Universidade Estadual Paulista “Júlio de Mesquita Filho”
Faculdade de Medicina Veterinária e Zootecnia

AVANÇOS NA REFRIGERAÇÃO DE SÊMEN ASININO (*EQUUS ASINUS*)

AMANDA CARVALHO SILVA

Botucatu, SP
Fevereiro - 2026

Universidade Estadual Paulista “Júlio de Mesquita Filho”
Faculdade de Medicina Veterinária e Zootecnia

AVANÇOS NA REFRIGERAÇÃO DE SÊMEN ASININO (*EQUUS ASINUS*)

Dissertação apresentada junto ao Programa de Pós-graduação em Biotecnologia Animal para obtenção do título de MESTRE

Amanda Carvalho SIlva
Orientador: Prof. Dr. Lorenzo G. T. M. Segabinazzi
Coorientador: Prof. Dr. Gabriel Augusto Monteiro

Botucatu, SP
Fevereiro - 2026

S586a

Silva, Amanda Carvalho
Atualizações na refrigeração de sêmen asinino (Equus asinus) / Amanda Carvalho Silva. -- Botucatu, 2026
78 p. : tabs.

Dissertação (mestrado) - Universidade Estadual Paulista (UNESP), Faculdade de Medicina Veterinária e Zootecnia, Botucatu
Orientador: Lorenzo Garrido Teixeira Martini Segabinazzi
Coorientador: Gabriel Augusto Monteiro

1. Diluente. 2. Jumento. 3. Refrigeração. 4. Sêmen. I. Título.

Nome do autor (a): Amanda Carvalho Silva

Título: Avanços na refrigeração de sêmen asinino (Equus asinus)

BANCA EXAMINADORA

Prof. Dr. Lorenzo G. T. M. Segabinazzi
Presidente e Orientador
Departamento de Cirurgia Veterinária e Reprodução Animal, FMVZ - UNESP - Botucatu/SP

Profa. Dra. Fernanda Saules Ignácio
Membro
Departamento de Cirurgia Veterinária e Reprodução Animal, FMVZ - UNESP - Botucatu/SP

Prof. Dr. Rodrigo Arruda de Oliveira
Membro
Faculdade de Agronomia e Medicina Veterinária, FAV – UnB, Brasilia/DF

Data da defesa: 24 de Fevereiro de 2025

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001

RESUMO

SILVA, A.C. **AVANÇOS NA REFRIGERAÇÃO DE SÊMEN ASININO (*EQUUS ASINUS*)**
Botucatu – SP. 2025. Defesa (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

Apesar da expansão do mercado de asininos e muares, as biotécnicas reprodutivas utilizadas ainda são amplamente extrapoladas da espécie equina, frequentemente resultando em eficiência subótima. Evidências indicam que diluentes convencionais à base de leite desnatado não mantêm adequadamente a viabilidade espermática em asininos e que a adição de colesterol ou o uso de diluentes contendo gema de ovo pode melhorar a preservação celular, porém estudos de fertilidade foram conduzidos predominantemente em éguas. Assim, este trabalho avaliou o efeito de diferentes diluentes comerciais, da retirada de plasma seminal e da adição de gema de ovo sobre a qualidade espermática e fertilidade em jumentas. No primeiro experimento, amostras de sêmen foram refrigeradas nos seguintes grupos: diluente à base de leite com colesterol (BS – BotuSêmen Special®; controle), diluente à base de caseinato de sódio com colesterol (BG – BotuSêmen Gold®), diluente à base de gema de ovo com crioprotetores (BC – BotuCrio®), BotuCrio® sem crioprotetores (BC-Sc) e BotuCrio® com substituição da gema de ovo por caseinato de sódio (BCC). Um experimento adicional comparou BG com BG sem plasma seminal (BG-sP). A fertilidade e a contagem de polimorfonucleares (PMN) foi testada em jumentas inseminadas com BG, BC, BC-Sc e BC-sP, que apresentaram melhor cinética espermática. Posteriormente, avaliou-se a adição de diferentes proporções de BotuCrio e gema de ovo ao BG: BG, BG+2% gema de ovo (BG2E), BG+10% gema de ovo (BG10E), BC, BG+2% BC (BG2BC), BG+10% BC (BG10BC) e BG+20% BC (BG20BC). As taxas de fertilidade foram comparadas entre BG e BG20BC. Por fim, um último experimento comparou a cinética do sêmen refrigerado a 5 °C em BG, BG sem plasma seminal (BG-P), BG+20% BC (BGBC), BGBC sem plasma seminal (BGBC-P), INRA 96 (IN), IN sem plasma seminal (IN-P), IN+20% BC (INBC) e INBC sem plasma seminal (INBC-P). Os resultados demonstraram que a gema de ovo foi mais determinante que o colesterol para melhorar e manter a qualidade espermática de sêmen asinino refrigerado e que a retirada do plasma seminal beneficiou os resultados de cinética espermática. A seguir, os resultados indicaram que a adição de 20% de BotuCrio associada à remoção do plasma seminal retornou os melhores resultados de cinética, enquanto a fertilidade e a contagem de PMN não foi afetada pelos diferentes tratamentos.

Palavras-chave: Diluente; jumento; refrigeração; semen.

ABSTRACT

SILVA, A.C. **ADVANCES IN DONKEY (EQUUS ASINUS) SEMEN COOLING** Botucatu – SP. 2025. Master's Thesis – São Paulo State University Júlio de Mesquita Filho, FMVZ – Botucatu Campus

Despite the expansion of the donkey and mule market, available reproductive biotechniques are still largely extrapolated from the equine species, frequently resulting in low efficiency. Evidence indicates that conventional skim milk-based extenders do not adequately maintain sperm viability in donkeys and that the addition of cholesterol or the use of egg yolk-containing extenders may improve cellular preservation; however, fertility studies have been conducted predominantly in mares. Thus, this study evaluated the effect of different commercial extenders, seminal plasma removal, and the addition of egg yolk on sperm quality and fertility in jennies. In the first experiment, semen samples were cooled in the following groups: milk-based extender with cholesterol (BS – BotuSêmen Special®; control), sodium caseinate-based extender with cholesterol (BG – BotuSêmen Gold®), egg yolk-based extender with cryoprotectants (BC – BotuCrio®), BotuCrio® without cryoprotectants (BC-Sc), and BotuCrio® with the replacement of egg yolk by sodium caseinate (BCC). An additional experiment compared BG with BG without seminal plasma (BG-sP). Fertility and polymorphonuclear (PMN) counts were tested in jennies inseminated with BG, BC, BC-Sc, and BC-sP, which exhibited superior sperm kinetics. Subsequently, the addition of different proportions of BotuCrio and egg yolk to BG was evaluated: BG, BG+2% egg yolk (BG2E), BG+10% egg yolk (BG10E), BC, BG+2% BC (BG2BC), BG+10% BC (BG10BC), and BG+20% BC (BG20BC). Fertility rates were compared between BG and BG20BC. Finally, a last experiment compared the kinetics of semen cooled at 5 °C in BG, BG without seminal plasma (BG-P), BG+20% BC (BGBC), BGBC without seminal plasma (BGBC-P), INRA 96 (IN), IN without seminal plasma (IN-P), IN+20% BC (INBC), and INBC without seminal plasma (INBC-P). The results demonstrated that egg yolk was more determinant than cholesterol in improving and maintaining the quality of cooled donkey sperm, and that the removal of seminal plasma benefited sperm kinetic outcomes. Furthermore, the results indicated that the addition of 20% BotuCrio associated with the removal of seminal plasma yielded the best kinetic results, while fertility and PMN counts were not affected by the different treatments.

Keywords: donkey; extender; cooled semen

LISTA DE SIGLAS

ABCJPêga: Associação Brasileira dos Criadores de Jumento Pêga

BSP: *binder of sperm protein*

CASA: *computer-assisted semen analysis*

CBRA: colégio brasileiro de reprodução animal

COX-2: ciclooxigenase-2

DNA: ácido desoxirribonucléico

EROs: espécies reativas de oxigênio

IA: inseminação artificial

IMP: integridade de membrana plasmática

LDL: *low density lipoprotein*

MP: motilidade progressiva

MT: motilidade total

O2-: ânion superóxido

PH: potencial hidrogeniônico

PMM: potencial de membrana mitocondrial

PMN: polimorfonuclear

PS: plasma seminal

PUFAs: ácidos gráxos poli-insaturados

RAP: porcentagem de espermatozoides rápidos

UHT: ultra high temperature

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO E JUSTIFICATIVA

Apesar da proximidade filogenética entre equinos (*Equus cabalus*) e asininos (*Equus asinus*), as espécies diferem em anatomia, fisiologia, comportamento e necessidades de manejo. Tais diferenças refletem nas biotécnicas aplicadas à reprodução. Apesar do crescimento exponencial do mercado de asininos e muares no mundo, as biotécnicas aplicadas a esses animais ainda são extrapoladas da espécie equina, o que algumas vezes desencadeia resultados frustrantes nessa cadeia produtiva.

Asininos e muares compuseram uma fração importante das tropas utilizadas para trabalho e transporte em todo o mundo. A mecanização do trabalho e o êxodo rural, no entanto, contribuiram para o desinteresse nesses animais para ao trabalho de campo (1). Apesar de o consumo da carne de jumento não ser tão comum, há um recente interesse da indústria pelo leite de jumentas, principalmente para uso na indústria cosmética (2).

Enquanto algumas raças correm risco de extinção, outros locais como o Nordeste Brasileiro, regiões áridas dos Estados Unidos e Caribe a superpopulação de jumentos é um problema (3). Ainda sob outra perspectiva, existem raças em crescimento exponencial principalmente devido à valorização dos seus produtos, como o jumento Pêga, raça brasileira que conta com associação (ABCJPêga – Associação Brasileira de Criadores do Jumento Pêga) e provas de morfologia e marcha espalhadas por todo o país.

Além disso, a produção de híbridos movimenta o mercado equestre e os centros de pesquisa em biotécnicas da reprodução. Cavalos e jumentos possuem números diferentes de cromossomos (64 e 62, respectivamente), enquanto os seus produtos apresentam 63 cromossomos e, como consequência, são estéreis. Mulas e burros, produzidos pelo cruzamento entre um macho de espécie asinina e uma fêmea de espécie equina, são amplamente utilizados na lida com o gado, em cavalgadas e em provas de marcha, e apesar de estéreis, mulas podem ser utilizadas como receptoras de embrião. Por outro lado, o cruzamento entre um macho da espécie equina e uma fêmea asinina, que produz um bardoto, desperta curiosidade pelas baixas taxas de fertilidade (4).

Apesar de apresentar ótimos resultados de fertilidade com sêmen fresco tanto em éguas quanto em jumentas, o sêmen refrigerado e congelado de

jumentos ainda não retorna resultados satisfatórios (5,6). Os processos de refrigeração e criopreservação de sêmen se apoiam em anos de estudo a respeito da composição, do funcionamento e das demandas nutricionais da célula espermática, além da compreensão da fisiologia reprodutiva das fêmeas que vão receber esse sêmen.

O crescente interesse na reprodução de asininos desencadeou a percepção de que as biotécnicas aplicadas à reprodução equina não retornam os mesmos resultados quando extrapoladas para a espécie asinina. Extrapolar biotécnicas desenvolvidas para equinos é ignorar a complexidade e a individualidade da espécie asinina e esperar os mesmos resultados sem o mesmo nível de compreensão da espécie (4–7).

A necessidade de controle populacional, proteção de raças ameaçadas e as demandas comerciais de raças valorizadas indicam a urgência de maior compreensão da anatomia, fisiologia e comportamento dessa espécie e do desenvolvimento de novas biotecnologias aplicáveis especificamente à espécie asinina. Dessa forma, diversos estudos são conduzidos há anos para desvendar os mistérios que permeiam a reprodução de jumentos (3–8).

Para asininos, a utilização de sêmen criopreservado ainda não é uma realidade. O sêmen asinino pode ser criopreservado, entretanto, as taxas de fertilidade quando utilizado para inseminar jumentas é muito baixa, impossibilitando a aplicação dessa biotécnica na espécie (5,9). Essa particularidade torna mais urgente a compreensão dos processos de refrigeração para a espécie, já que, além de ser a única alternativa viável até o momento, a compreensão dos desafios do sêmen asinino refrigerado pode ajudar a desvendar as dificuldades enfrentadas para a utilização desse mesmo sêmen criopreservado.

A utilização de sêmen refrigerado permite o estreitamento de fronteiras, a proteção contra a disseminação de doenças e facilita o manejo reprodutivo da fêmea, mas, para tal, se faz necessária a utilização de meios diluentes e sistemas de refrigeração. Aparentemente, o sêmen asinino é capaz de suportar a taxa de refrigeração aplicada à espécie equina, entretanto, os diluentes à base de leite desnatado utilizados para equinos não retornam bons resultados para asininos (6,7).

Estudos recentes indicam que a adição de fontes de colesterol pode ser

benéfica para a manutenção da qualidade espermática (3,6), além de que o sêmen asinino refrigera melhor quando exposto a caseína ou gema de ovo (10,11), entretanto, não existem ainda protocolos bem definidos ou diluentes comerciais para a refrigeração de sêmen asinino.

2. REVISÃO DE LITERATURA

2.1. Asininos

Apesar da semelhança com a espécie equina, asininos possuem características anatômicas, fisiológicas e comportamentais próprias e conhecer essas particularidades é determinante para os resultados no manejo reprodutivo dessa espécie. A organização social dos jumentos de vida livre é territorial, enquanto cavalos se organizam em haréns, o que impacta diretamente no seu comportamento reprodutivo. Enquanto os garanhões possuem éguas no seu harém, as jumentas andam em pequenos grupos e entram nos territórios dos machos quando receptivas à monta (3).

Cavalos e jumentos são da ordem dos perissodáctilos, que abrange também animais como rinocerontes, zebras e antas. Entretanto, é importante compreender que o grau de proximidade filogenética só pode ser avaliado a partir de um referencial dentro da árvore genealógica, isto é, ao se considerar toda a classe Mammalia, jumentos e cavalos estarão muito próximos, entretanto, ao observar apenas a família Equidae, essa proximidade deixa de ser tão importante (12).

Steiner e Ryder (2011) observaram que o *Equus asinus,* espécie de estudo em questão, se aproxima mais do asno selvagem africano e das zebras do que dos cavalos domésticos (Figura 1), “divergindo do ancestral comum com os cavalos há 2 milhões de anos atrás” (12). A proximidade entre as espécies e os conhecimentos a respeito da espécie equina direcionaram por muito tempo a compreensão da espécie asinina, mas estudos como esses embasam a necessidade de tratar jumentos como a espécie distinta que são e desenvolver trabalhos específicos para compreendê-la.

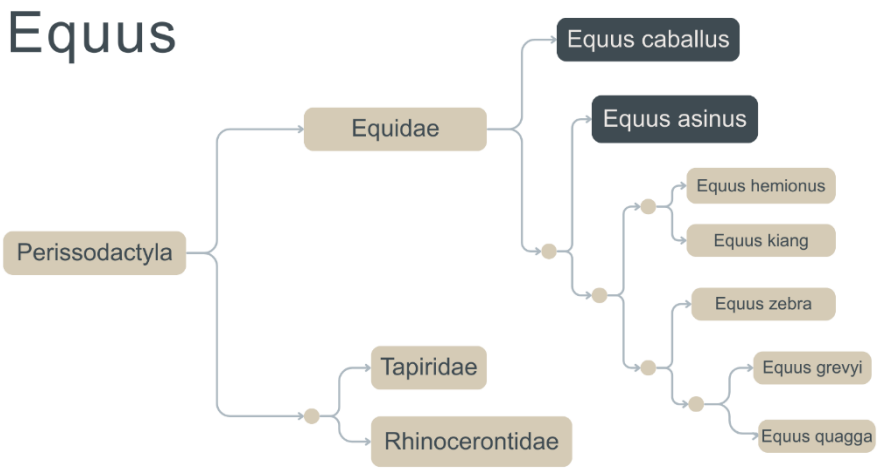


Figura 1: ilustração simplificada da genealigia das espécies *Equus caballus* e Equus asinus dentro do grupo Perissodactyla, baseada em Tougard et al., 2001, Steiner & Ryder, 2011 e Cozzuoul et al., 2013.

O período de côrte na espécie asinina se inicia com a aproximação da fêmea e pode durar de 30 a 90 minutos. Durante esse período, macho e fêmea vocalizam e o macho pode realizar a primeira monta sem ereção para garantir a receptividade da fêmea. As principais demonstrações de receptividade sexual na fêmea asinina são o comportamento de mascar, vocalizar, urinar e expor o clitóris, enquanto se mantém parada e com abdução dos membros pélvicos, mostrando-se receptiva à monta. É comum que o macho se afaste, tenha ereção enquanto afastado da fêmea e só então volte para realizar a cópula (3,13).

Quando comparados aos equinos, machos asininos possuem pênis mais longos, testículos maiores e de formato elipsóide, cauda do epidídimo maior e mais pronunciada e mamilos visíveis localizados em posição cranial aos testículos. Quanto às glândulas sexuais acessórias, o tamanho das glândulas bulbouretrais se destaca, apresentando-se maiores em relação às outras e em comparação às dos equinos. As fêmeas possuem anatomia de útero e ovários semelhante às éguas, se diferenciando principalmente na projeção caudal da cérvix, que pode apresentar diferentes formatos, como especificado por Canisso em 2019 (3).

Diferenças fisiológicas também impactam no manejo reprodutivo e na aplicação de biotécnicas nessa espécie. Fêmeas asininas não apresentam edema endometrial expressivo comparável ao edema observado em éguas, de

forma que não é possível se basear na imagem ultrassonográfica do útero para predizer cio ou ovulação. Além disso, autores relatam a presença de corpo *albicans* persistente e alta incidência de dupla ovulação em algumas raças asininas e consequente gestação gemelar. o que surpreendentemente não é um problema quando comparado a gestações gemelares em éguas. Jumentas comumente levam a termo gestações gemelares. A luteólise em jumentas causa contradição na literatura, alguns autores relatam boa luteólise com uma aplicação de prostaglandina (PGF2alfa) 3 dias após a ovulação, enquanto outros defendem a necessidade de duas aplicações e apenas após 5 a 6 dias de ovulação (14–17). Por fim, esses animais apresentam uma resposta inflamatória endometrial pós-cobertura mais exacerbada quando comparados a éguas, principalmente com sêmen criopreservado (3,13,18).

Devido ao longo período de côrte, é comum a utilização de PGF2alfa para diminuir o tempo de espera para a ereção nos jumentos, apesar de ser uma alternativa pouco ultilizada e não recomendada para a espécie equina (19–21). Panzani (2020) utilizou 125 µg de cloprostenol sódico e encontrou uma redução significativa no tempo de ereção (22). A PGF2alfa estimula a liberação de ocitocina e vasopressina, além de estimular a contração da musculatura do sistema reprodutor masculino (19,23).

Os jumentos tendem a ejacular maiores concentrações, entre 300 e 400 milhões por mL, e com melhor motilidade, de 80 a 90%, quando comparados aos cavalos. O volume é variável, entre 30 e 90 mL, com a característica de produzir pouco gel, também se diferenciando da espécie equina. A quantidade total de espermatozoides por ejaculado é maior, enquanto a média de patologias espermáticas é menor, entre 15% (3,8).

2.2. Célula espermática

O espermatozoide, gameta masculino produzido nos testículos, é uma célula complexa e altamente especializada para carrear o material genético masculino pelo trato reprodutivo da fêmea e realizar a fecundação (24). Anatomicamente, ele pode ser dividido entre cabeça e cauda ou flagelo, sendo a cabeça dividida em região acrossomal, lâmina pós-acrossomal e núcleo, e o flagelo divido em peça de conexão, peça intermediária, peça principal e peça final (25,26).

O acrossomo é uma organela externa formada a partir do complexo de Golgi que recobre a porção inicial do núcleo e se assemelha a um capuz, sendo delimitado por uma membrana contínua que se dobra sobre si e, didáticamente, se divide em membrana acrossomal externa e membrana acrossomal interna. O acrossomo é formado por enzimas hidrolíticas que atuarão, após a reação acrossomal, na ligação à zona pelúcida do oócito (24–26). O núcleo carrega o ácido desoxirribonucleico (DNA) condensado e é revestido pelo envelope nuclear e protegido pela teca perinuclear (24), sua porção caudal é coberta pela lâmina pós-acrossomal (26).

