UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA ANIMAL

CRIOPRESERVAÇÃO DO SÊMEN DE GARANHÕES DA RAÇA CRIOULA COM BAIXO E ALTO ESCORE DE CONDIÇÃO CORPORAL E REFRIGERAÇÃO DE SÊMEN EQUINO COM DILUENTES A BASE DE CASEÍNA.

GUILHERME NOVELLO

Botucatu – São Paulo
Junho/2020

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Orientador: Prof. Dr. Igor Frederico Canisso

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LISTA DE ABREVIATURAS

HSP	proteínas do plasma seminal equino
BSP	proteínas do plasma seminal bovino
pH	potencial hidrogeniônico
BG	botusêmen gold
IMP	integridade de membrana plasmática
HPM	potencial mitocondrial
MT	motilidade total
MP	motilidade progressiva
VCL	velocidade curvilinear
VAP	velocidade de trajeto
VSL	velocidade linear
RAP	espermatozoides rápidos
PS	plasma seminal
LDL	lipoproteínas de baixa densidade
DMSO	dimetilsulfóxido
ERO	espécies reativas do oxigênio
SM	síndrome metabólica
LH	hormônio luteinizante
FSH	hormônio folículo estimulante
10^6	milhão
<	menor
>	maior
≥	maior/igual
%	percentual
°C	graus Celsius
±	mais ou menos

SUMÁRIO

NOVELLO, G. CRIOPRESERVAÇÃO DO SÊMEN DE GARANHÕES DA RAÇA CRIOULA COM BAIXO E ALTO ESCORE DE CONDIÇÃO CORPORAL E REFRIGERAÇÃO DE SÊMEN EQUINO COM DILUENTES A BASE DE CASEÍNA. Botucatu – SP. 2020. 96p. Universidade Estadual Paulista "Júlio de Mesquita Filho" - Faculdade de Medicina Veterinária e Zootecnia.

RESUMO

A criopreservação (resfrigeração e congelamento) de sêmen equino é uma biotecnologia amplamente utilizada na indústria de criação de cavalos. No entanto, vários fatores podem afetar a qualidade seminal e a fertilidade do sêmen criopreservado. Claramente, as individualidades dos garanhões associadas à capacidade de resfriamento ou congelamento do sêmen, diluentes de sêmen, sistema de transporte e processamento são bem descritas como associadas a alterações na qualidade seminal. Nos homens, a obesidade e a síndrome metabólica têm sido associadas a efeitos adversos nos parâmetros espermáticos. Com isso, o objetivo deste estudo foi testar diferentes diluentes de sêmen, dispositivos de resfriamento, processamento, bem como comparar a capacidade de congelamento de sêmen de garanhões com baixo escore de condição corporal e alto escore de condição corporal. Capítulo 2: Primeiramente, foram testados os parâmetros de cinética espermática e as taxas de recuperação embrionária do sêmen diluído e refrigerado com diferentes extensores à base de caseína (INRA 96 ou BotuSêmen Gold) com ou sem plasma seminal e armazenados em três diferentes dispositivos comerciais de refrigeração. No experimento 1, 45 ejaculados de nove garanhões foram coletados, avaliados e igualmente divididos entre os diluentes e depois diluídos para 50 milhões de espermatozoides/mL. Em seguida, o sêmen diluído foi armazenado em três *containers* de refrigeração passiva (Equitainer, Equine Express II e BotuFlex) por 48 horas. No experimento 2, os mesmos ejaculados diluídos no experimento 1 foram centrifugados com cushion, o sobrenadante foi descartado e o "pellet" foi ressuspenso a 100 milhões de espermatozoides/mL com seu respectivo extensor. O sêmen foi então refrigerado como no experimento 1. Nos dois experimentos, os parâmetros de cinética espermática, integridade da membrana plasmática e o alto potencial da membrana mitocondrial foram avaliados às 0, 24 e 48 horas após o resfriamento. Para o experimento 3, 12 éguas (n = 24 ciclos) foram inseminadas com sêmen de um garanhão refrigerado por 48 horas. O sêmen foi processado como descrito no experimento 1. O lavado de embrião foi realizado 8 dias

após a ovulação. No experimento 1, o BotuSêmen Gold apresentou motilidade total e progressiva superior em relação ao INRA 96 (P <0,05). Não houve diferenças significativas entre os sistemas de refrigeração em nenhum dos experimentos. No experimento 2, os diluentes INRA 96 e BotuSêmen Gold apresentaram motilidade total e progressiva semelhante, no entanto o BotuSêmen Gold apresentou parâmetros superiores de velocidade espermática em todos os momentos. A recuperação embrionária foi idêntica para os dois extensores (50%). Finalmente, os resultados aqui obtidos sugerem que o BotuSêmen Gold é um diluente de sêmen equino a ser incluído nos testes frente ao INRA 96 na prática clínica. Em segundo lugar, foi avaliado o efeito do escore de condição corporal (ECC) na capacidade de congelamento de sêmen de garanhões da raça Crioula. Vinte garanhões foram alocados em 2 grupos de acordo com o ECC: os garanhões com ECC <7 foram caracterizados como Baixo-ECC (11/20) e os garanhões com ECC ≥7 como Alto-ECC (9/20). Foi obtida uma história clínica completa seguida de medidas morfométricas e de acúmulo de gordura subcutânea. As concentrações plasmáticas de glicose foram avaliadas após teste oral de tolerância a glicose. Para isso, os garanhões foram submetidos a jejum durante a noite e o xarope de milho foi administrado por via oral às 8h da manhã. Amostras de sangue foram coletadas para avaliação da glicose antes e após a administração oral de xarope de milho. Além disso, um ejaculado de cada garanhão foi coletado e diluído a 50 milhões de espermatozoides/mL com extensor à base de leite desnatado (BotuSêmen, Botupharma, Brasil). O sêmen foi centrifugado (600 × g / 10 min), o sobrenadante foi descartado e o "pellet" ressuspenso a 200 milhões de espermatozoides/mL com BotuCrio (Botupharma, Brasil) e submetido ao processo de criopreservação. A cinética espermática foi avaliada por CASA e integridade da membrana plasmática, ãnion superóxido, potencial mitocondrial, peroxidação lipídica e peróxido de hidrogênio pela citometria de fluxo em amostras de sêmen pós-descongelamento. O estudo morfométrico (peso corporal, ECC e circunferência do pescoço em 25% e 50%) foi superior em garanhões com alto-ECC (P <0,05). No entanto, não houve diferenças nos parâmetros espermáticos ou na concentração plasmática de glicose entre os grupos (P> 0,05). Em resumo, o alto ECC não afetou os parâmetros espermáticos e a capacidade de congelamento de sêmen de garanhões da Raça Crioula.

Palavras-chave: Sêmen; criopreservação; escore de condição corporal; diluente de sêmen equino.

NOVELLO, G. SEMEN CRYOPRESERVATION OF CRIOULO STALLIONS WITH LOW AND HIGH BODY CONDITION SCORE AND SEMEN COOLING USING CASEIN-BASED EXTENDERS. Botucatu – SP. 2020. 96p. Universidade Estadual Paulista "Júlio de Mesquita Filho" - Faculdade de Medicina Veterinária e Zootecnia.

ABSTRACT

Cryopresrvation (e.g. cooling and freezing) of equine semen is a widely used biotechnology in the horse breeding industry. However, several factors can affect sperm quality and fertility of the cryopreserved semen. Clearly, stallion individualities associated with semen cooling- or freezing-ability, semen extender, transport system and semen processing are well described to be associated with changes in sperm quality. Of interest, in men the obesity and metabolic syndrome have been associated with adverse effects in sperm parameters. Therefore, the objective of this study was to test different semen extenders, cooling devices, semen processing, as well as to compare the semen freezing ability of stallions with normal body condition score and those characterized as obese. Chapter 2: Firstly, semen parameters and embryo recovery rates of cooled stallion semen extended with different casein-based extenders (INRA 96 or BotuSemen Gold) with or without seminal plasma and stored in three commercial cooling devices were tested. In experiment 1, 45 ejaculates from nine mature stallions were collected, assessed, and equally split between both extenders and then extended to 50 million sperm/mL. Then, the extended semen was stored in three passive cooling containers (Equitainer, Equine Express II, and BotuFlex) for 48 hours. In experiment 2, the same ejaculates extended in experiment 1 were cushion-centrifuged, the supernatant was discarded, and the pellets were resuspended at 100 million sperm/mL with their respective extender. Semen was then cooled and stored as in experiment 1. In both experiments, sperm motility parameters, plasma membrane integrity, and high mitochondrial membrane potential were assessed at 0, 24, and 48 hours post cooling. For experiment 3, 12 mares (n = 24 cycles) were bred with 48 hour–cooled semen from one stallion. Semen was processed as described in experiment 1. Mares had embryo flushing performed by 8-day post-ovulation. In experiment 1, BotuSemen Gold displayed superior total and progressive motility relative to INRA 96 ($P<0.05$). There were no significant differences between the types of containers in any experiment. In experiment 2, INRA 96 and BotuSemen Gold extenders had similar total and progressive motility, but BotuSemen Gold had superior

sperm velocity parameters at all timepoints. Embryo recovery was identical for both extenders (50%). Finally, the results obtained herein suggest that BotuSemen Gold is a suitable alternative to be included in semen cooling tests against INRA 96 in clinical practice. Secondly, the effect of obesity in semen freezing ability of Crioulo stallions was evaluated. Twenty stallions were allocated into 2 groups according to their body condition score (BCS): stallions with BCS <7 were characterized as Low-BCS (11/20) and stallions with BCS ≥7 as High-BCS (9/20). A complete clinical history followed by morphometric and subcutaneous body fat measurements were obtained. Serum glucose concentrations were evaluated after oral sugar test. For this, the stallions were fasted overnight and corn syrup was orally administered at 8 am. Blood samples were collected for glucose assessment before and after oral administration of corn syrup. In addition, one ejaculated of each stallion was collected and extended at 50 million sperm/mL with skimmed-milk based extender (BotuSemen, Botupharma, Brazil). Semen was centrifuged (600 ×g/10 min), the supernatant discarded, the pellet resuspended at 200 million sperm/mL with BotuCrio (Botupharma, Brazil) and submitted to cryopreservation. Sperm kinetics were assessed by CASA and plasma membrane integrity, ROS, mitochondrial membrane potential and lipid peroxidation by flow cytometer in post-thawed semen samples. The morphometric study (e.g. body weight, BCS and neck circumference at 25% and 50%) were superior in obesity stallions ($P<0.05$). However, there were no differences in sperm parameters or serum glucose concentration between groups ($P>0.05$). In summary the high-BCS in Crioulo horse didn't affect sperm parameters and freezing ability of Crioulo stallions.

Keywords: Sperm, cryopreservation, body condition score, stallion semen extender.

CAPÍTULO 1

INTRODUÇÃO

Nos últimos anos, as tecnologias para a refrigeração de sêmen equino vêm evoluindo. Isso está acontecendo devido a uma alta porcentagem de garanhões que respondem mal ao processo de criopreservação, (VIDAMENT et al., 1997), também porque a grande maioria das raças permite a realização de inseminação artificial, beneficiando assim a reprodução de equinos e contribuindo com o progresso genético da espécie (AURICH; AURICH, 2006) e ainda pela praticidade no controle do ciclo estral da égua, sendo esse menos intensivo quando o sêmen refrigerado é utilizado. Isso tudo só é possível, pois a evolução das biotécnicas possibilitaram o transporte de sêmen por longos períodos, com diluentes e caixas de transportes que mantém o material genético em condições adequadas para manutenção da viabilidade espermática.

Com o uso da inseminação artificial a partir do sêmen refrigerado, destacam-se algumas vantagens como: a otimização do uso de garanhões com alto valor zootécnico, redução de gastos inerentes ao transporte e hospedagem, diminuição do estresse e de acidentes durante o transporte. Além da não exposição dos animais a patógenos de um novo ambiente, diminuindo a transmissão de doenças (BRINSKO; VARNER, 1992a).

No entanto, quando utilizamos a inseminação com sêmen refrigerado, vários são os fatores que influenciam no resultado final, ou seja, na taxa de concepção. O efeito individual do garanhão, a temperatura e o tempo de armazenamento, a caixa de transporte, a curva de refrigeração, o momento da inseminação, assim como o diluente de sêmen utilizado, o qual pode ser adequado conforme a sua composição e adaptação individual do garanhão (PUGLIESI, 2009).

Quando falamos em fatores individuais, por mais que não temos comprovação em equinos, sabemos que a obesidade em touros afeta a qualidade seminal devido ao deposito de gordura no escroto que consequentemente afetando a termorregulação. Em humanos já é comprovado que somente a obesidade já afeta a qualidade seminal e está relacionada com infertilidade. Homens com diagnóstico de síndrome metabólica também apresentam alteração não só nos padrões seminais como também hormonais.

Os diluentes mais utilizados no mundo, segundo Canisso et al., (2008) são a base de leite em pó desnatado, sendo que estes são derivações do diluente descrito por Kenney et al., (1975), que é a base de leite em pó desnatado, glicose, penicilina e estreptomicina. Alguns trabalhos apontam também, que quando esses diluentes são utilizados, a melhor faixa de temperatura para refrigeração do sêmen equino é entre 4 e 6°C quando

comparada a temperaturas entre 15 e 20°C (MORAN et al., 1992; VARNER et al., 1988, 1989).

Contudo, algumas pesquisas evidenciam a superioridade de novos diluentes que tem como base frações especificas do leite. Além de evitar interações indesejadas com as substâncias presentes no leite (POMMER; LINFOR; MEYERS, 2002), as frações especificas, como a caseína e o fosfocaseinato, são utilizadas nos diluentes de sêmen devido à capacidade de interagir com determinadas proteínas plasmáticas, aumentando a fertilidade dos espermatozoides (MANJUNATH, 2012).

Visto a importância do transporte de sêmen, o constante aprimoramento das técnicas de refrigeração associado aos produtos disponíveis no mercado visa melhorias na qualidade espermática, a fim de potencializar as taxas de fertilidade. Além de fatores individuais do garanhão, que ainda são poucos estudados, podem fazer com que nossos resultados finais caiam drasticamente. Com isso, o objetivo deste estudo foi avaliar os parâmetros de cinética, integridade de membrana plasmática, potencial mitocondrial e fertilidade frente a dois diluentes de sêmen equino, compostos por frações especificas do leite, sendo um composto por fosfocaseinato nativo (INRA 96), e outro a base de caseinato de sódio acrescido de colesterol (BotuSêmen Gold), na presença ou remoção do plasma seminal, submetidos ao processo de refrigeração em diferentes dispositivos de armazenamento à 5ºC ou 15ºC por 24 e 48 horas. Além também de avaliar o efeito do escore corporal de garanhões da raça Crioula, sobre a qualidade do sêmen congelado, cruzando esses dados com um estudo morfométrico e perfil de glicose desses animais.

REVISÃO DE LITERATURA

1. Célula espermática

O processo para produção da célula espermática é conhecido como espermatogênese, a qual ocorre dentro do parênquima testicular, mais especificamente dentro dos túbulos seminíferos, sendo dividida em três fases: espermatocitogênese, fase meiótica e espermiogênese (AMANN, 1993b). Após esse processo, se inicia a maturação nos ductos epidimários e armazenamento na cauda do epidídimo até que ocorra a ejaculação (SETCHELL, 1993). Quando a ejaculação acontece, temos uma célula relativamente simples, composta por cabeça e flagelo ou cauda, porém altamente especializada devido a sua função: fertilização (VARNER et al., 2015).

A cabeça do espermatozoide equino tem uma forma elíptica e é achatada em um plano dorsoventral, sendo mais espessa na porção posterior da cabeça do que na porção apical (VARNER et al., 2000). Pode ser dividida em região acrossomal, pós-acrossomal e fossa de implantação que demarca a junção entre a cabeça e a cauda. Na cabeça encontra-se o núcleo, onde está armazenado o material genético altamente condensado: o DNA (PESCH; BERGMANN, 2006; VARNER et al., 2015).

O acrossoma, estrutura essencial para que aconteça a fecundação, é uma espécie de capuz, derivado do complexo de Golgi (BURGOS; FAWCETT, 1955; PESCH; BERGMANN, 2006). Essa estrutura contém enzimas hidrolíticas, como, acrosina, hialuronidase, hidrolases e esterases, imprescindíveis para a lise da zona pelúcida e penetração no oócito (YAMAGATA et al., 1998).

Já a cauda, que é a estrutura que dá movimento a célula, pode ser dividida em peça de conexão, peça intermediaria, peça principal e peça terminal. A peça de conexão serve para conectar a cabeça a peça intermediaria, tendo como estrutura de desenvolvimento os centríolos que também dão origem ao axonema. O axonema é composto por uma par de microtúbulos centrais envolto por outros 9 pares de microtúbulos externos, formando assim uma configuração “9 + 2”, que deslizam sobre si promovendo movimento a célula (VARNERet al., 2015). Ainda, o axonema da peça intermediária é envolto pela bainha mitocondrial, composta pela matriz energética geradora de adenosina trifosfato (ATP), dando então a capacidade de movimento flagelar através do axonema (FERREIRA et al., 2002; PESCH; BERGMANN, 2006).

A estrutura espermática é envolta por uma membrana plasmática complexa, formada por uma bicamada lipídica, composta por fosfolipídios, glicolipídios, colesterol e proteínas de membrana (ALBERTS; BRAY; LEWIS, 1989). Cada um destes compostos se apresenta em diferentes proporções, sendo 57% de fosfolipídios, 37% de colesterol e 6% de glicolipídios. No entanto, os equinos apresentam uma relação de colesterol:fosfolipídio baixa, quando comparado a varrões e touros (DARIN-BENNET e WHITE, 1977). Essa composição da membrana plasmática forma uma barreira de permeabilidade altamente seletiva (ZÚCCARI, 1998), onde o colesterol promove uma maior estabilidade da membrana (CROSS, 1998; YEAGLE, 1985).

2. Efeitos da refrigeração sobre o espermatozoide equino

No momento da colheita a temperatura do sêmen é de aproximadamente 35°C, a partir daí a temperatura começa a cair, até atingir a temperatura ambiente. Segundo Moran et al. (1992), até que os espermatozoides atinjam a temperatura de 20°C os mesmos não sofrem alterações drásticas que comprometam sua viabilidade. No entanto, quando submetidos à refrigeração, se inicia um processo de estresse, até o momento em que a temperatura final de 5°C seja atingida (MORAN et al., 1992).

O processo de refrigeração afeta a estrutura espermática, sendo os componentes da membrana plasmática os mais afetados. Quando o sêmen atinge temperaturas que se aproximam aos 5°C, a membrana se torna mais susceptível a danos, que levam a alterações na permeabilidade associada a mudanças morfofuncionais, e afetam diretamente a motilidade e capacidade fecundante dos espermatozoides (AMANN; GRAHAM, 2011).

Quando os espermatozoides são submetidos ao processo de refrigeração, se inicia a fase de transição dos fosfolipídeos da membrana plasmática do estado liquido-cristalino para o estado de gel (AMANN; GRAHAM, 2011). Período esse classificado como crítico para o espermatozoide, o qual deve ter uma taxa de refrigeração de 0,05°C por minuto, entre as temperaturas de 19°C até 8°C, para que danos irreversíveis não ocorram às células espermáticas, causando queda no potencial fertilizante das mesmas (MORAN et al., 1992).

Outro fator alterado durante o processo de refrigeração é o metabolismo espermático. Logo após a colheita, enquanto o sêmen está em temperatura ambiente o metabolismo celular é alto, no entanto durante o processo de refrigeração, para cada 10°C de decréscimo na temperatura, o metabolismo espermático reduz cerca de 50%. Dessa forma, no momento em que a temperatura atinge 5°C, apenas 10% do seu metabolismo é utilizado para sobrevivência, possibilitando o armazenamento por períodos mais longos (SQUIRES et al., 1999).

3. Sistemas de refrigeração

Para a refrigeração de sêmen, existem sistemas passivos e ativos. A diferença entre esses dois sistemas se dá em relação às taxas de resfriamento e custo do equipamento. Os sistemas passivos são equipamentos de menor valor, no entanto a taxa de refrigeração

depende de alguns fatores como a temperatura ambiente, temperatura inicial e massa da amostra (VALLE et al., 1999). Já os sistema ativos apresentam valor mais elevado, porém com taxas de refrigeração pré-determinadas (VALLE et al., 1999) e lineares (KAYSER et al., 1992).

Por mais que apresentem taxas de refrigeração dependente, os sistemas passivos são os mais utilizados nacional e internacionalmente, que são feitos em caixas (*containers)* (SILVA FILHO; PALHARES; FONSECA, 1994). Para que seja classificado como um bom *container* é preciso que algumas características sejam atingidas, como: completo isolamento ambiente, baixo custo, ser inócuo para os espermatozoides, possuir curva de resfriamento lento, manutenção da temperatura para o período proposto, possuir estrutura resistente, simples e leve para aceitação pelos sistemas de transporte aéreo e terrestre, além de ser seguro contra violações entre outras situações adversas que possam comprometer o material (BRINSKO; CROCKETT; SQUIRES, 2000; SILVA FILHO, PALHARES, FONSECA, 1994).

A refrigeração do sêmen é feita em sistemas de refrigeração passivo, onde, caixas isotérmicas são utilizadas e o conteúdo é mantido próximo a uma fonte de frio (gelo reciclável), que de acordo com o sistema pode estabilizar a 15 – 20°C, ou 4 – 6°C. Essa redução de temperatura do sêmen auxilia na conservação, pois assim temos diminuição do crescimento bacteriano, redução do metabolismo espermático e redução também das espécies reativas de oxigênio (AURICH, 2008; KATILA, 1997).

Diversos são os modelos de dispositivos de refrigeração nacionais e internacionais, com diferentes taxas de refrigeração, temperatura final e tempo máximo de armazenamento (RAPHAEL, 2007). Essas caixas isotérmicas podem ser de poliestireno, como a BotuFlex® (Botupharma, Botucatu, SP, Brasil) ou Equine Express® II (MP& J Associates, DeMoines, IA, USA) ou de fibra, como o Equitaner® (Equitainer I; Hamilton Research, Inc., Ipswich, MA, USA), Butu-Box® (Botupharma, Botucatu, SP, Brasil) ou Botutainer® (Botupharma, Botucatu, SP, Brasil).

