. Posteriormente, foi realizada uma ANOVA one-way, seguida teste de Tukey para avaliar as diferenças entre os tratamentos (descongelados). Foi utilizado o software Statistical Analysis Software, versão 8.0 (SAS Institute Inc., Cary, NC, EUA), considerando $p<0,05$.

RESULTADOS E DISCUSSÕES

Os ejaculados apresentaram coloração normal, com um volume de 5,0±1,2mL, com concentração espermática de 461,0±59,2 espermatozoides/mL, e motilidade total de 95,3±0,8%. Os demais parâmetros morfofuncionais estão representados na Tab. 01.

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Tabela 01: Parâmetros espermáticos morfofuncionais (média±EP) de catetos antes e após a congelação de sêmen (n=10), no grupo controle e utilizando os antimicrobianos, gentamicina ou penicilinia/estreptomicina (P+E).

Parâmetros espermáticos	Fresco	Descongelado		
		Controle	Gentamicina	P + E
Morfologia normal (%)	80,0±4,3[a]	79,4±2,2[a]	75,1±3,5[a]	79,1±3,3[a]
Funcionalidade membrana (%)	69,4±8,62[a]	50,0±3,6[b]	59,4±6,1[b]	50,5±4,3[b]
Integridade de membrana (%)	80,9±2,2[a]	37,6±5,4[b]	40,1±5,8[b]	31,3±2,5[b]
Atividade mitocondrial (%)	79.3±2.8[a]	31.3±5.4[b]	34.9±5.9[b]	30.6±2.8[b]
Nº de espermatozoides ligados	212,0±22,6[a]	105,0±16,1[b]	100,7±8,1[b]	95,8±13,4[b]

[abc] Letras minúsculas sobrescritos indicam diferença significativa entre os grupos experimentais ($p<0,05$).

Conforme esperado, as amostras submetidas à congelação/descongelação tiveram seus parâmetros espermáticos reduzidos ($p<0,05$) em relação às amostras frescas, exceto quanto a morfologia espermática ($p>0,05$). Apesar disso, entre os tratamentos não houve diferenças significativa (Tab. 01), salientando que ambos os antimicrobianos não causaram danos aos parâmetros espermáticos morfofuncionais.

CONCLUSÕES

Pode-se concluir que a adição de antimicrobianos ao diluente seminal de catetos não tem efeito significativos sobre seus parâmetros morfofuncionais após o processo de congelação/descongelação. Assim, sugere-se que tanto a gentamicina como a associação penicilina/estreptomicina poderiam ser adicionados ao diluente para a criopreservação do sêmen de catetos.

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IMPACTO DE ANTIMICROBIANOS NOS PARÂMETROS CINÉTICOS DE ESPERMATOZOIDES CRIOPRESERVADOS DE CATETOS

(Impact of antimicrobials on the kinetic parameters of collared peccary frozen sperm)

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ABSTRACT

The objective was to verify the impact of the addition of antimicrobials on the kinetic parameters of sperm in the cryopreserved semen of collared peccaries. Ejaculates from 10 adult male, obtained by electroejaculation, were used. The samples had their kinetic parameters evaluated by computer analysis (CASA). Subsequently, they were cryopreserved in Tris plus egg yolk (20%) and glycerol (3%), whether or not (control) added gentamicin (70µg/mL) or the combination penicillin (1000 IU/mL) and streptomycin (1mgE/mL) (P+E). After one week, the samples were thawed and evaluated similarly to fresh semen. In fresh semen, total motility of 95.3±0.8% and 72.1±3.5% progressive motility were observed. After thawing, there were no differences between treatments, except for the cross-beat frequency (BCF) parameter, which was negatively influenced by P+E, in relation to fresh semen ($p<0.05$). In conclusion, it is suggested the use of gentamicin as an antimicrobial for the cryopreservation of semen from peccaries.

Key words: Biobank, semen, cryopreservation, antimicrobials.

INTRODUÇÃO

O cateto (*Pecari tajacu*) é um Tayassuídeo silvestre presente em diferentes biomas, desde o sul dos Estados Unidos até o norte da Argentina. Sua população é descrita como globalmente estável, mas em declínio no bioma Mata Atlântica (Gongora *et al*., 2011). Além de sua importância ecológica, destaca-se por seu potencial científico, sendo amplamente utilizado em pesquisas conservacionistas, que podem ser extrapoladas para espécies ameaçadas filogeneticamente próximas como o Taguá (*Catagonus wagneri*).

