UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

ESTRATÉGIAS PARA REDUÇÃO DO USO DE ANTIMICROBIANOS NA PRODUÇÃO DE DOSES INSEMINANTES DE SUÍNOS

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Tese apresentada como requisito parcial para obtenção de grau de Doutor em Ciências Veterinárias na área de Fisiopatologia da Reprodução.

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Estratégias para redução do uso de antimicrobianos na produção de doses inseminantes de suínos

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Resumo

Levando em consideração a mobilização mundial gerada pela resistência bacteriana, diversas estratégias vêm sendo desenvolvidas, com o intuito de reduzir o mau uso de antimicrobianos. No contexto da reprodução de suínos, devemos considerar que as doses inseminantes contêm antimicrobianos para o controle bacteriano, e que mais de 70% do volume infundido de uma dose sofre refluxo, propiciando o desenvolvimento de bactérias resistentes no ambiente. Considerando que a produção mundial de doses é estimada em aproximadamente $1{,}4 \times 10^7$ L/ano, a presença e disseminação de bactérias resistentes no ambiente após a IA é preocupante. Com o objetivo de reduzir a resistência bacteriana, a suinocultura está revendo o uso de antimicrobianos, buscando estratégias para o uso racional e prudente dessas drogas. O primeiro passo para redução do uso de antimicrobianos no processamento de doses de sêmen suíno é evocar a adoção de cuidados higiênicos com a contaminação bacteriana durante a coleta e processamento do ejaculado. Além disso, a redução da temperatura convencional (17°C) de armazenamento para um armazenamento de doses livres de antimicrobianos a 5°C também é uma estratégia bastante promissora, mas com desafios para os espermatozoides devido à sua sensibilidade os resfriamento. O intuito desse projeto foi, inicialmente, testar um método de coleta capaz de reduzir a contaminação bacteriana inicial durante a coleta de sêmen; além disso, objetivamos trabalhar os desafios que existem para que a redução da temperatura de armazenamento a 5 °C seja viabilizada na prática, incluindo a taxa de resfriamento e o transporte das doses inseminantes; finalmente, testamos a fertilidade de matrizes inseminadas com doses armazenadas a 5 °C, sem adição de antimicrobianos de forma a verificar a aplicação prática do uso da estratégia em condições de campo.

Palavras chave: Ejaculado, controle de qualidade, sêmen

Abstract:

Taking into account the worldwide mobilization generated by bacterial resistance, several strategies have been developed in order to reduce the misuse of antimicrobials. In the context of swine reproduction, we must consider that sêmen doses contain antimicrobials for bacterial control, and more than 70% of the infused volume undergoes reflux, favoring the development of resistant bacteria in the environment. Considering that the worldwide production of doses is estimated at approximately 1.4×10^7 L/year, the presence and dissemination of resistant bacteria in the environment after AI is of concern. In order to reduce bacterial resistance, the swine industry is reviewing the use of antimicrobials, seeking strategies for the rational and prudent use of these drugs. The first step is to encourage the adoption of hygienic care with bacterial contamination during the collection and processing of the ejaculate. Furthermore, reducing the conventional storage temperature (17°C) to storage of antimicrobial-free doses at 5°C is also a very promising strategy, but with challenges for sperm due to its sensitivity to cooling. The aim of this project was, initially, to test a collection method capable of reducing the initial bacterial contamination during semen collection; in addition, we aim to work on the challenges that exist so that the reduction of the storage temperature to 5°C is practically feasible, including the cooling rate and the transport of inseminant doses; finally, we tested the fertility of matrices inseminated with doses stored at 5 °C, without the addition of antimicrobials, in order to verify the practical application of using the strategy under field conditions.

Keywords: Ejaculate, quality control, semen

LISTA DE ABREVIATURAS E SIGLAS

µL	Microlitro
µm	Micrometro
ALH	Amplitude de deslocamento lateral da cabeça
BTS	*Beltsville Thawing Solution*
CASA	*Computer Assisted Semen Analysis*
CPS	Centrais de processamento de sêmen
DAP	Distância percorrida do trajeto médio
DCL	Distância percorrida do trajeto real
DI	Dose inseminante
DSL	Distância percorrida do trajeto em linha reta
IA	Inseminação artificial
IAPC	Inseminação artificial pós cervial
LIN	Linearidade: relação entre VSL/VCL
mL	Mililitros
MP	Motilidade progressiva
MT	Motilidade total
UFC	Unidades formadoras de colônia
VAP	Velocidade percorrida do trajeto médio
VCL	Velocidade percorrida do trajeto real
VSL	Velocidade percorrida do trajeto em linha reta
WOB	Wobble: relação entre VAP/VCL, oscilação do trajeto real em relação ao trajeto médio

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INTRODUÇÃO

A inseminação artificial é uma prática consolidada na suinocultura atual e seu sucesso depende da qualidade das doses inseminantes que serão utilizadas (BORTOLOZZO et al., 2015). Um ponto crítico para que sejam produzidas doses que atendam aos critérios mínimos de qualidade é o grau de contaminação bacteriana no ejaculado (WABERSKI et al., 2008). Além de a coleta de sêmen não ser um processo estéril (ALTHOUSE e LU, 2005) as doses são armazenadas a temperaturas de 15 a 17 °C (JOHNSON et al., 2000), o que pode promover a contaminação e proliferação de bactérias.