Sistemas como BotuFlex®, Equitaner® e Botutainer® podem atingir temperaturas finais de 15 e/ou 5°C. Para que a temperatura de 15°C seja atingida, apenas um bloco de gelo reciclável deve ser colocado na caixa. Já para se obter temperatura final de 5°C são necessários dois blocos de gelo reciclável. Sendo assim, para cada temperatura final existe um período máximo de manutenção de 24 e 48 horas, respectivamente. Quando utilizados os sistemas Equine Express® II ou Botu-Box®, é possível apenas o armazenamento com temperatura final de 15°C.

4. Plasma seminal

O sêmen é composto por duas frações: os espermatozoides, que compõem menos de 1% do volume total, e o plasma seminal, sendo sua principal função, servir como meio de transporte e manutenção para os espermatozoides (WITE, 1988). O plasma seminal consiste em um fluido proveniente da *rete testis*, epidídimo e glândulas sexuais acessórias. Em equinos, este fluido é expelido em frações durante a ejaculação (KARESKOSKI; KATILA, 2008).

A primeira fração é a pré-espermática, com aspecto aquoso e proveniente das glândulas bulbouretrais e próstata, com função de limpeza da uretra. A segunda fração é rica em espermatozoides, leitosa e composta por secreções do epidídimo e das ampolas dos ductos deferentes. Já a terceira fração é pobre em espermatozoides, porém com grandes quantidades de ácido cítrico e gel proveniente das vesículas seminais, tendo a função de carrear os espermatozoides remanescentes na uretra (AMANN; GRAHAM, 2011; KARESKOSKI; KATILA, 2008).

Devido ao grande volume e complexa composição do plasma seminal no ejaculado de garanhões, inúmeras pesquisas direcionadas ao estudo da proteômica do plasma seminal tem sido desenvolvidas (GAMBOA e RAMALHO-SANTOS, 2005; GUASTI et al., 2014; KARESKOSKI; KATILA, 2008; TÖPFER-PETERSEN et al., 2005). Segundo Töpfer-Petersen et al. (2005), as proteínas especificas do plasma seminal desempenham funções no processo de fertilização, como estabelecimento do reservatório espermático na tuba uterina, modulação da resposta imune no útero, regulação da capacitação, transporte espermático no trato reprodutivo da fêmea e a interação e fusão entre os gametas.

Gamboa e Ramalho-Santos, (2005) e Töpfer-Petersen et al. (2005) identificaram e caracterizaram proteínas especificas do plasma seminal de equinos, e associaram as mesmas com a fertilidade. Guasti et al. (2014) mostraram que a quantidade de proteínas presentes no plasma seminal de garanhões férteis era maior que no grupo dos subférteis, hipotetizando que essas proteínas poderiam melhorar o potencial fertilizante dos espermatozoides destes garanhões, no entanto isso não se repetiu quando as proteínas ligadas a membrana plasmática foram estudas, não havendo diferença no conteúdo proteico entre os dois grupos.

Calvete et al. (1994) isolaram e caracterizaram oito proteínas de baixa peso molecular as quais compõem mais de 70% do conteúdo proteico total do plasma seminal,

denominadas proteínas do plasma seminal equino (HSP-1 a HSP-8). Outros autores avaliaram o perfil eletroforético de proteínas do plasma seminal equino e observaram que as HSPs possuem propriedades biológicas semelhantes às BSPs, que são proteínas do plasma seminal de outros mamíferos, podendo ser utilizadas como marcadores de fertilidade (BRANDON et al., 1999; NOVAK et al., 2010). Dado que, no momento da ejaculação as BSPs entram em contato com os espermatozoides, modulando a reação de capacitação espermática a qual é essencial para a fertilização (MANJUNATH; THÉRIEN, 2002). No entanto, segundo Bergeron et al., (2006) e Manjunath et al., (2007) durante o processo de armazenamento essas proteínas se tornam prejudiciais, pois modificam a composição da membrana plasmática, extraindo fosfolipídios e colesterol da mesma.

Contudo, o plasma seminal ainda gera questionamentos em relação ao seu papel no processo de fertilização. Estudos mostram que espermatozoides recuperados da cauda do epidídimo de garanhões tem capacidade de fertilizar o oócito, mesmo sem nenhum contato prévio com as secreções provenientes das glândulas anexas (GUASTI et al., 2012; MONTEIRO et al., 2011b; PAPA et al., 2008).

Rigby et al. (2001) mostraram que garanhões que apresentavam queda acentuada da motilidade espermática após processo de refrigeração a 5°C pelo período de 24-48 horas, apresentavam aumento na cinética espermática quando o plasma seminal era parcialmente removido. Nesta mesma linha, Love et al. (2005) sugerem que para garanhões que mantem os padrões de cinética espermática, mas apresentam baixa fertilidade, seja retirado o plasma seminal. Os autores constataram que quando removiam o plasma seminal e submetiam o sêmen à refrigeração por 24-48 horas, havia uma melhor integridade do DNA espermático, quando comparado às amostras onde o plasma seminal não era removido e submetido as mesmas condições. Dessa forma, sugere-se que as substâncias que compõem o plasma seminal são prejudiciais à integridade do DNA espermático.

Para a congelação do sêmen equino, a centrifugação previa do sêmen para concentração dos espermatozoides, remoção do plasma seminal e seus possíveis efeitos deletérios, é considerado procedimento padrão (KARESKOSKI; KATILA, 2008). Moore et al. (2005) congelaram sêmen com concentrações de plasma seminal maiores que 80%, não notando diferença nos parâmetros de cinética espermática. Já Alghamdi et al. (2002) observaram que a remoção total ou a adição de concentrações entre 10 e 30% resultou na queda nos parâmetros de cinética espermática, no entanto quando foi adicionado baixa

quantidade de plasma seminal (<5%), foi possível observar efeito benéfico, com melhora na qualidade do sêmen descongelado.

Entre todas essas divergências, Aurich et al. (1996) observaram que efeitos maléficos à célula espermática podem ser reduzidos, pois quando adicionavam plasma seminal de garanhões que apresentavam altos padrões de cinética espermática pós-descongelação ao sêmen de garanhões com baixa congelabilidade, foi possível aumentar a motilidade e a integridade de membrana plasmática. O oposto aconteceu quando o plasma seminal de garanhões com baixa congelabilidade foi adicionado ao sêmen de garanhões com alta congelabilidade. Este estudo mostra a variabilidade individual na composição do plasma seminal de garanhões, podendo determinar a habilidade dos mesmos frente ao processo de criopreservação.

Embora a influência do plasma seminal sobre a viabilidade espermática esteja evidente, estudos mostram que espermatozoides colhidos da cauda do epidídimo se mostram mais resistentes frente ao processo de criopreservação quando comparado aos espermatozoides ejaculados (JOHNSON; AMANN; PICKETT, 1980; MONTEIRO et al., 2011a). Isso pode estar relacionado com as alterações no conteúdo e composição dos lipídeos da membrana plasmática quando os espermatozoides entram em contato com o plasma seminal, dando maior fluidez a membrana plasmática, o que leva a uma redução da resistência ao choque térmico (JOHNSON; AMANN; PICKETT, 1980).

Segundo Guasti et al. (2012), as divergências nos resultados de estudos envolvendo plasma seminal, estão relacionadas a diferentes metodologias aplicadas. Sendo que fatores como, temperatura, diluição, método de centrifugação, tempo e ainda as variações individuais entre garanhões, são os principais causadores dos conflitos ente os resultados (KARESKOSKI; KATILA, 2008).

5. Meios diluidores

Os meios diluidores de sêmen têm como principais funções a redução das concentrações de plasma seminal, dar aporte nutricional aos espermatozoides, estabilização do pH e osmolaridade, além da ação antimicrobiana (PUGLIESI, 2009). No entanto, o controle do pH e da osmolaridade são imprescindíveis para que a cinética espermática seja mantida, quando um destes se encontra fora dos padrões normais (pH: 7,7 e osmolaridade: 315 mOsm), temos redução na motilidade (GRIGGERS et al., 2001).

Os diluentes de sêmen são responsáveis pela manutenção dos espermatozoides durante o processo de refrigeração e possuem diversos constituintes, sendo açucares, eletrólitos, tampões, antibióticos, gema de ovo, leite e produtos de leite, cada um com suas funções. Os açucares são utilizados pelos espermatozoides para produção de ATP. Os eletrólitos e tampões garantem o controle da osmolaridade e pH. Já os antibióticos previnem o crescimento bacteriano durante o armazenamento. A gema de ovo o leite e os produtos do leite servem para proteção da célula espermática contra o choque frio (KATILA, 1997).

A ação protetora dos diluentes a base de gema de ovo se dá pelas lipoproteínas de baixa densidade (LDL) presentes nessa substância (FOULKES, 1977; KAMPSCHMIDT et al. 1953). Segundo Quinn et al. (1980) a fração fosfolipídica presente no LDL forma uma película protetora na superfície da membrana. Uma segunda hipótese é que as LDL permaneçam ligadas à membrana do espermatozoide, neutralizando potenciais agentes danosos presentes no plasma seminal (AMANN; GRAHAM, 2011). Em estudo realizado por Manjunath et al. (2002) foi visto que as LDLs se ligam às BSPs, impedindo o efeito prejudicial desta proteína sobre a membrana plasmática dos espermatozoides durante a criopreservação.

Quanto a proteção dada aos espermatozoides pelos diluentes a base de leite, segundo Amann e Graham (2011), estes parecem ser similares aos diluentes contendo gema de ovo, onde as proteínas do leite atum na proteção da membrana e também na estabilização de elementos proteicos na membrana do espermatozoide. Esta proteção é dada pelas micelas de caseína e proteínas presentes no leite desnatado, esses compostos se ligam diretamente as BSPs , neutralizando o efeito deletério das mesmas sobre os espermazoides (BERGERON; MANJUNATH, 2006; MANJUNATH, 2012).

No entanto, por mais eficientes que sejam na proteção dos espermatozoides durante o processo de criopreservação, o leite desnatado e a gema de ovo, possuem grande variabilidade em suas composições. Buscando soluções para isso, Batellier et al. (1997) testaram a adição de frações do leite a diluentes de sêmen equino. Mais recentemente, Garcia (2016) testou a substituição do leite desnatado pelo caseinato de sódio no diluente de sêmen equino, demostrando ser uma alternativa viável, pois o mesmo observou a manutenção das características e viabilidade espermática durante a refrigeração por 24 horas. O mesmo autor, pode ainda observar um aumento na taxa de prenhez, quando utilizado diluente composto por caseína em garanhões sensíveis ao processo de refrigeração. Nesta mesma linha Coutinho da Silva et al. (2012) observaram que quando

usaram caseinato de sódio na diluição do sêmen equino, houve um aumento no número de espermatozoides ligados à zona pelúcida, processo esse fundamental para a fertilização. Já Martins et al. (2016) observaram divergências em relação aos resultados anteriores, mostrando que apenas a adição de proteínas do leite, como as caseínas, ao diluente de sêmen equino, não melhorou a cinética espermática quando comparado com o diluente a base de leite.

Outra substância derivada do leite é o fosfocaseinato nativo, fração proteica, a qual já se mostrou eficaz na manutenção da viabilidade espermática e com capacidade de proteger a membrana plasmática (BATELLIER et al., 1997). No entanto, não se sabe como o fosfocaseinato age na proteção dos espermatozoides. Batellier et al. (2000) puderam observar que o fosfocaseinato nativo tem um efeito direto na proteção do espermatozoide, mas sem qualquer ligação com a membrana da célula espermática. Segundo Batellier et al. (2001), uma alternativa para melhorar a qualidade do sêmen refrigerado e aumentar a taxa de fertilidade é o armazenamento a 15°C, pois assim, poderiam ser diminuídas as lesões de membrana causadas pelo choque frio.

6. Obesidade

A alimentação de equinos em condições naturais é rica em fibras e pouco calórica, assim, quando o animal é submetido a uma situação em que há a ingestão de um excesso de fibras não estruturais contendo carboidratos, inicia-se a conversão em gordura durante a lipogênese (SILLENCE et al., 2007). Sendo que o excesso de deposição de tecido adiposo no organismo torna o animal obeso (WORLD HEALTH ORGANIZATION, 1998).

Além da função de armazenar energia, o tecido adiposo vem sido conhecido como um órgão endócrino, capaz de secretar diferentes substâncias conhecidas como adipocitocinas (GERHARDT et al., 2001). Segundo Braga (2014), essas substâncias têm diferentes papéis, atuando de forma autócrina, parácrina ou endócrina no organismo dos animais. Dentre elas, os adipócitos secretam TNF (fator de necrose tumoral), interleucina-6, leptina, adiponectina e resistina (BRAGA, 2014).

Em consequência à obesidade apresentada pelos equinos acometidos por síndrome metabólica, alterações endócrinas encontram-se associadas, dentre elas, baixos níveis de tiroxina, e elevados níveis de leptina e insulina são comumente observados nesses animais (VICK et al.; 2006). A resistência à insulina é caracterizada por uma

alteração na resposta do organismo à insulina, ou seja, quando os níveis hormonais estão normais ou até mesmo aumentados, e a resposta desenvolvida encontra-se diminuída (WILCOX, 2005).

A obesidade em animais causa inflamação crônica do organismo, a qual está relacionada ao desenvolvimento da resistência à insulina, isso ocorre pela secreção de TNF e citocina pró-inflamatória produzida pelo órgão adiposo que estimula a gliconeogênese hepática por atuar inversamente ao metabolismo de glicose (GUIMARÃES et al., 2007; RAYMOND, 2008). O TNF estimula o estabelecimento de hiperinsulinemia em decorrência da resistência à insulina gerada no organismo do animal (VOLP et al., 2008).

A interleucina-6 tem ação em respostas inflamatórias agudas (FANTUZZI, 2005). Além disso, é capaz de aumentar a circulação de ácidos graxos pela inibição dos receptores de insulina nos hepatócitos, ocasionando resistência à insulina tecidual (GUIMARÃES et al., 2007). O aumento da concentração de IL-6 encontra-se de forma proporcional ao aumento de peso corporal e inverso à sensibilidade à insulina (LACERDA; MALHEIROS; WILKEN-ABREU, 2016). Já a resistina é um hormônio altamente relacionado à resistência insulínica, encontrando-se em concentrações elevadas em animais diabéticos e obesos (LACERDA; MALHEIROS; WILKEN-ABREU, 2016).

6.1 Deposição de gordura

Em bovinos reprodutores jovens, dietas altamente energéticas geram um aumento de deposição de gordura escrotal, capaz de interferir na termorregulação, aumentando a temperatura dos testículos e do escroto, consequentemente a isso um maior número de alterações morfológicas e queda nos parâmetros de cinética espermática foram observadas nesses animais, quando comparados com animais que recebiam uma dieta menos energética (COULTER; COOL; KASTELIC, 1997).

Já em cordeiros, foi avaliada a capacidade da dieta materna em influenciar na histologia testicular, e a submissão às dietas altamente calóricas foi capaz de influenciar na estrutura testicular da prole, sendo observando um aumento no tamanho dos túbulos seminíferos (MOSSA et al., 2018). Em homens, a mensuração da gordura testicular é mais específica que o volume para diagnóstico de infertilidade (LI et al, 2019).

6.2 Síndrome Metabólica Equina

A síndrome metabólica (SM) é uma doença crônica, caracterizada pela obesidade e deposição de gordura localizada, causadora da resistência à insulina nos animais acometidos (KEEN, 2013). Desde 1985, Henneke e colaboradores relataram a influência da obesidade no manejo reprodutivo das éguas. Isso ocorre pela secreção de diferentes substâncias pelo tecido adiposo, capazes de influenciar na concentração de insulina no organismo desses animais (RADIN; SHRKEY; BETHANY, 2009).

Os sinais clínicos característicos de SME são comumente confundidos com disfunção da pars intermédia da glândula pituitária, entretanto, os cavalos acometidos por SM não apresentam hirsutismo (KEEN, 2013). As principais características atribuídas a um animal com SM são: sobrepeso e acúmulo de gordura de forma localizada na nuca, supraorbital, prepúcio ou glândula mamária e garupa, laminite recorrente ou crônica e infertilidade em éguas (KEEN, 2013). Além disso, animais acometidos por SM comumente apresentam hiperlipemia, hiperglicemia e hipertrigliceridemia, o que é sugerido como causa de subfertilidade em éguas e garanhões (DURHAM et al., 2019).

6.2.3 Resistência à insulina

A resistência à insulina nos equinos pode ocorrer por diferentes mecanismos, entretanto, está principalmente atribuída à obesidade, que gera lipotoxicidade, alterações na transdução dos sinais da insulina e inflamações sistêmicas resultantes da secreção de citocinas pró-inflamatórias pelo tecido adiposo (KACZMAREK; JANICKI; GLOWSKA, 2016).

Em um experimento realizado por Gómez-Elias e colaboradores (2019), ratos submetidos a uma situação semelhante à síndrome metabólica, tiveram seu aparelho reprodutor e sêmen analisados, apresentando uma diminuição do tamanho do epidídimo e o aumento de gordura gonadal, quanto à qualidade seminal, estes animais apresentaram uma contagem inferior de espermatozoides, ainda que, a viabilidade, morfologia, motilidade, reação acrossomal e fertilidade in vitro foram semelhantes às do grupo controle.

O desenvolvimento de uma inflamação crônica nos equinos portadores de SM é causado pela constante estimulação da produção de citocinas inflamatórias circulantes, as quais aumentam o estresse oxidativo no organismo do animal (KEEN, 2013). Em éguas,

o aumento das espécies reativas de oxigênio (ERO) devido a SM, foi descrito por Mota (2014) como prejudicial ao desenvolvimento de oócitos.

7. Alterações Reprodutivas

A infertilidade é um importante fator observado na indústria equina, e o estresse oxidativo parece interferir na fertilidade do garanhão. Segundo Ball (2009) a produção de espécies reativas de oxigênio pelos espermatozoides é capaz de interferir na sobrevivência e fertilidade espermática quando de forma exacerbada. A congelação ou resfriamento de sêmen parece aumentar as reações oxidativas, sugerindo sua relação no processo de fertilização (BALL, 2009).

As EROs, geram alterações epigenéticas na expressão gênica em decorrência de diferentes fatores, tais como a obesidade (GIBB et al., 2020). O sêmen de garanhões é altamente dependente da fosforilação oxidativa como fonte de ATP, e quando armazenado, é capaz de produzir altas concentrações de ERO, as quais acarretam em peroxidação lipídica, disfunção mitocondrial, danos ao DNA e redução da longevidade dos espermatozoides (GIBB; LAMBOURNE; AITKEN, 2014). O espermatozoide humano é altamente susceptível ao estresse oxidativo, isso ocorre por apresentar quarenta vezes menos catalase do que os garanhões (GIBB; LAMBOURNE; AITKEN, 2014).

Humanos com diabetes mellitus apresentam alterações na função testicular, as quais prejudicam o desenvolvimento espermático, dificultando o transporte de glicose por GLUTs, prejudicando o metabolismo de glicose, ocasionando estresse oxidativo, implicando em fragmentações de DNA mitocondrial e nuclear, e ainda, ocasionando apoptose (DIAS et al., 2014). Indivíduos com diabetes mellitus comumente apresentam baixa qualidade seminal, sendo que isso acontece principalmente pelas alterações hormonais que ocorrem no organismo, assim como na obesidade e na síndrome metabólica (DIAS et al., 2014).

Em um experimento realizado por Gibb, Lambourne e Aitken (2014), foi observado pela primeira vez relação positiva entre o estresse oxidativo e a fertilidade de garanhões, com isso, os autores ressaltam que o estresse oxidativo não é benéfico para o organismo, e que, apenas amostras de sêmen com baixas concentrações de ERO pode ser dita como altamente fértil.

Segundo Durham e colaboradores (2019), o IGF-1 parece estar relacionado ao desenvolvimento da laminite na SM em equinos. Os receptores de IGF-1 parecem sofrer

um estimulo ao estabelecimento de dietas ricas em carboidratos ou ainda em infusões de insulina (LANE et al., 2017). Em éguas, a concentração de insulina e de IGF-1 foi associada ao crescimento folicular e interação no desenvolvimento reprodutivo (BESEN et al., 2018). Segundo Roser (2008), a relação entre os níveis de IGF-1 e a fertilidade dos machos é variável, entretanto, os altos níveis de IGF-1 parecem estar associados às maiores taxas de prenhez, com isso, ainda que possa ocorrer um aumento de IFG-1 durante a SM, este não parece ser a causa de subfertilidade nos animais.

Segundo Agarwal e Said (2004), todas as células aeróbicas passam por um processo de exposição à ERO, entretanto, o excesso é que causa o estresse oxidativo, sendo o estresse oxidativo o principal responsável pela infertilidade em homens, estando associado ao processo de apoptose celular, constituindo assim um mecanismo molecular patogênico.

Em homens, a obesidade e o sobrepeso são determinantes no aumento da subfertilidade, sendo essa característica atribuída à diminuição de andrógenos, globulina de ligação de hormônios sexuais e aumento dos níveis de estrógeno (AGGERHOLM et al, 2008. Em garanhões, os receptores de IGF-1 foram encontrados nas membranas das células germinativas, células de Leydig e células de Sertolli, indicando que podem ser influenciados pela secreção de LH, FSH, sendo o LH o fator mais importante, além disso, o grau de obesidade é correlacionado à redução dos níveis séricos de inibina B, ainda que o aumento de FSH e LH compensatório não seja observado, mesmo assim é sugerido que uma desregulação do eixo hipotalâmico-pituitário-gonadal explica a infertilidade e alteração na qualidade seminal observada em humanos com este fenótipo (PLESSIS et al., 2010).

O mecanismo pelo qual a obesidade relaciona-se à infertilidade em humanos, foi descrito por envolver o desenvolvimento de hipotesteronemia, capaz de impactar na espermatogênese e função espermática dos indivíduos, além disso, o excesso de tecido adiposo na espécie está relacionado ao aumento de estrógenos e consequentemente alterações como diminuição da concentração espermática, além do aumento de lesões de DNA (KATIB, 2015). Entretanto, em equinos, ainda é desconhecido o impacto que a síndrome metabólica possa causar no estado reprodutivo de garanhões.

8. Referências

AGARWAL, A.; SAID, T. M. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. **Bju International**, p. 503-507, 2005.