A cauda do espermatozoide é formada pelo axonema, que consiste em nove pares de microtúbulos marginais e dois microtúbulos centrais. O axonema é responsável pela capacidade de movimentação do espermatozoide e se estende por todo o comprimento da cauda, cercado por fibras densas. (24,25). Os pares externos são conectados entre si por ligações de nexina e conectados ao par central por raios radiais. O par central, por sua vez, é conectado por uma ponte cruzada (25,27).

A peça de conexão abriga os centríolos e dá origem às fibras densas, enquanto na peça intermediária encontram-se as mitocondrias em forma de espiral ao redor das fibras densas externas, formando a bainha mitocondrial. Na peça principal as bainhas fibrosas externas envolvem as fibras densas e o axonema, enquanto a peça final se diferencia por possuir apenas o axonema (24–26).

Nos equinos a cabeça do espermatozoide é definida como oval (26) ou espatulada (25), achatada em plano dorsoventral e com comprimento de aproximadamente 60 µm (28). O espermatozoide asinino, entretanto, não tem a sua morfologia bem detalhada na literatura, indicando apenas que a célula espermática nessa espécie apresenta cabeça maior e mais arredondada (11), e peça intermediária maior, quando comparada à do espermatozoide equino (18).

Quanto à patologia espermática, os padrões definidos pelo Colégio Brasileiro de Reprodução Animal (CBRA, 2013) para sêmen equino fresco indicam como aceitáveis até 40% de defeitos, sendo no máximo 20% maiores. Não há diretrizes para asininos no CBRA, mas a média apresentada na literatura é de 15% de defeitos totais (29).

2.3. Membrana plasmática

A membrana plasmática do espermatozoide é uma estrutura complexa, composta por fosfolipídios, glicolipídios, colesterol e proteínas, responsável por manter a integridade celular e regular eventos fisiológicos como capacitação, reação acrossomal e fusão com o oócito (30). Um dos aspectos centrais da sua composição é a elevada proporção de ácidos graxos poli-insaturados, que conferem fluidez e flexibilidade à bicamada lipídica, mas também aumentam a vulnerabilidade à peroxidação lipídica. O equilíbrio entre colesterol e fosfolipídios regula a estabilidade estrutural da membrana e influencia diretamente a sua resistência a variações térmicas (31,32).

Durante a refrigeração, a membrana sofre transições de fase lipídica que resultam na perda de fluidez, aumento da permeabilidade e maior suscetibilidade ao estresse oxidativo, fenômenos descritos como choque frio (33). A diminuição da temperatura também promove redistribuição e saída de colesterol da membrana, desorganizando microdomínios lipídicos essenciais à função espermática (34). Essas alterações resultam em perda de motilidade, integridade de membrana e capacidade fecundante (35).

A composição lipídica da membrana está diretamente relacionada à sua fluidez, mas também à sua resistência a baixas temperaturas. Além disso, os lipídeos presentes na membrana atuam como estoque de energia para a célula espermática. O perfil lipídico da célula espermática equina se difere do observado na célula espermática asinina. Chaves (2015) identificou 101 íons lipídicos em cavalos e 105 em jumentos, sendo que os equinos apresentaram cinco exclusivos da espécie e os jumentos nove, além de apresentarem maior quantidade relativa de 18 fosfolipídeos, sendo a maioria insaturados (36).

Essas particularidades na composição lipídica da membrana plasmática dos asininos explicam sua alta sensibilidade ao resfriamento. Estudos de lipidômica identificaram que espermatozoides de jumento possuem elevada proporção de ácidos graxos poli-insaturados (PUFAs), característica que favorece a fluidez, mas aumenta a vulnerabilidade à peroxidação lipídica e ao dano oxidativo durante armazenamento a frio (37). Além disso, a remoção ou diluição do plasma seminal pode intensificar a instabilidade da membrana, dado que proteínas e antioxidantes presentes nesse fluido atuam como protetores contra o estresse oxidativo (38).

Nesse contexto, estratégias específicas vêm sendo testadas para aumentar a resistência da membrana espermática de jumentos à refrigeração. A suplementação de diluentes com colesterol por meio de ciclodextrinas demonstrou melhorar a estabilidade da membrana e a viabilidade espermática após o resfriamento (6). Tais evidências ressaltam que a compreensão da composição específica da membrana plasmática dos asininos é essencial para o desenvolvimento de protocolos mais eficazes de conservação do sêmen dessa espécie.

2.4. Plasma seminal

O plasma seminal (PS) é a porção não celular que compõe o sêmen, é formado por secreções testiculares, epididimarias e glandulares e contém proteínas, hormônios, açúcares, eletrólitos e enzimas. Tanto os seus efeitos na célula espermática quanto no trato reprodutivo da fêmea são contraditórios. Algumas proteínas do PS equino são capazes de se ligar ao espermatozoide para modular a capacitação, enquanto outras conferem proteção para a célula espermática e contribuem para a sua maturação e movimentação (39–41).

Outra contradição da sua função está na ação das proteínas ligantes (*binder of sperm proteins* – BSP) que são fundamentais para a capacitação, mas, com o tempo, passam a mobilizar fosfolipídeos e colesterol da membrana plasmática, tornando-se prejudiciais (42). Além disso, estudos com espermatozoides coletados da cauda do epididimo demonstram que o PS não é determinante para a capacidade fertilizante do espermatozoide (43). Dessa forma, a função do plasma seminal para a célula espermática segue não totalmente elucidada.

Quanto à sua ação no trato reprodutivo da fêmea, enquanto alguns autores defendem que proteínas e citocinas pró inflamatórias do PS podem induzir a migração de polimorfonucleares (PMNs) para o epitélio uterino (40,44), outros autores apontam sua função na modulação da resposta inflamatória, diminuição na ligação espermatozoide-PMN e regulação negativa na expressão de ciclooxigenase-2 (COX-2), enzima que atua na resposta inflamatória (44–47).

Em asininos, componentes antioxidantes do PS, capazes de proteger a membrana espermática da peroxidação lipídica, foram relacionados aos parâmetros de motilidade e integridade de membrana plasmática e à sua

capacidade de resistir à criopreservação (18,48). Miró e Papas (2018) apontam os níveis de melatonina presentes no PS asinino como um dos possíveis mecanismos de controle de espécies reativas de oxigênio (EROs).

Nesse sentido, não há também um consenso em relação à retirada ou não do plasma seminal para os processos de refrigeração e criopreservação do sêmen, ou retirada seguida de ressuspensão. Enquanto alguns estudos demonstram efeitos deletérios do PS ao sêmen e defendem a sua retirada (1,49,50), outros autores obtiveram melhora na qualidade seminal quando mantiveram ou adicionaram plasma seminal nas amostras de sêmen (51,52). Para asininos, o dilema se mantém. É possível encontrar resultados positivos (53–55), negativos (1) para a remoção do plasma seminal em processos de refrigeração.

2.5. Diluentes

A utilização de diluentes para aumentar o tempo de viabilidade do sêmen começou na bovinocultura de leite em 1940, pelo que data a literatura, e se deu primeiro com a gema de ovo (56). Em 1941, Salisbury; Fuller; Willett adicionaram o citrato de sódio para tamponar a gema de ovo e a partir de 1950 os estudos utilizando leite desnatado iniciaram (58–60).

Diluentes à base de leite já eram utilizados em programas de inseminação artificial na China, enquanto acreditava-se que o espermatozoide equino não seria capaz de tolerar a utilização de extensores com gema e de manter a sua motilidade. Em 1949, com a adição de gema de ovo à glicose, os primeiros resultados de manutenção de motilidade associada a satisfatórias taxas de prenhez motivaram as pesquisas para diluentes de sêmen equino (61). Com a utilização do leite desnatado em pó acrescido de glicose, surgiram os diluentes “Kenney”, que são até hoje a base para os diluentes comerciais para a espécie equina (61).

A base dos diluentes para sêmen equino é composta por açucares, para conferir energia à célula espermática e manter a motilidade; antibióticos, para diminuir a proliferação de bactérias durante o armazenamento. Além da utilização de tampões como HEPES (ácido N-2-hidroxietilpiperazina-N-2-etanesulfônico), TRIS (tris-hidrometil-aminometano) e bicarbonato de sódio (NaHCO3) para manter o pH (entre 6.8 e 7,2); e de açúcares como glicose,

frutose e lactose associados a citrato de sódio para manter a osmolaridade adequada (entre 300 e 360 mOsm/L). Gema de ovo, leite ou moléculas isoladas do leite como a caseína e a lactose também possuem ação estabilizadora de pH ou osmolaridade, mas são utilizados principalmente para proteger o espermatozoide das variações de temperatura (62,63).

Em diluentes de criopreservação, outros crioprotetores serão adicionados (64,65).

2.6. Crioproterores

Ao ser exposta a temperaturas baixas durante os processos de refrigeração e criopreservação, a célula espermermática sofre alterações significativas. Para minimizar os danos causados principalmente pelo processo de congelação, agentes crioprotetores são adicionados à solução. Esses agentes podem ser divididos em dois grupos: crioprotetores penetrantes e crioprotetores não-penetrantes (33).

Os crioprotetores não-penetrantes são macromoléculas de alto peso molecular que atuam no meio extracelular por diferença de osmolaridade, induzindo a desidratação da célula espermática e, consequentemente, impedindo a formação de cristais de gelo no seu interior; ou estabilizam a membrana plasmática conferindo maior proteção durante as alterações de temperatura. Os principais agentes crioprotetores não-penetrantes são açúcares, gema de ovo e proteínas do leite (66,67).

As micelas de caseína presentes no leite interagem com as proteínas da membrana plasmática, impedindo a perda de fosfolipídeos e colesterol, enquanto as lipoproteínas de baixa densidade (LDL) da gema de ovo protegem a célula espermática do choque frio. Ambos os mecanismos de proteção não estão bem esclarecidos na literatura, embora acredite-se que o LDL interaja com proteínas do plasma seminal (BSP) neutralizando o seu efeito tóxico à celula espermática (68), e impeça a perda excessiva de colesterol, além de minimizar a produção de EROs (69).

Por outro lado, crioprotetores penetrantes devem ser substâncias de baixo peso molecular e alta solubilidade. Seu mecanismo de ação se baseia na formação de pontes de hidrogênio com as moléculas de água, limitando a formação de cristais de gelo, e na alteração da osmolaridade da solução,

induzindo a saída de água da célula. Ao penetrar no meio intracelular, esses agentes lançam mão da sua propriedade coligativa para alterar o ponto crioscópico do meio e, com isso, proteger a célula dos danos causados pelos cristais. Álcoois e amidas são os mais utilizados para esse fim (66,70).

Entre os crioprotetores penetrantes, o glicerol e a metilformamida se destacam na espécie equina. O glicerol é um álcool com três grupos hidroxila (OH) em sua estrutura molecular, o que lhe confere propriedades hidroscópicas que, na célula espermática, desidratam e diminuem o ponto de congelamento. Porém, apesar de amplamente utilizado na criopreservação de sêmen, o glicerol pode ser tóxico à célula espermática se utilizado em concentrações mais altas que 5% para equinos (70–73).

As amidas são formadas por uma amina e um ácido carboxílico, o que lhes confere a capacidade de ligação com a água em três sítios diferentes. Apesar de ser metade da capacidade ligante do glicerol, as amidas causam menos dano osmótico na célula, sendo utilizadas em maior proporção para a espécie equina (73,74). Entre elas, destacam-se a dimetilformamida e a metilformamida, que é produto da biotransformação da primeira (75).

Vidament et al. (2009) demonstraram, em um experimento comparando diferentes concentrações de glicerol em sêmen de jumento, que apenas 2,2% de glicerol adicionado ao sêmen foi suficiente para diminuir as taxas de prenhez em jumentas de 64%, antes da adição do crioprotetor, para 0%, após a adição. Da mesma forma, ao adicionar 1,9% de glicerol no sêmen refrigerado, os autores obtiveram apenas 1/12 resultados positivos de prenhez.

2.6. Refrigeração de sêmen

A refrigeração de sêmen permite a diminuição do metabolismo espermático, o que favorece a manutenção da viabilidade por mais que 24 horas. Além disso, o uso de sêmen refrigerado facilita o manejo reprodutivo da fêmea e possibilita o envio de sêmen a longas distâncias (76,77). A taxa de refrigeração deve ser controlada para evitar choque frio, principalmente entre 19 ºC e 8 ºC, já que é nesse momento que há maior instabilidade dos lipídeos, favorecendo lesões nas membranas espermáticas (77,78).

Inicialmente, o sêmen deve ser estabilizado à temperatura ambiente (20 °C). Esta queda inicial não causa maiores danos aos espermatozoides, desde

que estejam diluídos em um meio adequadado (78). A fase crítica de refrigeração é o momento de transição dos fosfolipídios da membrana plasmática do estado líquido-cristalino para o estado de gel, entre 19 ºC e 8 ºC (67). Para impedir maiores danos à membrana espermática, uma taxa de refrigeração de 0,05 ºC por minuto deve ser respeitada, principalmente nesse período de transição, além da necessidade de diluição do sêmen com extensores específicos (78–80)

A refrigeração do sêmen pode acontecer de modo passivo ou ativo, sendo que a refrigeração passiva por meio de caixas isotérmicas é a mais utilizada para envio de sêmen refrigerado. Com o emprego de equipamentos comerciais específicos para refrigeração e transporte de sêmen, a taxa de refrigeração é controlada, possibilitando uma curva de refrigeração lenta e progressiva até a estabilização a 5 ºC. Através disso, é possível reduzir os efeitos estressantes e deletérios que são gerados aos espermatozoides e alcançar uma viabilidade de até 72 horas pós-ejaculação para a maioria dos garanhões (79)

Diferente do que é observado em equinos, o sêmen asinino não apresenta bons resultados quando refrigerado em diluentes comerciais à base de leite. Aparentemente, fontes extra de colesterol (gema de ovo ou colesterol associado a ciclodextrina) são necessárias para a manutenção da qualidade espermática nessa espécie. A adição de colesterol-ciclodextrina aumentou 3,5 vezes a chance de prenhez em éguas (6) e, da mesma forma, diluentes a base de gema de ovo parece beneficiar a fertilidade do sêmen asinino quando utilizado para inseminação de éguas (7).

Alguns autores sugerem que o plasma seminal do jumento contém proteínas capazes de remover o colesterol da membrana plasmática dos espermatozoides, e por esse motivo, fontes de colesterol seriam benéficas para a refrigeração do sêmen asinino (3). Em um trabalho recente, foi demonstrado que a utilização de um diluente comercial de congelação à base de gema de ovo com adição de crioprotetores melhora a qualidade e fertilidade de sêmen de jumentos utilizado para inseminação de éguas (7).

No entanto, não se sabe ao certo o efeito desses diluentes quando sêmen refrigerado é utilizado para a inseminação de fêmeas asininas, já que o efeito tóxico dos crioprotetores para o endométrio de jumentas tem sido hipotetizado como causa das baixas taxas de fertilidade de jumentas em protocolos de inseminação artificial (IA) com sêmen congelado (5). Os resultados de fertilidade

de sêmen asinino refrigerado variam de acordo com o diluente utilizado, a presença ou não de plasma seminal e a espécie da fêmea inseminada (Tabela 1).

Tabela 1: Sumário Comparativo dos Estudos de Fertilidade em Éguas e Asininas Inseminadas com Sêmen Refrigerado, Avaliando Diferentes Bases Diluidoras e a Presença de Plasma Seminal.

Referência	Diluente/Base do Diluente	Plasma Seminal (PS)	Temperatura (°C)	Tempo de Conservação	Espécie da Fêmea	MT (%)	Fertilidade (%)
Vidament et al., 2009	Leite UHT	Presente	4	>8h	Equina	-	45 (33/73)
					Asinina	-	45 (9/20)
Carvalho et al., 2017	Leite desnatado c/ glicose	Ausente	5	12h	Equina	69,6 ± 3,9	56,52 (13/23)
				24h		64,9 ± 4,5	56,52 (13/23)
	Lactose e gema de ovo, s/ glicerol			12h		45,2 ± 3,8	4,76 (1/21)
				24h		34,1 ± 4,7	4,76 (1/21)
Segabinazzi et al., 2021	Leite desnatado	Presente	5	24h	Equina	55,0 ± 10,0	13 (2/15)
	Leite desnatado + ciclodextrina c/ colesterol					75,0 ± 8,0	47 (7/15)
Gobato et al., 2022	Leite desnatado + ciclodextrina c/ colesterol	Presente	5	24h	Equina	70,2 ± 6,7	33 (9/27)
	Caseinato de sódio + ciclodextrina c/ colesterol					80,0 ± 5,5	67 (18/27)
	Gema de ovo					87,2 ± 5,9	89 (25/28)
	Leite desnatado + ciclodextrina c/ colesterol	Ausente	5	24h	Equina	76,4 ± 6,2	59 (16/27)
	Caseinato de sódio + ciclodextrina c/ colesterol					84,3 ± 4,5	89 (24/27)
	Gema de ovo					91,8 ± 3,0	74 (20/27)

MT= motilidade total; **UHT**= *ultra high temperature*;

Não é padronizado na literatura nem pelo Colégio Brasileiro de Reprodução Animal (CBRA) a dose inseminante para sêmen asinino. Os experimentos realizados por Vidament et al., (2009) e por Carvalho et al., (2017) utilizaram 400x10^6 spz, enquanto os experimentos realizados por Segabinazzi et al., (2021) e por Gobato et al., (2022) utilizaram 1 bilhão de espermatozoides. Vale ressaltar, entretanto, que apenas o experimento realizado por Vidament el at., (2009) realizou teste de fertilidade em jumentas.

Oliveira et al. (2016) testaram diferentes doses e demonstraram que jumentas são beneficiadas por doses inseminantes maiores (81). Dessa forma, apesar dos resultados obtidos por Vidament et al., (2009) serem iguais para éguas e jumentas, não é possível definir se os resultados de fertilidade nas jumentas não foi afetado pela dose inseminante.

Papa et al., (2015) avaliaram o efeito do glicerol na refrigeração de sêmen bovino e encontraram influência positiva do crioprotetor nas taxas de fertilidade, concordando com o que foi sugerido de que na célula espermática bovina o glicerol é metabolizado e convertido em estoque de glicose que vai ser usado no processo de fertilização (82–84).

3. CONSIDERAÇÕES FINAIS

A refrigeração de sêmen ainda é a técnica de manipulação de sêmen mais importante para a espécie asinina, apesar de muito pouco compreendida. Sabe-se que fontes extras de colesterol podem ser benéficas e que diluentes de criopreservação à base de gema de ovo conferem bons resultados à refrigeração de sêmen asinino. No entanto, ainda não existe um protocolo definido, tampouco um diluente desenvolvido para jumentos e poucos estudos têm focado na fertilidade do sêmen refrigerado nessa espécie.

4. HIPÓTESE E OBJETIVOS

4.1. Hipótese

A gema de ovo afeta positivamente a qualidade espermática de sêmen asinino refrigerado e as taxas de fertilidade em jumentas e a presença de crioprotetores não é prejudicial para os mesmos parâmetros.

4.2. Objetivos

4.2.1 Objetivo gerais

Obter melhores resultados de cinética e fertilidade na refrigeração de sêmen asinino por meio do desenvolvimento de meios de diluição mais eficazes para a espécie.

4.2.2 Objetivo especificos

1. Comparar os efeitos de diluentes à base de leite e gema de ovo sobre a cinética e integridade de membrana do sêmen asinino refrigerado por 72 horas;
2. Identificar um substituto comercial à gema de ovo eficiente para a refrigeração de sêmen de jumentos;
3. Avaliar o impacto da remoção do plasma seminal sobre a longevidade e o perfil oxidativo do sêmen asinino refrigerado;
4. Determinar as taxas de prenhez em jumentas inseminadas com sêmen refrigerado utilizando diferentes diluentes comerciais;
5. Comparar a fertilidade de jumentas inseminadas com sêmen refrigerado na presença ou ausência de plasma seminal;
6. Mensurar a resposta inflamatória uterina (citologia e contagem de PMNs) em jumentas inseminadas com sêmen refrigerado em diferentes meios base;
7. Estabelecer as concentrações ideais de gema de ovo para a preservação do sêmen asinino a 5 ºC;
8. Analisar os efeitos de diferentes crioprotetores sobre a qualidade espermática do sêmen asinino refrigerado;
9. Avaliar as taxas de gestação em jumentas e éguas inseminadas com diluente à base de leite acrescido de substituto comercial de gema de ovo;
10. Comparar o efeito da adição de diluente de criopreservação à base de gema de ovo e da retirada do plasma seminal em sêmen asinino refrigerado a 5 ºC em diluente à base de leite desnatado.