Com relação ao efeito negativo da bacteriospermia, é possível observar uma queda no percentual de espermatozoides móveis em doses contaminadas e um alto percentual de aglutinação espermática (MONGA & ROBERTS et al., 1994). Mesmo em amostras nas quais não se observa perda de motilidade, o efeito negativo do contato entre bactéria e espermatozoide pode ser verificado, pela maior incidência de danos e rupturas na membrana plasmática e acrossomas, que podem levar à perda da viabilidade espermática (DIEMER et al., 1996; GĄCZARZEWICZ et al., 2016). Por esse motivo, é uma prática comum adicionar antimicrobianos na composição dos diluentes de sêmen suíno.

Levando em consideração a mobilização mundial gerada pelo cenário de resistência bacteriana, diversas estratégias vêm sendo desenvolvidas, com o intuito de reduzir o uso de antimicrobianos (WHO, 2011). No contexto da reprodução de suínos, devemos considerar que as doses inseminantes contêm antimicrobianos para o controle bacteriano, e que mais de 70% do volume infundido de uma dose sofre refluxo, propiciando o desenvolvimento de bactérias resistentes no ambiente (STEVERINK et al., 2007). A produção mundial de doses é estimada em aproximadamente $1,4 \times 10^7$ L/ano, ou seja, a presença e disseminação de bactérias resistentes no ambiente após a IA é preocupante (WABERSKI, 2017). Além disso, laboratórios de processamento de sêmen suíno ocasionam a coexistência de bactérias com antimicrobianos e desinfetantes, o que, também, pode promover a resistência bacteriana nas doses (WABERSKI, 2017).

Com o objetivo de reduzir a resistência bacteriana, a suinocultura está revendo o uso de antimicrobianos, buscando estratégias para o uso racional e prudente dessas drogas. O primeiro passo para redução do uso de antimicrobianos

no processamento de doses de sêmen suíno é evocar a adoção de cuidados higiênicos com a contaminação bacteriana durante a coleta e processamento do ejaculado (SCHULZE et al., 2015). Além disso, a redução da temperatura convencional (17°C) de armazenamento para um armazenamento de doses livres de antimicrobianos a 5°C também é uma estratégia bastante promissora, (WABERSKI et al., 2019; MENEZES et al., 2020; PASCHOAL et al., 2020; JÄKEL et al., 2021), mas com desafios para os espermatozoides devido à sua sensibilidade ao resfriamento (WABERSKI et al., 1994).

Levando em conta o contexto atual e a mobilização global para redução no uso de antimicrobianos, o intuito desse projeto foi, inicialmente, testar um método de coleta capaz de reduzir a contaminação bacteriana inicial durante a coleta de sêmen; além disso, objetivamos trabalhar os desafios que existem para que a redução da temperatura de armazenamento a 5 °C seja viabilizada na prática, incluindo a taxa de resfriamento e o transporte das doses inseminantes; finalmente, testamos a fertilidade de matrizes inseminadas com doses armazenadas hipotermicamente a 5 °C, sem adição de antimicrobianos de forma a verificar a aplicação prática do uso da estratégia em condições de campo.

CAPITULO I - REVISÃO BIBLIOGRÁFICA

A inseminação artificial (IA) é a tecnologia reprodutiva mais comum na produção suína (BORTOLOZZO *et al.*, 2015). Esta biotécnica, com uso de sêmen líquido resfriado, é consolidada em rebanhos suínos tecnificados e tem contribuído para o aperfeiçoamento de vários aspectos na produção suinícola (KNOX, 2016). No entanto, para que a técnica seja eficiente, é imprescindível a utilização de doses inseminantes de ótima qualidade. Um ponto crítico para que as doses atendam aos critérios mínimos de qualidade é o grau de contaminação bacteriana (WABERSKI et al., 2008), visto que a bacteriospermia pode causar queda no percentual de espermatozoides móveis, aglutinações e rupturas ou danos na membrana plasmática e acrossoma (MONGA & ROBERTS et al., 1994; DIEMER et al., 1996; GĄCZARZEWICZ et al., 2016). Por esse motivo, são adicionados antimicrobianos na composição dos diluentes de sêmen suíno.

Levando em consideração a mobilização mundial gerada pelo cenário de resistência bacteriana, diversas estratégias vêm sendo desenvolvidas, com o intuito de reduzir o mau uso de antimicrobianos (WHO, 2011). Incialmente é importante considerar que para redução do uso de antimicrobianos no processamento de doses de sêmen suíno é necessário evocar a adoção de cuidados higiênicos com a contaminação bacteriana desde a coleta até o processamento do ejaculado (SCHULZE et al., 2015). Além disso, a redução da temperatura convencional (17°C) de armazenamento para um armazenamento de doses livres de antimicrobianos a 5°C também é uma estratégia bastante promissora, (WABERSKI et al., 2019; MENEZES et al., 2020; PASCHOAL et al., 2020; JÄKEL et al., 2021), mas com desafios para os espermatozoides devido à sua sensibilidade os resfriamento a aos efeitos das emissões de vibrações no transporte (WABERSKI et al., 1994; SCHULZE et al., 2018).