AGGERHOLM, A. S.; THULSTRUP, A. M.; TOFT, G.; RAMLAU-HANSEN, C. H.; BONDE, J. P. Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? **Fertility and Sterility**, v. 90, p. 619-626, 2008.

ALBERTS, B.; BRAY, D.; LEWIS, J. The plasma membrane. In: **Molecular biology of the cell**. 2. ed. New York: Garland Publishing, 1989. p. 275–340.

ALGHAMDI, A. S. et al. Effect of seminal plasma concentration and various extenders on postthaw motility and glass wool-Sephadex filtration of cryopreserved stallion semen. **American Journal of Veterinary Research**, v. 63, n. 6, p. 880–885, 2002.

AMANN, R. P. Functional Anatomy of the Adult Male. In: MCKKINON, A.O.; VOSS, J. L. (Ed.). **Equine Reproduction**. 1. ed. Philadelphia: Lea &Fabiger. p. 645–657.

AMANN, R. P.; GRAHAM, J. K. Spermatozoal function. In: MCKINNON, A. O. . et al. (Eds.). **Equine Reproduction**. 2. ed. Wiley-Blackwell, 2011. p. 1053–1084.

AMANN, R. P.; PICKETT, B. W. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. **Journal of Equine Veterinary Science**, 1987.

AURICH, C. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. **Animal Reproduction Science**, v. 89, n. 1- 4 SPEC. ISS., p. 65–75, 2005.

AURICH, C. Recent advances in cooled-semen technology. **Animal Reproduction Science**, v. 107, n. 3–4, p. 268–275, set. 2008.

AURICH, J.; AURICH, C. Developments in European horse breeding and consequences for veterinarians in equine reproduction. **Reproduction in Domestic Animals**, ago. 2006.

AURICH, J. E. et al. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. **Theriogenology**, v. 46, n. 5, p. 791–797, 1 out. 1996.

BALL, B. The effect of oxidative stress on equine sperm function. **Semen Storage & Stallion Fertility**, 2009.

BATELLIER, F. et al. Effect of milk fractions on survival of equine spermatozoa. **Theriogenology**, v. 48, n. 3, p. 391–410, ago. 1997.

BATELLIER, F. et al. Delayed insemination is successful with a new extender for storing fresh equine semen at 15°C under aerobic conditions. **Theriogenology**, v. 50, n. 2, p. 229–236, 15 jul. 1998.

BATELLIER, F. et al. Preservation of stallion sperm quality by native phosphocaseinate: a direct or indirect effect?. **Journal of reproduction and fertility. Supplement**, n. 56, p. 69–77, 2000.

BATELLIER, F. et al. Advances in cooled semen technology. **Animal reproduction science**. v. 68, n. 3–4, p. 181–90, 3 dez. 2001.

BERGERON, A.; MANJUNATH, P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. **Molecular Reproduction and Development**, v. 73, n. 10, p. 1338–1344, out. 2006.

BESEN, L. S.; PIMENTEL, A. M.; ESMERALDINO, A. T.; MEIKLE, A.; COSTA-MATTOS, R. Free IGF1 and insulin concentrations in the folicular fluid and follicle IGF1 receptor expression differs according to follicle size in the mare. **Journal of Equine Veterinary Science**, v. 66, p. 126-127, 2018.

BRAGA, C. S. **Hormônios do tecido adiposo.** Seminário apresentado na disciplina Bioquímica do Tecido Adiposo, Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul, 2014.

BRANDON, C. I. et al. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. **Theriogenology**, v. 52, n. 5, p. 863–873, 1 out. 1999.

BRINSKO, S. P.; CROCKETT, E. C.; SQUIRES, E. L. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. **Theriogenology**, v. 54, n. 1, p. 129–136, 1 jul. 2000.

BRINSKO, S. P.; VARNER, D. D. Artificial Insemination. In: MCKINNON, A. O.; VOSS, J. L. (Eds.). **Equine Reproduction**. Philadelphia: Lea & Febiger, 1992a. p. 790–792.

BRINSKO, S. P.; VARNER, D. D. Artificial insemination and preservation of semen. **The Veterinary clinics of North America. Equine practice**, v. 8, n. 1, p. 205–218, 1992b.

BURGOS, M. H.; FAWCETT, D. W. Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (Felis domestica). **The Journal of biophysical and biochemical cytology**, v. 1, n. 4, p. 287–300, 25 jul. 1955.

CALVETE, J. J. et al. Isolation and Biochemical Characterization of Stallion Seminal-plasma Proteins. **Reproduction in Domestic Animals**, v. 29, n. 5, p. 411–426, 1994.

CANISSO, I. F. et al. INSEMINAÇÃO ARTIFICIAL EM EQUINOS: sêmen fresco, diluído, resfriado e transportado. **Revista Acadêmica: Ciência Animal**, v. 6, n. 3, p. 389–398, 2008.

COULTER, G. H.; COOK, R. B.; KASTELIC, J. P. Effects of dietary energy on scrotal surface temperature, seminal quality, and sperm production in young beef bulls. **Journal of Animal Science**, v. 75, p. 1048-1052, 1997.

COUTINHO DA SILVA, M. A. et al. Effects of components of semen extenders on the binding of stallion spermatozoa to bovine or equine zonae pellucidae. **Reproduction (Cambridge, England)**, v. 143, n. 5, p. 577–85, maio 2012.

CROSS, N. L. Role of cholesterol in sperm capacitation1. **Biology of Reproduction**, v.

59, n. 1, p. 7–11, 1 jul. 1998.

DARIN-BENNETT, A.; WHITE, I. G. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. **Cryobiology**, v. 14, n. 4, p. 466–70, ago. 1977.

DELL'AQUA JUNIOR, J. A. et al. Association of sodium caseinate and cholesterol improved fertility of chilled stallion semen. **Journal of Equine Veterinary Science**, v. 66, p. 51, jul. 2018.

DIAS, T. R.; ALVES, M. G.; SILVA, B. M.; OLIVEIRA, P. F. Sperm glucose transport and metabolism in diabetic individuals. **Molecular and Celluar Endocrinology**, v. 396, p. 37-45, 2014.

DOUGLAS-HAMILTON, D. H. et al. A field study of the fertility of transported equine semen. **Theriogenology**, v. 22, n. 3, p. 291–304, set. 1984.

DURHAM, A. E.; FRANK, N.; MCGOWAN, C. M.; MENZIES-GOW, N. J.; ROELFSEMA, E.; VERVUERT, I.; FEIGE, K.; FEY, K. ECEIM consensus statement on equine metabolic syndrome. **Journal of Veterinary Internal Medicine**, p. 335-349, 2019.

ECOT, P. et al. Evaluation of a cushioned centrifugation technique for processing equine semen for freezing. **Animal reproduction science**, v. 89, n. 1–4, p. 245–8, out. 2005.

FANTUZZI, G. Molecular mechanisms in allergy and clinical immunology: Adipose tissue, adipokines, and inflammation. **American Acadey of Allergy, Asthma and Immunology** p. 911-919, Chicago, 2005.

FERREIRA DE CARVALHO, O. et al. Efeito oxidativo do óxido nítrico e infertilidade no macho. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 38, n. 1, 2002.

FOULKES, J. A. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. **Journal of Reproduction and Fertility**, v. 49, n. 2, p. 277–284, 1977.

GAMBOA, S.; RAMALHO-SANTOS, J. SNARE proteins and caveolin-1 in stallion spermatozoa: Possible implications for fertility. **Theriogenology**, v. 64, n. 2, p. 275–291, 15 jul. 2005.

GARCIA, V. F. C. **Adição de caseinato de sódio ao diluente de refrigeração de sêmen equino**. Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu/SP., 2016.

GERHARDT, C. C.; ROMERO, I. A.; CANCELLO, R.; et al. Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. **Mol. Cell. Endocrinol.**, v. 175, p. 81–92, 2001.

GIBB, Z.; LAMBOURNE, S. R.; AITKEN, R. J. The paradoxical relationship between stallion fertility and oxidative stress. **Biology of Reproduction**, v. 91, p. 1-10, 2014.

GIBB Z.; GRIFFIN R. A.; AITKEN R. J.; IULIIS G. N. D. Functions and effects of reactive oxygen species in male fertility. **Animal Reproduction Science**, 2020.

GÓMEZ-ELIAS, M. D. RAINERO CÁCERES, T. S.; GIACCAGLI, M.M.; GUAZZONE, V. A.; DALTON, G. N.; SIERVI, A.; CUASNICÚ, P. S.; COHEN, D. J.; ROS, V. G. Association between high-fat diet feeding and male fertility in high reproductive performance mice. **Scientific Reports**, Natureresearch, 2019.

GRIGGERS, S. et al. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. **Theriogenology**, v. 56, n. 4, p. 613–622, 1 set. 2001.

GUASTI, P. N. et al. Protein content of equine seminal plasma and sperm plasma membrane in subfertile stallions. **Journal of Equine Veterinary Science**, v. 34, n. 1, p. 84–85, jan. 2014.

GUASTI, P. N.; MONTEIRO, G. A.; PAPA, F. O. Componentes do plasma seminal e sua influência sobre a criopreservação e fertilidade de espermatozóides equinos. **Veterinária e Zootecnia**, p. 169–180, 2012.

GUIMARÃES, D. E. D.; SARDINHA, F. L. C., MIZURINI, D. M., CARMO, M. G. T. Adipocitocinas: uma nova visão do tecido adiposo. **Revista de Nutrição**, v. 20, n. 5, Campinas, 2007.

HARTWIG, F. P. et al. Use of cholesterol-loaded cyclodextrin : An alternative for bad cooler stallions. **Theriogenology**, v. 81, n. 2, p. 340–346, 2014.

HENNEKE, D. R., POTTER, G. D., KREIDER, J.L., YEATES, B. F. Relationship between condition score, physical measurements and body fat percentage in mares. **Equine Veterinary Journal**, p. 371-372, 1983.

JOHNSON, L.; AMANN, R. P.; PICKETT, B. W. Maturation of equine epididymal spermatozoa. **American Journal of Veterinary Research**, v. 41, n. 8, p. 1190–1196, ago. 1980.

KACZMAREK, K.; JANICKI, B.; GLOWSKA, M. Insulin resistance in the horse: a review. **Journal of Applied Animal Research**, v. 44, p. 424-430, 2016.

KAMPSCHMIDT, R. F.; MAYER, D. T.; HERMAN, H. A. Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. **Journal of Dairy Science**, v. 36, n. 7, p. 733–742, 1953.

KARESKOSKI, M.; KATILA, T. Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity**. Animal Reproduction Science**, set. 2008.

KATILA, T. Procedures for handling fresh stallion semen. **Theriogenology**, v. 48, n. 7, p. 1217–1227, nov. 1997.

KATIB, A. Mechanisms linking obesity to male infertility. **Central European Journal of Urology**, v. 68, p. 79-85, 2015.

KAYSER, J. P. et al. Effects of linear cooling rate on motion characteristics of stallion

spermatozoa. **Theriogenology**, v. 38, n. 4, p. 601–614, 1992.

KEEN, J. Equine metabolic syndrome/insulin resistance syndrome in horses In: WILEY; J. **Clinical Endocrinology of Companion Animals**. 1st ed. rev. Jonh Wiley & Sons, 2013.

KENNEY, R. et al. **Minimal contamination techniques for breeding mares: techniques and preliminary findings**, 1975.

LACERDA, M. S.; MALHEIROS, G. C.; WILKEN-ABREU, A. O. Tecido adiposo, uma nova visão: as adipocinas e seu papel endócrino. **Revista Científica da FMC**, v. 11, p. 25-31, 2016.

LANE, H. E.; BURNS, T. A.; HEGEDUS, O. C.; WATTS, M. R.; WEBER, P. S.; WOLTMAN, K. A.; GEOR, R. J.; MCCUCHEON, L. J.; EADES, S. C.; MATHES, L. E.; BELKNAP, J. K. Lamelar events related to insulin-growth factor-1 receptor signalling in two models relevant to endocrinopathic laminitis. **Equine Veterinary Journal**, v. 5, p. 643-654, 2017.

LEFRAPPER, L.; WALSTON, L.; WHISNANT, C. S. Comparison of various extenders for storage of cooled stallion spermatozoa for 72 hours. **Journal of Equine Veterinary Science**, v. 30, n. 4, p. 200–204, abr. 2010.

LI, Q.; YANG, F.; ZHOU, W.; CHENG, N.; TANG, W.; GUO, Y.; GUO, R. Quantification of testicular fat deposition in the evaluation of middle-aged overweight male infertility. **Magnetic Resonance Materials in Physics, Biology and Medicine**, v.33, p. 377-384, 2019.

LOVE, C. C. et al. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. **Theriogenology**, v. 63, n. 6, p. 1584–1591, 1 abr. 2005.

MANJUNATH, P. et al. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. **Biology of Reproduction**, v. 67, n. 4, p. 1250–1258, 1 out. 2002.

MANJUNATH, P. et al. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. **Society of Reproduction and Fertility supplement**, 2007.

MANJUNATH, P. New insights into the understanding of the mechanism of sperm protection by extender components. **Anim. Reprod.** v. 9, n. 4, p. 809-815, 2012.

MANJUNATH, P.; THÉRIEN, I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. **Journal of Reproductive Immunology**, v. 53, n. 1–2, p. 109–119, 2002.

MARTINS, H. S. et al. Milk, caseinate and lactoferrin addition to equine semen cooling extenders. **Andrologia**, v. 48, n. 9, p. 950–956, nov. 2016.

MONTEIRO, G. A. et al. Fertilidade de espermatozóides recuperados da cauda do epidídimo de garanhões subférteis. **Veterinária e Zootecnia**, v. 18, n. 2, p. 255–263,

2011a.

MONTEIRO, G. A. et al. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. **Animal Reproduction Science**, v. 127, n. 3–4, p. 197–201, set. 2011b.

MOORE, A. I.; SQUIRES, E. L.; GRAHAM, J. K. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. **Cryobiology**, v. 51, n. 3, p. 241–249, 2005a.

MOORE, A. I.; SQUIRES, E. L.; GRAHAM, J. K. Effect of seminal plasma on the cryopreservation of equine spermatozoa. **Theriogenology**, v. 63, n. 9, p. 2372–2381, 1 jun. 2005b.

MORAN, D. M. et al. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. **Theriogenology**, v. 38, n. 6, p. 999–1012, 1992.

MOSSA, F.; BEBBERE, D.; LEDDA, A.; BURRAI, G. P.; CHEBLI, I.; ANTUOFERMO, E.; LEDDA, S.; CANNAS, A.; FANCELLO, F.; ATZORI, A. S. Testicular development in male lambs prenattaly exposed to a hight-starch diet. **Molecular Reproduction Deveopment**, p. 1-11, 2018.

MOTA, L. H. C. M.; JÚNIOR MORAES, F. J.; CARVALHO, Y. N. T.; CASTELO BRANCO, M. A. SOUZA, J. A. T. Melatonina e desenvolvimento embrionário. **Revista Brasileira de Reprodução Animal**, v. 38, n. 4, p. 220-224, 2014.

NOVAK, S. et al. Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. **Theriogenology**, v. 74, n. 6, p. 956–967, out. 2010.

PAGL, R. et al. Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5 °C. **Theriogenology**, v. 66, n. 5, p. 1115–1122, 15 set. 2006.

PAPA, F. O. et al. Freezing of stallion epididymal sperm. **Animal Reproduction Science**, v. 107, n. 3–4, p. 293–301, set. 2008.

PEÑA, F. J.; BALL, B. A.; SQUIRES, E. L. A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples. **Cytometry Part B - Clinical Cytometry**, v. 94, n. 2, p. 302–311, 1 mar. 2018.

PESCH, S.; BERGMANN, M. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. **Micron**, out. 2006.

PLANTE, G. et al. Interaction of milk proteins and Binder of Sperm (BSP) proteins from boar, stallion and ram semen. **Reproductive biology and endocrinology : RB&E**, v. 13, p. 92, 15 ago. 2015.

PLESSIS, S. S. D.; CABLER, S.; McALISTER, D. A.; SEBANEGH, E.; AGARWAL, A. The effect of obesity on sperm disorders and male infertility. **Nature Reviews**, v. 7, p. 153-161, 2010.

POMMER, A. C.; LINFOR, J. J.; MEYERS, S. A. Capacitation and acrosomal exocytosis are enhanced by incubation of stallion spermatozoa in a commercial semen extender.

Theriogenology, v. 57, n. 5, p. 1493–1501, 2002.

PUGLIESI, G. **Viabilidade e fertilidade do sêmen equino resfriado a 5 °c por 24 horas com dois diluidores**. Universidade Federal de Viçosa, 2009.

QUINN, P. J.; CHOW, P. Y. W.; WHITE, I. G. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. **Journal of Reproduction and Fertility**, v. 60, n. 2, p. 403–407, 1980.

RADIN, M. J.; SHARKEY, L. C.; HOLYCROSS, B. J. Adipokines: a review of biological and analytical principles and na update in dogs, cats, and horses. **Veterinary Clinical Phatology**, v. 2, n. 28, p. 136-156, 2009.

RAPHAEL, C. F. **Efeitos da centrifugação nas características de movimento, integridade e peroxidação lipídica das membranas do espermatozóide eqüino refrigerado**. Universidade de São Paulo, 2007.

RAYMOND, J.G. Metabolic predispositions to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. **Journal of Equine Veterinary Science,** v. 12, p. 753–759, 2008.

RIGBY, S. L. et al. Advances in cooled semen technologies: seminal plasma and semen extender. **Animal reproduction science**, v. 68, n. 3–4, p. 171–80, 3 dez. 2001.

ROSER, J. F. Regulation of testicular function in the stallion: An intricate network of endocrine, paracrine and autocrine systems. **Animal Reproduction Science**, v. 107, p. 179-196, 2008.

SETCHELL, B. P. Male reproduction. In: KLNG, G. J., NEIMANN-SORE SEN, A., TRLBE, O. E. (Ed.). **World animal science - Reproduction in domesticated animals.** New York: Elsevier, 1993. p. 83–127.

SIEME, H.; KNOP, K.; RATH, D. Effects of cushioned centrifugation on sperm quality in stallion semen stored cooled at 5°C for 24h, and stored cooled for 2h or 24h and then frozen. **Animal Reproduction Science**, v. 94, n. 1–4, p. 99–103, ago. 2006.

SILLENCE, M.; ASPLIN, K.; POLLITT, C.; MCGOWAN, C. What causes equine laminitis? The role of impaired glucose uptake. Kingston: Rural Industries **Research and Development Corporation**, 2007.

SILVA FILHO, J.M., PALHARES, M.S.; FONSECA, F. A. Transporte e inseminação artificial com sêmen de equino. **Caderno Técnico da Escola de Veterinária da UFMG**, v. 11, p. 3–112, 1994.

SQUIRES EL et al. Cooled and frozen stallion semen. **Animal Reproduction and Biotechnology Laboratory**, p. 1–38, 1999.

TISCHNER, M. Artificial insemination in horses: a review of current status quo. **Equine Veterinary Education**, v. 4, n. 2, p. 89–92, 1992.

TÖPFER-PETERSEN, E. et al. The role of stallion seminal proteins in fertilisation. **Animal Reproduction Science**, v. 89, n. 1- 4 SPEC. ISS., p. 159–170, 2005.

TORRES, P.; SERRES, C. Effect of cholesterol-loaded cyclodextrin on motility and plasma membrane integrity of cooled stallion sperm. **Animal Reproduction Science.** v. 94, p. 148–151, 2006.

VALLE, G. R. et al. Utilização de um contêiner modelo Celle modificado para resfriamento e transporte de sêmen eqüino. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, v. 51, n. 5, p. 505–514, out. 1999.

VARNER, D. D. et al. Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. **Theriogenology**, v. 29, n. 5, p. 1043–1054, 1988.

VARNER, D. D. et al. Fertilizing capacity of equine spermatozoa stored for 24 hours at 5 or 20°C. **Theriogenology**, v. 32, n. 4, p. 515–525, 1989.

VARNER, D. D. et al. Techniques for evaluating selected reproductive disorders of stallions. **Animal Reproduction Science. Anais**...2 jul. 2000

VARNER, D. D.; GIBB, Z.; AITKEN, R. J. Stallion fertility: A focus on the spermatozoon. **Equine Veterinary Journal**, v. 47, n. 1, p. 16–24, 1 jan. 2015.

VICK, M. M.; SESSIONS, D. R.; MURPHY, B. A.; KENNEDY, E. L.; REEDY, S. E.; FITZGERALD, K. Y. Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. **Reproduction, Fertility and Development**, n. 18, p. 609-617, 2006.

VIDAMENT, M. et al. Equine frozen semen: Freezability and fertility field results. **Theriogenology**, v. 48, n. 6, p. 907–917, 15 out. 1997.

VOLP, A. C. P.; ALFENAS, R. C. G.; COSTA, N. M. B.; MINIM, V. P. R.; STRINGUETA, P. C.; BRESSAN, J. Capacidade dos biomarcadores inflamatórios em predizer a síndrome metabólica. **Arq. Bras. Endocrinal Metab.** p. 537-549, 2008.

WAITE, J. A. et al. Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. **Theriogenology**, v. 70, n. 4, p. 704–714, 1 set. 2008.

WILCOX, G. Insulin and Insulin Resistance. **The Clinical Biochemist Reviews**, v. 2, n. 26, p. 19-39, 2005.

WITE, R. G. Secreções do trato reprodutivo masculino e plasma seminal. In: **HAFEZ, E. S. E. Reprodução animal**. São Paulo: Monole, 1988. p. 212–228.

WORLD HEALTH ORGANIZATION. Obesity, preventing and managing the global epidemic. **Programme of Nutrition Family and Reproductive Health**, Geneva, 3-5 june 1998.

YAMAGATA, K. et al. Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. **Journal of Biological Chemistry**, v. 273, n. 17, p. 10470–10474, 24 abr. 1998.

YEAGLE, P. L. Cholesterol and the cell membrane. **BBA - Reviews on Biomembranes**, 9 dez. 1985.

ZAHN, F. S.; PAPA, F. O.; DELL'AQUA, J. A. Cholesterol incorporation on equine sperm membrane: effects on post-thaw sperm parameters and fertility. **Theriogenology**, v. 58, n. 2–4, p. 237–240, ago. 2002.

ZÚCCARI, C. E. S. N. **Efeito da criopreservação sobre a integridade estrutural da célula espermática eqüina.** Faculdade de Medicina Veterinária e Zootecnia – Universidade Estadual Paulista "Júlio de Mesquita Filho"., 1998.