5. REFERÊNCIAS*

1. Rota A, Magelli C, Panzani D, Camillo F. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology. 2008 May 15;69(2):176–85. doi:10.1016/j.theriogenology.2007.09.003 PubMed PMID: 17945340.

2. Camillo F, Rota A, Biagini L, Tesi M, Fanelli D, Panzani D. The Current Situation and Trend of Donkey Industry in Europe. Journal of Equine Veterinary Science. W.B. Saunders; 2018. p. 44–9. doi:10.1016/j.jevs.2017.11.008

3. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key Aspects of Donkey and Mule Reproduction. Veterinary Clinics of North America - Equine Practice. W.B. Saunders; 2019. p. 607–42. doi:10.1016/j.cveq.2019.08.014 PubMed PMID: 31672204.

4. Fanelli D, Tesi M, Monaco D, Diaz-Jimenez M, Camillo F, Rota A, et al. Deep-horn Artificial Insemination With Frozen Thawed Semen After re-extension in Autologous Seminal Plasma May Improve Pregnancy Rates in Jennies. J Equine Vet Sci. 2022 May 1;112. doi:10.1016/j.jevs.2022.103932 PubMed PMID: 35283216.

5. Vidament M, Vincent P, Martin FX, Magistrini M, Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim Reprod Sci. 2009 May;112(1–2):22–35. doi:10.1016/j.anireprosci.2008.03.016 PubMed PMID: 18502059.

6. Segabinazzi LGTM, Scheeren VF da C, Freitas-Dell'Aqua C de P, Papa FO, Alvarenga MA, Dell'Aqua JA, et al. Cholesterol-Loaded Cyclodextrin Addition to Skim Milk-Based Extender Enhances Donkey Semen Cooling and Fertility in Horse Mares. J Equine Vet Sci. 2021 Oct 1;105. doi:10.1016/j.jevs.2021.103719 PubMed PMID: 34607680.

7. M Gobato MLDPS, Miro J, Gaitskell-Phillips G, Carluccio A, T M Segabinazzi LG, M Gobato ML, et al. Ability of donkey sperm to tolerate cooling: Effect of extender base and removal of seminal plasma on sperm parameters and fertility rates in mares. 2022. Report.

8. Morais RNMRGVWG. REPRODUCTIVE BIOLOGY OF DONKEY. II. PHYSYCAL AND M ORFHOLOGYCAL CHARACTERISTICS OF SEMEN. 1994. Report.

9. Oliveira JV de et al. ,. Effect of cryoprotectant on donkey semen freezability and fertility. Anim Reprod Sci. 2006 Aug;94(1–4):82–4. doi:10.1016/j.anireprosci.2006.04.010

10. de Melo MIV et al. USO DE LIPOPROTEÍNAS DE BAIXA DENSIDADE E DE GEMA DE OVO NA PRESERVAÇÃO DO SÊMEN ASININO RESFRIADO A 6oCMaria Isabel Vaz de Melo1+,Yolanda deFátima Resende2, Elton Pavão Rezende2, Paola Per. 2012.

* Referências organizadas de acordo com as regras de estilo e formato para referências do *International Committee of Medical Journals Editor* (ICMJE) intitulada *Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly work in Medical Journals – Recomendations* ICMJE - *Vancouver*

11. Luiza M, Gobato M, Paulo BS. UNIVERSIDADE ESTADUAL PAULISTA FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA EFEITO DE DIFERENTES DILUENTES NA QUALIDADE E FERTILIDADE DO SÊMEN REFRIGERADO DE JUMENTO PÊGA (Equus asinus). Report.

12. Steiner CC, Ryder OA. Molecular phylogeny and evolution of the Perissodactyla. Zool J Linn Soc. 2011 Dec;163(4):1289–303. doi:10.1111/j.1096-3642.2011.00752.x

13. Mcdonnell SM. Reproductive behavior of donkeys Equus asinus. Applied Animal Behaviour Science. 1998. Report.

14. Segabinazzi LGTM, Landers M, Kent A, Peterson E, Gilbert R, French H. Efficacy and Side Effects of Low Single Doses of Cloprostenol Sodium or Dinoprost Tromethamine to Induce Luteolysis in Donkeys. J Equine Vet Sci. 2021 Dec 1;107. doi:10.1016/j.jevs.2021.103774 PubMed PMID: 34802629.

15. Segabinazzi LGTM, Gilbert RO, Ambrosia RL, Bergfelt DR, Samper JC, Peterson EW, et al. Structural and Functional Dynamics of the Ovary and Uterus during the Estrous Cycle in Donkeys in the Eastern Caribbean. Animals. 2023 Jan 1;13(1). doi:10.3390/ani13010074

16. Panzani D, Tardella M, Govoni N, Tesi M, Fanelli D, Rota A, et al. Effect of the administration of alfaprostol 3 or 6 days after ovulation in jennies: ultrasonographic characteristic of corpora lutea and serum progesterone concentration. Theriogenology. 2018 Nov 1;121:175–80. doi:10.1016/j.theriogenology.2018.08.014 PubMed PMID: 30165306.

17. Carluccio A, Panzani S, Contri A, Tosi U, De Amicis I, Veronesi MC. Luteal function in jennies following PGF2α treatment 3 days after ovulation. Theriogenology. 2008 Jul 1;70(1):121–5. doi:10.1016/j.theriogenology.2008.03.010 PubMed PMID: 18439666.

18. Miró J, Papas M. Post–Artificial Insemination Endometrial Inflammation and Its Control in Donkeys. Journal of Equine Veterinary Science. W.B. Saunders; 2018. p. 38–43. doi:10.1016/j.jevs.2017.11.007

19. Kreider JL, 0gg WL, Turner JW. INFLUENCE OF PROSTAGLANDIN F,a ON SPERM PRODUCTION AND SEMINAL CHARACTERISTItS OF THE STALiIONl. 1981. Report.

20. Mcdonnell SM. EJACULATION Physiology and Dysfunction. Vol. 8. 1992. Report.

21. Panzani D, Quaresma M, Fanelli D, Camillo F, Moroni R, Rota A, et al. Hastening time to ejaculation in donkey jacks treated with the pgf2α analog, cloprostenol sodium. Animals. 2020 Dec 1;10(12):1–11. doi:10.3390/ani10122231

22. Panzani D, Quaresma M, Fanelli D, Camillo F, Moroni R, Rota A, et al. Hastening time to ejaculation in donkey jacks treated with the pgf2α analog, cloprostenol sodium. Animals. 2020 Dec 1;10(12):1–11. doi:10.3390/ani10122231

23. Veronesi MC, De Amicis I, Panzani S, Kindahl H, Govoni N, Probo M, et al. PGF2α, LH, testosterone, oestrone sulphate, and cortisol plasma concentrations around sexual stimulation in jackass. Theriogenology. 2011 May;75(8):1489–98. doi:10.1016/j.theriogenology.2010.12.010 PubMed PMID: 21295832.

24. Cruz DF, Freitas MJ, Patrício A, Fardilha M. Vias de sinalização reguladoras das funções do espermatozoide. Revista Internacional de Andrologia. Ediciones Doyma, S.L.; 2014. p. 104–11. doi:10.1016/j.androl.2014.04.006

25. Varner DD, Gibb Z, Aitken RJ. Stallion fertility: A focus on the spermatozoon. Equine Vet J. 2015 Jan 1;47(1):16–24. doi:10.1111/evj.12308 PubMed PMID: 24943233.

26. Orsolini MF, Meyers SA, Dini P. An update on semen physiology, technologies, and selection techniques for the advancement of in vitro equine embryo production: Section i. Animals. MDPI; 2021. doi:10.3390/ani11113248

27. Brito LFC. Evaluation of Stallion Sperm Morphology. Clinical Techniques in Equine Practice. 2007 Dec;6(4):249–64. doi:10.1053/j.ctep.2007.09.004

28. Pesch S, Bergmann M. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. Micron. 2006. p. 597–612. doi:10.1016/j.micron.2006.02.006 PubMed PMID: 16621580.

29. Canisso IF, Andrade Souza F, Ribeiro De Carvalho G, Domingos Guimarães J, Capistrano Da Silva E, Linhares Lima A. Rev Bras Reprod Anim [Internet]. Vol. 4. Report. Available from: www.cbra.org.br

30. Amaral A, Castillo J, Estanyol JM, Ballesca JL, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. Molecular and Cellular Proteomics. 2013;12(2):330–42. doi:10.1074/mcp.M112.020552 PubMed PMID: 23161514.

31. Dutta S, Majzoub A, Agarwal A. Oxidative stress and sperm function: A systematic review on evaluation and management. Arab Journal of Urology. Taylor and Francis Ltd.; 2019. p. 87–97. doi:10.1080/2090598X.2019.1599624

32. Castro M, Leal K, Pezo F, Contreras MJ. Sperm Membrane: Molecular Implications and Strategies for Cryopreservation in Productive Species. Animals. Multidisciplinary Digital Publishing Institute (MDPI); 2025. doi:10.3390/ani15121808

33. Watson PF. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science [Internet]. 2000. Report. Available from: www.elsevier.comrlocateranireprosci

34. Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology. 2005 Dec;51(3):241–9. doi:10.1016/j.cryobiol.2005.07.004 PubMed PMID: 16122725.

35. Baumber J, Sabeur K, Vo A, Ball BA. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology. 2003 Oct 15;60(7):1239–47. doi:10.1016/S0093-691X(03)00144-4 PubMed PMID: 14511778.

36. Chaves M. UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO” FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA PERFIL LIPÍDICO DA MEMBRANA CITOPLASMÁTICA DE ESPERMATOZOIDES “IN NATURA” E CRIOPRESERVADOS DE JUMENTOS E CAVALOS MARIA MANOELA BARATA DE CASTRO CHAVES. 2015. Report.

37. Prasinou P, De Amicis I, Fusaro I, Bucci R, Cavallini D, Parrillo S, et al. The Lipidomics of Spermatozoa and Red Blood Cells Membrane Profile of Martina Franca Donkey: Preliminary Evaluation. Animals. 2023 Jan 1;13(1). doi:10.3390/ani13010008

38. Catalán J, Yánez-Ortiz I, Tvarijonaviciute A, González-Aróstegui LG, Rubio CP, Barranco I, et al. Seminal Plasma Antioxidants Are Related to Sperm Cryotolerance in the Horse. Antioxidants. 2022 Jul 1;11(7). doi:10.3390/antiox11071279

39. Wen X, Ren H, He Q, Yi M, Ulaangerel T, Bou G. Comparative Analysis of Proteomic Characteristics in Seminal Plasma Between Horses and Donkeys. Animals. 2025 Jun 1;15(11). doi:10.3390/ani15111532

40. Rodríguez-Martínez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ. Seminal plasma proteins: What role do they play? American Journal of Reproductive Immunology. 2011 Jul;66(SUPPL. 1):11–22. doi:10.1111/j.1600-0897.2011.01033.x PubMed PMID: 21726334.

41. Papas M, Arroyo L, Bassols A, Catalán J, Bonilla-Correal S, Gacem S, et al. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. Theriogenology. 2019 Dec 1;140:180–7. doi:10.1016/j.theriogenology.2019.08.032 PubMed PMID: 31479834.

42. Manjunath et al. Seminal plasma proteins: functions and interaction with protective agents during semen preservation [Internet]. 2007. Report. Available from: https://www.researchgate.net/publication/6193603

43. Monteiro GA, Papa FO, Zahn FS, Dellaqua JA, Melo CM, Maziero RRD, et al. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. Anim Reprod Sci. 2011 Sep;127(3–4):197–201. doi:10.1016/j.anireprosci.2011.08.002 PubMed PMID: 21890290.

44. Rodriguez-Martinez H, Martinez EA, Calvete JJ, Peña Vega FJ, Roca J. Seminal plasma: Relevant for fertility? International Journal of Molecular Sciences. MDPI; 2021. doi:10.3390/ijms22094368 PubMed PMID: 33922047.

45. Vilés K, Rabanal R, Rodríguez-Prado M, Miró J. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. Theriogenology. 2013 Apr 15;79(7):1019–26. doi:10.1016/j.theriogenology.2013.01.006 PubMed PMID: 23453786.

46. Miró J, Vilés K, García W, Jordana J, Yeste M. Effect of donkey seminal plasma on sperm movement and sperm-polymorphonuclear neutrophils attachment in vitro. Anim Reprod Sci. 2013 Aug;140(3–4):164–72. doi:10.1016/j.anireprosci.2013.06.007 PubMed PMID: 23891218.

47. Miró J, Catalán J, Marín H, Yánez-Ortiz I, Yeste M. Specific seminal plasma fractions are responsible for the modulation of sperm–pmn binding in the donkey. Animals. 2021 May 1;11(5). doi:10.3390/ani11051388

48. Catalán J, Yánez-Ortiz I, Tvarijonaviciute A, González-Arostegui LG, Rubio CP, Yeste M, et al. Impact of Seminal Plasma Antioxidants on Donkey Sperm Cryotolerance. Antioxidants. 2022 Feb 1;11(2). doi:10.3390/antiox11020417

49. Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology. 2005 Apr 1;63(6):1584–91. doi:10.1016/j.theriogenology.2004.05.030 PubMed PMID: 15763103.

50. Brinsko SP, Crockett LEC, Squires EL. EFFECT OF CENTRIFUGATION AND PARTIAL REMOVAL OF SEMINAL PLASMA ON EQUINE SPERMATOZOAL MOTILITY AFTER COOLING AND STORAGE. Report.

51. Alghamdi AS, Foster DN, Troedsson MHT. Equine seminal plasma reduces sperm binding to polymorphonuclear neurophils (PMN's) and improves the fertility of fresh semen inseminated into inflamed uteri. Reproduction. 2004;127(5):593–600. doi:10.1530/rep.1.00096 PubMed PMID: 15129015.

52. Aurich JE, Ktibne JA, Hoppe3 H, Auricbz C. SEMINAL PLASMA AFFECTS MEMBRANE INTEGRITY AND MOTILITY OF EQUJNE SPERMATOZOA AFTER CRYOPRESERVATION. 1996. Report.

53. Serres C, Rodriguez A, Alvarez A, Santiago I, Gabriel J, Go Âmez-Cue Âtara C, et al. Effect of centrifugation and temperature on the motility and plasma membrane integrity of Zamorano-Leone Âs donkey semen. 2002. Report.

54. Rodrigues et al. LT. Efeito de diferentes diluentes e da remoção do plasma seminal na refrigeração do sêmen asinino (Equus asinus). Revista Acadêmica: Ciência Animal. 2017 Jul 21;15:143. doi:10.7213/academica.15.s01.2017.71

55. Dalmau CS. UNIVERSIDAD COMPLUTENSE DE MADRID ASNO ZAMORANO-LEONES MEMORIA PARA OPTAR AL GRADO DE DOCTOR PRESENTADA POR Consuelo Serres Dalmau Bajo la dirección del Doctor. 2004. Report.

56. Phillips PH, Lardy HA. A Yolk-Buffer Pabulum for the Preservation of Bull Semen. J Dairy Sci. 1940;23(5):399–404. doi:10.3168/jds.S0022-0302(40)95541-2

57. Salisbury GW, Fuller HK, Willett EL. Preservation of Bovine Spermatozoa in Yolk-Citrate Diluent and Field Results from its Use. J Dairy Sci. 1941;24(11):905–10. doi:10.3168/jds.S0022-0302(41)95476-0

58. Almquist JO, Wickersham EW. Diluents for Bovine Semen. XII. Fertility and Motility of Spermatozoa in Skimmilk with Various Levels of Glycerol and Methods of Glycerolization. J Dairy Sci. 1962;45(6):782–7. doi:10.3168/jds.S0022-0302(62)89488-0

59. Thacker DL, Almquist JO. Diluters for Bovine Semen. I. Fertility and Motility of Bovine Spermatozoa in Boiled Milk. J Dairy Sci. 1953;36(2):173–80. doi:10.3168/jds.S0022-0302(53)91474-X

60. O'Dell WT, Almquist JO. Freezing Bovine Semen. I. Techniques for Freezing Bovine Spermatozoa in Milk Diluents. J Dairy Sci. 1957;40(12):1534–41. doi:10.3168/jds.S0022-0302(57)94674-X

61. Brinsko SP. Antibiotics and other additives for semen extenders to enhance fertility 1. 2009. Report.

62. Müller Y. Effects of substituting sodium chloride and omitting antibiotics in stallion semen diluents for prolonged storage at elevated temperatures. 2019. Report.

63. Trentin JM, Centeno LAM, De Souza Balestrin T, Minela T, Zanatta GM, Pessoa GA, et al. Sodium bicarbonate and HEPES as buffer components for cooling the semen of pony stallions. Semina:Ciencias Agrarias. 2018 Mar 1;39(2):631–42. doi:10.5433/1679-0359.2018v39n2p631

64. Batellier F, Vidament M, Fauquant J, Duchamp G, Arnaud G, Yvon JM, et al. Advances in cooled semen technology. Animal Reproduction Science. 2001. Report.

65. Conde DC, Souza GV de, Almeida Filho PR de O, Shimoda E, Silva JFS. Características seminais de dois diluentes de simples preparação e acessibilidade de seus componentes para o resfriamento de ejaculado equino. Brazilian Journal of Development. 2025 Jun 23;11(6):e80541. doi:10.34117/bjdv11n6-064

66. Pickett BW, Amann RP. EXTENSION AND STORAGE OF STALLION SPERMATOZOA: A REVIEW. 1987. Report.

67. Graham JK. CRYOPRESERVATION OF STALLION SPERMATOZOA. 1996. Report.

68. Manjunath P, Thérien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. Thérien / Journal of Reproducti6e Immunology [Internet]. 2002. Report. Available from: www.elsevier.com/locate/jreprimm

69. Dalal J, Chandolia RK, Pawaria S, Kumar A, Kumar D, Selokar NL, et al. Low-density lipoproteins protect sperm during cryopreservation in buffalo: Unraveling mechanism of action. Mol Reprod Dev. 2020 Dec 1;87(12):1231–44. doi:10.1002/mrd.23434 PubMed PMID: 33140487.

70. Nouri Gharajelar S, Ali Sadrkhanloo R, Onsori M, Saberivand A, Nouri Gharajelar DVM S. A comparative study on the effects of different cryoprotectants on the quality of canine sperm during vitrification process. ARTICLE Veterinary Research Forum. 2016. Report.

71. Oliveira SN de. EFEITO DO GLICEROL E AMIDAS EM DIFERENTES CURVAS DE REFRIGERAÇÃO SOBRE A QUALIDADE DO SÊMEN CONGELADO EQUINO. 2020. Report.

72. Macías García B, Ortega Ferrusola C, Aparicio IM, Miró-Morán A, Morillo Rodriguez A, Gallardo Bolaños JM, et al. Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology. 2012 Apr 15;77(7):1280–9. doi:10.1016/j.theriogenology.2011.10.033 PubMed PMID: 22326587.

73. Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros ASL. Amides as cryoprotectants for freezing stallion semen: A review. Anim Reprod Sci. 2005;89(1-4 SPEC. ISS.):105–13. doi:10.1016/j.anireprosci.2005.07.001 PubMed PMID: 16099609.

74. Squires EL, Keith SL, Graham JK. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology. 2004 Sep 15;62(6):1056–65. doi:10.1016/j.theriogenology.2003.12.024 PubMed PMID: 15289047.

75. Van Den Bulcke M, Rosseel MT, Wijnants P, Buylaert W, Belpaire FM. Metabolism and hepatotoxicity of N,N-dimethylformamide, N-hydroxymethyl-N-methylformamide, and N-methylformamide in the rat. Arch Toxicol. 1994. Report.