1.1 Efeito da bacteriospermia na qualidade seminal

Altos níveis de contaminação bacteriana do sêmen (bacteriospermia) estão diretamente associados com o comprometimento da qualidade espermática de doses inseminantes (ALTHOUSE et al., 2000; ALTHOUSE & LU, 2005). Os efeitos deletérios gerados pela bacteriospermia podem ser ocasionados pela ação direta das bactérias, as quais liberam substâncias tóxicas aos espermatozoides (ALTHOUSE

et al., 2000), como é o exemplo da *Escherichia coli*, ou por eventos indiretos, como a alteração de pH do meio, devido aos metabólitos resultantes da proliferação bacteriana que podem acidifica-lo (RIDEOUT et al., 1982). Além disso, vale ressaltar, ainda, que as doses inseminantes são compostas por sêmen e diluente, que é rico em substratos como a glicose, que têm como função nutrir os espermatozoides. Em situações de alta contaminação bacteriana, as bactérias competem pelos substratos presentes na dose, resultando em uma redução da fonte de energia utilizada pelos espermatozoides (RIDEOUT et al., 1982).

Os efeitos negativos da bacteriospermia incluem uma queda no percentual de espermatozoides móveis em doses contaminadas, oriunda ou não da aderência das bactérias à superfície das células espermáticas, que promove a aglutinação espermática (MONGA & ROBERTS et al., 1994). No caso de aglutinações que não geram uma redução na motilidade, seu efeito negativo pode acontecer, ainda, por danos e rupturas na membrana plasmática e acrossomas, que podem levar à perda da viabilidade espermática (DIEMER et al., 1996; GĄCZARZEWICZ et al., 2016).

Controversamente ao que foi relatado em estudos anteriores (MONGA & ROBERTS et al., 1994; DIEMER et al., 1996; GĄCZARZEWICZ et al., 2016), em um estudo mais recente (WABERSKI et al., 2019), a integridade da membrana espermática foi avaliada em doses diluídas e armazenadas a 5 °C sem antimicrobianos, e em doses armazenadas a 17 °C com antimicrobianos. As doses armazenadas a 5 °C tiveram maior crescimento bacteriano, no entanto, o percentual de células com membranas intactas não diferiu entre os tratamentos às 72 h de armazenamento. Vale ressaltar que nesse estudo um diluente de alto potencial protetivo foi utilizado, o que poderia ter neutralizado o efeito negativo da bacteriospermia até 72 h de armazenamento.

Uma proporção de 1:1 (espermatozoide:bactéria), e em alguns casos de 1:100, pode ser suficiente para gerar efeitos indesejáveis nas doses e, consequentemente, no desempenho reprodutivo do rebanho (TAMULI et al., 1984; AUROUX et al., 1991; ALTHOUSE et al., 2000). Althouse et al. (2000) utilizaram culturas bacterianas comumente isoladas no sêmen (*Alcaligenes xylosoxydans*, *Burkholderia cepacia*, *Enterobacter cloacae*, *E. coli*, *Serratia marcenscens* e *Stenotrophomonas* (*Xanthomonas*) *maltophilia*) para confirmar a atividade espermicida dessas bactérias (motilidade inferior a 30%). Foram transferidas de 10

a 15 unidades formadoras de colônias (UFC) de cada cultura bacteriana em cada dose de 80 mL contendo um total de 3,5 $\times 10^9$ de espermatozoides. Os autores observaram valores de motilidade abaixo de 30% em todas as amostras avaliadas, sendo que em 54 das 56 amostras inoculados a motilidade teve valores inferiores a 5%.

Ao passo em que alguns autores indicam que os efeitos negativos da contaminação bacteriana estão associados não apenas com o grau de contaminação, mas com o tempo de armazenamento (GĄCZARZEWICZ et al. 2016; GOLDBERG et al., 2017), outros indicam que a qualidade espermática pode se manter alta durante longos períodos de armazenamento (144 horas), mesmo quando há crescimento bacteriano (WABERSKI et al., 2019). Gączarzewicz et al. (2016) verificaram que durante 5 dias de armazenamento a motilidade espermática nas doses reduziu, ao passo que a proliferação de bactérias aeróbicas aumentou. Concomitante, Goldberg et al. (2017) verificaram efeitos deletérios aos espermatozoides em amostras contaminadas após 108 e 168 horas de armazenamento. Nesse estudo, houve redução da motilidade e aumento do percentual de defeitos acrossomais em amostras com a contaminação superior a 1,4 $\times 10^4$ UFC/mL, quando comparadas às amostras com 3,3 $\times 10^2$ UFC/mL ($P < 0,05$) de mesófilos totais. No entanto, em um estudo mais recente (WABERSKI et al., 2019) apesar do aumento da contaminação bacteriana de doses armazenadas a 5 °C sem antimicrobianos, a motilidade espermática se manteve superior a 80% durante 144 h de armazenamento.

Avaliando resultados *in vivo* da contaminação bacteriana, Martin et al. (2010) verificaram uma redução significativa no tamanho da leitegada, quando a contaminação do sêmen foi superior a 3,5 $\times 10^3$ UFC/mL. Nesse estudo, se observou uma contaminação majoritária das amostras (79%) com *Escherichia coli*. Os autores afirmaram que há uma correlação positiva entre a concentração dessa bactéria em amostras de sêmen com a aglutinação espermática, e uma correlação negativa com o tamanho da leitegada. Por outro lado Waberski et al. (2019) observaram que ao inseminar matrizes suínas com doses sem antimicrobianos armazenadas a 5°C ou com antimicrobianos, armazenadas a 17 °C, apesar do aumento de crescimento bacteriano nas doses do primeiro grupo, nenhum prejuízo foi observado nos índices produtivos de porcas e leitoas. Nesse estudo a

contaminação das doses armazenadas sem antimicrobianos chegou à magnitude de 7,6 $\times 10^3$ UFC/mL em 24 horas de armazenamento.