HIPÓTESE

O uso de um diluente de sêmen equino a base de caseinato de sódio acrescido de colesterol, pode apresentar resultados superiores em relação a cinética espermática e viabilidade, após a refrigeração a 5 e 10°C por até 48 horas, quando comparado a um diluente já difundido no mercado que possui em sua composição fosfocaseinato nativo.

O alto escore de condição corporal, sem consequências secundárias, como a síndrome metabólica equina não afeta a qualidade seminal de garanhões da raça Crioula.

OBJETIVOS

- Comparar dois diferentes diluentes, um a base de caseína acrescido de colesterol e um segundo composto por fosfocaseinato nativo, em sua capacidade de manutenção da cinética e viabilidade espermática durante o processo de refrigeração a 5 e 10°C por até 48 horas.

- Avaliar se há efeito do plasma seminal sobre a cinética e viabilidade dos espermatozoides diluídos com dois diferentes meios um a base de caseína e um segundo a base de fosfocaseinato nativo.

- Determinar a fertilidade do sêmen diluído em dois diferentes meios um a base de caseína e um segundo com fosfoscaseinato nativo.

- Comparar a qualidade do sêmen congelado de garanhões com alto e baixo escore de condição corporal.

CAPÍTULO 2

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STALLION SEMEN COOLING USING NATIVE PHOSPHOCASEINATE BASED EXTENDER AND SODIUM CASEINATE CHOLESTEROL LOADED CYCLODEXTRIN BASED EXTENDER

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Conflicts of interest: none

Abstract

The objective of this study was to compare semen parameters and embryo recovery rates of cooled stallion semen extended with INRA 96 or BotuSemen Gold. In experiment 1, 45 ejaculates from nine mature stallions were collected, assessed, and equally split between both extenders and then extended to 50 million sperm/mL. Then, the extended semen was stored in three passive cooling containers (Equitainer, Equine Express II, and BotuFlex) for 48 hours. In experiment 2, the same ejaculates extended in experiment 1 were cushion-centrifuged, the supernatant was discarded, and the pellets were resuspended at 100 million sperm/mL with their respective extender. Semen was then cooled and stored as in experiment 1. In both experiments, sperm motility parameters, plasma membrane integrity, and high mitochondrial membrane potential were assessed at 0, 24, and 48 hours post cooling. For experiment 3, 12 mares (n = 24 cycles) were bred with 48 hours–cooled semen from one stallion. Semen was processed as described in experiment 1. Mares had embryo flushing performed by 8-day post-ovulation. In experiment 1, BotuSemen Gold displayed superior total and progressive motility relative to INRA 96 ($P < 0.05$). There were no significant differences between the types of containers in any experiment. In experiment 2, INRA 96 and BotuSemen Gold extenders had similar total and progressive motility, but BotuSemen Gold had superior sperm velocity parameters at all timepoints. Embryo recovery was identical for both extenders (50%). Finally, the results obtained herein suggest that BotuSemen Gold is a suitable alternative to be included in semen cooling tests against INRA 96 in clinical practice.

Keywords: semen extender; horses; passive semen cooling device

1.Introduction

Cooled-transported stallion semen is a widely used approach to breed mares in North America, Western Europe, Australia, New Zealand, and Brazil. It first became popular in the 1990s, when most American breed registries began accepting cooled-transported equine semen. In the 1970s, a skim milk-based extender was introduced by Dr. Robert Kenney to be infused in the uterus of mares immediately before mating [1]. After this, the extender was used for on-farm artificial insemination with fresh semen, for the cooling and transporting semen, and as a centrifugation extender before semen freezing. Kenney's extender has been commercialized with a variety of antibiotics (e.g. amikacin, penicillin, and gentamicin) added by the manufacturer or to be added to the extender immediately before use. Stallions would have semen collected and extended in Kenney's extender containing different combinations of antibiotics to determine the best antibiotic(s) to sustain sperm motility longevity during cooling for a given stallion [2]. Kenney's extender spread worldwide and was the main extender used to transport stallion cooled semen until the early 2000s.

In the 1990s, French investigators working at the Institut National de La Recherche Agronomique (INRA) identified that native phosphocaseinate was cryoprotective for stallion sperm during cooling [3], and this molecule was used to replace the skim milk component in INRA 82, a traditional French extender, to create the novel extender INRA 96 [3]. About a decade later, the extender became commercially available and then widely used around the world. Given the superior results extending stallion semen with INRA 96 over any Kenney combination, many practices and breeding centers discontinued cooling tests [4,5].

As other extenders (e.g., Ghent and Equi-Pro) were introduced, investigators performed cooling tests [5]; however, INRA 96 remained the superior and most widely used extender overall. One concern with INRA 96 is that the concentration of antibiotics present may be insufficient to prevent bacterial overgrowth during cooling [6]. Pseudomonas aeruginosa in particular, a highly contagious cause of chronic endometritis in mares, may not be inhibited; thus, it became an industry standard to include ticarcillin with clavulanic acid, an anti-Pseudomonas beta-lactam [7]. Additionally, while INRA 96 is a suitable extender for most stallions, there is still a portion of stallions that do not cool well with this extender. Therefore, an alternative extender to serve the needs of this stallion population is warranted.

Recently, a new semen cooling extender was introduced containing sodium caseinate in combination with cholesterol loaded-cyclodextrin (BotuSemen Gold). Caseins are milk proteins that can presumably prevent sperm cryodamage by competitively binding to seminal plasma proteins involved in this process [8,9]. Caseins also interact with ionized calcium, which plays a paramount role in tyrosine phosphorylation during sperm capacitation [10,11]. Cyclodextrin works as a carrier to incorporate cholesterol into the plasma membrane, thus presumably improving sperm survival during cooling [12,13]. Stallions with poor semen cooling are thought to have lower levels of cholesterol in the sperm plasma membrane [14,15], and cholesterol loaded cyclodextrin has been suggested as an alternative to prevent sperm damage during cooling in this type of stallion [12,17-19]. However, to date, a study comparing this novel extender with INRA 96 is lacking.

Equitainer, a passive cooling device, was developed in the mid-80s to transport equine semen [20]. It provides excellent temperature insulation and a suitable cooling curve for equine sperm, and it maintains the temperature for up to 48h [20,21]. Since the

Equitainer is expensive, cheaper alternative passive cooling semen devices (e.g., Equine Express II) were developed. While less expensive than the Equitainer, disposable boxes have questionable insulation from extreme temperature variations [21]. Recently, another passive cooling device was developed using high-density Styrofoam material (BotuFlex), which can presumably allow for a cooling curve similar to the Equitainer [22]. However, these three passive cooling devices have not been simultaneously compared.

The objective of this study was to compare the most widely used semen extender based on native phosphocaseinate (INRA 96) and a new extender based on sodium caseinate associated with cholesterol loaded cyclodextrin (BotuSemen Gold), and three semen cooling containers by assessing semen parameters and embryo recovery rates of cooled stallion semen. We hypothesized that the combination of cholesterol and sodium casein results in superior parameters for cooled stallion semen than native phosphocaseinate.

2. Materials and Methods

The experimental protocols were approved by the Animal Care and Use Committee, the Institutional Animal Care Unit Committee of the University of Illinois, under protocols # 19134 and #17140. Three experiments were carried out at the College of Veterinary Medicine of the University of Illinois Urbana-Champaign, IL, USA. Experiments 1 and 2 were conducted between August and September 2019, and Experiment 3 was conducted from November to December 2019. Seven out of nine stallions were client-owned, and all the owners signed a consent form permitting us to use their animals in this study.

2.1. Stallions, semen collection, and initial evaluation

Nine mature stallions (four Quarter Horses, two Standardbreds, one Paint Horse, one Morgan, and one Arabian), (ranging from 8 to 17 years), were enrolled in the study. Stallions were kept in stalls at the Illinois Veterinary Teaching Hospital in Urbana, IL, for the duration of the study. All animals were fed with mixed alfalfa-grass hay, with free access to water. Prior to the beginning of the study, three wash-out semen collections were performed. Thereafter, semen collections were performed at 48-72 h intervals on a dummy mount in the presence of an estrus mare. A Missouri model (Nasco, Fort Atkinson, WI, USA) artificial vagina coupled with a collection bag (Whirl-Pak, Nasco) and inline filter (Har-Vet, Spring Valley, WI, USA) was used. The artificial vagina was lubricated with a non-spermicidal gel (Clarity® A.I. Lubricating Jelly Aurora Pharmaceutical, LLC, Northfield, MN, USA).

Immediately after semen collection, the total gel-free volume of the ejaculate was weighed and loaded in 50 mL conical tubes (Corning®, Centristar™, Corning, NY, USA). Then, raw semen was immediately extended at 1:1 (v:v) ratio with temperature-matched BotuSemen Gold (Botupharma USA, Phoenix AZ, USA) or INRA 96 (IMV, Maple Grove, MN, USA). The sperm concentration was determined using an automated cell counter (Nucleocounter® SP-100™, Chemometec, Denmark) following the manufacturer instructions. Briefly, 50 μL of semen was diluted in 5 mL of lysis buffer (Reagent S100, Chemometec, Denmark) and loaded into the cassettes before the assessment. After the assessment of concentration, semen was further extended to 50 million sperm/mL with INRA 96 or BotuSemen Gold.

Experiment 1. Stallion semen cooling extended with INRA 96 or BotuSemen Gold

The extended semen was hermetically packed in a disposable plastic bag Whirl-Pak® and stored in three different containers, Equitainer (Equitainer II; Hamilton Research, Inc., Ipswich, MA, USA), Equine Express II (Exodus Breeder Supply, York, PA, USA) or BotuFlex (Botupharma USA). Two units of each container were prepared for evaluations at 24 and 48h post-cooling. Each passive cooling device was prepared according to manufacturer recommendations.

Equitainer's ice cans and ice packs for BotuFlex and Equine Express II were deep-frozen for at least 24h at -20ºC. The Equitainer (46 × 25 cm, height × width, 5.4 kg) consists of two metal frozen cans, whereas, the BotuFlex (30×24.5×24.5 cm, length × width × height, 1.4 kg) consists of two ice packs to be placed on each side of the device and a well in the center of the device where semen can be placed (Figure 1). Equine Express II (35.5 × 25 × 20 cm, length × width × height, 1.8 kg) consists of only one large ice pack (Figure 1). According to the manufacturer, the Equitainer is designed to have a cooling curve of -0.03 ºC/min and to maintain semen at 4-8°C for 48h. BotuFlex is designed to have a curve of 0.05ºC/min and to maintain semen at 5 ºC for ~48h when two ice packs are included. Whereas, Equine Express II is designed to have a cooling curve of - 0.035 ºC/min to maintain semen below 10ºC for 48h. Worth noting that Equitainer is supposed to have 120 to 180 mL of semen inside the isothermolizer to have the proper cooling curve, whereas BotuFlex 150 to 200 mL volume of semen is needed in the well for a proper cooling curve. The ideal volume for a cooling curve is not disclaimed by the manufacturer of Equine Express II, but it holds the capacity to have two syringes of 60 mL.

For this experiment, sperm motility parameters, intact plasma membrane, and percentage of sperm with intact plasma membrane and high mitochondrial potential were assessed at times 0, 24, and 48h after cooling-storage, as described below.

Figure 1. Passive cooling devices used to transport cooled stallion semen. (A) Equitainer (Hamilton Research, Inc., Ipswich, MA, USA), * denotes the two frozen cans and ± isothermolizer; (B) Equine Express II (Exodus Breeder Supply, York, PA, USA), *denotes the icepacks, and the two arrows denotes the pit where semen can be placed for cooled-shipped; (C) BotuFlex (Botupharma, Phoenix, AZ, USA). * indicate the ice packs, and the arrow is pointing to the semen well.

Sperm motility evaluations

Assessment of the sperm motility parameters was carried out using computer-assisted sperm analysis (CASA) using default settings recommended by the manufacturer (Spermvision, Minitube of America, Verona, WI, USA) for equine sperm. The preset values for the CASA were: static cell area 14-80 μm^2; straightness threshold for progressive motility 90%; average path velocity threshold for static cell <9.5 μm/s; cell intensity 106; light-emitting diode illumination intensity 1800-2550. Each sample was incubated for 10 min at 37°C before each evaluation. A small aliquot (10 μL) of extended semen was placed on a pre-heated slide with a coverslip for the assessments. Total percent of sperm motility (%), progressive sperm motility (%), sperm velocity parameters assessed included curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), and straight-line velocity (VSL, μm/s).

Membrane integrity and mitochondrial potential

The evaluation of the percentage of sperm with intact membrane and percentage of sperm with intact plasma membrane and high mitochondrial potential was conducted using a spectral flow cytometer as previously described [22]. Briefly, the staining solution of Zombie Green dye (#423112 Biolegend, San Diego, CA, USA) was freshly prepared with 100 μL of DMSO added to each vial of dye; similarly, MitoTracker Deep Red FM (M22426, Molecular Probes, Eugene, OR, USA) stock solution was prepared by adding DMSO to have a solution of 10 μM. The stock solution was aliquoted and frozen at -20°C until the use.

One milliliter containing 50 million sperm/mL was centrifuged (600*g* × 10 min) and then resuspended in PBS to a concentration of 3-5 million sperm/mL. An aliquot of 100 μL of this solution was stained with 1 μL of Zombie Green and 1 μL MitoTracker Deep Red. After mixing, the sample was incubated for 30 min at room temperature in the dark. The incubation was followed by a centrifugation 400*g* × 5 min. At this point, the samples were fixed with 500 μL of buffered formalin 2% until flow cytometry evaluation. The fixed samples were stored in the dark at room temperature until the assessment within 72 h from the fixation. Before the flow cytometric analyses, samples were washed with 1 mL of PBS, centrifuged at 400 *g* × 5 min, and resuspended in PBS (250 μL). The analyses of the stained samples were conducted using a full-spectrum detector based (filter-less) Cytek Aurora Flow Cytometer (Cytek Biosciences Inc., Fremont, CA, USA). The analysis was concluded when at least 10,000 fluorescent gated events were recorded. Zombie Green was excited and detected with a 488nm fluorescence detector, whereas MitoTracker Deep Red was excited with a 644/665 nm detector. Unstained and single stained controls were used to unmix the signals. As previously described [22] four subpopulations of sperm were identified. The populations of sperm with intact (low Zombie Green signal) or damaged (high Zombie Green signal) plasma membrane were subdivided into low or high mitochondrial membrane potential based on the intensity of the signal given by Mitotracker Deep Red staining. Debris was manually excluded based on the minimal emitted fluorescence. Data from the flow cytometer were exported and analyzed with FlowJo (FlowJow v. 10 Software; Ashland, OR, USA). The percentage of sperm with intact plasma membrane and the percentage of sperm with intact plasma membrane and high mitochondrial membrane potential were accounted for comparisons across groups.

Experiment 2. Cooling test of stallion semen, cushion-centrifuged and re-extended with INRA 96 and BotuSemen Gold

The same 45 ejaculates obtained in experiment 1, were further processed with cushion centrifugation after being initially extended to 50 million sperm/mL in INRA 96 or BotuSemen Gold. Cushion centrifugation was performed as previously described [23–26]. Briefly, extended semen in both extenders were loaded in 50 mL conical tubes (Corning®, Centristar™) and then added 1 mL of cushion fluid (Red-Cushion, Botupharma) placed at the bottom of the tube with a blunted spinal needle (18G × 13.5 cm). Centrifugation was carried at 1000*g* × 20 min at room temperature. Following centrifugation, the supernatant and cushion solution were discarded. The concentration of the remaining pellet was assessed with Nucleocounter and then resuspended in fresh extenders, INRA 96 or BotuSemen Gold, to a final concentration of 100 million sperm/mL. Thereafter, the extended semen was loaded in the three types of containers as described in experiment 1. Cooled semen was then assessed for sperm motility parameters, plasma membrane integrity, and sperm with intact plasma membrane and high mitochondrial potential were assessed at 0, 24, and 48h after cooling-storage, as described above for experiment 1.

According to BotuSemen Gold's manufacturer, once stallion semen is centrifuged, it should be resuspended to at least 100 million sperm/mL. If a lower dilution is used, the high cholesterol/seminal plasma ratio may prevent sperm from undergoing capacitation, despite satisfactory motility. There are no manufacturer recommendations regarding sperm concentration after centrifugation with INRA 96.

Experiment 3. Embryo recovery rates of 48h-cooled stallion semen extended in INRA 96 or BotuSemen Gold

A 13 years-old Quarter Horse stallion enrolled in experiments 1 and 2 had semen collected for a fertility test. This stallion was kept at the Illinois Veterinary Teaching Hospital, and fed with mixed alfalfa-grass hay, with free access to water. Semen collections and initial evaluations were performed as previously described. After collection, two semen samples were extended with BotuSemen Gold or INRA 96 at 50 million sperm/mL and then stored in an Equitainer for 48 h before insemination.

Twenty-four estrous cycles (BotuSemen Gold n=10 cycles and INRA 96 n=14 cycles) were used to inseminate fourteen light breed mares (ranging from 5 to 18 years old). The mares were kept on grass pasture and monitored by transrectal ultrasonography every other day until at least one pre-ovulatory follicle (35 mm of diameter in the presence of endometrial edema) was detected. Then mares were checked daily, and ovulation was then hastened with histrelin acetate (500 μg/IM, Strelin®, Botupharma USA). At 24 h post-induction of ovulation, all mares were artificially inseminated with 1 billion total sperm previously-stored for 48 h in Equitainer. Mares were examined 24 h after insemination to check for ovulation and the presence of free intrauterine fluid accumulation. Mares were treated if necessary, with oxytocin or uterine lavage. Uterine flushing for embryo recovery was performed with 4 L of sterile Lactated Ringer's solution 8 days post-ovulation. Immediately after uterine flushing, mares received an injection of 250 μg of cloprostenol sodium.

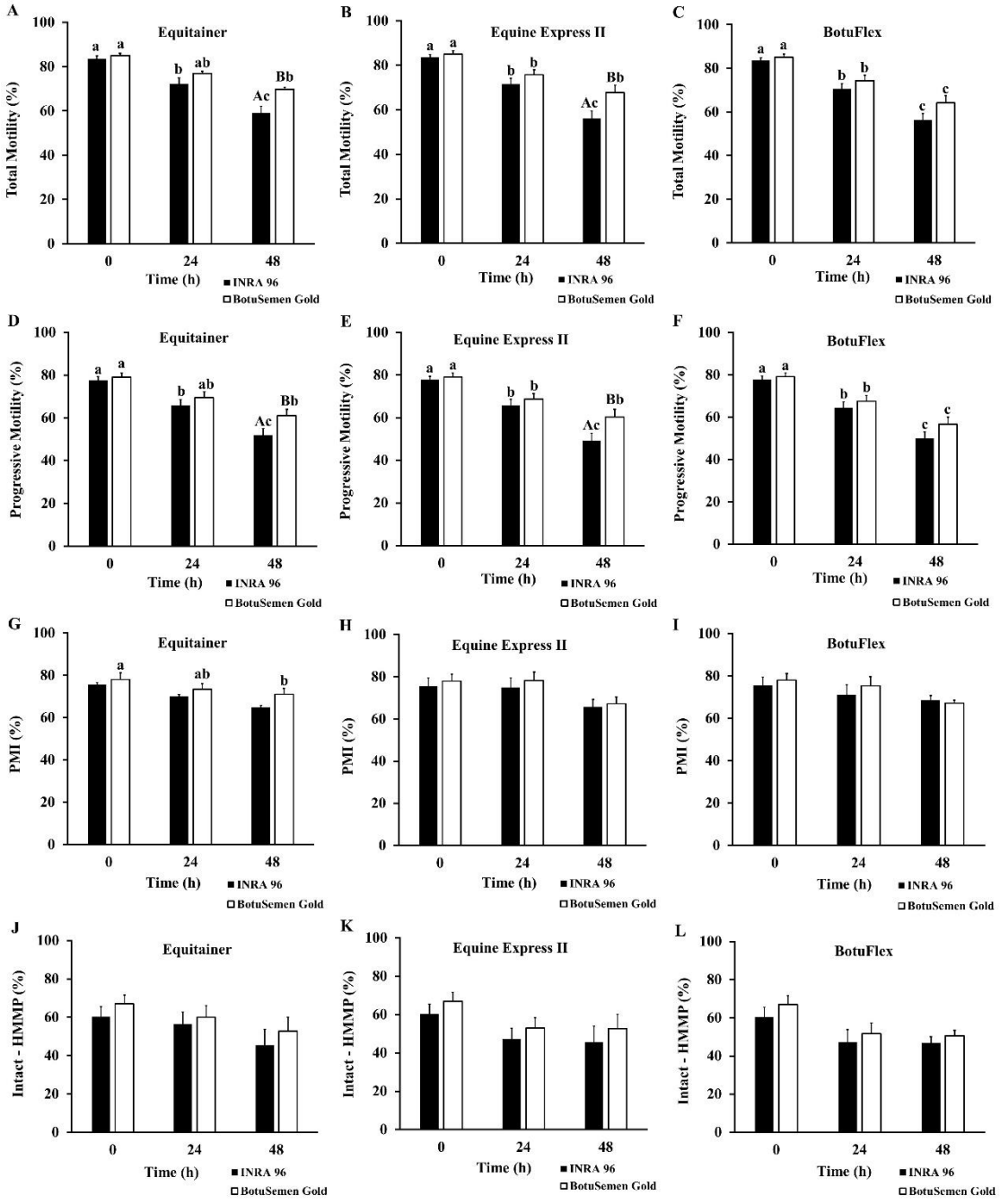
2.2. Statistical analyses

Data analyses were carried out with RStudio v 0.99.489 (RStudio Team, Boston, MA). Data were analyzed by mixed models, with extender, passive cooling devices, and time considered as fixed effects and stallion and ejaculate number as random effects. Tukey's test was used for post-hoc comparisons. Data are expressed as mean ± SEM. Statistical significance was set at $p<0.05$. The embryo recovery rates were identical between extenders, and results were descriptively presented below.