A criopreservação do sêmen tem sido a principal biotécnica aplicada na conservação de material genético da espécie (Souza *et al*., 2016). A execução do processo pode acarretar contaminações microbianas que podem afetar a qualidade das amostras armazenadas. Para controlar crescimento bacteriano, vários agentes, dentre eles a gentamicina, e a associação penicilina/estreptomicina, têm sido utilizados nos diluentes seminais. Entretanto, os próprios antimicrobianos, a depender de sua concentração, podem afetar a motilidade e a viabilidade espermáticas (Rurangwa *et al*., 2004).

Os sistemas de análise computadorizada (CASA) permitem uma avaliação objetiva não só da motilidade, mas também de demais parâmetros cinéticos do espermatozoide. Ele pode fornecer informações acuradas, precisas e significativas a respeito do movimento individual de cada célula bem como de suas subpopulações espermáticas (AMANN e KATZ, 2004). Desse modo, possibilitando a identificação de sutis efeitos de variáveis sobre o sêmen,

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como por exemplo o uso de drogas adicionadas aos diluentes. Assim, objetivou-se verificar o impacto dos antimicrobianos sobre os parâmetros cinéticos dos espermatozoides de catetos após congelação/descongelação de sêmen.

MATERIAL E MÉTODOS

Os protocolos experimentais foram aprovados pelo Comitê de Ética em Experimentação Animal da UFERSA (nº. 05/2020). Foram utilizados 10 ejaculados provenientes de machos sexualmente maduros, mantidos em grupo, e alimentação para suínos e frutas, além de água à vontade. Na ocasião da coleta, os animais foram contidos com um puçá, e anestesiados com 5mg/kg propofol (Propovan®, Cristália, Fortaleza, Brasil) em bolus endovenoso. O sêmen foi coletado por eletroejaculação conforme protocolo estabelecido para a espécie (Castelo *et al*., 2010).

Os ejaculados foram avaliados imediatamente quanto aos seus parâmetros cinéticos por meio da análise computadorizada de sêmen (CASA – IVOS 7.4G, Hamilton-Thorne ResearchTM, Beverly, MA, USA), em câmaras Leja de quatro canais (20µm), com configurações pré-estabelecidas para a espécie (Souza *et al*., 2016). Os parâmetros avaliados incluíram: número de células contadas, motilidade total (%), motilidade progressiva (%), velocidade média da trajetória (VAP; mm/s), velocidade linear progressiva (VSL; mm/s), velocidade curvilínea (VCL; mm/s), amplitude lateral da cabeça (ALH; mm), frequência de batimento cruzado (BCF; Hz), índice de progressão (STR; %) e índice de linearidade (LIN; %). A população de espermatozoides foi subdividida em quatro categorias: rápida, com VAP> VMV; médio, com (VLV <VAP <VMV); lento, com VAP < VLV; e estáticos (Souza *et al*., 2016).

As amostras foram congeladas em Tris + gema (20%) + glicerol (3%) (controle sem antimicrobianos) ou este diluente adicionado de 70µg/mL de gentamicina (G) ou 1000 UI/mL de penicilina/1mgE/mL de estreptomicina (P+E), e armazenadas em nitrogênio líquido (Souza *et al*., 2016). Após uma semana, as amostras foram descongeladas em banho maria a 37 °C por 1 minuto e submetidas novamente à avaliação dos seus parâmetros cinéticos, conforme descrito para o sêmen fresco.

Os dados foram expressos em média±erro padrão (média±SEM). O teste de Dunnett foi aplicado para comparar o sêmen fresco com os demais tratamentos. Posteriormente, foi realizada ANOVA one-way, seguida do teste de Tukey para avaliar as diferenças entre os tratamentos (descongelados). Foi utilizado o software Statistical Analysis Software, versão 8.0 (SAS Institute Inc., Cary, NC, EUA), considerando $p<0,05$.

RESULTADOS E DISCUSSÕES

O sêmen fresco dos animais, conforme mostrado na Tab. 01, apresentou parâmetros cinéticos com valores dentro da normalidade para a espécie (Castelo *et al*., 2010).

Conforme esperado, verificou-se que a descongelação provocou a redução de alguns dos parâmetros cinéticos (Tab. 01) quando comparados ao sêmen fresco ($p<0,05$), exceto a VCL, ALH, STR e subpopulação espermática média e lenta ($p>0,05$). Os parâmetros cinéticos obtidos pelo sistema CASA são importantes para predizer a fertilidade de machos,

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destacando-se a motilidade total e a motilidade progressiva, as quais não sofreram influência da ação dos antimicrobianos no presente estudo (Tab. 01). Resultados semelhantes também foram verificados em búfalos (ANDRABI *et al*., 2016).