Os estudos indicam que existe um efeito não apenas do grau de contaminação bacteriana, mas também do tipo de bactéria contaminante, visto que algumas delas podem ser mais agressivas aos espermatozoides. Nesse sentido, uma abordagem exclusivamente quantitativa apresenta limitações, pois não considera o aspecto qualitativo, ou seja a identificação dos agentes que causam efeitos mais ou menos deletérios aos espermatozoides.

Dependendo da intensidade, a contaminação bacteriana de doses inseminantes também pode levar a prejuízos ao desempenho reprodutivo devido à ocorrência de patologias no trato genito-urinário das matrizes, que incluem endometrites, infecções sistêmicas nas matrizes e de morte embrionária (MAES et al., 2008; BUSSALLEU et al., 2011). Em programas de inseminação artificial, pode haver altas taxa de retorno ao estro e de ocorrência de secreções vulvares quando as matrizes são inseminadas com doses altamente contaminadas (3,5 $\times 10^3$ UFC/mL de *E. coli*), reduzindo o desempenho do rebanho (ALTHOUSE et al., 2000; MARTIN et al., 2010).

1.2 Fontes de contaminação do sêmen e de doses inseminantes

Apesar de o sêmen suíno ser considerado livre de bactérias, o processo de coleta e a produção de doses resultam, frequentemente, em contaminação (ALTHOUSE & LU, 2005). No caso de ejaculados de reprodutores suínos saudáveis se espera que as fontes de contaminação se originem do animal, o que inclui como fonte de contaminação as fezes, secreções prepuciais, secreções respiratórias, pelos e pele (ALTHOUSE e LU, 2005; MAES et al., 2008; WABERSKI, 2017). Existem ainda causas relacionadas à fontes não animais, que incluem principalmente ambiente, material empregado na coleta e processamento do ejaculado e água utilizada no diluente (GOLDBERG et al., 2013; SCHULZE et al., 2015).

Em estudo realizado por Althouse et al. (2000) foram identificasdas como fontes de contaminação animal fezes, fluídos da cavidade prepucial e pêlos. Concomitante com o resultado deste estudo, foi observado por Goldberg et al. (2013) que quando o líquido oriundo do divertículo prepucial entra em contato com

a luva do coletador e escorre para o copo de coleta há maior razão de chance (4,3; P = 0,001) para que ejaculados tenham contagem de mesófilos totais superior a 220 unidades formadoras de colônias por mililitro (UFC mL^{-1}). Além disso, ainda nesse estudo, se constatou que quando a duração é superior a sete minutos aumenta-se em 2,2 vezes a razão de chance de se obter ejaculados com contaminação superior a 220 UFC mL^{-1} (P = 0,035). (Tabela 1). As fontes de contaminação do sêmen suíno também incluem também o coletador, com contaminação oriunda dos cabelos, pele, secreções respiratórias e luva por eles utilizada (MAES et al., 2008; GOLDBERG et al., 2013).

A variedade de bactérias contaminantes do sêmen suíno é bastante vasta, sendo bactérias Gram negativas da família Enterobacteriaceae as mais comuns (ALTHOUSE e LU, 2005). Ao avaliar 88 amostras de sêmen suíno processado, Althouse et al. (2008) verificaram que 57% das amostras apresentavam bactérias Gram negativas, das quais 54% eram da família Enterobacteriaceae e 30% da família Pseudomonadacea. Entre as Gram positivas, a mais frequentemente isolada foi a *Leifsonia aquatica* (20%). Gączarzewicz et al. (2016) realizaram um estudo no qual 99% das amostras de ejaculados suínos avaliados tiveram presença de bactérias aeróbicas, com amplitude de 8×10^1 a $3,7 \times 10^8$ UFC/mL. Althouse & Lu (2005) identificaram as bactérias *Alcaligenes* spp *xylosoxydans*, *Burkholderia cepacia*, *Enterobacter cloacae*, *Serratia marcescens* e *Xanthomonas maltophilia* como as mais comumente encontradas nos ejaculados suínos em estudo realizado na América do Norte. Em estudo realizado na Europa, de um total de 60 ejaculados suínos de 13 rebanhos europeus, foi observada contaminação em 63% das amostras, das quais foram isoladas cepas de *Escherichia coli*, *Serratia marcescens*, *Staphylococcus epidermidis* e *Staphylococcus. aureus*, *Proteus* spp., *Streptococcus* spp. e *Pseudomonas aeruginosa* (BRESCIANI et al., 2014).

Para verificar as principais fontes de contaminação de doses inseminantes presentes dentro do ambiente laboratorial de unidades de difusão genética (UDG), Schulze et al. (2015) elencaram pontos críticos de higiene não associados aos reprodutores ou coleta de sêmen, mas sim à linha de fluxo de trabalho durante a produção na central (Tabela 1). Foram avaliados estufas de aquecimento dos copos de coleta, sistema de transporte do ejaculado do local de coleta ao laboratório, os diluentes, a face interna dos tanques de diluição, os corantes de identificação das doses, elementos de manipulação (quadros, telefone, teclado dos computadores),

superfícies das bancadas do laboratório, sistema de tratamento da água, pias ou drenos de escoamento e a água utilizada no diluente (SCHULZE et al., 2015).