76. Avanzi BR. Efficiency of different cooling and storage systems for maintaining equine semen viability in a hot environment. Anim Reprod Sci. 2006 Aug;94(1–4):152–4. doi:10.1016/j.anireprosci.2006.03.042

77. Canisso IF, Andrade Souza F, Capistrano Da Silva E, Carvalho GR, Domingos Guimarães J, Linhares Lima A. INSEMINAÇÃO ARTIFICIAL EM EQUINOS: sêmen fresco, diluído, resfriado e transportado Artificial insemination in equines: diluted, cooled and transported race semen. Ciênc. Agrár. Ambient. 2008. Report.

78. Moran DM, Jasko DJ, Squires EL, Amann RP. DETERMINATION OF TEMPERATURE AND COOLING RATE WHICH INDUCE COLD SHOCK IN STALLION SPERMATOZOA. 1992. Report.

79. Nunes. Fatores relacionados ao sucesso da inseminação artificial de éguas - Nunes 2006.

80. Kayser JP, Amann RP, Shideler RK, Squires EL, Jasko DJ, Pickett BW. ISFECTS OF LINEAR coowNG RATE ON MOTION CEARACIERISTICS OF !ZTALUON SPERhtA'lOZOA. 1992. Report.

81. Oliveira JV de, Oliveira PV de LF, Melo e Oña CM, Guasti PN, Monteiro GA, Sancler da Silva YFR, et al. Strategies to improve the fertility of fresh and frozen donkey semen. Theriogenology. 2016 Apr 15;85(7):1267–73. doi:10.1016/j.theriogenology.2015.12.010 PubMed PMID: 26806444.

82. Papa PM, Maziero RD, Guasti PN, Junqueira CR, Freitas-Dell'Aqua CP, Papa FO, et al. Effect of glycerol on the viability and fertility of cooled bovine semen. Theriogenology. 2015 Jan 1;83(1):107–13. doi:10.1016/j.theriogenology.2014.08.009 PubMed PMID: 25441498.

83. Mohri H, Masaki J. GLYCEROKINASE AND ITS POSSIBLE ROLE IN GLYCEROL METABOLISM OF BULL SPERMATOZOA'. Report.

84. T. MANN & I. G. WHITE. Metabolism of Glycerol, Sorbitol and Related Compounds by Spermatozoa.

CAPÍTULO 2

ARTIGO CIENTÍFICO 1

Impact of permeable cryoprotectants and seminal plasma on donkey fertility with cooled-stored semen

Silva, A. C.[1], Zanardi, C. S[1], Monteiro, G. A. [1], Segabinazzi, L. G. T. M.[1,2]

[1]Sao Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu, SP, Brazil

[2]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St. Kitts, West Indies

Highlights

- Egg yolk is beneficial to donkey spermatozoa.
- The removal of cryoprotectants from the freezing extender reduced pregnancy rates in jennies.
- The removal of seminal plasma prior to cooling improved pregnancy rates in jennies.

ABSTRACT

It is well-established that cholesterol sources can benefit asinine spermatozoa and that the use of egg yolk-based cryopreservation extenders is a viable alternative for semen cooling in this species. However, the specific importance of egg yolk, cryoprotectants, and the extender base during the refrigeration process remains unclear. In this context, the present study compared the sperm kinetics of skim milk-based extenders containing cholesterol (BotuSêmen Special - BSS) and a skim milk-based extender with casein and cholesterol (BotuSêmen Gold - BG), an egg yolk-based cryopreservation extender (BotuCrio - BC), BC without cryoprotectants (BCsC), and a cryopreservation extender using sodium caseinate as a substitute for egg yolk (BCC). A parallel experiment compared kinetic results between the BG group and a group that underwent centrifugation for seminal plasma removal prior to resuspension in BotuSêmen Gold (BG-P).

Furthermore, fertility rates were evaluated in jennies inseminated using the BG, BC, BCsC, and BG-P groups. Regarding sperm kinetics, the BC and BCsC groups were superior ($P < 0.05$) in Experiment 1, while the group without seminal plasma (BG-P) outperformed the group with seminal plasma (BG) in Experiment 2. Fertility results did not differ significantly between groups. The kinetic data corroborate existing literature, supporting egg yolk as the superior alternative for semen cooling. However, the fertility data diverge from the hypothesis that cryoprotectants are responsible for the low fertility rates in jennies inseminated with cryopreserved donkey semen, as the group without cryoprotectants yielded results similar to the group containing cryoprotectants.

Keywords: Cooling; donkey; extender; semen.

1. Introduction

Historically, donkeys and mules have represented a significant proportion of the global working and draft animal population. However, the mechanization of labor and the rural exodus have diminished the demand for these animals, leading to a decline in their relevance within the broader equine market (1). Despite limited meat consumption, there has been a burgeoning industrial interest in donkey milk, particularly within the cosmetic sector (2). Additionally, hybrid production remains vital for livestock management, especially in the Brazilian Midwest and the United States, where these animals are indispensable for field operations (3). Despite the close phylogenetic relationship between horses (*Equus caballus*) and donkeys (*Equus asinus*), distinct differences in anatomy, physiology, behavior, and management requirements directly impact the efficacy of reproductive biotechnologies. While fresh donkey semen yields satisfactory fertility rates in both mares and jennies, the use of cooled and frozen-thawed jack semen continues to produce inconsistent results (4,5). This discrepancy arises largely because donkey reproductive techniques are often extrapolated from equine protocols. Notably, cryopreserved donkey semen yields significantly lower fertility rates when used in jennies (6–8); consequently, cooled semen remains the only viable alternative for breeding animals across different locations.

Donkey spermatozoa appear to tolerate cooling rates similar to those used for horses. Nevertheless, the skim milk-based extenders standard in equine practice

do not provide adequate preservation for asinine semen. Recent evidence suggests that the addition of cholesterol sources and egg yolk may enhance sperm quality maintenance. However, further research is required to develop extenders capable of preserving donkey semen at a standard comparable to cooled equine semen (5,9,10).

Furthermore, current literature regarding fertility predominantly reports pregnancy rates in mares inseminated with cooled donkey semen (5,6,10,11). One hypothesis for the limited success of cryopreserved semen in jennies is an exacerbated uterine inflammatory response attributed to penetrating cryoprotectants, while other authors suggest this reaction is due to the removal of seminal plasma (8).

In this regard, the present study aims to elucidate the impact of egg yolk and penetrating cryoprotectants on sperm kinetics, and to evaluate the effects of egg yolk, cryoprotectants, and seminal plasma on fertility in jennies inseminated with cooled donkey semen.

2. Materials and Methods

This study was reviewed and approved by the Comitê de Ética no Uso de Animais (CEUA) of São Paulo State University (UNESP), Botucatu, São Paulo, Brazil, under protocol no. 0569/2023. The experiment was conducted from February 2024 to August 2025.

2.1. Experiment 1 – Effects of cryoprotectant removal and egg yolk substitution in a cryopreservation extender on the viability of cooled donkey semen

2.1.1. Animals

Seven Pêga jacks, aged between 6 and 14 years, with proven fertility and housed at a private farm in Laranjal Paulista, São Paulo, were selected for the experiment. The animals were kept in stalls, fed hay, and had free access to water. To deplete sperm reserves, 3 consecutive semen collections were performed, followed by a rest day before the start of experimental procedures. All collections were performed using a Botucatu model artificial vagina (Botupharma®, Botucatu, São Paulo, Brazil) filled with water at 55ºC, in the presence of an estrous jenny. To reduce the waiting time for erection, all animals

received 0,5 mL of sodium D-cloprostenol (Botupharma®, Botucatu, São Paulo, Brazil, 120 µg/mL) intramuscularly .

Four ejaculates were collected per animal (n=28) on alternate days. After collection, semen was immediately filtered (to remove the seminal gel). Next, a 10 µl aliquot of semen was mixed with 490µl of distilled water. This dilution (1:50) was used to determine sperm concentration in a Neubauer chamber. Based on the determined concentration, the remaining semen was diluted and divided into five experimental groups according to the experimental design to begin treatments.

2.1.2. Experimental Design

Each ejaculate was divided into five aliquots and diluted to a final concentration of 50x10^6 spz/mL in the respective extenders: skim milk-based extender containing cholesterol (BotuSêmen Special, Botupharma®, Brazil – BS, control group); sodium caseinate-based extender containing cholesterol (BotuSêmen Gold, Botupharma®, Brazil – BG); egg yolk-based extender containing cryoprotectants (BotuCrio, Botupharma®, Brazil – BC); modified BotuCrio® extender where egg yolk was replaced, in the same percentage, by sodium caseinate (BCC); and BotuCrio® without cryoprotectants (BCnC) (Figure 1).

After dilution, samples were stabilized at 22ºC for 20 minutes in an air-conditioned laboratory, stored in a semen cooling container (BotuFlex®, Botupharma®) at 5ºC for 24 h, and subsequently transferred to a semen storage refrigerator (Minitube®), where they were maintained from 24 to 72 h. Semen samples were evaluated at 0, 24, 48, and 72 h of storage regarding total motility (TM), progressive motility (PM), percentage of rapid sperm (RAP), plasma membrane integrity (PMI), high mitochondrial membrane potential (HMMP), and intracellular superoxide production (ROS). For all evaluations, samples were homogenized and warmed to 37ºC in a dry block heater for 10 minutes.

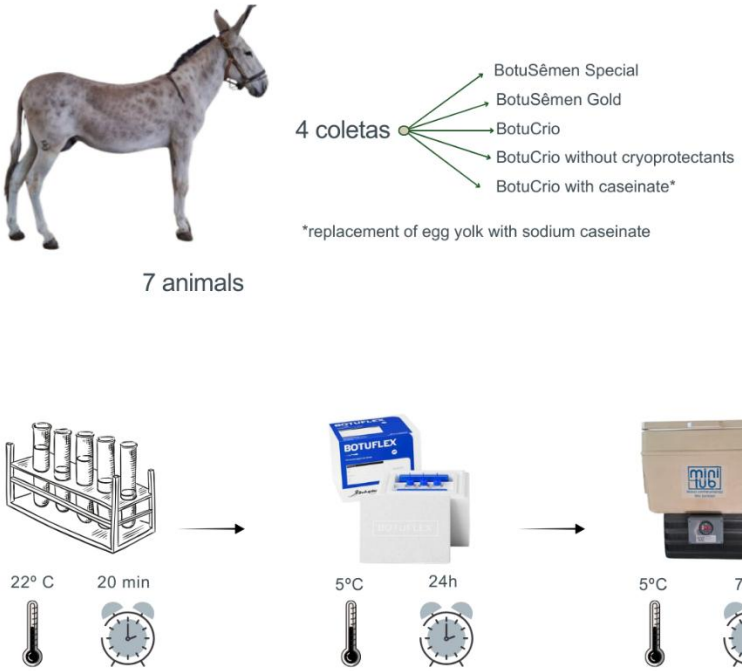


Figure 1: Experimental design of donkey semen processing and cooling protocols. Semen was collected from seven donkeys, with four collections made from each animal (N=28). Each ejaculate was divided into five experimental groups: BotuSêmen Special (BS), BotuSêmen Gold (BG), BotuCrio (BC), BotuCrio without cryoprotectants (BC-Sc), and BotuCrio with sodium caseinate replacing egg yolk (BCC). Samples were stabilized at 22°C for 20 minutes, followed by refrigeration at 5°C for 24 hours (BotuFlex) and up to 72 hours (Minitube cooling box).

2.1.3. Extenders

BotuSêmen Special® (Botupharma, Brazil)	Skimmed milk-based extender (data provided: pH 6.8–7.2 and osmolarity 355–375) with undefined composition containing 20 g/L of skimmed milk, CLC, sugar, antioxidant, 1 g/L of gentamicin sulfate and 1 g/L of sodium penicillin.
BotuSemen Gold® (Botupharma, Brazil)	Sodium caseinate-based extender (data provided: pH 6.8–7.2 and osmolarity 355–375) with undefined composition containing 20 g/L of sodium caseinate, CLC, sugar, antioxidant, 1 g/L of gentamicin sulfate and 1 g/L of sodium penicillin.
BotuCrio® (Botupharma, Brazil)	Egg yolk-based extender (data provided: pH 6.8–7.2 and osmolarity 1,200) with undefined composition containing 10% of egg yolk, buffers, sugars, antioxidant, 500 mg/L of gentamicin, 1% of glycerol and 4% of formamid.
BotuCrio without cryoprotectants	Egg yolk-based extender (data provided: pH 6.8–7.2 and osmolarity 1,200) with undefined composition containing 10% of egg yolk, buffers, sugars, antioxidant and 500 mg/L of gentamicin
BotuCrio with sodium caseinate	Egg yolk-based extender (data provided: pH 6.8–7.2 and osmolarity 1,200) with undefined composition containing 20 g/L of sodium caseinate, buffers, sugars, antioxidant, 500 mg/L of gentamicin, 1% of glycerol and 4% of formamid.

Adaptado de Gobato et al., (2022)

2.2. Experiment 2 – Effects of cryoprotectant removal and egg yolk substitution in a cryopreservation extender on the viability of cooled donkey semen

2.2.1. Animals

A parallel trial was conducted using the same experimental design and animals, following the authors' observation of the need to evaluate the effects of seminal plasma removal. For this parallel assessment, the treatment groups consisted of a sodium caseinate-based extender containing cholesterol (BotuSêmen Gold, Botupharma®, Brazil – BG) and a sodium caseinate-based extender containing cholesterol (BotuSêmen Gold, Botupharma®, Brazil) devoid of seminal plasma (BG-P). For the seminal plasma-free group, samples were initially diluted in a skim milk-based extender (BotuSêmen®, Botupharma, Brazil) and processed as described in Experiment 1. Subsequently, 1 million spz/mL were subjected to centrifugation at 600 x g (2,200 RPM) for 10 minutes using an Excelsa II 206 BL centrifuge (Fanem®). Immediately following centrifugation, the supernatant was discarded, and the pellet was resuspended in BotuSêmen Gold to a final concentration of 50 million spz/mL. A 20% sperm loss during centrifugation was assumed for experimental calculations. Assessments of sperm kinetics and plasma membrane integrity were performed as described in Experiment 1.

2.3. Experiment 2 – Effects of cryoprotectant and seminal plasma removal on fertility rates of jennies inseminated with cooled-stored donkey semen

2.3.1 Animals

Four cycles of 16 Jennies (61 cycles) were enrolled in a crossover design. The jennies were monitored via rectal palpation and ultrasonography every 3–4 days (or longer, depending on the estrous cycle phase and follicular size). Once estrus was detected (follicle > 25mm in diameter, absence of a corpus luteum, and receptivity to the male), evaluations became daily. When the largest follicle reached >30mm in diameter, ovulation was induced with 1 mL of histrelin acetate. Jacks were collected after inductions so that jennies were inseminated 24 h post-induction with semen also cooled for 24 h at 5ºC.

Two donkeys was used to fertility test and the semen was processed as described in Experiment 1 and 2. The best kinect groups from these experiments were used for the fertility test: BotuSêmen Gold (BG), BotuCrio (BC), BotuCrio without cryoprotectants (BCnC), and BotuGold without seminal plasma (BG-SP). Inseminations were performed with doses of 1 x 10^9 spz/mL into the uterine body using a rigid artificial insemination pipette (Minitube).

Additionally, endometrial cytologies were performed at moments 0 (at the time of ovulation induction) and 6, 24, and 48 hours post-insemination to evaluate the uterine inflammatory response.

Pregnancy diagnosis was performed 14 days post-ovulation and re-evaluated at 16 days via transrectal ultrasonography and all jennies received 0.5 mL of d-cloprostenol sodium to return to the cycle. All jennies underwent a rest cycle (washout) without insemination between treatment cycles to avoid carryover effects. Females that failed to ovulate were excluded from the cycle and re-enrolled in the subsequent one. It is noteworthy that anovulatory abnormalities are not uncommon and are not detrimental to the general health of the females.

2.4. Evaluations

2.4.1. Endometrial cytologies

Cytologies were performed using a disposable cytobrush (Minitube, USA); two slides were prepared for each sample, stained Diff-Quick (Siemens Healthcare Diagnostics Inc., Deerfield, IL, EUA), and examined under a light microscope at 400x magnification. For cytology evaluation, a slide scanning pattern was defined (Figure 2), and endometrial cells and polymorphonuclear cells (PMNs) were counted until reaching a total of 100 cells. The final result was considered the mean percentage of PMNs from the two slides referring to the same examination. Red blood cells, when present, were disregarded from the count, considering their presence to be of iatrogenic origin.

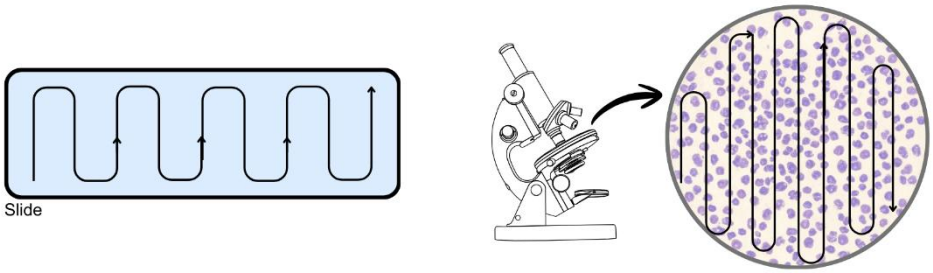


Figure 2: Representative description of the slide scanning pattern and cell counting within the microscopic field of view.

2.4.2. Sperm kinect

Sperm kinetic evaluations were performed using a Computer-Assisted Sperm Analysis (CASA) system (IVOS 12, Hamilton Thorne Inc., Beverly, MA, USA) as described by Guasti, 2013. All samples were stabilized at 37°C for 10 minutes; subsequently, a 10 µl aliquot was placed in a Makler counting chamber (Irvine Scientific, Santa Ana, CA). All fields were visually inspected to ensure homogeneity and the absence of bubbles or debris. Measurements were then conducted across five random fields, with a minimum of 1000 cells evaluated per sample. For all samples, the parameters recorded included total motility (TM, %), progressive motility (PM, %), and the percentage of rapid spermatozoa (RAP, %).

2.4.3. Epifluorescence microscopy

Plasma membrane integrity (PMI) was assessed using epifluorescence microscopy (Leica Microsystems-DMLB, Germany). Samples were homogenized and incubated at 37°C in a dry block heater for 10 minutes prior to evaluation. To determine PMI, a fluorescent staining solution was prepared using 1 mL of sodium citrate, 20 µL of formol saline, 40 µL of carboxyfluorescein diacetate (CFDA; 0.46 mg/mL diluted in dimethyl sulfoxide, stored at -20°C in the dark; C5041, Sigma-Aldrich Brasil Ltda., SP, Brazil), and 20 µL of propidium iodide (PI; 0.5 mg/mL diluted in saline solution, stored at -20°C in the dark; P4170, Sigma-Aldrich Brasil Ltda., SP, Brazil).

Semen samples were mixed with the staining solution at a 1:1 ratio, stabilized for 10 minutes in a light-protected environment, and mounted on slides for evaluation under a fluorescence microscope at 400x magnification. Spermatozoa displaying

green fluorescence were classified as intact, while cells displaying any red fluorescence—regardless of the lesion site—were classified as damaged. One hundred cells were evaluated per sample, and results were expressed as the percentage of intact cells. To minimize intra-observer subjectivity, a standardized slide scanning pattern was utilized (Figure 3).

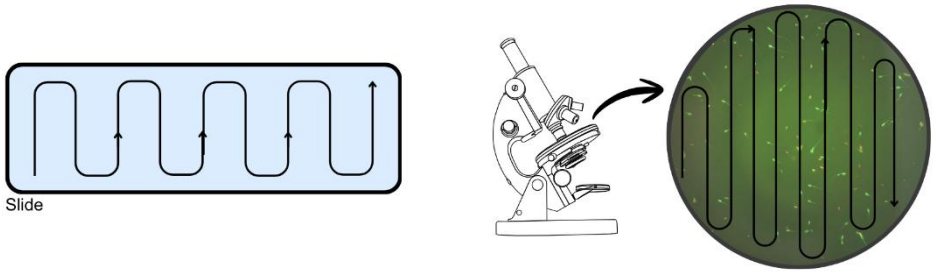


Figure 3: Representative description of the slide scanning pattern and cell counting within the microscopic field of view.