Tabela 01: Padrões cinéticos (média±EP) da motilidade de espermatozoides criopreservados em Tris-gema-glicerol adicionado ou não (Controle) dos antibióticos gentamicina ou penicilina/estreptomicina (P+E) em catetos (n=10).

Parâmetros	Sêmen Fresco	Sêmen Descongelado		
		Controle	Gentamicina	P + E
Motilidade total (%)	95,3±0,8^{A}	34,1±3,7^{Ba}	37,2±6,1^{Ba}	32,6±4,0^{Ba}
Motilidade Progressiva (%)	72,1±3,5^{A}	20,2±2,4^{Ba}	23,2±4,6^{Ba}	20,0±2,8^{Ba}
Veloc. média da trajetória (mm/s)	69,2±4,8^{A}	46,1±2,7^{Ba}	47,7±2,8^{Ba}	45,8±2,5^{Ba}
Veloc. linear progressiva (mm/s)	56,8±4,4^{A}	35,7±3,1^{Ba}	36,0±3,4^{Ba}	33,2±3,0^{Ba}
Veloc. curvilínea (mm/s	119,4±8,0^{A}	100,6±5,5^{Aa}	100,2±4,4^{Aa}	99,7±5,0^{Aa}
Amplitude lateral da cabeça (mm)	5,3±0,3^{A}	5,4±0,2^{Aa}	5,6±0,1^{Aa}	5,7±0,2^{Aa}
Frequência de batimento cruzado (Hz)	37,2±0,6^{A}	35,6±0,9^{Aa}	34,1±0,5^{Bab}	31,7±1,0^{Bb}
Índice de progressão (%)	77,5±2,0^{A}	72,7±2,4^{Aa}	70,9±3,5^{Aa}	69,0±2,2^{Aa}
Índice de linearidade (%)	47,0±2,4^{A}	34,4±1,5^{Ba}	34,8±2,3^{Ba}	33,3±1,5^{Ba}
Subpopulações espermáticas				
Rápido (%)	81,0±3,6^{A}	23,5±3,0^{Ba}	27,7±4,9^{Ba}	23,4±3,0^{Ba}
Médio (%)	14,4±3,0^{A}	10,6±1,8^{Aa}	9,4±1,4^{Aa}	9,1±1,6^{Aa}
Lento (%)	2,0±0,2^{A}	2,7±0,7^{Aa}	2,6±0,4^{Aa}	2,4±0,6^{Aa}
Estático (%)	3,0±0,6^{A}	63,1±4,2^{Ba}	60,3±6,4^{Ba}	65,0±4,1^{Ba}

^{AB}Letras maiúsculas sobrescritos indicam diferença significativa entre os grupos frescos e descongelados ($p<0,05$); ^{ab}Letras minúsculas sobrescritos indicam diferença significativa entre os grupos experimentais ($p<0,05$).

Apenas sobre a BCF, observou-se um efeito negativo da adição de penicilina/estreptomicina em relação ao grupo controle sem antimicrobianos ($p<0,05$). Segundo Gil *et al*. (2009), a BCF, em associação com ALH e VCL são considerados parâmetros indicadores do vigor espermático, sendo importantes para a fertilidade.

Neste caso, a gentamicina, por ser um aminoglicosídeo de amplo espectro de ação, atua sobre bactérias gram-negativas, especificamente sobre sua síntese proteica. Desta forma, seu mecanismo de ação se dá em virtude de erros causados ao RNA mensageiro, o qual contribui para a morte celular (Tortora *et al*., 2017), sem causar danos aos espermatozoides.

CONCLUSÕES

Neste sentido, sugere-se a utilização da gentamicina (70µg/mL) como antimicrobiano a ser adicionado ao diluente Tris-gema-glicerol para a criopreservação do

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sêmen de catetos, no intuito de se obter uma maior eficiência na conservação de todos os parâmetros cinéticos espermáticos.

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ANEXOS

RESUMOS PUBLICDOS EM EVENTOS NACIONAIS

O trabalho intitulado **CRIOPRESERVAÇÃO DE SÊMEN DE CATETOS (PECARI TAJACU) EM DILUENTE TRIS ADICIONADO DE SUPERÓXIDO DISMUTASE**, de autoria de **Tayná Moura Matos** , **Samara Sandy Jerônimo Moreira** , **Luana Grasiele Pereira Bezerra** , **Romário Parente dos Santos** , **Ana Glória Pereira** e **ALEXANDRE RODRIGUES SILVA** foi aprovado na modalidade Resumo, para apresentação no evento 42º Congresso Brasileiro da Anclivepa a ser realizado 10/04/2023.