Um fator de extrema importância quando se pensa em doses é a qualidade da água de deve ser purificada e livre de bactérias, pois a disseminação do agente bacteriano poderia comprometer um volume significativo das doses produzidas. Para isso a água passa por diversos processos que removem partículas grosseiras, com filtração e ultrafiltração, além de deionização e osmose reversa (SILVA et al., 2006; MEDINA et al., 2010).

Tabela 1: Principais fatores de risco de origem animal ou não animal que contribuem de forma significativa com a contaminação bacteriana em unidades de difusão genética de acordo com diferentes estudos.

Autores	**Fator**
Althouse et al., 2000	Fezes
	Fluídos do divertículo prepucial
	Pele/ pelos
	Coletador
	Água (sistemas de destilação e osmose reversa)
	Ar/sistema de ventilação
Althouse e Lu, 2005	Secreções prepuciais
	Secreções respiratórias
	Pelo e pele
Maes et al., 2008	Pele e pelos
	Coletador
	Luvas de coleta
Goldberg et al., 2013	Unidade de difusão genética
	Líquido prepucial que escorre pela luva do coletador
	Duração da coleta superior a 7 minutos
	Pênis escapou durante a coleta manual
	Higiene da luva de coleta
	Idade do cachaço
Schulze et al., 2015	Unidade de difusão genética
	Estufas de aquecimento
	Sistema de transporte: coleta – laboratório
	Diluente

Tanque de diluição
Corantes de identificação de doses
Elementos de manipulação
Sistema de água (pias e ralos)
Água utilizada no diluente

1.3 Uso de antimicrobianos nas doses e a resistência bacteriana

Considerando a existência de diversas fontes de contaminação (GOLDBERG et al., 2013; SCHULZE et al., 2015) e que as doses têm o ambiente rico em substratos, ideal para o crescimento bacteriano (RIDEOUT et al., 1982), torna-se necessária a adição de antimicrobianos aos diluentes para que a proliferação bacteriana nas doses seja controlada (ALTHOUSE et al., 2008; SCHULZE et al., 2014; SPECK et al., 2014). É importante considerar a preocupação com a resistência bacteriana frente aos antimicrobianos. No contexto da reprodução de suínos, devemos considerar que as doses inseminantes contêm antimicrobianos para o controle bacteriano oriundo da coleta e processamento, e que mais de 70% do volume de uma dose sofre refluxo propiciando o desenvolvimento de bactérias resistentes no ambiente (Steverink et al., 2007). Considerando que a produção mundial de doses é estimada em aproximadamente $1{,}4 \times 10^7$ L/ano, a presença e disseminação de bactérias resistentes no ambiente após a IA é preocupante (Waberski, 2017).

Nesse sentido, ao avaliar 37 doses de centrais norte americanas, Althouse et al. (2000) observaram que todas as bactérias isoladas foram resistentes à gentamicina. Em outro estudo realizado por Schulze et al. (2015) entre os anos de 2010 e 2011, foi observado que 88 das 334 amostras de sêmen diluído tiveram resultados positivos quando testadas a contaminação bacteriana. Levando em consideração as amostras contaminadas, 57% (50/88) das bactérias identificadas foram Gram-negativas e todas elas foram resistentes à gentamicina. Vale salientar que os antimicrobianos mais comumente utilizados na formulação de diluentes de sêmen suíno são aminoglicosídeos, enfatizando a gentamicina (ALTHOUSE & LU, 2005; ÚBEDA et al., 2013).

Em um estudo retrospectivo, Althouse e Lu (2005) encontraram evidências de resistência a antimicrobianos para outros antimicrobianos, incluindo

amoxicilina, lincomicina, tilosina e espectinomicina. Mais recentemente, Schulze et al. (2015) também identificaram resistência bacteriana para uma ampla gama de antimicrobianos utilizados tanto em saúde animal quanto humana. As bactérias foram resistentes às drogas: ampicilina, sulfato de amicacina, cefotaxima, clindamicina, enrofloxacina, neomicina, penicilina, sulfato de polimixina, sulfonamidas, espectinomicina trimetoprima, tetraciclina e tilosina.

Devido à baixa eficiência, para que seu efeito seja potencializado, tem se tornado cada vez mais comum a associação de dois ou mais antimicrobianos. As diretrizes do conselho da União Europeia 90/429/EEC estabelecem algumas concentrações máximas para adição dos antimicrobianos: 500 µg/mL de estreptomicina, 500 UI/mL de penicilina, 150µg/mL de lincomicina e 300 µg/mL de espectinomicina. No entanto, alguns dos princípios ativos citados anteriormente já são considerados como criticamente importantes em humanos segundo a organização mundial de saúde (WHO - 2011) pelo alto risco de desenvolver multirresistência.