2.4.4. Flow cytometry

Flow cytometric analyses were performed using an LSR Fortessa flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with blue (488 nm, 100 mW), red (640 nm, 40 mW), and violet (405 nm, 100 mW) lasers. Samples were incubated at 37°C for 10 minutes prior to each evaluation and diluted in TALP-PVA containing Hoechst 33342 (7 µM in distilled water). For each sample, at least 10,000 events were recorded, and data were extracted using the system's software (BD FACSDiva™ v6.1). The samples were evaluated for high mitochondrial membrane potential (HMMP, %), and reactive oxygen species production (ROS, %).

2.5. Statiscal analisys

All statistical analyses were performed using RStudio (version 2022.07.1). A Mixed model was arranged for PMN counting the jennies as a random effect and cycle order, day of the cycle, treatment groups as fixed effects. Similarly, a mixed model was arranged for sperm parameters, counting the treatment groups and ejaculate order as fixed effects, and the donkeys as a random effect. The residual normality of the model was tested using the Shapiro-Wilk test. Non-parametric data (e.g., PMN counts) were transformed using the Box-Cox method when

necessary. Parametric data (e.g., sperm motility parameters) were assessed by mixed models and Bonferroni post hoc comparisons (function “lsmeans” in the R “emmeans” package). Logistic regression (function “logistf” in the R “logistf” package) was used to assess pregnancy rates between groups. Significance was set at $P<0.05$ for all tests, and statistical trend was defined as $P < 0.1 > 0.05$. All data are presented as mean ± SD or SEM when stated.

3. Results

3.1. Experiment 1

Regarding sperm kinetics (TM, PM, RAP) and PMI, no differences were observed among groups at 0 h ($P>0.05$). However, BC and BCnC exhibited higher values than the other groups at 24, 48, and 72 h for TM, PM, and RAP ($P<0.05$). Furthermore, BC and BCnC better maintained TM values, with no significant differences between 0 and 24 h ($P>0.05$) or between 24 and 48 h ($P>0.05$). PM was higher for BC and BCnC at all time points ($P<0.05$), although BCnC did not differ from BG at 24 h ($P>0.05$). Both BC and BCnC maintained PM values between 0, 24, and 48 h ($P>0.05$), with a decline observed only at 72 h ($P<0.05$). Regarding RAP, BC and BCnC were superior to the other groups at all time points ($P<0.05$) and also maintained consistent values between 0, 24, and 48 h ($P>0.05$). The results are illustrated in Figure 4.

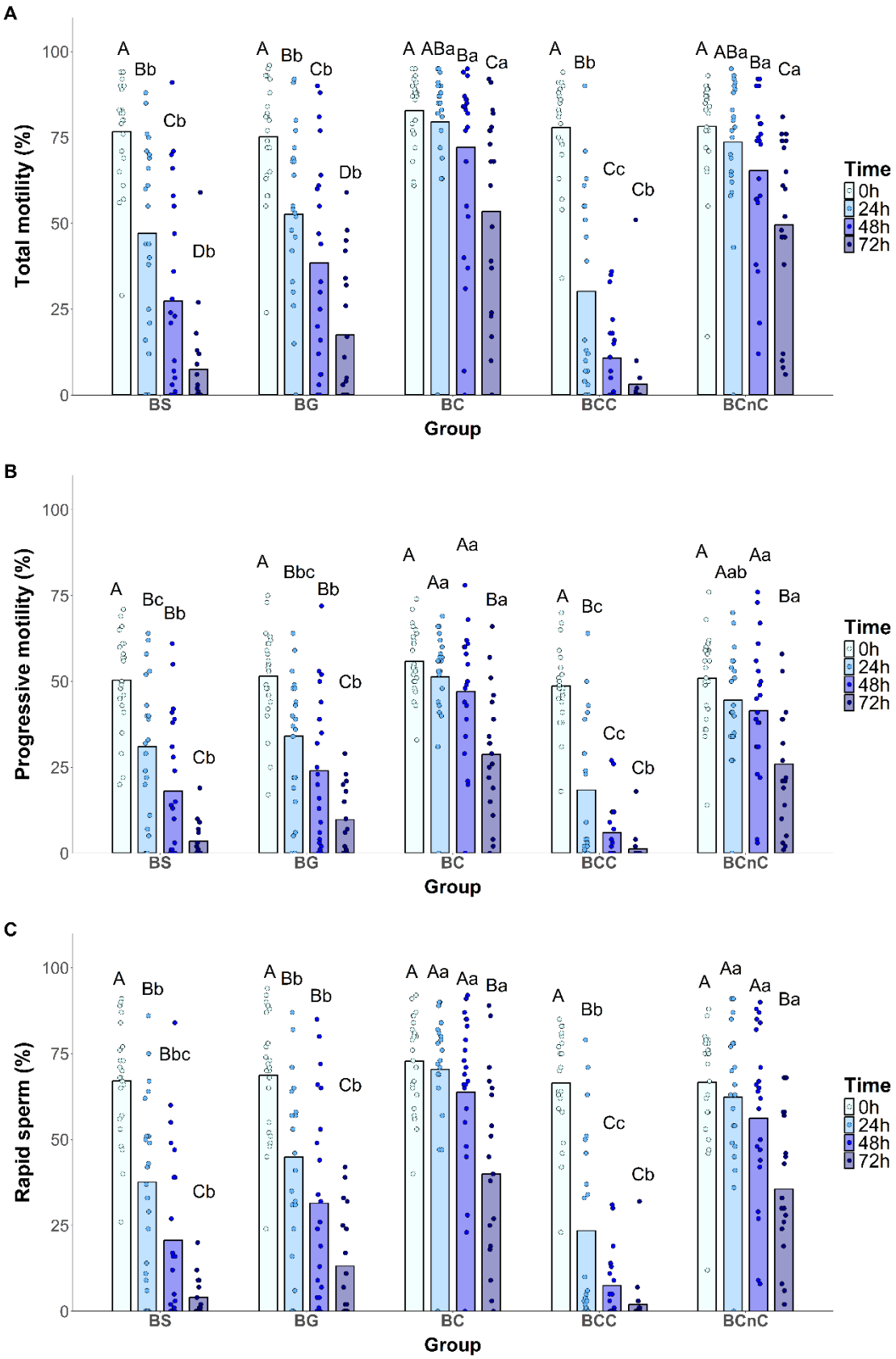


Figure 4: Total Motility (A), Progressive Motility (B), and Rapid Sperm Motility (C) of donkey semen cooled for up to 72 h in different experimental groups: BotuSêmen (BS), BotuSêmen Gold (BG), BotuCrio (BC), BotuCrio with sodium caseinate replacing egg

yolk (BCC), and BotuCrio without cryoprotectants (BCnC). Bars represent the mean, and circles represent individual values for each biological replicate. Different lowercase letters (a, b, c) indicate significant differences ($P < 0.05$) among groups within the same cooling time. Different uppercase letters (A, B, C) indicate significant differences ($P < 0.05$) over time within the same group.

Results for PMI were similar across all groups at 0, 48, and 72 h ($P>0.05$); notably, at 48 h, BCnC, BC, BS, and BG showed superior results compared to BCC ($P<0.05$) and were similar to each other ($P>0.05$), while BC, BS, BG, and BCC also showed no significant differences ($P>0.05$). The results for HMMP differed among groups starting at 0 h. BCnC and BC showed the best results ($P<0.05$); however, no differences were observed between BC and BS ($P>0.05$), followed by BS, BG, and BCC. At 24, 48, and 72 h, BCnC and BC were similar to each other ($P>0.05$) and superior to BG, BS, and BCC ($P<0.05$), with no differences among the latter ($P>0.05$). BC and BCnC were also more efficient in maintaining HMMP results, which remained similar between 0, 24, and 48 h ($P>0.05$). ROS production was lower in BC and BCnC at all time points ($P<0.05$), being similar to BS only at 0 h ($P>0.05$). The results are illustrated in Figure 5.

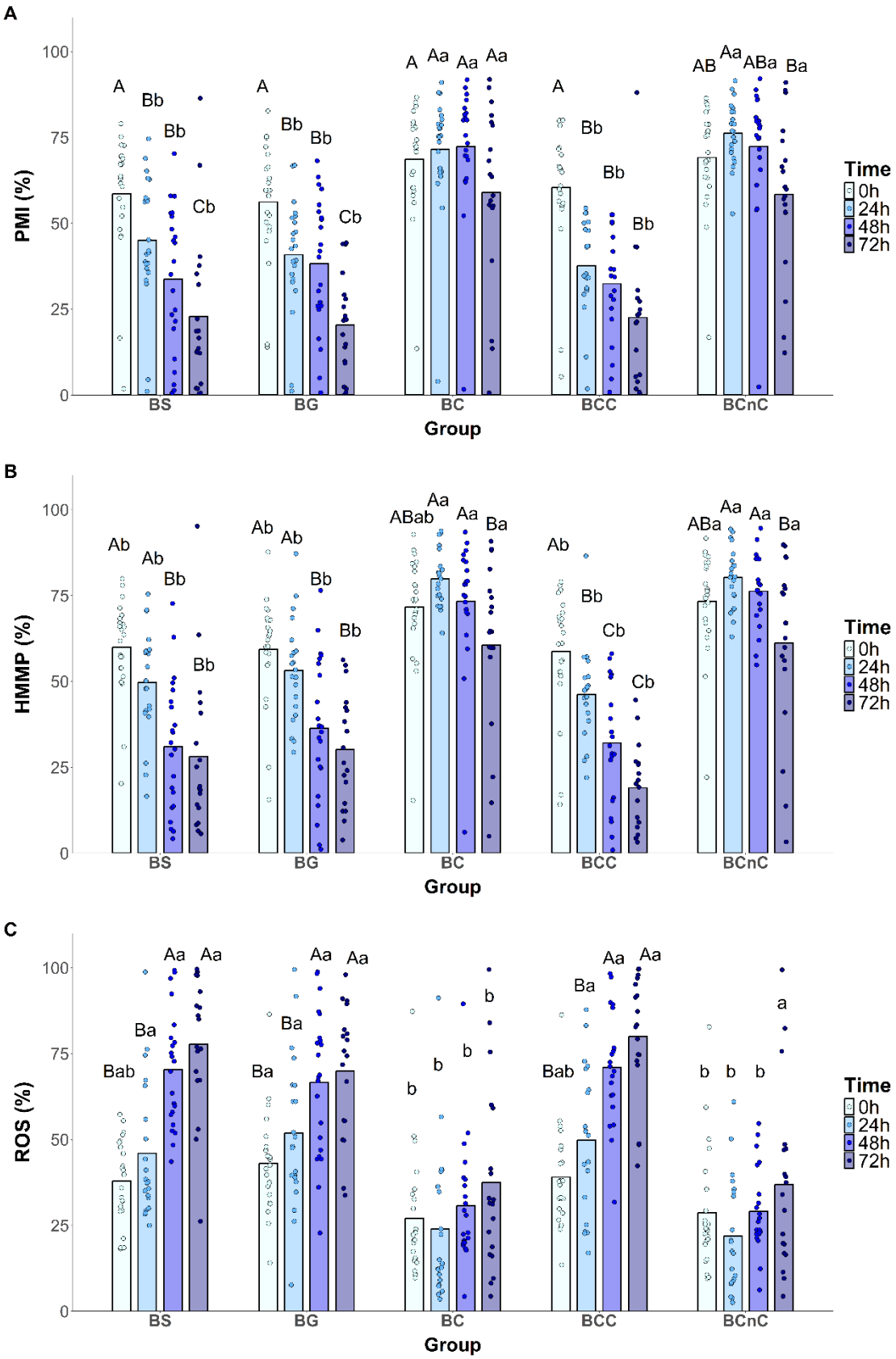


Figure 5: Plasma Membrane Integrity (PMI), Mitochondrial Membrane Potential (MMP), and Intracellular Superoxide Production (ROS) of donkey semen cooled for up to 72 h in different experimental groups: BotuSêmen (BS), BotuSêmen Gold (BG), BotuCrio (BC),

BotuCrio with sodium caseinate replacing egg yolk (BCC), and BotuCrio without cryoprotectants (BCnC). Bars represent the mean, and circles represent individual values for each biological replicate. Different lowercase letters (a, b, c) indicate significant differences (P < 0.05) among groups within the same cooling time. Different uppercase letters (A, B, C) indicate significant differences (P < 0.05) over time within the same group.

3.1.1. Experiment 1.1

Regarding TM and PM, values did not differ between BG and BG-P at 0h; however, they were significantly higher in the BG-P group at 24h, 48h, and 72h (P < 0.0001). The BG-P group also exhibited higher PMI and RAP values from 24h onwards compared to the BG group (P < 0.05). For VAP, no differences were observed between groups at 0h, 24h, and 48h, but BG-P maintained superior results at 72h (P = 0.0059). No significant differences were found between groups or over time for VCL values. Conversely, VSL showed a progressive decline over time, with BG-P maintaining higher values than BG at 48h (P = 0.001) and 72h (P < 0.0001). These results are illustrated in Figure 6.

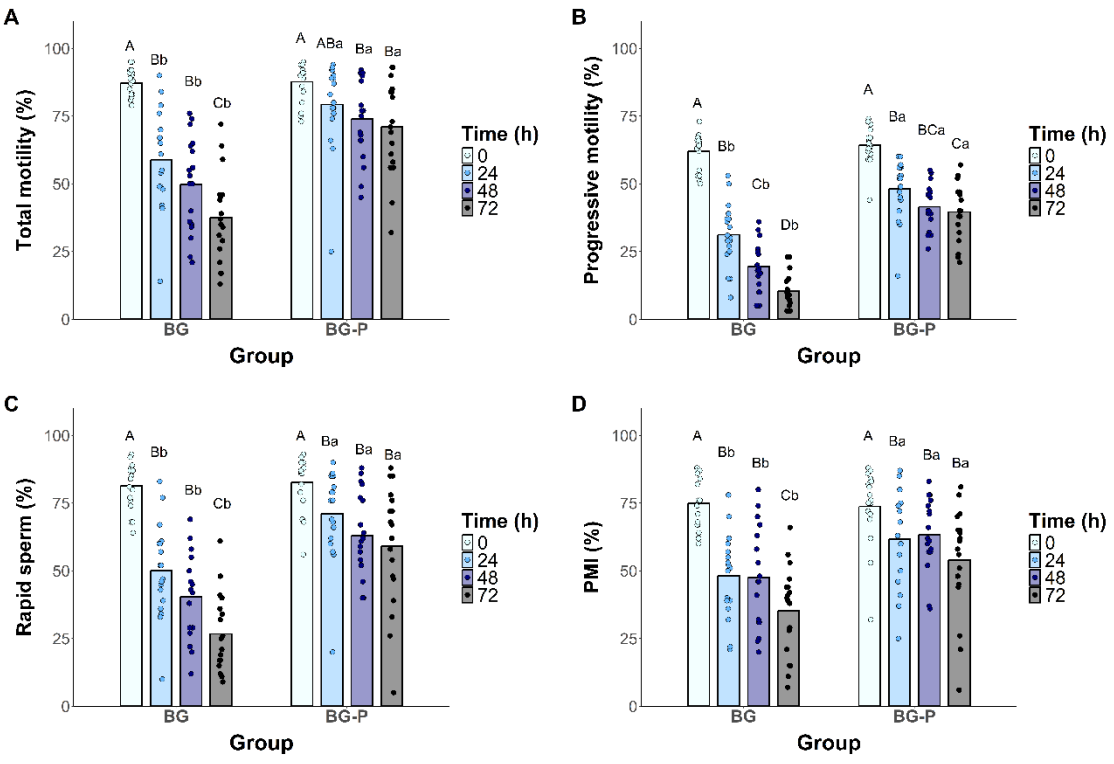


Figure 6: Total Motility (A), Progressive Motility (B), Rapid Sperm Motility (C), and Plasma Membrane Integrity (PMI) of donkey semen cooled for up to 72 h in different experimental groups: BotuSêmen Gold (BG) and BotuSêmen Gold without seminal plasma (BG-P). Bars represent the mean and circles represent individual values for each biological replicate. Different lowercase letters (a, b, c) indicate significant differences ($P < 0.05$) among groups within the same cooling time. Different uppercase letters (A, B, C) indicate significant differences ($P < 0.05$) over time within the same group.

3.2. Experiment 2

Regarding fertility, jennies inseminated with BG and BC exhibited the same pregnancy rates (9/15; 60%), followed by the BG-SP group (8/15; 53.3%), and the BCnC group (7/16; 43.75%), without difference between the groups. (Figure 7).

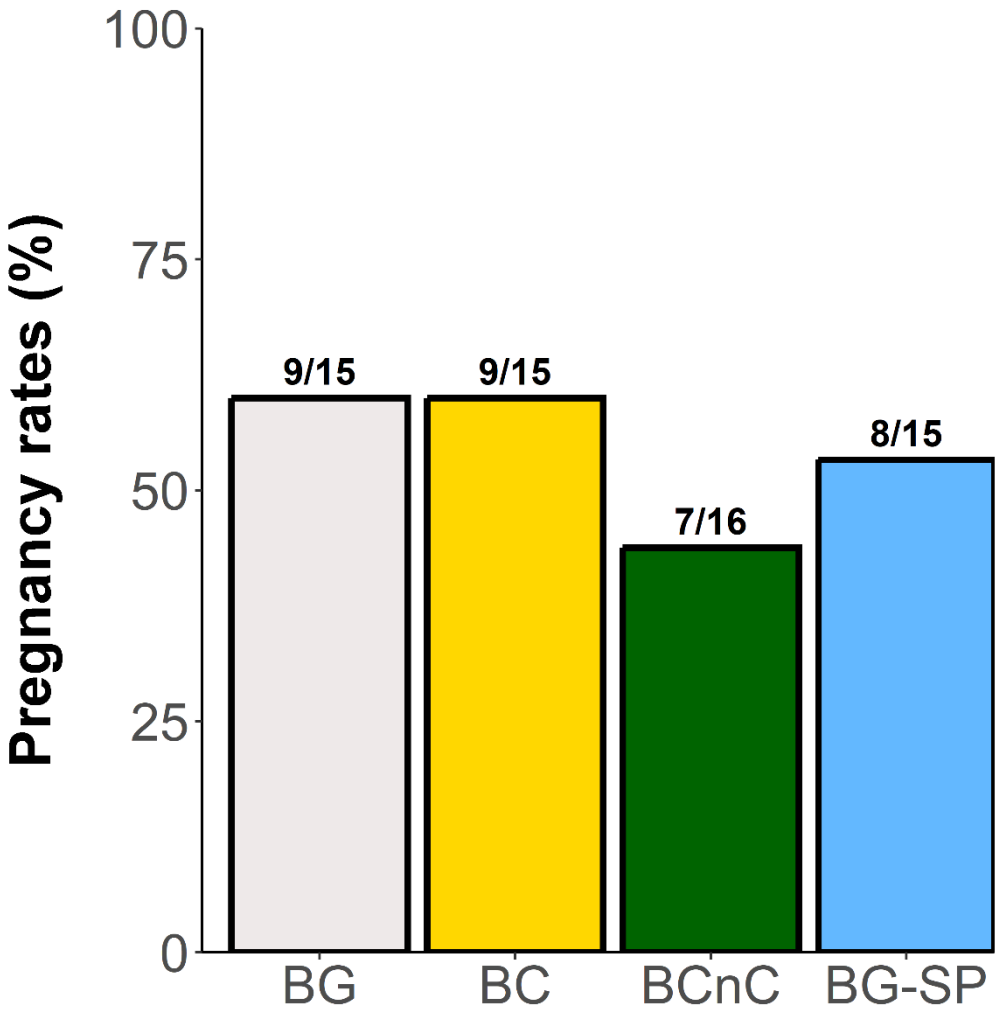


Figure 7: Pregnancy rates of jennies inseminated with cooled donkey semen (5ºC for 24 h) using different extenders: BotuSêmen Gold (BG); BotuCrio (BC); BotuCrio without cryoprotectants (BCnC); and BotuSêmen Gold without seminal plasma (BG-sP). The numbers above each bar indicate the obtained pregnancy proportion (pregnant animals/inseminated animals).