3. Results

Experiment 1

Total motility and progressive motility decreased over time for semen extended in BotuSemen Gold and INRA 96 ($p<0.05$) (Figures 2A-F). Overall there were no differences between the three passive cooling devices used for the transport of cooled stallion semen ($p>0.05$). Semen extended with BotuSemen Gold, stored in Equitainer or Equine Express II, had superior sperm motility when compared to INRA 96 at 48 h ($p<0.05$) (Figures 2AB, 2DE). There were no differences between INRA 96 and BotuSemen Gold when semen was stored in BotuFlex ($p>0.05$) (Figures 2C and 2F). There were no effects due to time, passive cooling device, or extender in the percentage of sperm with intact plasma membrane or sperm with intact plasma membrane with high mitochondrial membrane potential ($p>0.05$) (Figure 2G-L). There were no differences in curvilinear velocity, average path velocity, straight-line velocity across passive cooling devices, extenders, or time ($p>0.05$) (Table 1). Representative images of spectral flow cytometry for plasma membrane integrity and mitochondrial membrane potential are portrayed in Figure 3.

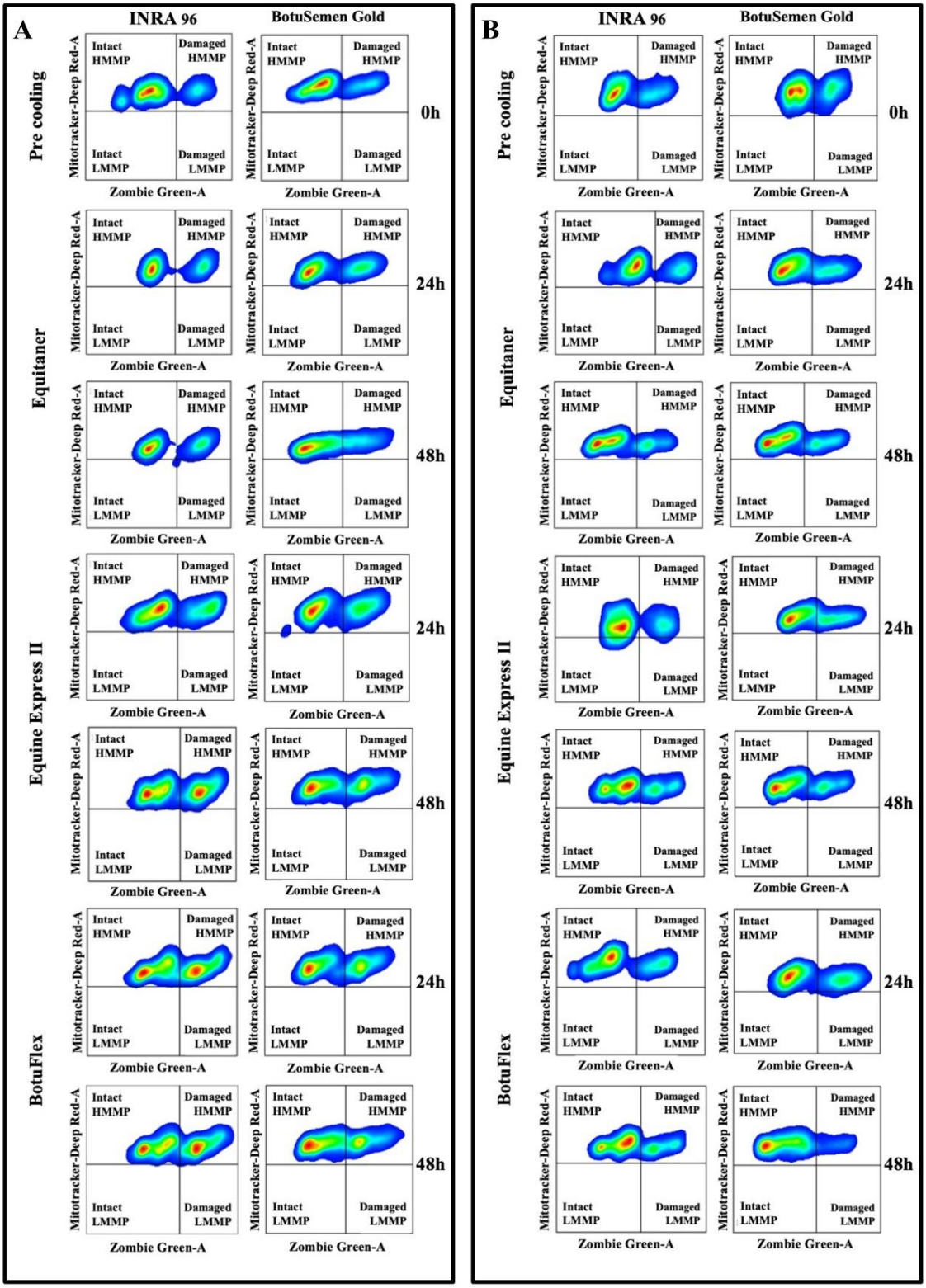


296 **Figure 2**. Parameters for stallion semen extended in INRA96 or BotuSemen Gold and
297 cooled for 48 h in three different passive cooling devices (Equitainer, Equine Express II,
298 and BotuFlex, rows 1-3). (A-C) Total sperm motility; (D-F) Percentage of progressive
299 motility; (G-I) Intact plasma membrane; and (J-L) Percentage of sperm with intact plasma
300 membrane with high mitochondrial membrane potential (Intact HMMP). Different
301 superscripts denote effects of time (^{abc}) and differences between extenders for within each
302 time point (^{AB}) ($p<0.05$).

303 **Table 1.** Motility parameters for stallion sperm extended in INRA 96 or BotuSemen Gold (BG) cooled in three passive cooling devices
304 (Equitainer, Equine Express II, and BotuFlex).

		VCL		VAP		VSL	
	Time (h)	INRA 96	BG	INRA 96	BG	INRA 96	BG
Equitainer	0	146.0 ± 2.7	153.0 ± 2.3	80.0 ± 1.9	88.0 ± 1.9	62.2 ± 1.6	68.2 ± 2.0
	24	141.0 ± 3.5	153.0 ± 3.0	77.0 ± 2.2	85.2 ± 1.8	61.0 ± 1.8	67.6 ± 1.6
	48	143.0 ± 4.3	150.0 ± 3.7	74.6 ± 2.4	81.8 ± 2.2	57.7 ± 2.1	63.8 ± 2.0
Equine Express II	0	146.0 ± 2.7	153.0 ± 2.3	80.0 ± 1.9	88.0 ± 1.9	62.2 ± 1.6	68.2 ± 2.0
	24	142.0 ± 3.8	152.0 ± 3.5	77.0 ± 2.3	84.7 ± 2.2	61.3 ± 1.8	67.4 ± 1.9
	48	138.0 ± 4.8	152.0 ± 4.4	73.8 ± 2.6	82.9 ± 2.7	57.7 ± 2.2	64.8 ± 2.4
BotuFlex	0	146.0 ± 2.7	153.0 ± 2.3	80.0 ± 1.9	88.0 ± 1.9	62.2 ± 1.6	68.2 ± 1.9
	24	144.0 ± 3.5	151.0 ± 3.4	78.5 ± 2.2	85.2 ± 2.2	63.3 ± 1.9	68.8 ± 1.9
	48	142.0 ± 4.8	143.0 ± 4.1	75.2 ± 2.7	78.1 ± 2.4	60.0 ± 2.4	62.2 ± 2.0

305 VCL: curvilinear velocity (μm/s); VAP: average path velocity (μm/s); VSL: straight-line velocity (μm/s)



306 **Figure 3**. Representative flow cytometry images of one stallion ejaculate extended in
307 INRA96 or BotuSemen Gold and cooled in three passive cooling devices (Equitaner,
308 Equine Express II, and BotuFlex) for 48 hours. (A) semen was not cushion-centrifuged;
309 (B) semen was cushion centrifuged. On each subset of images, the samples were stained

with Mitotracker Deep Red and Zombie Green. The left quadrants display sperm with intact plasma membrane and high (HMMP) (upper), or low (LMMP) mitochondrial membrane potential (lower), the right quadrants display sperm with damaged plasma membrane and high (HMMP) (upper) or low (LMMP) mitochondrial membrane potential (lower). Abbreviations. Intact: sperm with intact plasma membrane PMI: plasma membrane integrity; Damaged: sperm with damaged plasma membrane; HMMP: high membrane mitochondrial membrane potential; LMMP: low membrane mitochondrial membrane potential.

Experiment 2

Total motility and progressive motility decreased over time for semen extended in BotuSemen Gold and INRA 96 ($p<0.05$) (Figures 4A-F). There were no differences between the three passive cooling devices used for the transport of cooled stallion semen ($p>0.05$). There were no effects due to from time, passive cooling device, or extender in the percentile of sperm with intact plasma membrane or percentile of sperm with intact plasma membrane and high mitochondrial membrane potential ($p>0.05$) (Figure 4 G-L). The only exception being that semen extended in BotuSemen Gold stored in Equitainer had a reduction in plasma membrane integrity between 0 and 48 h of cooled storage (Figure 4G). Curvilinear velocity, average path velocity and straight-line velocity were similar across passive cooling devices ($p>0.05$); however, BotuSemen Gold had superior sperm velocity parameters relative to INRA 96 for all timepoints assessed in all three passive cooling devices ($p<0.05$) (Table 2).

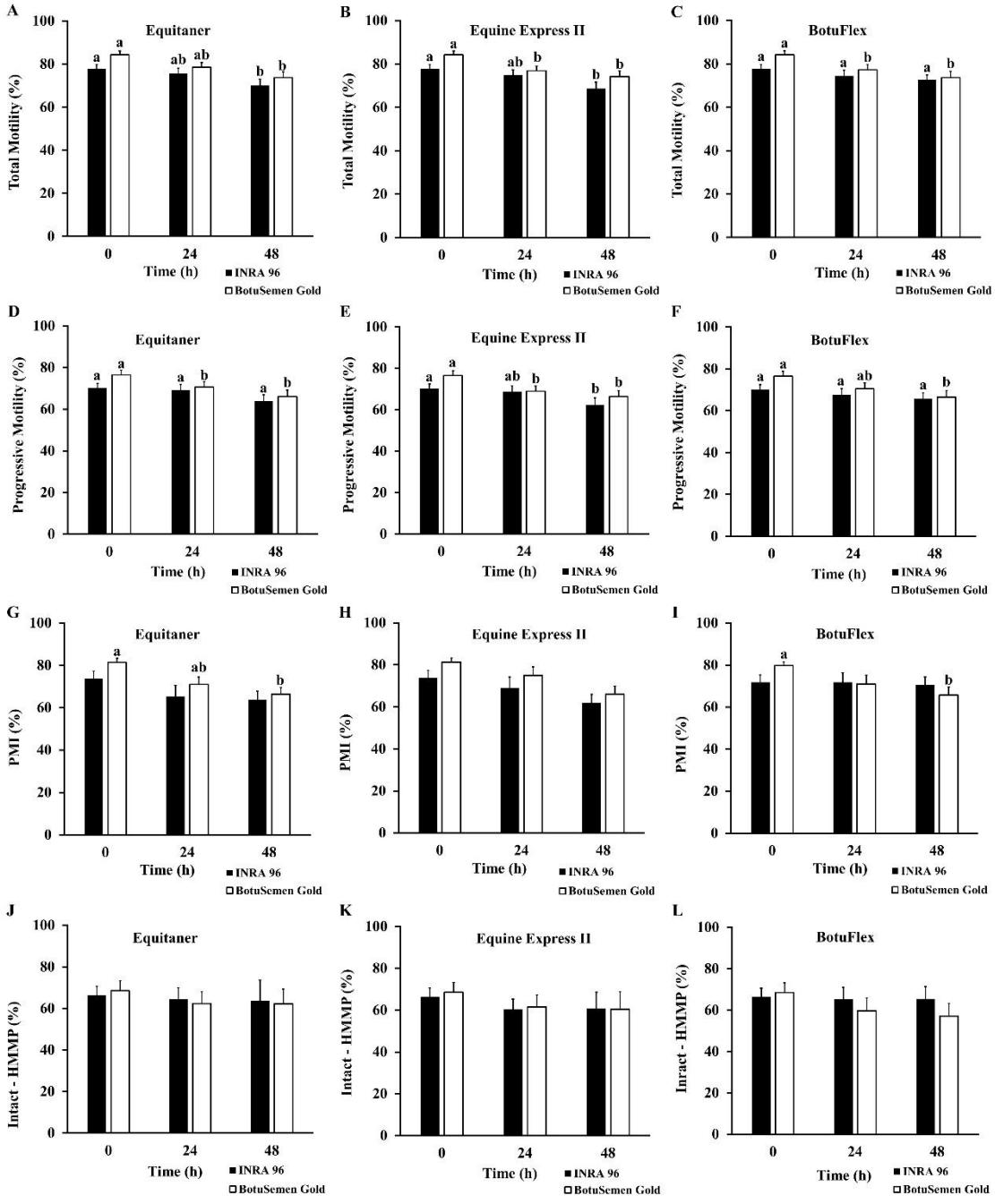


Figure 4. Parameters of stallion semen cushion-centrifuged extended in INRA96 or BotuSemen Gold and cooled for 48h in three different passive cooling devices (Equitainer, Equine Express II, and BotuFlex, rows 1-3). (A-C) Total sperm motility; (D-F) Progressive motility; (G-I) Percentage of sperm with intact plasma membrane; and (J-L) Percentage of sperm with intact plasma membrane and high mitochondrial membrane potential (Intact HMMP). Different superscripts denote effects of time (^{abc}) ($p<0.05$)

340 **Table 2**. Motility parameters for stallion sperm after cushion centrifugation and resuspension in INRA 96, and BotuSemen Gold (BG). Semen
341 was stored in three passive cooling devices (Equitainer, Equine Express II, and BotuFlex) for 48h.

		VCL		VAP		VSL	
	Time (h)	INRA 96	BG	INRA 96	BG	INRA 96	BG
Equitainer	0	128.0 ± 3.1^{A}	148.0 ± 1.6^{B}	66.6 ± 1.7^{A}	85.0 ± 1.2^{B}	50.8 ± 1.3^{A}	66.0 ± 1.3^{B}
	24	129.0 ± 2.9^{A}	148.0 ± 2.5^{B}	68.1 ± 1.6^{A}	83.4 ± 1.7^{B}	54.9 ± 1.5^{A}	66.8 ± 1.6^{B}
	48	133.0 ± 2.8^{A}	146.0 ± 3.2^{B}	70.7 ± 1.9^{A}	80.7 ± 2.0^{B}	57.6 ± 1.7^{A}	65.1 ± 1.8^{B}
Equine ExpressII	0	128.0 ± 3.1^{A}	148.0 ± 1.6^{B}	66.6 ± 1.6^{A}	85.0 ± 1.2^{B}	50.8 ± 1.2^{A}	66.0 ± 1.3^{B}
	24	130.0 ± 2.6^{A}	149.0 ± 2.2^{B}	68.4 ± 1.6^{A}	82.8 ± 1.6^{B}	55.1 ± 1.4^{A}	66.7 ± 1.5^{B}
	48	129.0 ± 3.0^{A}	147.0 ± 2.4^{B}	69.0 ± 1.9^{A}	80.6 ± 1.6^{B}	56.6 ± 1.6^{A}	64.5 ± 1.5^{B}
BotuFlex	0	128.0 ± 1.6^{A}	148.0 ± 1.6^{B}	66.6 ± 1.6^{A}	85.0 ± 1.2^{B}	50.8 ± 1.3^{A}	65.9 ± 1.3^{B}
	24	135.0 ± 2.3^{A}	147.0 ± 2.4^{B}	70.5 ± 1.4^{A}	83.0 ± 1.6^{B}	55.9 ± 1.2^{A}	67.4 ± 1.4^{B}
	48	135.0 ± 2.5^{A}	148.0 ± 2.3^{B}	71.7 ± 1.7^{A}	81.5 ± 1.7^{B}	58.2 ± 1.5^{A}	66.1 ± 1.5^{B}

342 VCL: curvilinear velocity (μm/s); VAP: average path velocity (μm/s); and VSL: straight-line velocity (μm/s). Different superscripts (^{AB})
343 denote difference between extenders ($p<0.05$).

Experiment 3

Embryo recovery rates were identical between extenders 50%; (Figure 5). All embryos recovered were expanded grade 1 blastocysts. While this was not an endpoint assessed in the present study, mares did appear to develop a similar post-breeding inflammatory response to both extenders.

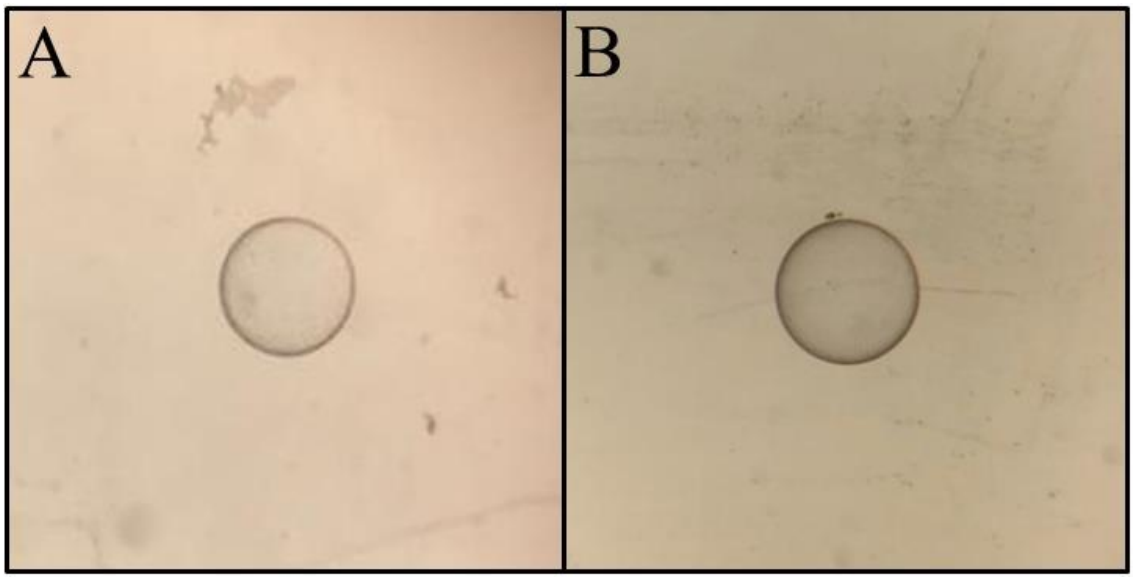


Figure 5. Representative blastocysts harvested from a mare bred with 48h-cooled stallion semen extended with INRA 96 (A) or BotuSemen Gold (B)

4. Discussion

This is the first study aimed to compare the most widely used equine extender, INRA 96, and BotuSemen Gold, a new commercially available equine semen extender. In addition, the present study examined the most traditional passive semen cooling device (i.e., Equitainer), with the most widely used device (i.e., Equine Express II), and a new device (i.e., BotuFlex). In addition, embryo recovery rates were assessed in mares bred with 48h-cooled equine semen. The results obtained with both extenders were largely similar for most endpoints assessed, except that BotuSemen Gold was superior to INRA

96 for total and progressive motility during cooled-storage when semen was not centrifuged, and BotuSemen Gold had superior sperm velocity parameters to INRA 96 after cushion-centrifugation for all time points in all three passive cooling devices. These findings suggest that there was an interaction between components of BotuSemen Gold and seminal plasma, as once seminal plasma was removed via cushion-centrifugation, the differences for total and progressive motility between extenders disappeared. However, removal of seminal plasma-enhanced all sperm velocity parameters for cooled semen extended in BotuSemen Gold when compared to INRA 96 in the various time points. While we cannot be certain of the mechanisms of action, centrifugation is a known method to remove membrane-bound membranes in other species [29,30]. Sperm velocity has been suggested to be essential for the assessment of frozen stallion semen [31], but its relevance for cooled-transported semen has not been clear.

The mechanism by which total and progressive motility were enhanced in the cooled semen extended with BotuSemen Gold not undergoing centrifugation was not studied herein. Presumably, the cholesterol present in this extender can be responsible for the differences in extenders. It has previously been demonstrated that cholesterol-loaded cyclodextrin helps to promote cryotolerance in horses, cattle, sheep, goats, and dogs [12,32-35]. Therefore, shifting the lipid composition of the sperm plasma membrane can be useful to enhance the cryotolerance of sperm at low temperatures [18]. Cholesterol/phospholipid ratio in the plasma membrane is essential to maintain the fluidity and stability of the sperm membrane at low temperatures [17-35]. Higher concentrations of cholesterol in the plasma membrane are known to result in a reduction in cryodamage through the transition phase during cooling [14]. Sperm from certain species (e.g., rabbits and dogs), have higher cholesterol/phospholipid ratio in the plasma membrane and display higher resistance to freezing than stallions [37]. In addition, sperm

quality and longevity are highly variable across animals [12,38] which may, in part, explain the variability of sperm resistance to the cooling in this species. Thus, since native phosphocaseinate present in INRA 96 and sodium caseinate present in BotuSemen Gold are similar molecules, these two molecules may provide equivalent protection during cooling, and the addition of cholesterol via cyclodextrin in BotuSemen Gold could partially explain the difference in results for total and progressive motility. Regardless of the differences in motilities, both extenders supported excellent semen parameters up to 48 h of cooled-storage.

Surprisingly, plasma membrane integrity and percentage of sperm with both intact plasma membranes and high mitochondrial membrane potentials were not different between extenders with or without centrifugation. Other studies have shown a strong correlation between these variables and sperm motility parameters [39]; however, the previous studies used different staining methods for flow cytometric analyses. Herein, we have used a novel protocol using Mitotracker Red and Zombie green, which was fixed and read within 72h as stated above and recommended by the original study [24]. It is possible that the lack of difference for sperm with both intact plasma membrane and high mitochondrial membrane potential was due to the fact that the mechanism enhancing motility parameters for semen extended in BotuSemen Gold was not directly mediated by plasma membrane or mitochondrial functions. Alternatively, the flow cytometric technique used herein may not have been sensitive enough to detect subtle differences between extenders.

The cholesterol-loaded cyclodextrin extender used in the present extender was reported to be a useful extender for stallions displaying poor semen cooling ability when compared to a standard skim milk-based extender [12]. However, since none of the

411 stallions enrolled in this experiment had poor semen cooling ability, we were unable to
412 compare INRA 96 and BotuSemen Gold for such a population of stallions.