Em termos práticos, precisamos considerar que além do volume de dose inseminante que permanece no trato reprodutivo da matriz e entra em contato direto com a fêmea, a resistência bacteriana pode ser preocupante quando se pensa no refluxo de doses inseminantes de suíno que ao refluir do trato reprodutivo das fêmeas durante a inseminação artificial e entra em contato com bactérias não resistentes do ambiente. O volume de doses de sêmen suíno varia de 50 a 100 mL (MORREL & WALLGRE, 2014) e, de acordo com Steverink et al. (1998), as matrizes têm um refluxo médio de 70% do volume infundido durante as duas primeiras horas após a inseminação artificial transcervical. No caso da inseminação pós-cervical aproximadamente 60% do volume infundido sofre refluxo, valor inferior, mas ainda assim bastante alto (SBARDELLA et al., 2014).

Nesse sentido, é importante considerar que bactérias resistentes oriundas do laboratório e uma subdosagem de antimicrobiano (que está presente na dose) entram em contato direto com o ambiente que é rico em matéria orgânica e bactérias não resistentes. Isso inclui dois processos extremamente importantes para o desenvolvimento e propagação da resistência: Uma subdosagem de antimicrobianos sendo inserida diretamente no meio, que favorece o desenvolvimento de resistência em cepas não resistentes e a transferência de genes

resistentes para o ambiente, o que promove um aumento da transferência horizontal para bactérias presentes no ambiente (HEUER AND SMALLA 2007; JECHALKE et al., 2014).

1.4 Armazenamento hipotérmico de doses suínas a 5°C

A redução da temperatura de armazenamento de 17 para 5 °C é uma estratégia promissora para viabilizar a redução do uso de antimicrobianos em doses inseminantes suínas (WABERSKI et al., 2019; PASCHOAL et al., 2020; JÄKEL et al., 2021). O armazenamento hipotérmico a 5 °C promove uma redução do crescimento bacteriano em amostras armazenadas sem antimicrobianos a 5°C quando comparadas àquelas armazenadas a 17 °C ($P < 0,05$) durante 120 horas de armazenamento (MENEZES et al., 2020). No entanto, o resfriamento a 5°C significa um grande desafio para a qualidade espermática devido à sensibilidade dos espermatozoides suínos ao resfriamento a temperaturas abaixo de 15 °C (WATSON et a., 1985; WABERSKI et al., 2019; PASCHOAL et al., 2020).

A redução da temperatura está associada com a mudança na organização e na composição da bicamada lipídica da membrana espermática (BUHR et al., 1994). Buhr et al. (1994) explicam que a medida em que a temperatura decresce, os fosfolipídios adquirem restrição no seu movimento lateral visto que as cadeias laterais de ácidos graxos tendem a se tornar menos móveis. Fosfolipídios puros em bicamadas demonstram uma transição de estado fluido para estado de gel ao atingirem a temperatura de transição, próxima aos 15 a 10 °C, abaixo da qual formam uma matriz hexagonal (PARKS e LYNCH, 1992). No entanto, quando a natureza dos fosfolipídios é mista, como no caso dos suínos, há uma grande variedade de composições das cadeias laterais, resultando em diferentes temperaturas de transição. Se os grupos lipídicos têm diferentes temperaturas de transição pode ocorrer a separação de fases, na qual algumas proteínas ficam agrupadas enquanto outras ficam excluídas das matrizes hexagonais de lipídios gelificados (DE LEEUW et al., 1990).

O colesterol presente nas membranas espermáticas também pode influenciar o comportamento lipídico (PARKS & LYNCH, 1992). A ação do colesterol é ampliar as transições de fase o que acaba minimizando a alteração e separação de fases (WATSON, 2000). Sendo assim, a proporção colesterol/fosfolipídio tem um papel importante na fluidez nas membranas plasmáticas, e espécies com maior

concentração de colesterol são menos susceptíveis aos danos causados pela baixa temperatura. No caso das células espermáticas de suínos, a proporção colesterol/fosfolipídio é menor (0,20) quando comparada à de outras espécies como carneiros (0,85), touros (0,83) e garanhões (0,36), o que resulta em mais danos ao resfriamento do sêmen pela baixa tolerância ao choque térmico pelo frio (AMANN & GRAHAM, 1993; MACK et al., 1986; PARKS e GRAHAM, 1992; WATSON, 2000; PURDY, 2006). Nesse sentido, diversos estudos têm sido desenvolvidos para avaliar estratégias para minimizar as consequências do resfriamento, como a diluição bitérmica do ejaculado (FERREIRA et al., 2005; LÓPEZ-RODRIGUEZ et al., 2012; SCHULZE et al., 2013; ALMEIDA et al., 2015) e a implementação de diferentes estratégias de resfriamento até a temperatura de armazenamento (KATZER et al., 2005).

Atualmente, um diluente que promete a preservação da qualidade espermática submetida à baixa de temperatura de armazenamento está sendo estudado. Utilizando esse diluente, Menezes et al. (2020) verificaram que doses diluídas com antimicrobianos (apramicina e de ampicilina) e armazenadas a 17 °C apresentaram motilidade total, progressiva e integridade de acrossoma superiores quando comparadas às doses diluídas sem antimicrobianos e armazenadas a 5 °C. No entanto, a motilidade espermática total e progressiva observada em doses armazenadas a 5°C foi superior (85,0% ± 1,0 e 77,7% ± 1,2, respectivamente) ao limiar mínimo aceitável (70%) para inseminação artificial (JOHNSON et al., 2000; FEITSMA, 2016). Ao avaliar os resultados de fertilidade de 817 porcas e 158 leitoas inseminadas duas vezes ao dia, com doses contendo 4 $\times 10^9$ e 2,2 $\times 10^9$ espermatozoides em 90 em 50 mL, respectivamente e com doses armazenadas a 17 °C com antimicrobianos e 5 °C sem antimicrobianos, Waberski et al. (2019) verificaram que a taxa de retorno ao estro, taxa de parto e tamanho de leitegada foram semelhantes entre os grupos.