Regarding uterine cytology, no significant differences were observed between groups at 0 h, 24 h, and 48 h ($P>0.05$). However, at 6 h, BCnC, BC, and BG-SP yielded the lowest polymorphonuclear leukocyte (PMN) counts ($P<0.05$), with no significant differences among them ($P>0.05$). Conversely, no differences were detected between the BC, BG-SP, and BG groups ($P>0.05$). For BG, BC, and BCnC, no significant differences were found between the 24 h and 48 h time points ($P>0.05$), whereas a significant difference was observed for BG-SP

(P<0.05). These results are illustrated in Figure 8.

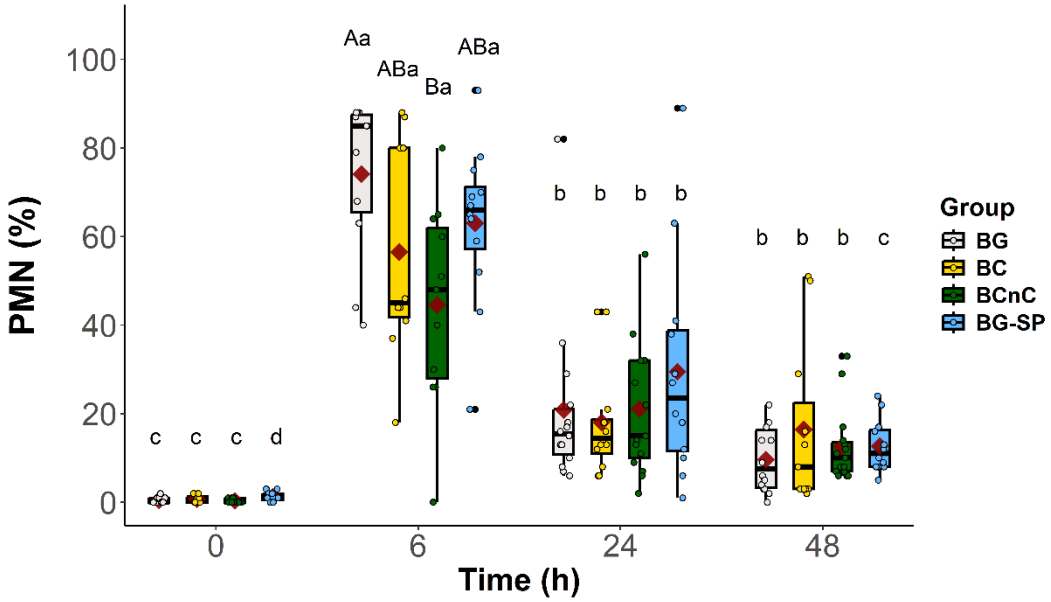


Figure 8: Box-plots representing the percentage of uterine polymorphonuclear neutrophils (PMN) at 0, 6, 24, and 48 h post-insemination. The data are expressed as medians (horizontal line within the box), means (red diamonds), and interquartile ranges (25º and 75º percentiles). Individual data points are represented by open circles. Different uppercase letters (A, B) indicate significant differences among groups within the same time point (P<0.05). Different lowercase letters (a, b, c, d) indicate significant differences within the same group over time (P<0.05).

4. Discussion

The kinetic results obtained provide evidence that egg yolk is indispensable for donkey semen cooling, agreeing with previously published data and indicating that milk-based extenders, even when supplemented with cholesterol and casein, although better, are not yet ideal for maintaining and protecting the donkey sperm cell (5,12–14). However, it was expected that cryoprotectant removal would be beneficial for cooling and fertility, but this result was not observed.

It is known that cryoprotectants have toxic effects on the sperm cell and that equine spermatozoa are especially sensitive to glycerol. Furthermore, authors suggest that in the asinine species, toxic effects occur not only on the sperm cell but also in the uterus after insemination (15–17). However, the BC and BCnC

groups obtained the best results for all semen evaluation parameters, being similar to each other (P>0.05). It was not possible to observe positive or negative effects caused by cryoprotectants on kinetics. On the other hand, fertility results indicate the absence of negative effects from cryoprotectants.

Regarding seminal plasma, the results agree with the literature and previous studies (5,9,18) which indicate that seminal plasma removal is one of the alternatives for cooling donkey semen and for its fertility in mares. The findings presented here demonstrate that these results are also consistent in jennies. However, these same results contradict the hypothesis that the absence of seminal plasma is responsible for the low fertility rates in jennies inseminated with cryopreserved donkey semen (19).

However, the highlight lies in the group with the lowest fertility rates. While the literature points to cryoprotectants as the main culprits for fertility issues in jennies (17,20), the results obtained in Experiment 3 indicate that the presence of cryoprotectant was not detrimental to the fertility of jennies inseminated with cooled donkey semen and the presence of cryoprotectants did not affect the PMN count.

5. Conclusion

Donkey spermatozoa benefit from seminal plasma removal and cholesterol present in egg yolk, and the amount present in BSS and BG extenders is not sufficient for spermatozoa of this species. It is not possible to definitively conclude whether the presence of cryoprotectants influenced sperm kinetics or fertility; however, the results demonstrate that their presence did not modulate the endometrial inflammatory response.

6. References

1. Rota A, Magelli C, Panzani D, Camillo F. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology. 2008 May 15;69(2):176–85.

2. Camillo F, Rota A, Biagini L, Tesi M, Fanelli D, Panzani D. The Current Situation and Trend of Donkey Industry in Europe. Vol. 65, Journal of Equine Veterinary Science. W.B. Saunders; 2018. p. 44–9.

3. Larry Sawers. The Mule, the South, and Economic Progress. 2004.

4. Fanelli D, Tesi M, Monaco D, Diaz-Jimenez M, Camillo F, Rota A, et al. Deep-horn Artificial Insemination With Frozen Thawed Semen After re-extension in Autologous Seminal Plasma May Improve Pregnancy Rates in Jennies. J Equine Vet Sci. 2022 May 1;112.

5. M Gobato MLDPS, Miro J, Gaitskell-Phillips G, Carluccio A, T M Segabinazzi LG, M Gobato ML, et al. Ability of donkey sperm to tolerate cooling: Effect of extender base and removal of seminal plasma on sperm parameters and fertility rates in mares. 2022.

6. Vidament M, Vincent P, Martin FX, Magistrini M, Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim Reprod Sci. 2009 May;112(1–2):22–35.

7. Oliveira PVLF, Segabinazzi LGTM, Crespilho AM, Oliveira J V., Silva AC, Freitas-Dell'Aqua CP, et al. Autologous seminal plasma and post-breeding uterine lavage as alternative options to improve fertility rates of jennies inseminated with cryopreserved jack semen. J Equine Vet Sci. 2025 Jul 1;150.

8. Rota A, Panzani D, Sabatini C, Camillo F. Donkey jack (Equus asinus) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology. 2012 Nov;78(8):1846–54.

9. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key Aspects of Donkey and Mule Reproduction. Vol. 35, Veterinary Clinics of North America - Equine Practice. W.B. Saunders; 2019. p. 607–42.

10. Segabinazzi LGTM, Scheeren VF da C, Freitas-Dell'Aqua C de P, Papa FO, Alvarenga MA, Dell'Aqua JA, et al. Cholesterol-Loaded Cyclodextrin Addition to Skim Milk-Based Extender Enhances Donkey Semen Cooling and Fertility in Horse Mares. J Equine Vet Sci. 2021 Oct 1;105.

11. Carvalho LE, Silva Filho JM, Palhares MS, Sales ALR, Gonczarowska AT, Oliveira HN, et al. Physical characteristics and fertility of fractionated donkey semen cooled at 5°C. Arq Bras Med Vet Zootec. 2017;69(1):29–38.

12. de Melo, Maria Isabel Vaz, et al. "Uso de lipoproteínas de baixa densidade e de gema de ovo na preservação do sêmen asinino resfriado a 6o C." Brazilian Journal of Veterinary Medicine 34.4 (2012): 288-294

13. Alonso C, Castañeira C, Bragulat AF, Losinno L. Effect of egg yolk-based extender and seminal plasma removal on sperm viability of cooled donkey semen. Braz J Vet Res Anim Sci. 2021 Mar 10;58(Special Issue).

14. Oliveira JV de, Oliveira PV de LF, Melo e Oña CM, Guasti PN, Monteiro GA, Sancler da Silva YFR, et al. Strategies to improve the fertility of fresh and frozen donkey semen. Theriogenology. 2016 Apr 15;85(7):1267–73.

15. Macías García B, Ortega Ferrusola C, Aparicio IM, Miró-Morán A, Morillo Rodriguez A, Gallardo Bolaños JM, et al. Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology. 2012 Apr 15;77(7):1280–9.

16. Santiago et al. Comparison of different cryoprotectants for freezing donkey jack (Equus asinus) semen. 2023;

17. Vidament M, Vincent P, Yvon JM, Bruneau B, Martin FX. Glycerol in semen extender is a limiting factor in the fertility in asine and equine species. Vol. 89, Animal Reproduction Science. 2005.

18. Troncarelli Rodrigues et al. L. Efeito de diferentes diluentes e da remoção do plasma seminal na refrigeração do sêmen asinino (Equus asinus). Revista Acadêmica: Ciência Animal. 2017 Jul 21;15:143.

19. Miró J, Papas M. Post–Artificial Insemination Endometrial Inflammation and Its Control in Donkeys. Vol. 65, Journal of Equine Veterinary Science. W.B. Saunders; 2018. p. 38–43.

20. Bruno SL, Neild DM, Plaza JP, Ferrante AA, Carretero MI, Olivieri GEA, et al. Comparison of different cryoprotectants for freezing donkey (Equus asinus) semen. J Equine Vet Sci. 2024 May 1;136.

21. Papa PM, Maziero RD, Guasti PN, Junqueira CR, Freitas-Dell'Aqua CP, Papa FO, et al. Effect of glycerol on the viability and fertility of cooled bovine semen. Theriogenology. 2015 Jan 1;83(1):107–13.

22. Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. Modes of Interaction of Cryoprotectants with Membrane Phospholipids during Freezing. Vol. 24, CRYOBIOLOGY. 1987.

23. Patrick Williams W, Quinn PJ, Tsonev L, Rumiana Koynova aad D. The effects of glycerol on the phase behaviour of hydrated distearoylphosphatidylethanolamine and its possible relation to the mode of action of cryoprotectants. Vol. 062, Biochimica et Biophysica Acta. 1991.

24. Oliveira CH, Vasconcelos AB, Souza FA, Martins-Filho OA, Silva MX, Varago FC, et al. Cholesterol addition protects membrane intactness during cryopreservation of stallion sperm. Anim Reprod Sci. 2010 Apr;118(2–4):194–200.

25. Travis AJ, Kopf GS. The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. Journal of Clinical Investigation. 2002 Sep 15;110(6):731–6.

ARTIGO CIENTÍFICO 2

Optimizing a Cost-Effective Approach for the Cooling and Transportation of Donkey Semen

Silva, A. C.[1], Zanardi, C. S[1], Monteiro, G. A. [1], Segabinazzi, L. G. T. M.[1,2]

[1]Sao Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu, SP, Brazil

[2]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St. Kitts, West Indies

Highlights

- Egg yolk is beneficial to donkey spermatozoa.
- The addition of 20% egg yolk-based freezing extender may be sufficient to improve sperm parameters and fertility rates in donkeys

Abstract

Kinetic and fertility results for chilled donkey semen remain suboptimal, as commercial extenders have been primarily developed for horses and do not meet the specific requirements of asinine spermatozoa. Given that fertility outcomes with cryopreserved semen currently render this technique unfeasible for the species, it is urgent to understand the requirements of the asinine sperm cell to optimize these results. It is well-established that the addition of cholesterol sources is beneficial for donkeys, with egg yolk serving as a viable alternative. However, the use of freezing extenders makes the procedure highly costly. In this context, the present study aimed to evaluate the effect of egg yolk on chilled donkey semen. To this end, different extenders were prepared by combining a commercial milk-based extender containing casein and cholesterol with egg yolk or cryopreservation extenders in varying proportions. The results indicate that the addition of 2% egg yolk associated with cholesterol may be an alternative for donkey semen chilling, and that the presence of a cryoprotectant was determinant in optimizing the outcomes. Based on the results of the first

experiment, a second trial was conducted comparing two extenders (BotuSêmen Gold – BG and INRA 96 - I) with and without seminal plasma (BG, I, BG-P, I-P), with the addition of 20% BotuCrio (BGBC and IBC), and with the addition of 20% BotuCrio without seminal plasma (BGBC-P and IBC-P). The results indicate similar values for both extenders and suggest that the addition of BC is equivalent to seminal plasma (SP) removal, while the groups with BC and without SP yielded the best results.

Keywords: donkey; egg yolk; extender; semen; refrigeration,

1. Introduction

Donkeys and mules represent a significant fraction of working animals within cattle ranching, particularly in Brazil and the United States (1). Furthermore, numerous groups and associations exist worldwide for the valorization, trade, and protection of these animals, aiming both at the maintenance of their genetic material and commercial interests. Recent studies highlight diverse applications for donkey milk, while countries such as China utilize donkey skin for Ejiao production, in addition to the cultural aspects related to the species (2).

Despite the exponential growth of the donkey and mule market worldwide, reproductive biotechnologies used for these animals are still extrapolated from the equine species, generating frustrating results and management difficulties. Horses *(Equus caballus*) and donkeys (*Equus asinus*) belong to the same phylogenetic order, which confers similarities between them. However, within this order, donkeys are more similar to African wild asses and zebras than to horses (3). In this sense, it is expected that the extrapolation of biotechnologies does not yield satisfactory results; however, it is urgent to understand that failures are due to a misunderstanding of the species and a lack of specific studies, rather than an inability to apply these biotechnologies to donkeys.

It is known that asinine spermatozoa require extra sources of cholesterol and that better cooling results are obtained using egg yolk-based cryopreservation extenders or by adding an egg yolk-based cryopreservation extender to a milk-based cooling extender (4–6). However, the effect of these extenders on fertility rates in jennies remains unknown, as previous studies evaluating fertility were conducted in mares (4,5,7,8). Moreover, there is no defined protocol for donkey semen cooling, and the usage of cryopreservation extenders significantly increases procedure costs.

The addition of cholesterol-loaded cyclodextrin to a skim milk-based extender improved total motility of sperm cooled at 5ºC for 24 h by compared to the use of a skim milk-based extender alone, and improved fertility rates in mares (4). Furthermore, the use of an egg yolk-based extender and the removal of seminal plasma are beneficial for the total motility of donkey semen cooled for 24 h at 5ºC (9–12), in addition to improving fertility rates in mares when compared to the use of a skim milk-based extender containing cholesterol (5).

However, the effect of these extenders on fertility rates in jennies is unknown, as

the only study found that evaluated fertility in jennies compared it only with UHT milk dilution and obtained equal pregnancy rates between species (7). Additionally, the use of cryopreservation extenders renders procedure costs unfeasible for field application. It is known that the addition of egg yolk or an egg yolk-based freezing extender to a skim milk-based cooling extender can improve kinetic results (9–12), however, this possibility has not been extensively explored. Furthermore, it is believed that the cryoprotectant is the main factor responsible for the disastrous fertility rates in jennies inseminated with cryopreserved donkey semen (9,13).

Therefore, the present study aimed to gather existing data in the literature and use it as a basis to investigate and understand the requirements of the asinine sperm cell to withstand the cooling process, as well as to evaluate the effect of egg yolk and cryopreservation extenders on the cooling and fertility of donkey semen used to inseminate jennies.

2. Materials And Methods

2.1. Experiment 1 – Effects of adding cryopreservation extender and egg yolk to a cholesterol-containing caseinate-based extender on the viability of cooled donkey semen

2.1.1. Animals

The experiment was conducted in 2024 at the School of Veterinary Medicine and Animal Science (FMVZ) of São Paulo State University (UNESP), Botucatu campus. Three mixed-breed jacks aged between 6 and 14 years with proven fertility were used for the study. All animals were housed at the University, kept in stalls, fed hay, and had free access to water. The animals were clinically healthy and subjected to consecutive collections prior to the start of the experiment to deplete sperm reserves. All collections were performed using a phantom and a Botupharma model artificial vagina (Botupharma®, Botucatu, São Paulo, Brazil) filled with water at 55ºC. To reduce the waiting time for erection, all animals received 0.5 mL of sodium D-cloprostenol (Botupharma®, Botucatu, São Paulo, Brazil, 120 µg/mL, experimental batch) intramuscularly.

2.1.2. Experimental Design

Six ejaculates from each animal were used in the experiment (n=18). In all

collections, semen was filtered to remove the gel fraction, and then a solution of 490 µL of distilled water and 10 µL of semen was prepared to determine sperm concentration in a Neubauer chamber. Based on the concentration, each ejaculate was divided into seven aliquots and diluted to a final concentration of 5x10^6 spz/mL in the following extenders: BotuSêmen Gold (Botupharma®, Brazil – BG) as the control solution; BotuCrio (Botupharma®, Brazil – BC); BotuSêmen Gold with the addition of 2% BotuCrio (BG2BC); BotuSêmen Gold with the addition of 10% BotuCrio (BG10BC); BotuSêmen Gold with the addition of 20% BotuCrio (BG20BC); BotuSêmen Gold with the addition of 2% egg yolk (BG2E); and BotuSêmen Gold with the addition of 10% egg yolk (BG10E).

After dilution, samples were stabilized at 22ºC for 20 minutes, stored in a semen cooling container (BotuFlex®, Botupharma®) at 5ºC for 24 h, and subsequently transferred to a semen storage refrigerator (Minitube®), where they were maintained from 24 to 144 h. Samples were evaluated before cooling and every 24 hours regarding total motility (TM), progressive motility (PM), percentage of rapid sperm (RAP), plasma membrane integrity (PMI), high mitochondrial membrane potential (HMMP), and intracellular superoxide production (ROS).

2.1.3. Extenders

For dilutions, a commercial skim milk-based extender containing casein and cholesterol (BotuSêmen Gold), a commercial egg yolk-based freezing extender (BotuCrio), and non-commercial extenders prepared for the experiment were used. Extenders were prepared using a precision balance and micropipettes. For the preparation of BG2BC, BG10BC, and BG20BC, only the commercial extenders were used, based on a ratio calculation, such that: for BG2BC, the solution was 98:2 (BG:BC); for BG10BC, the solution was 90:10 (BG:BC); and for BG20BC, the solution was 80:20 (BG:BC).

For extenders supplemented with egg yolk, fresh hen eggs were used; the yolk was separated and cleaned to remove all albumen, the vitelline membrane was ruptured and removed, and only the yolk content was isolated. The BG2E and BG10E extenders were also prepared using ratio calculations, such that BG2E was prepared with 98% BotuSêmen Gold and 2% egg yolk, and BG10E was prepared with 90% BotuSêmen Gold and 10% egg yolk. In extenders prepared with egg yolk, Orvus Es Paste (OEP) was added at a concentration of 30%

relative to the egg yolk percentage. In BG2E, 0.6 mL of OEP was added, and in BG10E, 3 mL of OEP was added. The extenders were centrifuged at 5000 RPM for 30 minutes, and only the supernatant was used.

2.2. Experiment 2 – Effects of adding cryopreservation extender to a cholesterol-containing caseinate-based extender on fertility rates of jennies and mares inseminated with cooled donkey semen

2.2.1. Animals

Sixteen Caribbean jennies, ranging from 7 to 12 years of age, and 6 mares were utilized for the study. All females were healthy and of proven fertility. The animals are part of the herd at the Ross University School of Veterinary Medicine (RUSVM) in St. Kitts, West Indies, during the year 2024. Both mares and jennies were housed in grass paddocks with ad libitum access to shade, water, and mineral salt, and were fed New Guinea grass (Megathyrsus maximus).