413 Cryodamage results in capacitation-like changes that alters the sperm structure
414 and metabolism, which results loss of sperm motility [40]. Caseins can prevent
415 cryodamage by the competitively binding with seminal plasma proteins, such
416 sequestering binder sperm proteins, which are involved in modifications on sperm
417 membrane lipids during capacitation [9,41-43]. In pigs, the addition of casein to semen
418 extenders prevented the cholesterol efflux from sperm membranes caused by seminal
419 plasma proteins [44]. As both extenders assessed in the present study contain caseins, we
420 believe that a similar mechanism of protection happens for both extenders, but the exact
421 mechanism remains to be determined [8].

422 Centrifugation of semen pre-cooling is routinely applied for semen from stallions
423 presenting less than 100 million sperm/mL in raw semen, and for stallions known to
424 produce semen with poor cooling ability [45]. In the present study, cushion centrifugation
425 was applied to test these two extenders under typical practical settings. However, because
426 none of the stallions needed centrifugation before cooling, it is unknown if we would
427 have obtained different results with the present experimental design if stallions with low
428 semen concentration or semen with poor tolerance to cooling were used. In the present
429 study, only one horse had slightly worse semen quality than the remaining, even so his
430 semen was not poor enough to be classified as having poor semen cooling.

431 Previously, semen cooling tests were widely applied in North America to
432 determine the best extender and antibiotics for stallion semen. This practice has been
433 largely discontinued by most breeding centers since INRA 96 outperforms other
434 extenders and became the most popular extender used commercially. The findings of the
435 present place the BotuSemen Gold extender at the similar level than INRA 96, which in

436 suggest that a semen cooling test could be beneficial to determine the most suitable
437 extender for a given stallion. In addition, the results obtained with BotuSemen Gold
438 provides evidence to have this extender included in any semen cooling test against other
439 extenders available in the market.

440 The embryo recovery obtained in the present study was satisfactory for 48h-
441 cooled semen. The present results were similar to those obtained by French investigators
442 [3], breeding mares with 48h-cooled semen extended with INRA 96. As the mares used
443 in the present experiment had been known to have marginal fertility, the results could
444 have been superior if mares of high fertility were used.

445 In conclusion, our results indicated that INRA 96 and BotuSemen Gold result in
446 satisfactory semen parameters upon cooled-storage in all three passive cooling devices
447 tested herein. However, for the group of stallions used in our study, BotuSemen Gold
448 extended semen had superior total and progressive motility than INRA 96 without
449 cushion-centrifugation. Cushion-centrifugation resulted in similar total and progressive
450 motility between extenders. However, BotuSemen Gold had superior sperm velocity
451 parameters for all time points assessed in all three passive cooling devices. Cushion-
452 centrifugation and its absence in the processing of semen resulted in similar plasma
453 membrane integrity and sperm with intact plasma membrane and high mitochondrial
454 membrane potential. In addition, embryo recovery rates of 48h-cooled semen resulted in
455 similar percentiles for both extenders. It remains to be determined if semen from stallions
456 with poor cooling ability behave similarly to those with satisfactory semen cooling
457 ability. Nonetheless, the present study suggests that BotuSemen Gold could be included
458 in semen cooling tests against INRA 96 and other extenders to optimize the identification
459 of the best extender for a given stallion.

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5. References

[1] Kenney R, Bergman R, Cooper WF, Morse G. Minimal contamination techniques for breeding mares: techniques and preliminary findings. In: Proceedings of American Association of Equine Practitioners, Boston: 21st AAEP 1975;21:327-36.

[2] Varner DD, Scanlan CM, Thompson JA, Brumbaugh GW, Blanchard TL, Carlton CM, et al. Bacteriology of preserved stallion semen and antibiotics in semen extenders. Theriogenology 1998;50:559–73.

[3] Batellier F, Magistrini M, Fauquant J, Palmer E. Effect of milk fractions on survival of equine spermatozoa. Theriogenology 1997;48:391–410.

[4] Batellier F, Gérard N, Courtens JL, Palmer E, Magistrini M. Preservation of stallion sperm quality by native phosphocaseinate: a direct or indirect effect? J Reprod Fertil Suppl 2000;56:69–77.

[5] LeFrapper L, Walston L, Whisnant CS. Comparison of various extenders for storage of cooled stallion spermatozoa for 72 hours. J Equine Vet Sci 2010;30:200–4.

[6] Ramires Neto C, Sancler da Silva YFR, Resende HL, Guasti PN, Monteiro GA, Papa PM, et al. Control methods and evaluation of bacterial growth on fresh and cooled stallion semen. J Equine Vet Sci 2015;35:277–82.

485 [7] Dean CJ, Hobgood AM, Blodgett GP, Love CC, Blanchard TL, Varner DD. The
486 addition of ticarcillin-clavulanic acid to INRA 96 extender for stallion semen cooling.
487 Equine Vet J Suppl 2012;44:95–9.
488 [8] Manjunath P. New insights into the understanding of the mechanism of sperm
489 protection by extender components. Anim Reprod 2012;9:809-15.
490 [9] Plante G, Lusignan M-F, Lafleur M, Manjunath P. Interaction of milk proteins
491 and Binder of Sperm (BSP) proteins from boar, stallion and ram semen. Reprod Biol
492 Endocrinol 2015;13:92.
493 [10] Pagl R, Aurich JE, Müller-Schlösser F, Kankofer M, Aurich C. Comparison of an
494 extender containing defined milk protein fractions with a skim milk-based extender for
495 storage of equine semen at 5 °C. Theriogenology 2006;66:1115–22.
496 [11] Coutinho da Silva MA, Seidel GE, Squires EL, Graham JK, Carnevale EM.
497 Effects of components of semen extenders on the binding of stallion spermatozoa to
498 bovine or equine zonae pellucidae. Reproduction 2012;143:577–85.
499 [12] Hartwig FP, Lisboa FP, Hartwig FP, Monteiro GA, Maziero RRD, Freitas-
500 Dell'Aqua CP, et al. Use of cholesterol-loaded cyclodextrin : An alternative for bad
501 cooler stallions. Theriogenology 2014;81:340–6.
502 [13] Klein U, Gimpl G, Fahrenholz F. Alteration of the myometrial plasma membrane
503 cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin
504 receptor. Biochemistry 1995;34:13784–93.
505 [14] Amann RP, Pickett BW. Principles of cryopreservation and a review of
506 cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7:145–73.
507 [15] Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian
508 spermatozoa on susceptibility to cold-shock. Cryobiology 1977;14:466–70.
509 [16] Zahn FS, Papa FO, Dell'Aqua JA. Cholesterol incorporation on equine sperm

membrane: effects on post-thaw sperm parameters and fertility. Theriogenology 2002;58:237–40.

[17] Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 2005;51:241–9.

[18] Torres P, Serres C. Effect of cholesterol-loaded cyclodextrin on motility and plasma membrane integrity of cooled stallion sperm. Anim Reprod Sci 2006;94:148–51.

[19] Douglas-Hamilton DH, Osol R, Osol G, Driscoll D, Noble H. A field study of the fertility of transported equine semen. Theriogenology 1984;22:291–304.

[20] Brinsko SP, Rowan KR, Varner DD, Blanchard TL. Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. Theriogenology 2000;53:1641–55.

[21] Peña FJ, Ball BA, Squires EL. A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples. Cytom B Clin Cytom 2018;94:302–11.

[22] Roach J, Schnobrich M, Ellerbrock R, Feijo L, Bradecamp E, Alvarenga MA, et al. Comparison of cushioned centrifugation and SpermFilter filtration on longevity and morphology of cooled-stored equine semen. Vet Rec 2016;178:241.

[23] Ecot P, Decuadro-Hansen G, Delhomme G, Vidament M. Evaluation of a cushioned centrifugation technique for processing equine semen for freezing. Anim Reprod Sci 2005;89:245–8.

[24] Waite JA, Love CC, Brinsko SP, Teague SR, Salazar JL, Mancill SS, et al. Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 2008;70:704–14.

[25] Sieme H, Knop K, Rath D. Effects of cushioned centrifugation on sperm quality in stallion semen stored cooled at 5°C for 24h, and stored cooled for 2h or 24h and then

frozen. Anim Reprod Sci 2006;94:99–103.

[26] Manjunath P, Nauc V, Bergeron A, Ménard M. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. Biol Reprod 2002;67:1250–8.

[27] Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res 2005;322:43–52.

[28] Vidament M. French field results (1985-2005) on factors affecting fertility of frozen stallion semen. Anim Reprod Sci 2005;89:115–36.

[29] Murphy C, English AM, Holden SA, Fair S. Cholesterol-loaded-cyclodextrins improve the post-thaw quality of stallion sperm. Anim Reprod Sci 2014;145:123–9.

[30] Purdy PH, Graham JK. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 2004;48:36–45.

[31] Mocé E, Purdy PH, Graham JK. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Anim Reprod Sci 2010;118:236–47.

[32] Konyali C, Tomás C, Blanch E, Gómez EA, Graham JK, Mocé E. Optimizing conditions for treating goat semen with cholesterol-loaded cyclodextrins prior to freezing to improve cryosurvival. Cryobiology 2013;67:124–31.

[33] Inanc ME, Tekin K, Olgac KT, Yilmaz B, Cil B, Tasdemir U, et al. Effect of cholesterol loaded cyclodextrin on semen cryopreservation of Aksaray Malakli shepherd dogs of different ages. Anim Reprod Sci 2018;193:191–200.

[34] Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology 1977;14:466–70.

[35] Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 2000;54:129–36.

[36] Dorado J, Alcaráz L, Duarte N, Portero JM, Acha D, Demyda S, et al. Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 2011;76:381–5.

[37] Yanagimachi R. Mammalian fertilization. In: Knobil, E & Neill J, editor. Physiol. Reprodution. 2nd ed., New York: E-Plublishing Inc; 1994, p. 189–317.

[38] Bergeron A, Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol Reprod Dev 2006;73:1338–44.

[49] Manjunath P, Thérien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 2002;53:109–19.

[40] Manjunath P, Bergeron A, Lefebvre J, Fan J. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. Soc Reprod Fertil Suppl 2007;65:217–28.

[41] Tatemoto H, Oshiro R, Shimada H, Konno T, Yamanaka K, Ashizawa K. Addition of casein to the diluents during semen transportation improves the post-thaw qualities of Okinawan Native Agu pig spermatozoa. Nihon Danc Chikusan Gakkaihou 2015;58:75–86.

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HIGH OR LOW BODY FAT DEPOSITION IN THE PRESENCE OF A NORMAL ORAL SUGAR TEST IS NOT ASSOCIATED WITH POST-THAW SEMEN PARAMETERS IN STALLIONS

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Abstract

This study compared the post-thaw semen parameters of stallions with high and low body condition scores (BCS) and evaluated associations between body morphometric parameters and post-thaw semen parameters. Twenty stallions were split into Low-BCS (BCS<7, n=11) and High-BCS (BCS ≥7, n=9) groups, and underwent a complete morphometric analysis (e.g., neck scores and circumference, crest neck height, body weight, and height), and subcutaneous body fat thickness (SFT) at the tail head, withers, shoulders, and retroperitoneal space. A fasted oral sugar test was conducted on all stallions. One ejaculate was obtained from each stallion and diluted to 50 million sperm/mL with a skim milk-based extender. Semen was centrifuged, the supernatant discarded, the pellet resuspended at 200 million sperm/mL with an egg yolk-based extender, and then frozen. Post-thaw sperm motility parameters were assessed by CASA. Similarly, post-thaw plasma membrane integrity, mitochondrial membrane potential, hydrogen peroxide, intracellular superoxide production, and lipid peroxidation were analyzed with fluorescent probes and flow cytometer. The circumference at 25% and 50% of the neck's length were larger for High-BCS stallions ($p<0.05$). There were no differences between groups for the neck crest height ($p>0.05$). Stallions with High-BCS had greater SFT at the tail head than stallions with Low-BCS ($p<0.05$); however, there were no differences between groups in the SFT at the shoulders and withers ($p>0.05$). The retroperitoneal fat tended to be increased in the High-BCS group ($p=0.06$). All stallions had resting blood glucose <105 mg/dL which was below the suggested cutoff for equine metabolic syndrome. There were no differences between groups for resting glucose concentrations or for peak blood glucose concentration at 30 or 60 min after initiation of the oral sugar test ($p>0.05$). There were no differences in motility, sperm velocity parameters, plasma membrane integrity, mitochondrial membrane potential,

hydrogen peroxide, intracellular superoxide production, or lipid peroxidation between groups ($p>0.05$). Collectively, the findings of the present study suggest that High- or Low-BCS alone cannot explain post-thaw semen parameters in stallions. Further studies should address the associations between body fat deposition overtime and post-thaw semen parameters.

Keywords: Sperm, cryopreservation, metabolic syndrome, obesity, horse.

1. Introduction

The Crioulo is the most numerous horse breed in South America. The breed evolved from Spanish horses brought to the continent by settlers in the 16th century from the Iberian Peninsula [1]. These stock horses were widely used for farm work such as plowing plantation fields, transportation, and herding cattle [1]. Over the past few years, the wide use of this breed in various equestrian modalities has resulted in the increasing popularity and economic value of these animals, leading to remarkable changes in animal husbandry practices [2,3]. Previously, these horses were kept free-range on nutritionally poor native grassland, which forced them to adapt and to thrive under harsh conditions [1]. However, with their ever-increasing values, these horses are increasingly housed on temperate climate pasture or confined in stalls and fed energy-dense diets [3].

Anecdotally, the Crioulo seems to have a high proportion of animals classified as metabolically efficient ("easy keepers") compared with other horse breeds such as the Thoroughbred raised under similar conditions. Metabolically efficient horses tend to deposit fat in the neck, shoulders, and rump when fed at maintenance levels [4]. This phenotype is associated with insulin dysregulation and equine metabolic syndrome, however, not all horses with high body condition scores are affected, and clinical examination coupled with blood tests (e.g., baseline glucose or tolerance testing) can be used to identify horses with equine metabolic syndrome [4]. Horses affected with this condition are prone to a variety of clinical conditions ranging from endocrinophatic laminitis to hyperlipidemia, preputial, or mammary gland edema, mesenteric lipomas, and inappropriate lactation [4]. High body fat deposition has been a concern for broodmares and stallions of all breeds, and little has been done to study its effect on reproduction, though subfertility has been anecdotally one of the primary concerns

associated with equine obesity [4]. It has not been critically assessed whether if obese stallions with or without equine metabolic syndrome have semen quality affected. Specifically, it is unknown if stallions with high body condition scores have poor semen freezing ability in comparison with stallions of the same breed with lower body condition scores.

It is well-documented that high body condition score can impair semen quality in ruminants [5]. In bulls and rams, overfeeding presumably results in fat deposition around the scrotum neck, which increases testicular temperature and impairs spermatogenesis [5,6]. In humans, obesity alone does not appear to influence sperm quality [7], but other conditions associated with obesity (e.g., diabetes) have been suggested to affect semen quality [8,9]. A controversy exists in practice regarding whether stallions should be maintained at a higher (e.g., >7, scale 1-9) or lower (<7) body condition score (BCS) during the breeding season. To date, there are no studies determining the ideal stallion body condition score during the reproductive season or examining the association between body fat deposition and semen quality in stallions.

The objective of this study was to compare post-thaw semen parameters of stallions with high and low body condition scores and to determine the associations between body morphometric parameters and post-thaw semen parameters. We hypothesized that obesity alone in absence of equine metabolic syndrome does not affect post-thaw semen quality.

2. Materials and Methods

The experimental protocols were reviewed and approved by the Ethics Committee for Animal Use, São Paulo State University, under protocol # 0167/2019. This study was

carried out from September of 2019 to March of 2020, during the physiological breeding season of the Southern hemisphere. Privately-owned stallions (n=20, 10.5 ± 5.2 years old) housed in seven farms in the Rio Grande do Sul state, Brazil were enrolled in this study. All stallions enrolled in the study were registered as purebred with the Brazilian Association of Crioulo Horse Breeders. The animals were fed according to industry standards, which consisted of ad libitum alfalfa hay, trace minerals, and commercial grain (2 to 5 kg/day). None of the stallions in this study had a history of laminitis. All stallions were client-owned, and all eleven owners signed a consent form permitting us to use their stallions in this study.

2.1. Semen collection and freezing

During the present study, all animals were being actively collected for breeding mares or semen freezing. Sexual rest varied from 2 to 4 days before enrollment in the present study. One stallion was scheduled to breed 80 mares in the season and the rest were scheduled for less than 20 mares. One ejaculate was obtained from each stallion enrolled in the study. Semen collection was performed with a rigid artificial vagina (Botupharma, Botucatu, São Paulo, Brazil) off an estrus mare. Immediately after semen collection, each ejaculate was filtered with a paper filter (Minitube, Porto Alegre, Rio Grande do Sul, Brazil) to remove debris and the gel fraction. On-field, sperm motility was subjectively assessed via light microscopy and gel-free semen volume recorded. Sperm concentration was determined with a hemocytometer (Kasvi, São José dos Pinhais, Paraná, Brazil) using a 1:20 dilution with distilled water.

Semen was extended to 50 million sperm/mL with a commercial skim milk-based extender (BotuSemen, Botupharma), and the extended semen was centrifuged at 600×g for 10 min at room temperature (Excelsa II, Fanem, São Paulo, São Paulo, Brazil). Following centrifugation, the supernatant was discarded, the pellet was resuspended in a

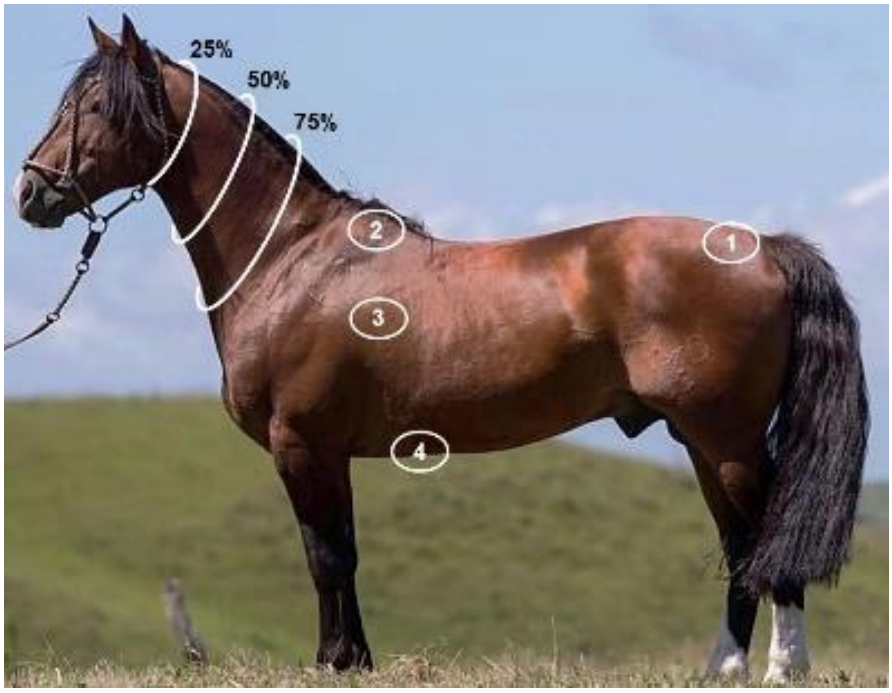
commercially available, egg-yolk-based semen freezing extender (Botucrio, Botupharma) at a concentration of 200 million sperm/mL. The diluted semen was manually loaded in 0.5 mL straws and maintained for 20 min in a passive cooling device (BotuFlex, Botupharma) with internal temperature previously stabilized at 5 °C [10]. Afterward, the straws were placed 6 cm above liquid nitrogen for 20 min and then plunged into liquid nitrogen [11]. The straws were placed in goblets and racks and then transported in liquid nitrogen to the Sao Paulo State University for post-thaw semen analyses as described below.

2.2. Morphometric measurements

Bodyweight (kg), height at the withers, thoracic circumference, neck length, neck crest height, and neck circumference at 25% (N 25), 50% (N 50), and 75% (N 75) along the neck was measured in centimeters using a metric measuring tape (Figure 1). Neck length was measured from the poll to the highest point of the withers [12]. Crest height was measured at 50% of neck length, and to differentiate the crest (tissue apparent above the ligamentum nuchae) from the neck muscles, palpation, and visual assessment were performed [13]. All measurements were taken while the neck was held in a relaxed position, approximately at a 45° angle. The BCS and crest neck scores (CNS) were determined by two experienced clinicians. The BCS was rated from 1 to 9 as previously described [14]; whereas CNS were graded from 0 to 5 [13]. Scores were rated to the nearest half score increment, and the median of each horse's scores was used for data analysis.

149 2.3. Body fat determination

150 Subcutaneous fat thickness (SFT) was measured with the use of ultrasound
151 coupled with a 7.5-MHz linear transducer (Mindray´s DP 2200, Mindray, São Paulo,
152 Brazil). Subcutaneous fat thickness was measured at 4 different sites (Figure 1). Sites 1
153 and 2 were located approximately 5 cm lateral from the midline on the left side; the first
154 was on the flat area on the rear of the rump approximately 7-8 cm cranial to the tail head,
155 and the second site was at the withers [15]. Site 3 was located immediately behind the
156 scapula (shoulder SFT) on the left side, approximately 25 cm lateral to the dorsal midline.
157 The last site was the area immediately caudal to the xiphoid process and lateral to the
158 midline (retroperitoneal fat), with the transducer in a parallel position to the ventral
159 midline, as previously described [16].



160 **Figure 1.** Representative image of the measurement sites in the stallions enrolled in the
161 present study. Neck circumferential measurements were performed as shown above at
162 25%, 50%, and 75% of the neck's length. Sites used to determine subcutaneous fat
163 thickness measurements are represented in circled numbers 1 to 4.

164 2.4. Oral sugar test

165 All stallions were subjected to an oral sugar test (OST) to assess their risk for
166 equine metabolic syndrome. The OST was conducted the day after semen collection as
167 described elsewhere [17]. Briefly, the stallions fasted overnight. In the night before OST,
168 each horse received one flake of alfalfa hay at 10 pm. At 7 am, an intravenous catheter
169 was placed in the left jugular vein. Blood samples were obtained at time 0 h (7:45 am)
170 for baseline glucose determination. Corn syrup (Yoki, General Mills Brazil Food, São
171 Paulo, Brazil) was orally administered (0.15 mL/kg) at 8 am. Blood samples were
172 obtained at 30, 60, 75, 90, 120,150, 180, 210, and 240 minutes after corn syrup
173 administration for glucose determination. Care was taken to keep each horse calm and
174 undisturbed in its stall during the sampling time. Glucose concentration was determined
175 immediately after each collection using a handheld glucometer (Accu-Chek Performa,
176 Roche Diabetes Care, São Paulo, São Paulo, Brazil) [18]. Glucose concentrations were
177 plotted over time for each horse and the glucose curve generated was interpreted as
178 previously described [11].