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Data do Aceite:10/04/2023

O trabalho intitulado **EFEITO DA ENZIMA CATALASE NA MOTILIDADE ESPERMÁTICA DO SÊMEN CRIOPRESERVADO DE CATETOS (PECARI TAJACU)**, de autoria de **Tayná Moura Matos** , **Samara Sandy Jerônimo Moreira** , **Luana Grasiele Pereira Bezerra** , **Yuri Gonçalves Matos** , **Náyra Rachel Nascimento Luz** e **ALEXANDRE RODRIGUES SILVA** foi aprovado na modalidade Resumo, para apresentação no evento 42º Congresso Brasileiro da Anclivepa a ser realizado 10/04/2023.

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ANEXOS

RESUMOS PUBLICDOS EM EVENTOS INTERNACIONAIS

Animal Reproduction

Cryopreservation of collared peccary semen using Tris-based extender supplemented of sodium dodecyl sulfate detergents

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Semen cryopreservation represents the main tool used for the conservation and maintenance of the genetic material of wild species. Use of sodium dodecyl sulfate (SDS) detergents has been highlighted for improving sperm longevity after thawing in various mammals, but its effects lack to be investigated in collared peccaries (*Pecari tajacu* Linnaeus, 1758). Therefore, we aim to evaluate the effects of SDS detergents on the longevity of peccary frozen-thawed semen. Ejaculates from 10 mature individuals from the Wild Animal Multiplication Center (CEMAS-UFERSA) were obtained by electroejaculation (Autojac®, Neovet, Campinas, SP, Brazil) under anesthetic restraint (5 mg/kg; Propovan®, Cristália, São Paulo, Brazil). Fresh semen was evaluated for sperm motility using a computerized system (CASA – IVOS12.0, Hamilton-Thorne, Beverly, USA), membrane integrity and mitochondrial activity using fluorescent probes (Hoechst 342 – Sigma-Aldrich, St Louis, MO, USA; Mito Tracker red® – CMXRos, Molecular Probes; propidium iodide – Sigma-Aldrich, Co., St Louis, MO, USA), and membrane functionality using distilled water as the hypoosmotic solution (0 mOsm / L). Samples were diluted in a Tris-based extender plus egg yolk (20%) and divided in different aliquots, among which, one remained without detergent supplementation, as a negative control group. The other aliquots were supplemented with Equex STM 0.5% that is a commercial SDS-derived detergent (positive control), or with purified SDS at different concentrations (0.1%, 0.3% and 0.5%). They were then refrigerated to 5 °C, added of 3% glycerol to reach a final concentration of 100 x 10^6 sperm/mL, packed in 0.25-mL plastic straws, and stored in liquid nitrogen. After one week, samples were thawed in a water bath at 37°C/1 min and subjected to a thermo-resistance test (TRT) at 37 °C for up 60 min, being reevaluated every 30 min. Data was expressed as means ± SEM. Treatments were compared by ANOVA followed by Tukey's test ($P < 0.05$). Fresh samples presented 87.8 ± 4.4% motile sperm, with 87.6 ± 2.2% membrane integrity, 87.4 ± 2.1% mitochondrial activity and 86.0 ± 3.3% functional membrane. Immediately after thawing, all the treatments provided a sperm motility higher than 40%, except the group containing SDS 0.5% that presented lower values (31.2 ± 6.3%) than controls ($P < 0.05$). During TRT, a significant decrease on sperm motility values was found for all the groups ($P < 0.05$) that provided values lower than 10% after 60 min. Regarding other parameters, all the treatments provided proximately 40% sperm presenting membrane integrity, mitochondrial activity, and membrane functionality immediately after thawing. At 30 min, these parameters were effectively preserved at the presence or absence of SDS-derived detergents, but after 60 min, lowest values for membrane integrity (26.6 ± 5.4%) and mitochondrial activity (12.9 ± 3.3%) were observed at the use of the SDS 0.5% ($P < 0.05$). Apparently, the peccary sperm presents a short longevity after thawing and the use of Tris-extender supplemented with SDS detergents is not effective to provide an improvement on its qualitative parameters. On the contrary, the increase of SDS concentration to 0.5% impairs peccary post-thawing sperm quality. Financial support: CAPES and CNPq

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