2.2.2. Experimental Design

16 jennies and 6 mares were monitored via transrectal ultrasonography for estrus detection, which was characterized by follicles >25 mm in diameter, absence of a corpus luteum, and receptivity to the male. When follicles reached >30 mm in diameter, females received 1 mL of histrelin acetate (Strelin, Botupharma, Brazil) to induce ovulation. Following induction, semen was collected, processed according to the groups BG and BG20BC, and cooled to 5ºC in a passive cooling box (BotuFlex, Botupharma, Brazil). Insemination was performed 24 h after induction with semen also cooled for 24 h. Ovulation was monitored by rectal palpation and transrectal ultrasonography. In jennies, pregnancy diagnosis was performed 14 days post-ovulation and re-evaluated 2 days later, whereas in mares, embryo recovery was conducted using Ringer's lactate solution 8 days post-ovulation.In cases of ovulatory failure, the cycle was discarded, and all females underwent rest cycles (washout) between insemination cycles.

2.3. Experiment 3 - Effects of cryopreservation extender addition and seminal plasma removal in egg yolk-supplemented caseinate-based

extender containing cholesterol on the viability of chilled donkey semen

2.3.1. Animals

The experiment was conducted in 2024 at the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), Botucatu campus. Two mixed-breed donkeys and six Pêga donkeys, aged between 6 and 14 years with proven fertility, were used in this study. The first two animals were housed at the University, while the remaining six were kept at a private stud farm in Laranjal Paulista; all animals were maintained in stalls, fed hay, and provided with ad libitum access to water. The animals were clinically healthy and underwent successive collections prior to the start of the experiment to deplete extragonadal sperm reserves. All collections were performed using a breeding phantom and a Botupharma® model artificial vagina (Botupharma®, Botucatu, São Paulo, Brazil) filled with water at 55 °C. To reduce the time to erection, all animals received 0.5 mL of sodium D-cloprostenol (Botupharma®, Botucatu, São Paulo, Brazil, experimental lot) via intramuscular injection.

2.3.2. Experimental Design

30 ejaculates and 7 donkey jacks were used in this study. In all collections, the semen was filtered to remove the gel fraction. Subsequently, a solution of 490 µL of distilled water and 10 µL of semen was prepared to determine sperm concentration using a Neubauer chamber. Based on the concentration, each ejaculate was divided into seven aliquots and diluted to a final concentration of 100 x 10^6 spz/mL in the following extenders: BotuSêmen Gold (Botupharma®, Brazil – BG); INRA 96 (IMV Technologies®, France – I); BotuSêmen Gold without seminal plasma (BG-P); INRA 96 without seminal plasma (I-P); BotuSêmen Gold supplemented with 20% BotuCrio (BGBC); INRA 96 supplemented with 20% BotuCrio (IBC); BotuSêmen Gold without seminal plasma supplemented with 20% BotuCrio (BGBC-P); and INRA 96 without seminal plasma supplemented with 20% BotuCrio (IBC-P).

After dilution, samples were stabilized at 22 °C for 20 minutes, stored in a passive cooling container (BotuFlex®, Botupharma®) at 5 °C for 24 h, and subsequently transferred to a semen storage refrigerator (Minitube®), where they were maintained for 24 to 72 h. Samples were evaluated before cooling and every 24 h thereafter for total motility (TM), progressive motility (PM), percentage of rapid

spermatozoa (RAP), and plasma membrane integrity (PMI).

2.3.3. Extenders

For dilution, a commercial skim milk-based extender containing casein and cholesterol (BotuSêmen Gold), a commercial extender formulated with native phosphocaseinate fractions (INRA 96), and an egg yolk-based cryopreservation extender containing cryoprotectants (BotuCrio) were used. In the groups with seminal plasma (SP), the semen was only filtered and diluted to 100 x 10^6 spz/mL. For the groups without seminal plasma, the semen was pre-diluted in the base extenders (BG or I), centrifuged at 600 x g using an Excelsa II 206 BL centrifuge (Fanem®) for 10 minutes, and the resulting pellet was resuspended in the same extenders (BG or I) to a concentration of 100 x 10^6 spz/mL.

For the groups supplemented with BotuCrio, the extenders were prepared prior to collection in an 80:20 (v/v) ratio (BG:BC or I:BC); the semen was then filtered and diluted to 100 x 10^6 spz/mL. In the groups combined with BC and without SP, the semen was diluted in the base extenders (BG or I), centrifuged at 600 x g for 10 minutes, and the pellet was resuspended in the pre-mixed 80:20 solution (BG:BC or I:BC). For all centrifuged groups, a 20% sperm loss during centrifugation was assumed for concentration calculations.

2.4. Evaluations

2.4.1. Sperm kinect

Sperm kinetic evaluations were performed using a Computer-Assisted Sperm Analysis (CASA) system (IVOS 12, Hamilton Thorne Inc., Beverly, MA, USA). All samples were stabilized at 37°C for 10 minutes; subsequently, a 10 µl aliquot was placed in a Makler counting chamber (Irvine Scientific, Santa Ana, CA). All fields were visually inspected to ensure homogeneity and the absence of bubbles or debris. Measurements were then conducted across five random fields, with a minimum of 1000 cells evaluated per sample. For all samples, the parameters recorded included total motility (TM, %), progressive motility (PM, %), and the percentage of rapid spermatozoa (RAP, %) as described by Guasti, 2013.

2.4.2. Epifluorescence microscopy

Plasma membrane integrity (PMI) was assessed using epifluorescence

microscopy (Leica Microsystems-DMLB, Germany). Samples were homogenized and incubated at 37°C in a dry block heater for 10 minutes prior to evaluation. To determine PMI, a fluorescent staining solution was prepared using 1 mL of sodium citrate, 20 µL of formol saline, 40 µL of carboxyfluorescein diacetate (CFDA; 0.46 mg/mL diluted in dimethyl sulfoxide, stored at -20°C in the dark; C5041, Sigma-Aldrich Brasil Ltda., SP, Brazil), and 20 µL of propidium iodide (PI; 0.5 mg/mL diluted in saline solution, stored at -20°C in the dark; P4170, Sigma-Aldrich Brasil Ltda., SP, Brazil).

Semen samples were mixed with the staining solution at a 1:1 ratio, stabilized for 10 minutes in a light-protected environment, and mounted on slides for evaluation under a fluorescence microscope at 400x magnification. Spermatozoa displaying green fluorescence were classified as intact, while cells displaying any red fluorescence, regardless of the lesion site, were classified as damaged. One hundred cells were evaluated per sample, and results were expressed as the percentage of intact cells. To minimize intra-observer subjectivity, a standardized slide scanning pattern was utilized (Figure 1).

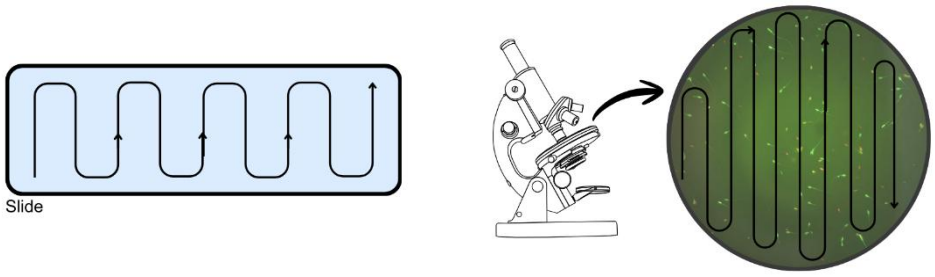


Figure 1: Representative description of the slide scanning pattern and cell counting within the microscopic field of view.

2.5. Statistic analisys

All statistical analyses were performed using RStudio (version 2022.07.1). A Mixed model was arranged for sperm parameters, counting the treatment groups and ejaculate order as fixed effects, and the donkeys as a random effect. The residual normality of the model was tested using the Shapiro-Wilk test. Parametric data (e.g., sperm motility parameters) were assessed by mixed models and Bonferroni post hoc comparisons (function “lsmeans” in the R “emmeans” package). Logistic regression (function “logistf” in the R “logistf”

package) was used to assess pregnancy rates between groups. Significance was set at $P<0.05$ for all tests, and statistical trend was defined as $P < 0.1 > 0.05$. All data are presented as mean ± SD or SEM when stated.

3. Results

3.1. Experiment 1

As expected, total motility (TM), progressive motility (PM), rapid sperm motility (RAP), and plasma membrane integrity (PMI) parameters did not differ among groups at 0 h ($P>0.05$). At 24 and 48 h, BC, BG20BC, BG10BC, BG10E, and BG2E exhibited the highest TM values ($P<0.05$), with no significant differences among them ($P>0.05$), followed by BG2E and BG, which were statistically similar ($P>0.05$). At 72 h, BG20BC, BG2E, BC, and BG10E showed the best TM results ($P<0.05$), with no differences observed among BG2E, BC, BG10E, and BG10BC ($P>0.05$). At 96 and 120 h, BG20BC, BG2E, and BC maintained the highest results ($P<0.05$), and BG10BC also joined this group at 120 h. By 144 h, only BG20BC, BG10BC, and BG2E displayed the highest TM values ($P<0.05$), with no differences between BG10BC, BG2E, and BC ($P>0.05$). Regarding the extenders, BG20BC demonstrated the greatest capacity to maintain TM results, sustaining values with no significant differences between 0, 24, 48, and 72 h ($P>0.05$).

BG20BC, BG10BC, BG10E, BG2E, and BC achieved the highest PM values ($P<0.05$) at 24, 48, and 72 h. At 96 h, BG20BC and BG2E stood out with the highest values ($P<0.05$), with no differences observed among BG2E, BG10BC, and BC ($P>0.05$). At 120 and 144 h, BG20BC, BG10BC, and BG2E comprised the group with the best results ($P<0.05$).

Regarding RAP, BC, BG20BC, BG10E, and BG10BC were the superior groups ($P<0.05$); however, no differences were found between BG20BC, BG10E, BG10BC, and BG2E ($P>0.05$). At 48 h, only BG2BC and BG were not part of the group with the highest results ($P<0.05$), while at 72 and 96 h, BG20BC and BG2E outperformed the other groups ($P<0.05$). At 120 and 144 h, BG10BC joined BG20BC and BG2E with the highest RAP values ($P<0.05$). BG20BC maintained RAP values without significant differences between 0, 24, 48, and 72 h ($P>0.05$). The results are illustrated in the following graph (Figure 2).

1

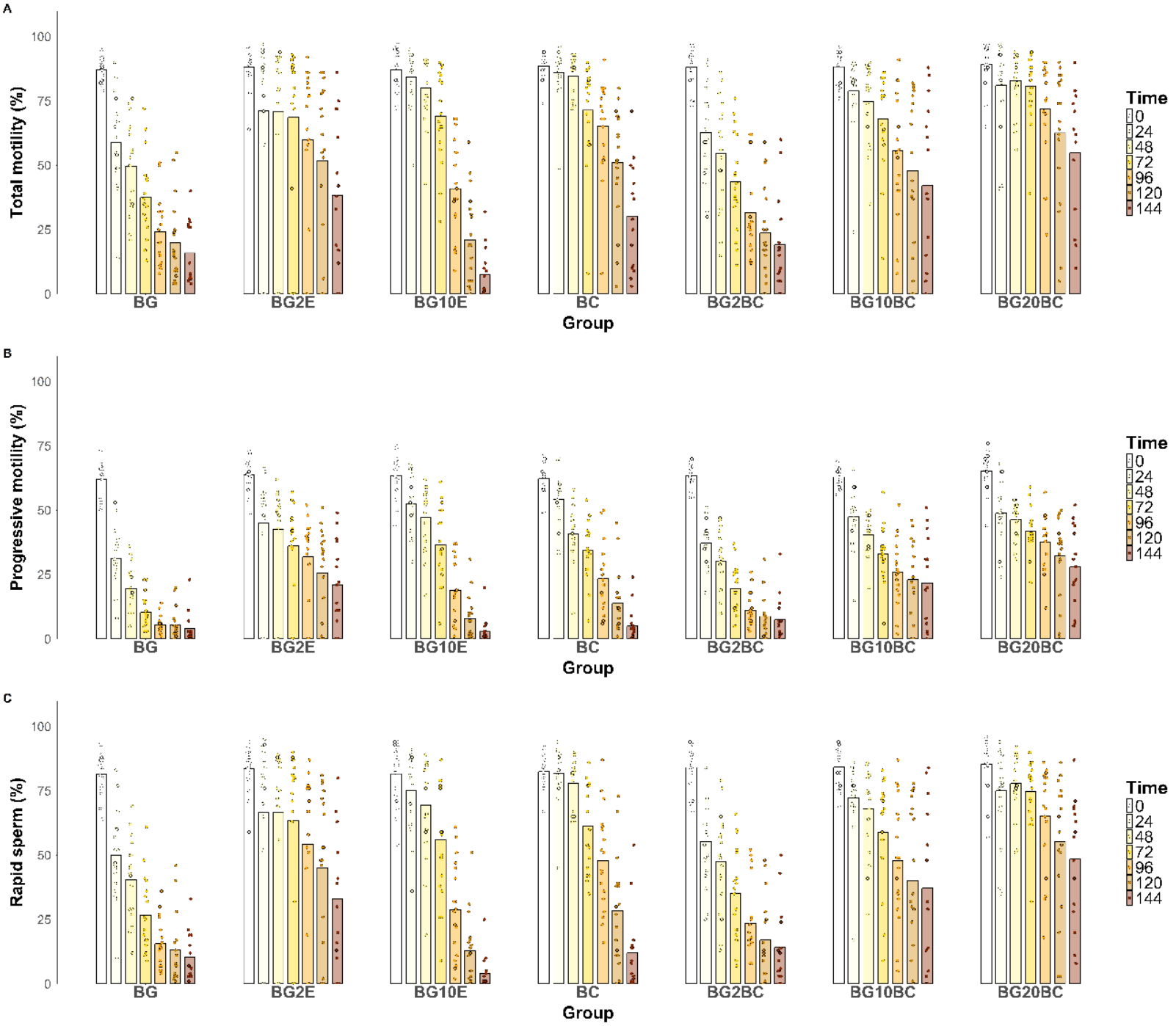
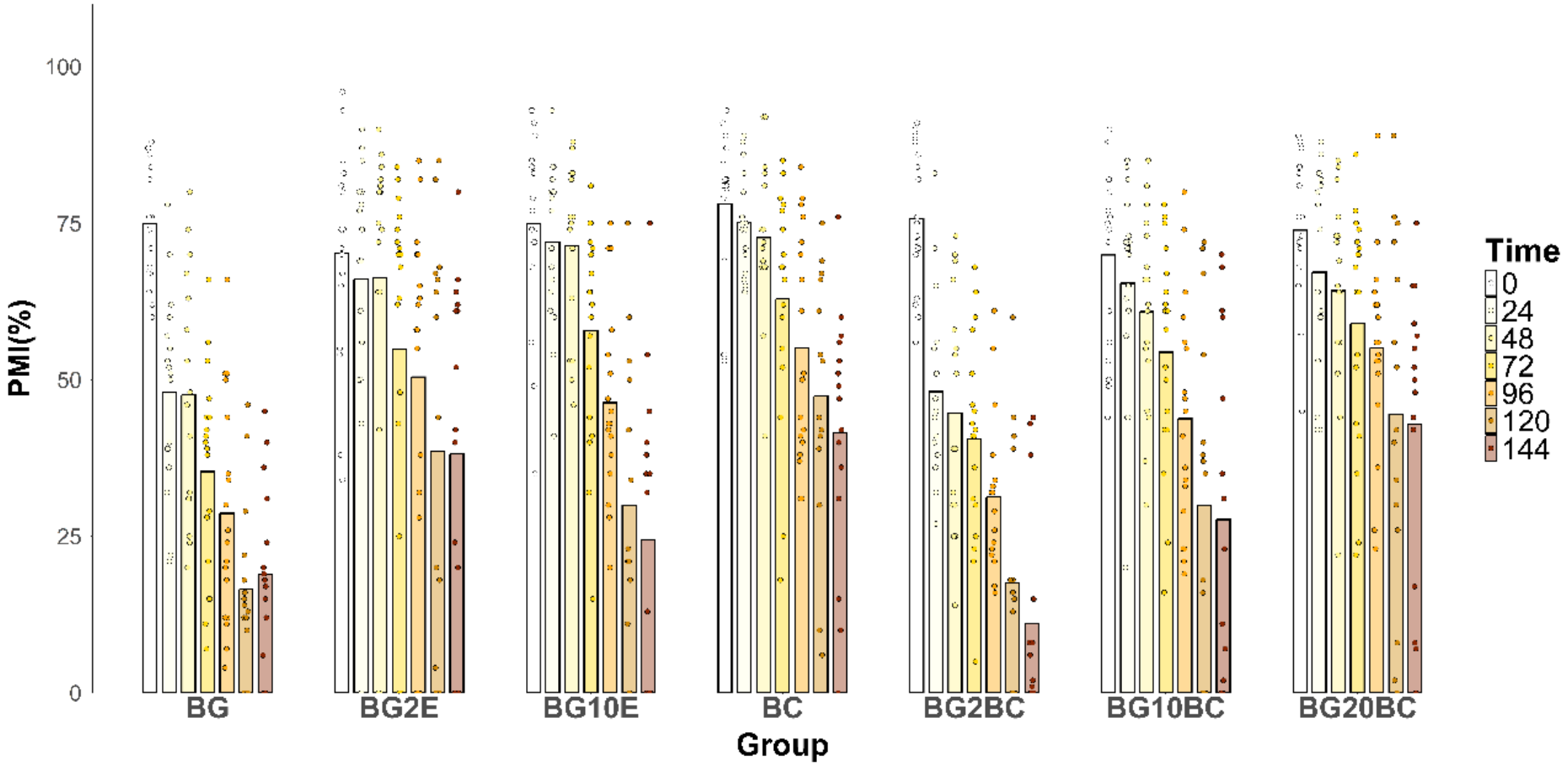

A
Total motility (%)
100
75
50
25
0
BG
BG2E
BG10E
BC
BG2BC
BG10BC
BG20BC
Group
Time
0
24
48
72
96
120
144
B
Progressive motility (%)
100
75
50
25
0
BG
BG2E
BG10E
BC
BG2BC
BG10BC
BG20BC
Group
Time
0
24
48
72
96
120
144
C
Rapid sperm (%)
100
75
50
25
0
BG
BG2E
BG10E
BC
BG2BC
BG10BC
BG20BC
Group
Time
0
24
48
72
96
120
144

Figure 2: Total Motility (A), Progressive Motility (B), and Rapid Sperm Motility (C) of donkey semen cooled for up to 144 h in different experimental groups: BotuSêmen Gold (BG), BotuSêmen Gold with 2% egg yolk (BG2E), BotuSêmen Gold with 10% egg yolk (BG10E), BotuCrio (BC), BotuSêmen Gold with 2% BotuCrio (BG2BC), BotuSêmen Gold with 10% BotuCrio (BG10BC) and BotuSêmen Gold with 20% BotuCrio (BG20BC). Bars represent the mean, and circles represent individual values for each biological replicate.

PMI values were similar for BC, BG10E, BG2E, BG20BC, and BG10BC ($P>0.05$), which comprised the group with the highest results ($P<0.05$) at all time points. In contrast, BG and BG2BC consistently represented the group with the lowest PMI values ($P<0.05$). These results are demonstrated in Figure 3.


PMI(%)
100
75
50
25
0
BG
BG2E
BG10E
BC
BG2BC
BG10BC
BG20BC
Group
Time
0
24
48
72
96
120
144

1

Figure 3: Plasma Membrane Integrity (PMI) of donkey semen cooled for up to 144 h in different experimental groups: BotuSêmen Gold (BG), BotuSêmen Gold with 2% egg yolk (BG2E), BotuSêmen Gold with 10% egg yolk (BG10E), BotuCrio (BC), BotuSêmen Gold with 2% BotuCrio (BG2BC), BotuSêmen Gold with 10% BotuCrio (BG10BC) and BotuSêmen Gold with 20% BotuCrio (BG20BC). Bars represent the mean, and circles represent individual values for each biological replicate.

3.2. Experiment 2

There was not difference in pregnancy rates between Jjennies (A) and mares (B) inseminated with BG20BC (A - 6/10, 60%; B - 6/8, 75%) and BG group (A - 6/13, 46.15%; B - 7/11, 63.73%) (Figure 4).