179

180 2.5 Post-thaw semen analyses

181 Two straws were thawed at 38°C for 30 s in a water bath. Immediately after
182 thawing, the content of the straws was transferred to a 2 mL tube, diluted (temperature-
183 match 1:1) with the same egg-yolk semen freezing extender for immediate sperm motility
184 analyses with a computer assisted-sperm analysis (HTM-IVOS 12, Hamilton Thorne
185 Research, Beverly, MA, USA). Five random fields were assessed for each sample. The
186 percentage of total sperm motility (TM), progressive motility (PM), average path velocity
187 (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and fast-moving
188 spermatozoa (RAP) were evaluated using customized settings for equine sperm. The

CASA-established settings were as follows: image capture (frames per second: 60Hz), image capture (number of frames: 45), detected cells (minimum contrast: 30), detected cells (minimum 30 pixels), defaults (cell size 5 pixels), defaults (cell intensity: 40), progressive cells (VAP 70 μm/s), progressive cells (STR 80%), slow cells (VAP cutoff 30 μm/s), slow cells (VSL cutoff 20 μm/s), illumination intensity (2,200), illumination photometer (125), video source (darkfield 60 Hz), static intensity gates (min/max: 0.48 and 1.45), static elongation gates (min/max: 0 and 97), chamber-type (Makler1), temperature (38°C), field selection (automatic).

Post-thaw all semen samples were submitted to fluorescent staining using a Fortessa LSR equipment (Becton Dickinson, Mountain View, CA, USA) equipped with blue (488-nm, 100 mW), red (640 nm, 40 mW) and violet (405-m, 100 mW) lasers. For flow cytometry assays, all the samples were extended in TALP-PVA containing Hoechst 33342 (7μM) to discard the non-cellular particles [19]. The composition of the TALP-PVA medium was as follows: 100 mM NaCl, 3.1 mM KCl, 25.0 mM NaHCO3, 0.3 mM NaH 2 PO 4, 21.6 mM DL 60% sodium lactate, 2.0 mM CaCl 2, 0.4 mM MgCl2, 10.0 mM Hepes-free acid, 1.0 mM sodium pyruvate, 1.0 mg/mL polyvinyl alcohol-PVA and 25 μg/mL gentamicin. For each assay, at least 10,000 cells per sample were analyzed.

For the evaluation of intracellular hydrogen peroxide (H_2O_2) production CM-H_2DCFDA (C6827, Life Technologies) was used in combination with propidium iodide thus, in 500 μL of the diluted semen solution, 1.5 μM of propidium iodide and 1 μM of CM-H_2DCFDA were added, the incubation was performed for 20 minutes at 37ºC. Assessments of mitochondrial membrane potential, intracellular superoxide (O_2^-) production and plasma membrane stability were carried out, with the association of YoPRO (YP; labeling for cells with destabilized plasma membrane), MitoStatus Red (MST; mitochondrial potential) and MitoSOXTM Red (MSR; generation of superoxide

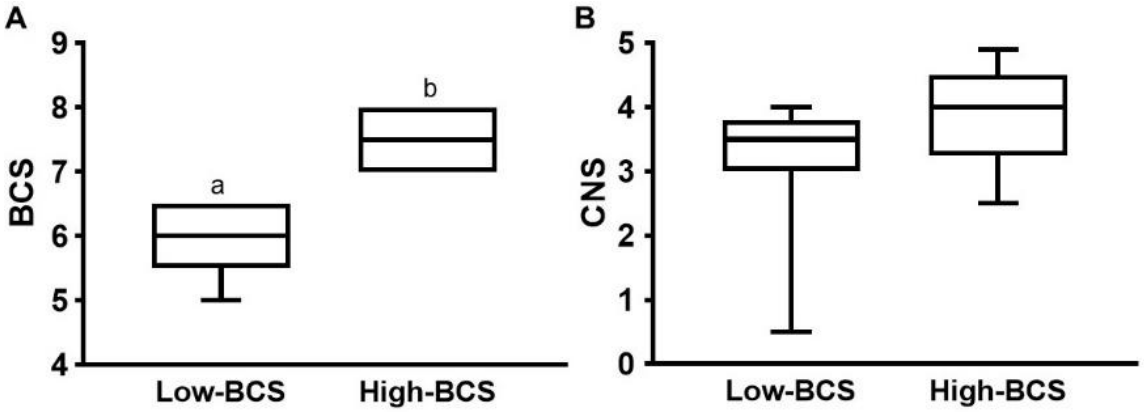
anion in the mitochondrial matrix) [20]. Thus, in a 500µL sample of diluted semen, 25nM YP, 20µM MST, and 2µM MSR were added, incubated at 37ºC for 20 minutes. Propidium iodide (PI), and FITC-PSA (Pisum sativum agglutinin conjugated to fluorescein isothiocyanate) were used to assess the integrity of the plasma and acrosomal membrane [21]. A sample of 200µL of semen was diluted in 1.5µM of PI and 2ng of FITC-PSA and incubated for 15 min at 37ºC while protected from light

Lipid peroxidation assessments were carried out with the fluorescent probe C11 BODYPY (D-3861; Molecular Probes, Carlsbad, CA, USA). A sample of the semen was diluted in TALP-PVA to a concentration of 5×10^6 sperm/mL with a final volume of 500µL. These samples were stained with 5 µM C11BODIPY581/591, 1.5 µM IP, and incubated for 30 minutes at 37ºC. After incubation, 2 consecutive washes were performed by centrifugation at 300g for 5 minutes with TALP-PVA and TALP-PVA and the pellet resuspended in 300µL of TALP-PVA [22]. After all the flow cytometric assays, data were extracted using the manufacturer's software (BD FACSDiva™ v6.1).

2.6 Statistical analyses

Statistical analysis was performed using GraphPad Prism (Istat 8.0 software, GraphPad Software Inc. USA). Stallions were grouped as high body condition score (>7) (High-BCS, n=9) or low body condition score (Low-BCS n=11) <7 (scale 1-9). To evaluate the Gaussian distribution, morphometric and fat measurements, glucose levels, and sperm parameters were subjected to a Shapiro-Wilk normality test. T-test (parametric variables) or Mann-Whitney U tests (non-parametric variables) were used to compare groups. Fisher's exact test was used to assess the proportion of stallions peaking glucose concentrations at 30 and 60 minutes after initiation of the oral sugar test. Pearson's coefficient of correlation was calculated for all variables assessed. Significance was set

239 at $p<0.05$. The statistical tendency was defined as $0.05 \leq p < 0.10$. Data are represented as
240 means and standard error means.
241
242 **3. Results**
243
244 3.1 Morphometric study and subcutaneous fat thickness
245 The group with High-BCS had greater body weight (Table 1) and higher body
246 condition scores than the Low-BCS group ($p<0.05$) (Figure 2), though there were no
247 differences in height between groups ($p>0.05$) (Table 1). The circumference at 25% and
248 50% of the neck's length were larger for the High-BCS group ($p<0.05$) (Table 1). There
249 were no differences between groups for the CNS and neck crest height ($p>0.05$) (Figure
250 2). Stallions with High-BCS had greater subcutaneous fat thickness than Low-BCS
251 stallions at the tail head ($p<0.05$); however, there were no differences between groups in
252 the subcutaneous fat measured at the shoulders and withers ($p>0.05$); whereas the
253 retroperitoneal fat tended to be increased in the High-BCS group (Table 2).
254 Representative images of the neck and whole body for stallions in the High-BCS and
255 Low-BCS groups are depicted in Figures 3 and 4.



256

257 **Figure 2.** Box plots for body condition scores (BCS) (A) and crest neck scores (CNS)
258 (B). The stallions were group in Low-BCS (<7, n=11) or High-BCS (≥7, n=9) (scale 1-
259 9). The box represents the 25% quartile, median, and 75% quartile, whereas the lower
260 whiskers represent the 25% quartile – 1.5 × (interquartile range) and the upper whisker
261 represents the 75% quartile + 1.5 × (interquartile range). Different superscripts$^{(a,b)}$ denote
262 effects of groups ($p<0.05$).

263 Table 1. Morphometric parameters for stallions classified as Low Body Condition Scores
264 (Low-BCS, n=11; <7) and High Body Condition Scores (High-BCS, n=9; ≥7).

Variables	Group		p-values
	Low-BCS	High-BCS	
Age (years)	11.2 ± 1.75	9.8 ± 1.7	0.60
Withers height (cm)	143.0 ± 0.4	143.4 ±0.6	0.50
Body weight (kg)	400.1 ± 6.9[a]	450.0 ± 17.1[b]	0.01
Neck crest height (cm)	8.0 ± 0.3	9.4 ± 0.9	0.11
NC 25 (cm)	75.7 ± 0.8[a]	82.0 ± 0.7[b]	0.0001
NC 50 (cm)	97.1 ± 2.4[a]	105.4 ± 1.8[b]	0.01
NC 75 (cm)	121.0 ± 2.1	124.2 ± 1.1	0.21

265 NC neck circumference at 25%, 50%, and 75% of the neck's length, respectively. Data
266 expressed as means ± SEM. Different superscript letters[(a,b)] within row indicate
267 differences between groups ($p < 0.05$).

268

269 Table 2. Subcutaneous fat thickness measured at 4 different sites in stallions classified as
270 Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition Scores
271 (High-BCS, n=9; ≥7).

Sites	Group		p-values
	Low-BCS	High-BCS	
Tailhead	1.5 ± 0.1[a]	2.2 ± 0.1[b]	0.0008
Withers	0.6 ± 0.09	0.5 ± 0.09	0.23
Shoulder	0.3 ± 0.03	0.4 ± 0.1	0.61
Retroperitoneal	0.9 ± 0.07	1.1 ± 0.05	0.06

272 Different superscript letters[(a,b)] in the row indicate differences between groups ($P < 0.05$).

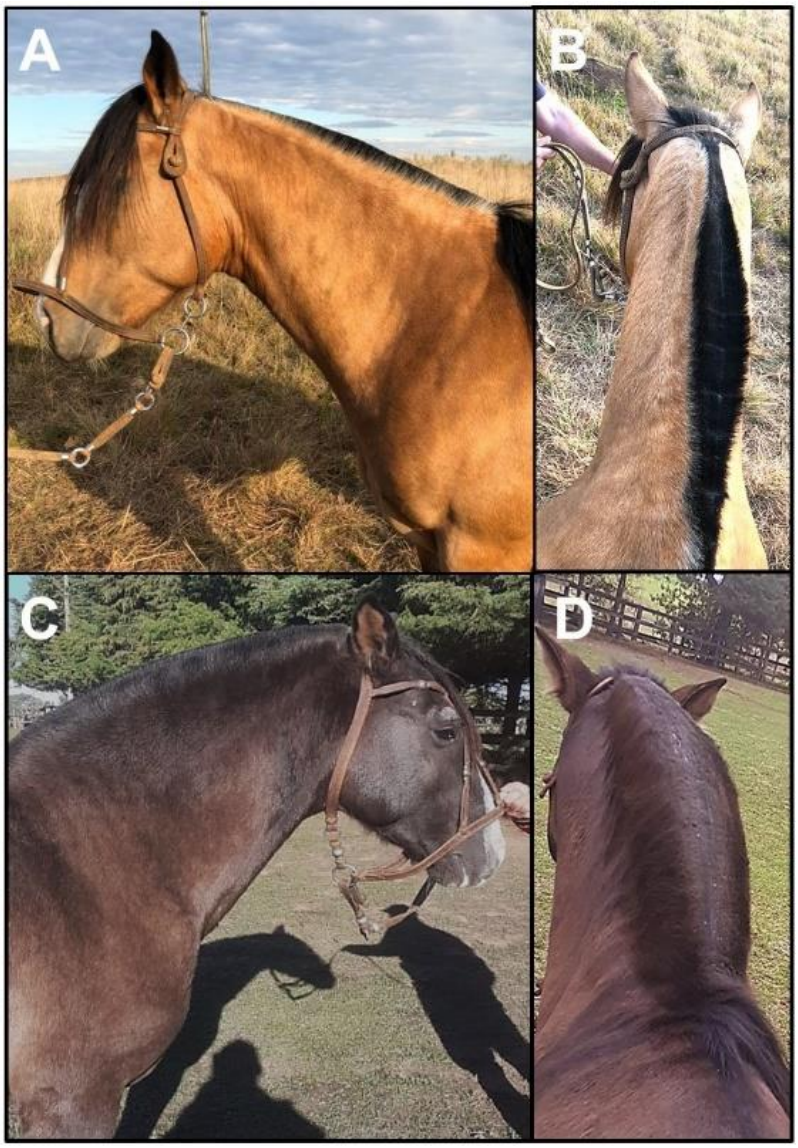


Figure 3. Representative images of the neck of a stallion in the group with a Low Body Condition score (A lateral view and B dorsolateral view) and a stallion with a High-Body Condition Score (C lateral view and D dorsolateral view).

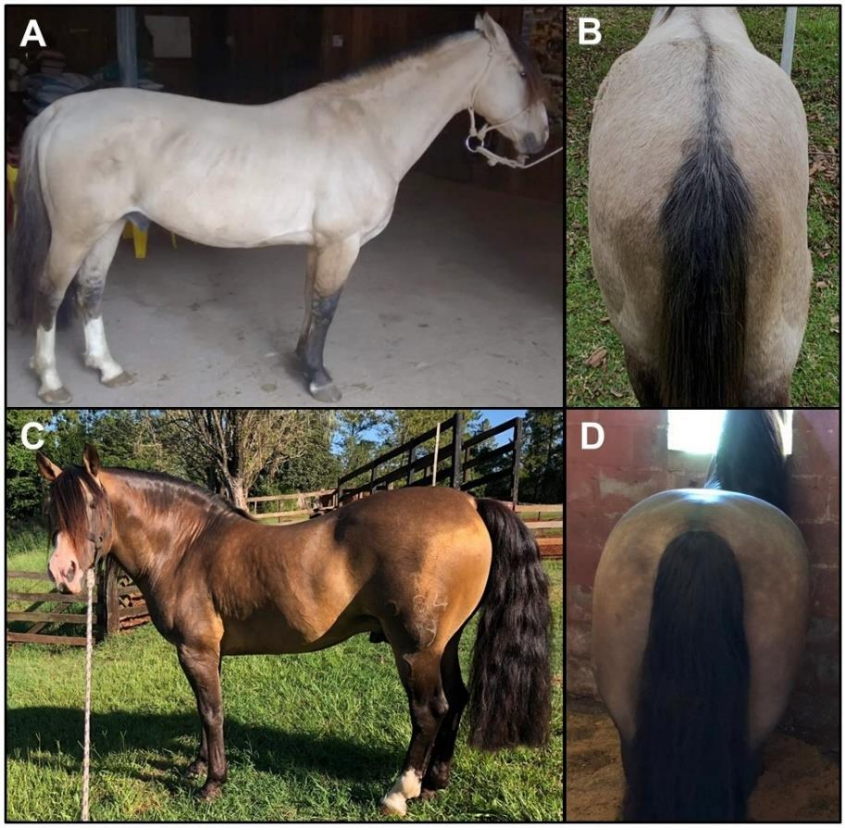


Figure 4. Representative images of the tailhead and other sites evaluated of a stallion in the group with a Low-Body Condition score (A and B) and a stallion with a High-Body Condition Score (C and D).

3.2 Oral sugar test

All stallions had resting blood glucose <105 mg/dL, which is below the suggested cutoff for equine metabolic syndrome [17]. There were no differences between groups for resting glucose concentrations (Table 3). For all stallions except one, glucose concentration peaked at 30 or 60 minutes after the administration of corn syrup (Table 3). There were no differences between groups for peak glucose concentrations 30 or 60 minutes after initiation of the OST ($p>0.05$).

287 Table 3. Mean glucose concentrations in stallions classified as Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition
288 Scores (High-BCS, n=9; ≥7) subjected to oral sugar test.

	Resting glucose (mg/dL)		Peak glucose (mg/dL) 30 min*			Peak glucose (mg/dL) 60 min**			Glucose (mg/dL) at 75 min	
	Mean ± SEM	Ranges	Mean ± SEM	Ranges	Proportion	Mean ± SEM	Ranges	Proportion	Mean ± SEM	Ranges
Low-BCS	85.64 ± 2.7	74-100	108.4 ± 4.8	94-111	7/11	103.0 ± 2.9	95-108	4/11	91.8 ± 2.9	79-113
High-BCS	83.67 ± 1.9	77-95	115.5 ± 8.1	96-148	6/9	105.7 ± 10.4	89-125	3/9	99.1 ± 5.1	75-125

There were no differences between groups for any of the endpoints assessed ($p>0.05$). *Mean glucose concentration for stallions that reached maximal blood glucose concentration within 30 minutes of the oral glucose bolus. **Mean glucose concentration for stallions that reached maximal blood glucose concentration at 60 minutes of the oral glucose bolus.

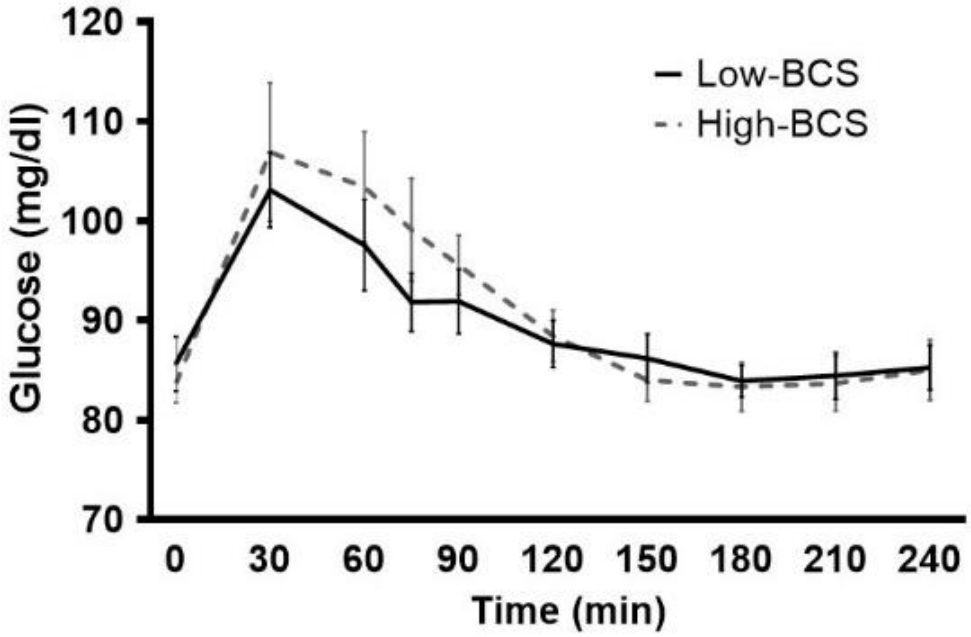


Figure 5. Plasma glucose concentrations from stallions classified as Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition Scores (High-BCS, n=9; ≥7). There were no differences between groups ($p>0.05$).

3.3 Post-thaw semen parameters

There were no differences between Low BCS and High BCS stallions in total motility, progressive motility (Figure 6), or sperm velocity parameters ($p>0.05$) (Table 4). Plasma membrane integrity, mitochondrial membrane potential, lipid peroxidation, intracellular superoxide production, and hydrogen peroxide were not different between groups ($p>0.05$) (Figures 7 and 8).