1.5 Desafios frente ao armazenamento de doses inseminantes a 5°C

Quando pensamos em estratégias substitutivas ao uso de antimicrobianos em doses, o resfriamento a 5 °C surge como uma alternativa bastante promissora, no entanto, nos deparamos com desafios que incluem, principalmente o

processamento, com ênfase na taxa de resfriamento e o transporte, que gera vibrações e choque mecânico ao espermatozoide.

Cuidados no resfriamento de doses de sêmen suíno

A resistência do espermatozoide ao estresse do resfriamento em situação de descrésimo lento de temperature já é conhecida (Watson 1996), apesar de os mecanismos ainda não serem completamente elucidados. Manter o semen diluído por até 24 h a tamperaturas próximas a 15 °C estabiliza a arquitetura lipidica da membrane plasmática (CASA & ALTHOUSE, 2013) e eleva o nível de fosforilação de resíduos de diversas proteínas espermáticas, incluindo a proteína de choque térmico 70 (YESTE et al., 2014). Nesse sentido, os protocolos de criopreservação de sêmen implementam um período extenso de estabilização, o que também está sendo recomendado para o armazenamento a 5 °C (CASA & ALTHOUSE 2013). No entanto, períodos extensor de estabilização de doses à temperaturas superiores a 15 °C podem favorecer a contaminação bacteriana, o que gera a falha no armazenamento de doses a 5 °C. Sendo assim, o ideal seria promover o resfriamento com curvas balanceadas, evitando, assim os efeitos negativos da injúria celular ocasionada pelo resfriamento abrupto (como já discutido nessa sessão) e o crescimento bacteriano. As curvas de resfriamento ideais para promover um cenário ótimo de resfriamento de doses inseminantes suínas ainda não foram reportadas. Mas esse é um passo essencial para a incorporação do armazenamento hipotérmico de doses livres de antimicrobianos na prática.

Efeito do transporte na qualidade de doses inseminantes

Além dos desafios associados à taxa de resfriamento, é importante considerar que a consolidação da inseminação artificial levou à necessidade de otimização do uso dos reprodutores e a uma consequente centralização da produção de doses inseminantes em poucos centros (UDGs). Esse processo levou ao aumento do volume de doses sendo transportadas para as granjas e também à execução de trajetos de longas distâncias, visto que a produção está sendo concentrada em um volume menor de polos (SCHULZE et al., 2018). Apesar dos benefícios gerados com a especialização de mão de obra e otimização de uso dos machos, essa centralização tem demonstrado um desafio significativo associado a manutenção da qualidade espermática durante o transporte.

O efeito negativo gerado do transporte das doses está associado com as condições das rodovias, aceleração e vibrações (DAPONTE et al., 2013). De forma geral, o delineamento de estudos que avaliem o efeito do transporte leva em consideração um limiar do desvio padrão de mensurações geradas em aparelhos celulares incorporados com acelerômetros para detecção de buracos, fissuras, colisões ou outras anormalidades de estradas (LANE et al., 2010; KALRA et al., 2014). Todavia, muita variação nesse tipo de abordagem é observada.

Pensando avaliar de forma precisa os efeitos negativos do transporte em doses inseminantes de sêmen suíno, Schulze et al. (2018) desenvolveram um estudo com o uso de aplicativo (Transport Log 1.0) desenvolvido especialmente para detectar as emissões de vibração geradas pelo transporte. Nesse estudo doses armazenadas a 17 °C foram submetidas a emissões de vibrações (simulação do transporte de UDG até as granjas) e se observou que as emissões exerciam um efeito dependente da frequência na motilidade espermática de doses inseminantes de suínos. O efeito negativo foi observado em doses diluídas com BTS, submetidas às emissões de vibração horizontais e circulares durante 6 horas com frequência de 300 rpm.

Devido ao uso de um diluente pouco complexo (BTS), as emissões de vibração podem causar uma má regulação do pH, visto que o diluente apresenta um sistema tampão simples (JOHNSON et al., 1982). Além disso, as emissões de vibração estão associadas com a perda de CO2 da fase líquida para o ar presente nas doses, alcalinizando o meio de preservação (VYT et al., 2007). Alguns estudos indicam que a alcalinização das doses inseminantes pode ser responsável pela perda de motilidade espermática (GATTI et al., 1993; VYT et al., 2007). Há ainda evidências de redução da atividade mitocondrial e integridade do acrossoma e membrana plasmática em doses submetidas à rotação e homogeneização durante o armazenamento (SCHULZE et al., 2015; MENEGAT et al., 2017). As emissões de vibração também podem aumentar o estresse oxidativo nos espermatozoides, levando à perda de motilidade ou alterações nas propriedades de membrana devido à força de cisalhamento gerada durante o transporte (SCHULZE et al., 2018).