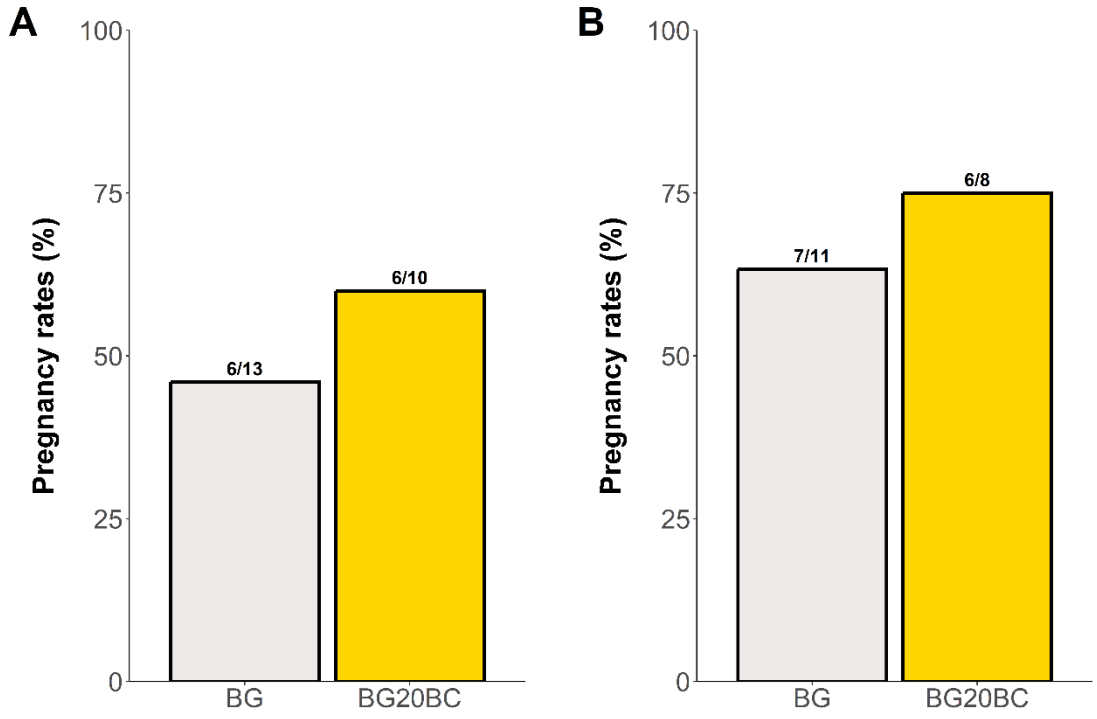


Figure 4: Pregnancy rates of jennies (A) and mares (B) inseminated with cooled donkey semen (5ºC for 24 h) using BotuSêmen Gold (BG) and BotuSêmen Gold with 20% BotuCrio (BG20BC). The numbers above each bar indicate the obtained pregnancy proportion (pregnant animals/inseminated animals).

3.3. Experiment 3

The groups did not differ from each other for any evaluation at 0 h (P>0.05). From 0 h onwards, for all parameters (TM, PM, RAP, and PMI), BGBC-SP and INBC-SP exhibited the best performance (P<0.05). Results for all evaluations (TM, PM, RAP, and PMI) indicate similar performance among extenders up to 24 h, as no statistical differences were observed between them for the same manipulations (BG and IN; BG-SP and IN-SP; BGBC and INBC; BGBC-SP and INBC-SP) (P>0.05).

TM was superior for INBC-SP and BGBC-SP at 48 h (P<0.05), followed by BGBC, which did not differ from BGBC-SP (P<0.05). At 72 h, BG-SP approached BGBC and BGBC-SP (P<0.05), followed by BGBC-P and INBC-SP with the highest values (P<0.05). INBC, IN-SP, BGBC, and BG-SP were similar to each other across all time points (P>0.05).

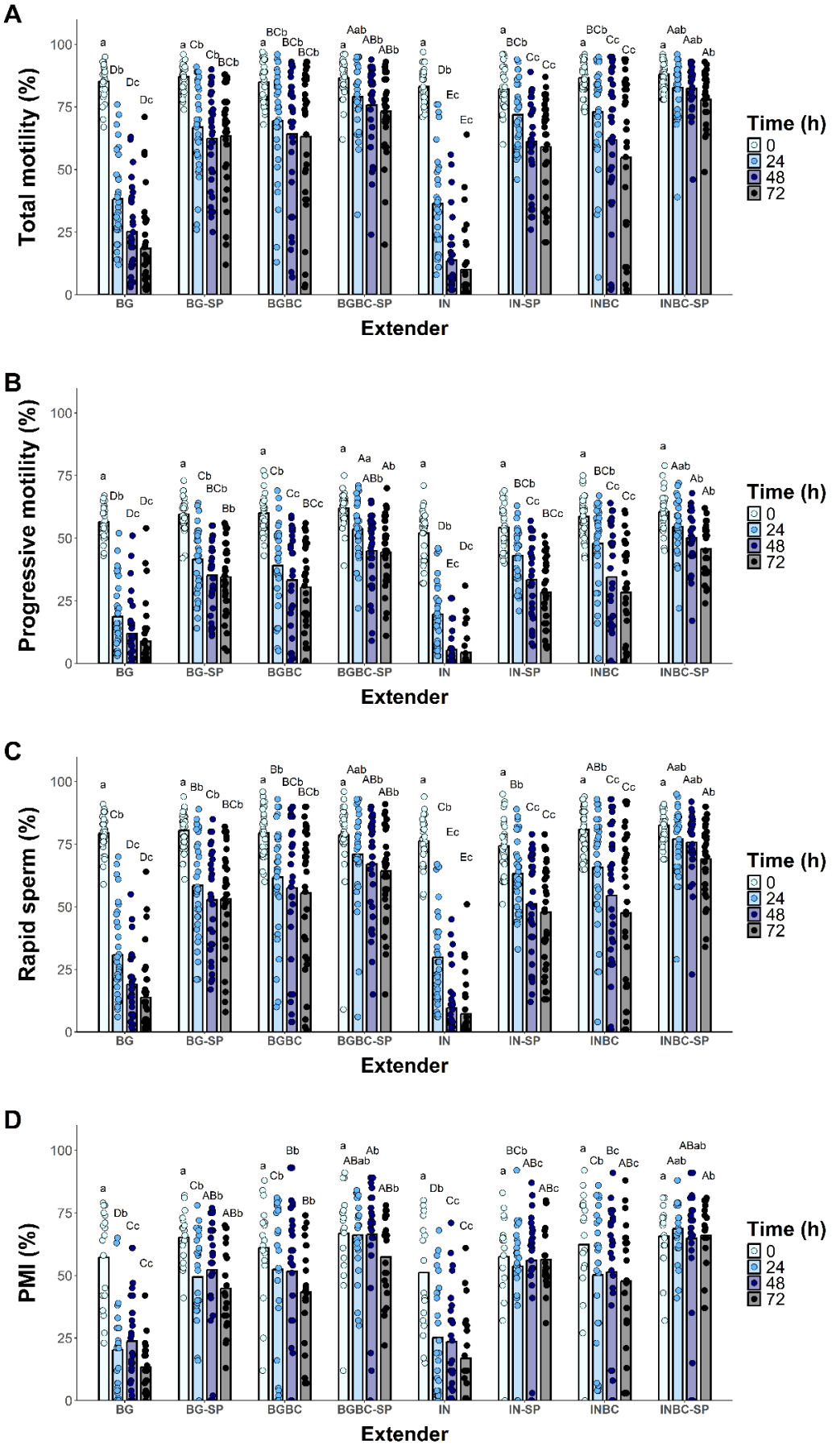
In terms of PM, INBC-SP, BGBC-SP, and INBC comprised the group with the best results at 24 h (P<0.05); there was no difference between BGBC-SP, INBC, and INBC-SP (P>0.05), forming the second-best group (P<0.05). At 48 and 72 h, only INBC-SP and BGBC-SP showed the best results (P<0.05), although at 48 h, no difference was observed between BGBC-SP and BG-SP (P>0.05). The INBC, BGBC, IN-SP, and BG-SP groups were similar to each other at 24 and 48 h (P>0.05), while at 72 h, similarity was observed between BG-SP, IN-SP, and BGBC (P>0.05) and between IN-SP, BGBC, and INBC (P>0.05); however, BG-SP was superior to INBC (P<0.05).

Concernig RAP results at 24 h were superior for INBC-SP, BGBC-SP, and INBC (P<0.05), with no significant difference between them (P>0.05). At 48 h, INBC-SP and BGBC-SP presented the best results (P<0.05), but BGBC-SP did not differ from BGBC (P>0.05). At 72 h, BGBC-SP, BGBC, and BG-SP composed the second-best group (P<0.05), with no difference between them (P>0.05). Across all time points, the BGBC, BG-SP, INBC, and IN-SP groups were similar to each other (P>0.05).

With respect to PMI, BGBC-SP and INBC-SP showed the best results at 24 h (P<0.05), but no difference was found between BGBC-SP and IN-P (P>0.05). At 72 h, BGBC-SP, INBC-SP, BG-SP, and IN-SP achieved the best results (P<0.05) with no significant difference among them (P>0.05). At the 72 h mark, INBC-SP was numerically superior to the others, but no difference (P>0.05) was found between INBC-SP, BGBC-SP, IN-SP, BG-SP, and INBC for the highest values

(P<0.05), while BGBC-SP, IN-SP, INBC, BG-SP, and BGBC were also similar to each other (P>0.05).

As fot the extenders, the BG groups maintained TM and RAP results more effectively over time (no difference between 24, 48, and 72 h for BG-SP, BGBC, and BGBC-P; P<0.05) compared to the INRA groups (no difference between 24, 48, and 72 h only for INBC-SP; P<0.05). Meanwhile, PMI results were similar at 24, 48, and 72 h for BG, BG-SP, BGBC, BGBC-SP, IN-SP, INBC, and INBC-SP (P>0.05). The results are presented in Figure 5.


A
Total motility (%)
B
Progressive motility (%)
C
Rapid sperm (%)
D
PMI (%)
Extender
BG
BG-SP
BGBC
BGBC-SP
IN
IN-SP
INBC
INBC-SP
Time (h)
0
24
48
72

1

Figure 5: Total Motility (A), Progressive Motility (B), Rapid Sperm Motility (C), and Plasma Membrane Integrity (PMI) of donkey semen cooled for up to 72 h in different experimental groups: BotuSêmen Gold (BG) and BotuSêmen Gold without seminal plasma (BG-SP), BotuSêmen Gold with BotuCrio (BGBC), BotuSêmen Gold with BotuCrio and without seminal plasma (BGBC-SP), INRA 96 (IN), INRA 96 without seminal plasma (IN-SP), INRA 96 with BotuCrio (INBC) and INRA 96 with BotuCrio and without seminal plasma (INBC-SP) Bars represent the mean and circles represent individual values for each biological replicate. Different uppercase letters (A, B, C) indicate significant differences ($P < 0.05$) between groups within the same cooling time. Different lowercase letters (a, b, c) indicate significant differences ($P < 0.05$) over time within the same group.

4. Discussion

It is known that skim milk is insufficient to protect the donkey sperm membrane (2,4,5), and the obtained results corroborate data found in the literature. The BG group presented the lowest TM and PMI values after 24 h of cooling, despite being an extender supplemented with casein and cholesterol. Although casein and cholesterol act as membrane protectants and stabilizers and yield optimal results for the equine species (14,15), the low sterol/phospholipid molar ratio of the donkey sperm membrane confers greater fluidity, but also greater vulnerability to thermal shock and oxidative stress (16–18). Low-density lipoproteins (LDL) present in egg yolk provide greater stabilization to the lipid bilayer and protect the cell from these temperature variations (6,19).

The results obtained comparing the BC and BG groups corroborate literature data indicating that the use of an egg yolk-based cryopreservation extender is a better alternative for donkey semen cooling compared to the use of a milk-based cooling extender (5,6). However, results are not yet optimal, which may be attributed to glycerol toxicity to the sperm cell (7,20,21). Despite having the membrane stabilized by the presence of egg yolk, the sperm cell was exposed to the cytotoxic and osmotic toxicity of glycerol present in BotuCrio (5,6).

The associations were made using a skim milk-based extender with casein and cholesterol (BG) with the addition of egg yolk (casein, cholesterol, and yolk association) or with the addition of BotuCrio (casein, cholesterol, yolk, and cryoprotectant association).

The BG10E group obtained higher immediate results than the BG2E group for all kinetic evaluations but failed to sustain these results, whereas the BG2E group was more consistent in maintaining them. However, when comparing the BG10E group with the BC group, considering that BotuCrio has 10% egg yolk in its composition, the BC group was more competent in maintaining values, indicating a possible protection conferred by cryoprotectants, despite their toxicity.

The comparison among the BG2BC, BG10BC, and BG20BC groups agrees with the comparison between BG2E and BG10E that 2% egg yolk, within the studied groups, was the ideal concentration to confer cell protection. Although egg yolk lipids are essential for membrane protection, the excess of polyunsaturated fatty acids (PUFAs) may also serve as a substrate for lipid peroxidation (17,22). Apparently, the use of 10% egg yolk saturated the amount necessary for cell protection and favored the formation of reactive oxygen species (ROS), rendering the environment toxic to the sperm cell.

The association of casein, cholesterol, 1% or more egg yolk and cryoprotectants (BG10BC and BG20BC) presented the best results, with the BG20BC group standing out compared to all others. The presence of cryoprotectants appears to be toxic in high proportions but important in lower concentrations. The best results were obtained by diluting the cryopreservation extender and decreasing the proportion of cryoprotectants.

It is not possible to define whether cholesterol and casein were determinant in the association, since other milk-based extenders without these two components were not tested. However, it is known that cholesterol plays a determinant role in donkey semen cooling (4).

Regarding fertility, the obtained results did not differ between the groups, but were better than the results found by Vidament (2009).

Based on the findings of Experiment 1, Experiment 3 compared the supplementation of 20% BC in two distinct cooling extenders (BotuSêmen Gold and INRA 96) and assessed the impact of seminal plasma removal. The results indicate similarity between the extenders when used for cooled donkey semen; however, they reinforce the premise that extenders based solely on skim milk are insufficient to maintain adequate sperm quality (6,7).

On the other hand, the similarity observed among groups with BC supplementation and those with seminal plasma removal (INBC, IN-P, BGBC,

and BG-P) supports the hypothesis that donkey seminal plasma promotes cholesterol efflux from the plasma membrane (3). This is evidenced by the fact that seminal plasma removal and the addition of exogenous cholesterol sources yielded equivalent results. Nevertheless, the potential effect of cryoprotectants observed in Experiment 1 cannot be disregarded.

The synergistic effect of these treatments (INBC-P and BGBC-P) resulted in the highest kinematic parameters across all experimental groups. The absence of seminal plasma, combined with the addition of egg yolk, could potentially lead to membrane cholesterol saturation, a mechanism previously suggested in the comparison between BG2E and BG20BC. However, fertility rates were not evaluated in Experiment 3. Consequently, the precise impact of cryoprotectants on the cooled donkey sperm plasma membrane remains to be fully elucidated, and it is not yet possible to definitively state that seminal plasma sequesters membrane cholesterol, nor to quantify this loss.

5. Conclusion

The fertility of groups supplemented with BotuCrio and subjected to seminal plasma removal remains to be proven. However, based on the results obtained in this study and the current literature, it can be concluded that the highest kinematic parameters for donkey semen cooled at 5°C are achieved with the addition of 2% egg yolk and the removal of seminal plasma.

References

1. Larry Sawers. The Mule, the South, and Economic Progress. 2004.

2. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key Aspects of Donkey and Mule Reproduction. Vol. 35, Veterinary Clinics of North America - Equine Practice. W.B. Saunders; 2019. p. 607–42.

3. Steiner CC, Ryder OA. Molecular phylogeny and evolution of the Perissodactyla. Zool J Linn Soc. 2011 Dec;163(4):1289–303.

4. Segabinazzi LGTM, Scheeren VF da C, Freitas-Dell'Aqua C de P, Papa FO, Alvarenga MA, Dell'Aqua JA, et al. Cholesterol-Loaded Cyclodextrin

Addition to Skim Milk-Based Extender Enhances Donkey Semen Cooling and Fertility in Horse Mares. J Equine Vet Sci. 2021 Oct 1;105.

5. M Gobato MLDPS, Miro J, Gaitskell-Phillips G, Carluccio A, T M Segabinazzi LG, M Gobato ML, et al. Ability of donkey sperm to tolerate cooling: Effect of extender base and removal of seminal plasma on sperm parameters and fertility rates in mares. 2022.

6. Alonso C, Castañeira C, Bragulat AF, Losinno L. Effect of egg yolk-based extender and seminal plasma removal on sperm viability of cooled donkey semen. Braz J Vet Res Anim Sci. 2021 Mar 10;58(Special Issue).

7. Vidament M, Vincent P, Martin FX, Magistrini M, Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim Reprod Sci. 2009 May;112(1–2):22–35.

8. Carvalho LE, Silva Filho JM, Palhares MS, Sales ALR, Gonczarowska AT, Oliveira HN, et al. Physical characteristics and fertility of fractionated donkey semen cooled at 5°C. Arq Bras Med Vet Zootec. 2017;69(1):29–38.

9. Catalán J, Yánez-Ortiz I, Tvarijonaviciute A, González-Arostegui LG, Rubio CP, Yeste M, et al. Impact of Seminal Plasma Antioxidants on Donkey Sperm Cryotolerance. Antioxidants. 2022 Feb 1;11(2).

10. Miró J, Vilés K, García W, Jordana J, Yeste M. Effect of donkey seminal plasma on sperm movement and sperm-polymorphonuclear neutrophils attachment in vitro. Anim Reprod Sci. 2013 Aug;140(3–4):164–72.

11. Rota A, Magelli C, Panzani D, Camillo F. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology. 2008 May 15;69(2):176–85.

12. Rodrigues et al. LT. Efeito de diferentes diluentes e da remoção do plasma seminal na refrigeração do sêmen asinino (Equus asinus). Revista Acadêmica: Ciência Animal. 2017 Jul 21;15:143.

13. Rota A, Panzani D, Sabatini C, Camillo F. Donkey jack (Equus asinus) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology. 2012 Nov;78(8):1846–54.

14. Batellier F, Vidament M, Fauquant J, Duchamp G, Arnaud G, Yvon JM, et al. Advances in cooled semen technology. Vol. 68, Animal Reproduction Science. 2001.

15. Pagl R, Aurich JE, Müller-Schlösser F, Kankofer M, Aurich C. Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5 °C. Theriogenology. 2006 Sep 15;66(5):1115–22.

16. Parks JE, Lynch' D V. Lipid Composition and Thermotropic Phase Behavior of Boar, Bull, Stallion, and Rooster Sperm Membranes. Vol. 29, CRYOBIOLOGY. 1992.

17. Prasinou P, De Amicis I, Fusaro I, Bucci R, Cavallini D, Parrillo S, et al. The Lipidomics of Spermatozoa and Red Blood Cells Membrane Profile of Martina Franca Donkey: Preliminary Evaluation. Animals. 2023 Jan 1;13(1).

18. de Melo, Maria Isabel Vaz, et al. "Uso de lipoproteínas de baixa densidade e de gema de ovo na preservação do sêmen asinino resfriado a 6o C." Brazilian Journal of Veterinary Medicine 34.4 (2012): 288-294

19. Dalal J, Chandolia RK, Pawaria S, Kumar A, Kumar D, Selokar NL, et al. Low-density lipoproteins protect sperm during cryopreservation in buffalo: Unraveling mechanism of action. Mol Reprod Dev. 2020 Dec 1;87(12):1231–44.

20. Vidament M, Vincent P, Yvon JM, Bruneau B, Martin FX. Glycerol in semen extender is a limiting factor in the fertility in asine and equine species. Vol. 89, Animal Reproduction Science. 2005.

21. Macías García B, Ortega Ferrusola C, Aparicio IM, Miró-Morán A, Morillo Rodriguez A, Gallardo Bolaños JM, et al. Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology. 2012 Apr 15;77(7):1280–9.

22. Anton M, Gandemer G. Composition, Solubility and Emulsifying Properties of Granules and Plasma of Egg Yolk. Vol. 62, 484-JOURNAL OF FOOD SCIENCE. 1997.