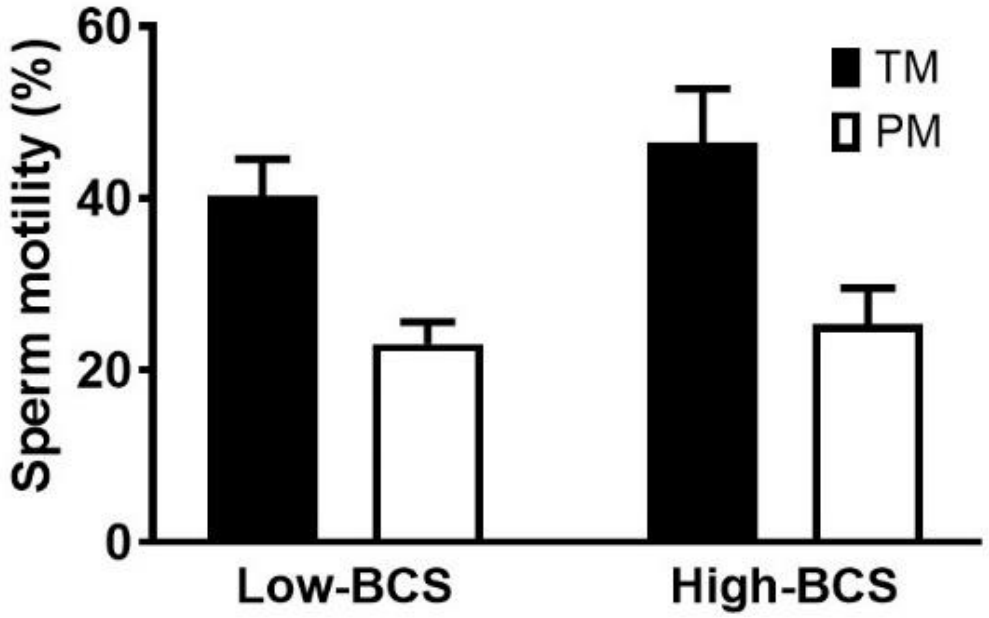
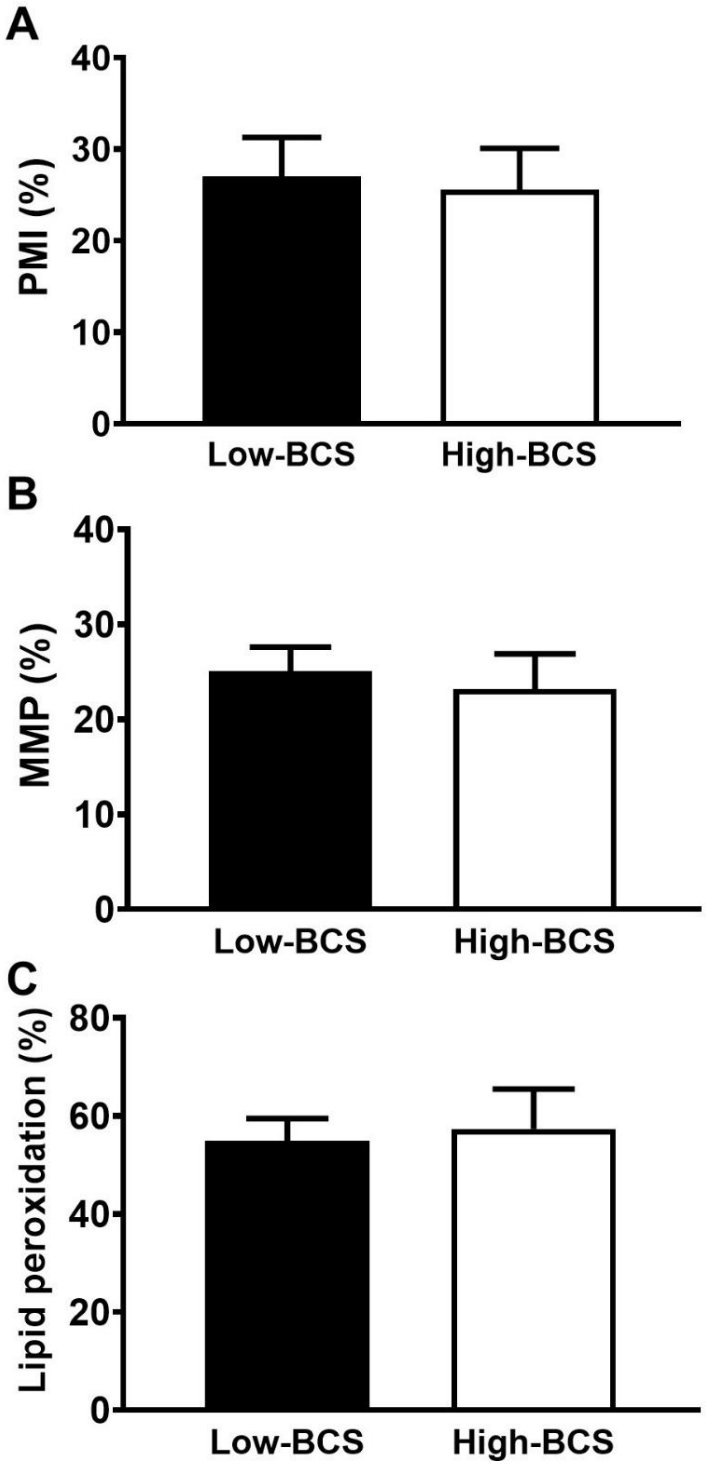


Figure 6. Total and progressive motility for stallions classified as Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition Scores (High-BCS, n=9; ≥7). There were no differences between groups for either endpoint assessed (p>0.05).

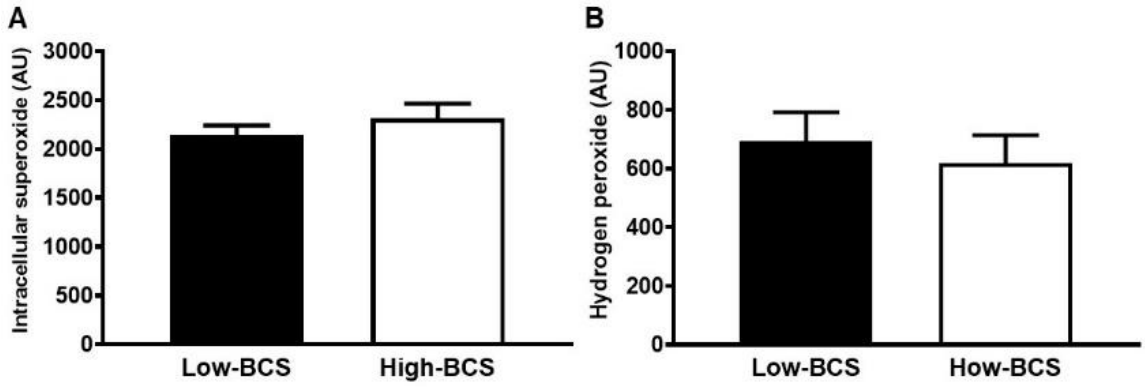
Table 4. Post-thaw sperm velocity parameters for stallions classified as Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition Scores (High-BCS, n=9; ≥7).

Groups	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	RAP (μm/s)
L-BCS	82.3 ± 2.2	70 ± 2	160 ± 4.5	30.0 ± 3.5
H-BCS	78.0 ± 3.1	66.3 ± 2.7	155 ± 7.0	30.4 ± 5.1

Average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and spermatozoa with rapid movement (RAP). Data expressed as Mean ± SEM. There were no differences between groups for any of the endpoints assessed (p>0.05).



311 **Figure 7**. Plasma membrane integrity (A), mitochondrial membrane potential (B), and
312 lipid peroxidation (C) for stallions classified as Low Body Condition Scores (Low-BCS,
313 n=11; <7) and High Body Condition Scores (High-BCS, n=9; ≥7). There were no
314 differences between groups for any of the endpoints assessed ($p>0.05$).



315 **Figure 8.** Intracellular superoxide (A), hydrogen peroxide (B), for stallions classified as
316 Low Body Condition Scores (Low-BCS, n=11; <7) and High Body Condition Scores
317 (High-BCS, n=9; ≥7). There were no differences between groups for any of the endpoints
318 assessed (p>0.05). AU, arbitrary unit.

319

320 3.4 Coefficient of variations

321 The neck crest height was moderately correlated (r= -0.5 to -0.6) with sperm
322 motility parameters such as progressive motility, average path velocity, straight-line
323 velocity, and sperm with rapid movement (Table 5). Fat deposition at withers was
324 moderately correlated (r= -0.5 to -0.6) with progressive motility. Sperm velocity and
325 motility parameters were highly correlated, as well as superoxide production and
326 mitochondrial membrane potential (Table 5).

Table 5. Coefficient of correlation for all endpoints evaluated.

	BCS	CNS	Age	WH	BW	NCH	NC25	NC50	NC75	TLD	WTR	SDR	RTP	BG	TM	PM	VAP	VSL	VCL	RAP	PMI	MMP	O_2^-	H_2O_2	LP
BCS	1.0																								
CNS	0.38	1.0																							
Age	-0.15	NC	1.0																						
WH	0.16	-0.11	NC	1.0																					
BW	0.33	NC	NC	0.14	1.0																				
NCH	0.17	0.78	NC	NC	-0.15	1.0																			
NC25	0.60	0.34	-0.24	0.13	0.60	0.24	1.0																		
NC50	0.48	0.46	-0.23	NC	0.30	0.34	0.69	1.0																	
NC75	0.30	0.18	0.26	NC	0.14	0.13	0.36	0.58	1.0																
TLD	0.53	NC	-0.11	-0.13	0.70	-0.20	0.65	0.40	0.20	1.0															
WTR	NC	0.23	0.13	-0.12	-0.41	0.19	-0.32	NC	-0.15	-0.25	1.0														
SDR	0.30	NC	-0.30	NC	NC	-0.23	0.29	0.34	0.40	0.16	0.11	1.0													
RTP	0.31	NC	NC	NC	0.15	NC	0.28	0.28	0.13	0.24	NC	0.26	1.0												
BG	-0.34	-0.58	-0.19	0.23	-0.12	-0.25	-0.12	-0.16	-0.12	NC	NC	NC	0.42	1.0											
TM	0.16	-0.17	NC	NC	0.31	-0.38	NC	0.13	0.18	0.37	-0.36	NC	0.14	NC	1.0										
PM	NC	-0.41	NC	NC	0.33	-0.52	NC	NC	NC	0.32	-0.56	-0.16	NC	NC	0.86	1.0									
VAP	-0.14	-0.50	-0.15	0.14	NC	-0.58	-0.18	NC	-0.15	-0.13	-0.35	NC	-0.18	NC	0.19	0.54	1.0								
VSL	-0.17	-0.58	-0.15	0.16	NC	-0.57	-0.15	-0.24	-0.28	NC	-0.47	-0.14	-0.13	0.17	NC	0.52	0.92	1.0							
VCL	0.11	-0.18	-0.14	NC	-0.27	-0.11	NC	0.26	NC	NC	0.20	NC	-0.18	0.11	NC	NC	0.53	0.35	1.0						
RAP	NC	-0.31	NC	NC	0.33	-0.54	-0.15	NC	0.12	0.27	-0.39	NC	NC	-0.16	0.88	0.92	0.55	0.42	0.14	1.0					
PMI	0.16	NC	0.25	0.13	NC	NC	0.18	NC	0.15	-0.17	-0.16	NC	NC	-0.38	NC	NC	0.34	0.34	NC	0.16	1.0				
MMP	NC	-0.25	NC	0.26	NC	-0.23	0.17	NC	NC	-0.12	-0.12	0.18	-0.23	-0.17	-0.31	-0.19	0.21	0.21	NC	-0.14	0.55	1.0			
O_2^-	NC	0.22	NC	NC	NC	0.19	-0.19	0.24	NC	NC	NC	-0.20	NC	NC	0.46	0.33	NC	NC	0.27	0.33	-0.50	-0.87	1.0		
H_2O_2	NC	NC	NC	NC	-0.36	NC	-0.20	-0.48	-0.34	-0.23	0.31	0.26	0.34	0.36	-0.39	-0.47	-0.31	-0.17	-0.31	-0.53	-0.16	0.15	-0.31	1.0	
LP	0.12	NC	0.20	NC	0.40	NC	NC	NC	NC	0.39	-0.42	-0.56	-0.29	-0.19	0.45	0.45	NC	NC	NC	0.39	-0.22	-0.38	0.45	-0.50	1.0

BCS, body condition score; CNS, crest neck score; WH, withers height; BW, body weight; NCH, neck crest height; NC, NC neck circumference; TLD, tailhead; WTR, withers; SDR, shoulder; RTP, retroperitoneal; BG, baseline glucose; TM, total motility; PM, progressive motility; VAP, average path velocity; (VCL) and VSL, straight-line velocity VCL, curvilinear velocity; RAP, spermatozoa with rapid movement PMI, plasma membrane integrity; MMP, membrane mitochondrial potential; O_2^-, intracellular superoxide; H_2O_2, hydrogen peroxide; LP, lipid peroxidation. The shaded area indicates moderate to strong correlations ($r > 0.5$). NC: no correlation or $r \leq 0.1$.

4. Discussion

The present study is the first to assess the associations between body morphometric parameters, fat deposition, and in stallions. The Crioulo breed was used herein as a model because the breed has been the focus of concerns regarding high body fat deposition [2,3]. However, the results can be extrapolated to other horse breeds kept under similar husbandry practices. While there were no associations between Low-BCS or High-BCS with post-thaw semen parameters, none of the stallions enrolled in the present study had a history of endocrinopathy-induced laminitis, abnormal baseline glucose concentration or glucose dysregulation after OST. It is possible that if stallions with glucose dysregulation were used (i.e., affected with equine metabolic syndrome), the results could have been different. Collectively, the findings of the present study suggest that High- or Low-BCS alone cannot explain post-thaw semen quality in stallions. It is worth noting that only a single time point was evaluated and the effects of high or low bodyfat deposition may show detrimental effects over time rather than in a single time point. Since all the stallions used herein had stable routines and diets, it unknown if having High- or Low-BCS for multiple seasons can have detrimental cumulative effects on semen freezing ability or reproductive longevity. Further studies are needed to address the effects of adiposity overtime on semen freezing characteristics.

Studies of horses with abnormal oral sugar test reported that animals with clinical signals of equine metabolic syndrome (e.g., obese and laminitis) have peak glucose concentrations starting at 75 minutes after sugar administration [3]. In the present study, the stallions had peak glucose concentrations between 30 and 60 minutes, suggesting that they were able to quickly metabolize glucose after its oral administration. One study involving horses with Low-BCS and High-BCS similar to ours suggested that low-BCS

358 horses should have fasting baseline glucose around 87 mg/dL and peak around 108
359 mg/dL, however, non-equine metabolic Crioulo horses with High-BCS had baseline
360 glucose of 92 mg/dL and then peak around 124 mg/dL after OST [3]. Horses with equine
361 metabolic syndrome are reported to have resting glycemia >110 mg/dL [17].

362 High-BCS in ruminants is associated with fat deposition in the scrotum neck,
363 resulting in problems with testicular thermoregulation and abnormal spermatogenesis
364 [5,6]. However, stallions tend to deposit fat around the external lamina of the prepuce,
365 rather than the scrotum. Therefore, increased BCS/adiposity does not appear to interfere
366 with testicular thermoregulation. If body condition/adiposity can affect semen quality and
367 fertility in stallions it would be due to mechanisms other than deposition of fat in the
368 scrotum. The present study is consistent with findings in man, where high-fat deposition
369 alone does not result in poor semen quality [7], however, other conditions associated with
370 obesity such as diabetes and hyperestrogenism, hypo-gonadotropic hypogonadism,
371 sexual dysfunctions, and sperm epigenetics disturbances can impair semen quality [23].

372 A controversy exists in practice regarding whether stallions should be kept with
373 higher (e.g., >7, scale 1-9) or lower body condition scores (e.g., <7) during the breeding
374 season. The argument against high body condition score is that popular stallions having
375 to breed or collect semen multiple times per week may struggle to do so effectively due
376 to lack of fitness and perceived lower libido than leaner stallions. Conversely, stallions
377 that are too thin or that lose too much weight during the breeding season tend to
378 experience a reduction in semen quality, thus, if the stallion starts the season with a Low-
379 BCS it could experience a further reduction in semen quality. In the present study
380 conducted in the peak of the breeding season in the Southern Hemisphere, BCS did not
381 affect semen quality, which agrees with our hypothesis. Although fertility was not

assessed in the present study, it unlikely that BCS similar to the ones in the present study would affect fertility.

When assessing BCS the typical points assessed are neck, withers, retroperitoneal space, behind the shoulders, and tail head as done in the current study. Our results indicated that subcutaneous fat deposition at the tail head and neck thickness are the variables best correlated with BCS in stallions. In the present study, the variation in BCS within groups was small (5-8). It is possible if stallions with a wider range of BCS were used, we may have yielded different results.

It is unknown if chronic insulin dysregulation and endocrinopathy-induced laminitis can affect testicular function, spermatogenesis, and sperm function in stallions. As none of the horses used in the current study had equine metabolic syndrome or endocrinopathy-induced laminitis, our findings do not allow us to make inferences about insulin metabolism and laminitis and semen quality. In addition, neither Low- nor High-BCS appeared to affect plasma membrane metabolism or oxidative stress in stallions. In bulls, thermal dysregulation is associated with increase morphologic sperm defects [5] and suggested a reduction in plasma membrane integrity [25]. Obesity in man is associated with increased oxidative stress on sperm and increased lipid peroxidation [26–28]. However, in horses with High-BCS in absence of equine syndrome metabolic, there was no increase in oxidative stress herein.

In conclusion, our findings suggest that High- or Low-BCS alone cannot explain post-thaw semen quality in stallions. It is possible that if stallions with glucose dysregulation were used (i.e., affected with equine metabolic syndrome), the results could have been different. Our results suggest that if a stallion has High-BCS, and does not have insulin-dysregulation or endocrinopathy-induced laminitis, its semen quality will likely not be affected by the high body fat deposition; however, it remains to be determined if

there is a long-term detrimental effect of High-BCS and post-thaw semen quality in stallions. Further studies are warranted to determine the chronic body fat deposition in stallions and the effects of the equine metabolic syndrome and insulin resistance on sperm parameters and reproductive efficacy of stallions.

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Author contributions

IFC and JAD designed the study. Animal sampling was completed by GN and FPL. Post-thaw semen analyses GN, CFD, LEC, and LGTMS. Data analyses, interpretation, and drafting of the manuscript GN, LGTMS, and IFC. All authors revised the manuscript before submission. IFC served as a major adviser and JAD as a co-adviser for GN’s graduate master’s committee in Animal Biotechnology.

5. References

[1] Brazilian Association of Crioulo Horse Breeders (ABCCC). Breeders manual. Pelotas: 2014,7-9. http://www.cavalocrioulo.org.br/admin/assets/upload/manuais/manual.pdf. Accessed in June 2020.

[2] Paz CFR, Paganela JC, Santos CA, Nogueira CEW, Faleiros RR. Relação entre obesidade, insulina plasmática e posicionamento da falange distal em equinos da raça crioula. Arq Bras Med Vet e Zootec 2013;65:1699–705.

[3] Cantarelli C, Dau SL, Stefanello S, Azevedo MS, De Bastiani GR, Palma HE, et al. Evaluation of oral sugar test response for detection of equine metabolic syndrome in obese Crioulo horses. Domest Anim Endocrinol 2018;63:31–7.

[4] Durham AE, Frank N, McGowan CM, Menzies-Gow NJ, Roelfsema E, Vervuert I, et al. ECEIM consensus statement on equine metabolic syndrome. J Vet Intern Med 2019;33:335–49.

[5] Coulter GH, Cook RB, Kastelic JP. Effects of dietary energy on scrotal surface temperature, seminal quality, and sperm production in young beef bulls. J Anim Sci 1997;75:1048–52.

[6] Fourie PJ, Schwalbach LM, Neser FWC, Van Der Westhuizen C. Scrotal, testicular and semen characteristics of young Dorper rams managed under intensive and extensive conditions. Small Rumin Res 2004;54:53–9.

[7] Alahmar AT, Ali Z, Muhsin Z, Qasim H. The impact of obesity on seminal fluid in men with infertility. Middle East Fertil Soc J 2018;23:346–9.

[8] Niwas Jangir R, Chand Jain G. Diabetes mellitus induced impairment of male reproductive functions: a review. Current Diabetes Reviews 2014;10:147-57.

[9] La Vignera S, Condorelli RA, Vicari E, D'Agata R, Salemi M, Calogero AE. High

456 levels of lipid peroxidation in semen of diabetic patients. Andrologia 2012;44:565–70.
457 [10] Cruz Garcia VF, Oliveira PVLF, Campos GA, Caneiro JAM, Andrade LRP, Silva
458 LFMC, et al. Alternative cooling curves for stallion semen cryopreservation. J Equine
459 Vet Sci 2016;43:S66–7.
460 [11] Papa FO, Melo CM, Fioratti EG, Dell'Aqua JA, Zahn FS, Alvarenga MA. Freezing
461 of stallion epididymal sperm. Anim Reprod Sci 2008;107:293–301.
462 [12] Frank N, Elliott SB, Brandt LE, Keisler DH. Physical characteristics, blood
463 hormone concentrations, and plasma lipid concentrations in obese horses with insulin
464 resistance. J Am Vet Med Assoc 2006;228:1383–90.
465 [13] Carter RA, Geor RJ, Burton Staniar W, Cubitt TA, Harris PA. Apparent adiposity
466 assessed by standardised scoring systems and morphometric measurements in horses and
467 ponies. Vet J 2009;179:204–10.
468 [14] Henneke DR, Potter GD, Kreider JL, Yeates BF. Relationship between condition
469 score, physical measurements and body fat percentage in mares. Equine Vet J
470 1983;15:371–2.
471 [15] Gentry LR, Thompson DL, Gentry GT, Del Vecchio RP, Davis KA, Del Vecchio
472 PM. The relationship between body condition score and ultrasonic fat measurements in
473 mares of high versus low body condition. J Equine Vet Sci 2004;24:198–203.
474 [16] Dugdale AHA, Curtis GC, Cripps P, Harris PA, Argo MM. Effect of dietary
475 restriction on body condition, composition and welfare of overweight and obese pony
476 mares. Equine Vet J 2010;42:600–10.
477 [17] Frank N. Equine Metabolic Syndrome. Vet Clin North Am Equine Pract
478 2011;27:73–92.
479 [18] Novello G, Sutili CM, Mazzuchini MP, Machado OS, Ribas LM, Rizzo FA,
480 Lisboa. FP. Comparação entre glicosímetro humano e veterinário para mensuração de

glicose durante teste oral de tolerância à glicose em equinos. XX Conferêncial Anu. ABRAVEQ, 2019, p. 160–1.

[19] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1998;38:1171-80.

[20] Freitas-Dell'Aqua CP, Guasti PN, Papa FO, Canisso IF, Dell'Aqua Junior JA. Superoxide anion is reduced in gradient selected cryopreserved stallion semen despite high mitochondrial potential. J Equine Vet Sci 2018;66:57.

[21] Freitas-Dell'aqua CP, Guasti PN, Monteiro GA, Maziero RRD, Dell'Aqua Jr JA PF. Flow cytometric analysis of fertile and subfertile frozen stallion spermatozoa. Anim Reprod 2012;9:941.

[22] Guasti PN, Freitas-Dell'Aqua CP, Maziero RRD, Hartwig FP, Monteiro GA, Lisboa FP, Papa FO. Validation of flow cytometry for assessment of membrane lipid peroxidation of equine spermatozoa. Anim Reprod 2012;9:929.

[23] Craig JR, Jenkins TG, Carrell DT, Hotaling JM. Obesity, male infertility, and the sperm epigenome. Fertil Steril 2017;107:848–59.

[24] Adcock SM, Ellerbrock R, Rothrock L, Muller V, Canisso IF. Can exogenous insulin improve sperm motility in cooled-stored stallion semen? Proc. Soc. Theriogenology Annu. Conf., 2016.

[25] Reis LSLS, Ramos AA, Camargos AS, Oba E. Integrity of the plasma membrane, the acrosomal membrane, and the mitochondrial membrane potential of sperm in Nelore bulls from puberty to sexual maturity. Arq Bras Med Vet e Zootec 2016;68:620–8.

[26] Abbasihormozi S, Babapour V, Kouhkan A, Naslji AN, Afraz K, Zolfaghary Z, et al. Stress hormone and oxidative stress biomarkers link obesity and diabetes with reduced fertility potential. Cell J 2019;21:307–13.

[27] Palmer NO, Bakos HW, Fullston T, Lane M. Impact of obesity on male fertility,

506 sperm function and molecular composition. Spermatogenesis 2012;2:253–63.

507 [28] Pearce KL, Hill A, Tremellen KP. Obesity related metabolic endotoxemia is

508 associated with oxidative stress and impaired sperm DNA integrity. Basic Clin Androl

509 2019;29:6.