Levando em consideração essas informações, fica claro que as doses inseminantes armazenadas a 5 °C são submetidas a um primeiro desafio que é a taxa de resfriamento e armazenamento hipotérmico e a um segundo desafio, o

transporte a longas distâncias. Em um estudo preliminar, Paschoal et al. (2019) avaliaram o efeito da temperatura de transporte durante emissões de vibração em doses de sêmen suíno armazenadas a 5°C. Nesse estudo doses inseminantes foram submetidas a 4 horas de emissões de vibrações logo após o envase, a 22°C ou 24 horas após o envase a 5 °C. Não houve redução da motilidade das doses submetidas a emissões de vibrações logo após o envase a 22 °C comparadas com doses submetidas a emissões de vibrações 24 horas após o envase, 5 °C (77.7 ± 2.7%, 77.3 ± 3.9%, respectivamente; $P > 0.05$). Os autores concluíram que a temperatura na qual as doses são submetidas às vibrações não influenciam na qualidade espermática, no entanto, os autores enfatizem o fato de terem utilizado um diluente com protetores de membrana em sua composição (Androstar® Premium).

Desenvolver estratégias promissoras, que visam substituir o uso de antimicrobianos, como é o caso do armazenamento hipotérmico a 5 °C, é imprescindível para reduzir a resistência gerada e/ou propagada pelas doses inseminantes. No entanto, avaliar os desafios associados ao uso dessa estratégia, assim como saná-los é um passo importante para a consolidação do uso do armazenamento a essa baixa temperatura. Sendo assim, o objetivo da tese é buscar estratégias para reduzir o uso de antimicrobiano em doses de sêmen suíno. A primeira estratégia inclui a redução da contaminação bacteriana ainda durante a coleta de sêmen e o estabelecimento de alguns pontos críticos de higiene durante a coleta semi-automática. A segunda inclui desobstar os desafios associados com o armazenamento hipotérmico a 5 °C: taxa de resfriamento e transporte e verificar a fertilidade de matrizes inseminadas com doses produzidas de acordo com as taxas de resfriamento ideais, sob condições mínimas de estresse com o transporte.

CAPÍTULO II – PRIMEIRO MANUSCRITO

ADJUSTED METHOD OF PENIS FIXATION DURING BOAR SEMI-AUTOMATIC SEMEN COLLECTION AIMING TO REDUCE BACTERIAL CONTAMINATION

MANUSCRITO PUBLICADO NO PERÍODICO
REPRODUCTION IN DOMESTIC ANIMALS
(de acordo com as normas do periódico)

Adjusted method of penis fixation during boar semi-automatic semen collection aiming to reduce bacterial contamination

Short running title: Reduction of ejaculate contamination using the adjusted penis fixation

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Abstract

Semen collection has an essential role in the initial bacterial load in boar ejaculates and extended semen. The study aimed to explore the efficacy of an adjusted penis fixation in a semi-automatic collection system on reducing bacterial contamination of ejaculates in two boar studs with different scenarios. Historically, stud A had low levels of bacterial load in raw semen, while stud B had a high level of contamination. A total of 56 mature boars had their semen collected using two methods of penis fixation: 1) Traditional: the penis was fixed directly with the artificial cervix and transferred to the adjustable clamp; 2) Adjusted: the fixation was performed with one gloved-hand, and after exteriorization, the penis was gripped using the artificial cervix with the other gloved-hand and transferred to the adjustable clamp. The bacterial load ($P = 0.0045$) and the occurrence of ejaculates > 231 CFU/mL ($P = 0.0101$) were reduced in the Adjusted compared to the Traditional method. Bacterial load was reduced when using the Adjusted method in stud B ($P = 0.0011$), which showed a greater occurrence of critical factors for bacterial contamination ($P \leq 0.0034$). The Adjusted method reduced the occurrence of ejaculates > 231 CFU/mL when the preputial ostium was dirty ($P = 0.016$) and the duration of semen collection was > 7 min ($P = 0.022$) compared to the Traditional method. In conclusion, the Adjusted penis fixation was efficient in reducing bacterial load of ejaculates, mainly in boar studs with high contamination challenges.

Keywords: Aerobic mesophiles, Bacterial load, Ejaculate, Sperm, Swine

1. Introduction

Boar ejaculates usually contain bacteria due to contamination during semen collection (Althouse & Lu, 2005; Goldberg et al., 2013) that may impair *in vitro* quality (motility, agglutination, membrane integrity), and fertility (Martín et al., 2010); therefore, antimicrobials are standard components in semen extenders (Knox, 2016). However, there is an increasing worldwide bacterial resistance to conventional antimicrobials used in boar semen extenders (Schulze, Ammon, Rüdiger, Jung, & Grobbel, 2015). In this sense, some efforts have been developed to determine critical factors for contamination to reduce bacterial load in extended boar semen (Schulze et al., 2015). Establishing hygiene management guidelines for semen collection (Althouse & Lu, 2005; Goldberg et al., 2013; Schulze et al., 2015) is an essential first step to avoid the misuse of antimicrobial substances.

Due to the interest in maximizing efficiency, different types of semi-automated collection systems have been designed, allowing simultaneous boar collection, and a reduction in the number of technicians per boar and labor costs (Aneas, Gary, & Bouvier, 2008; Lellbach, Leiding, Rath, & Staehr, 2008; Terlouw et al., 2008). The number of ejaculates collected per hour has been reported as 5.7 for the manual method ("gloved-hand"), and up to 12.6 using semi-automated (Lellbach et al., 2008). Some manufacturers state that semi-automatic semen collection is a promising tool to reduce bacterial contamination (Rodriguez, Van Soom, Arsenakis, & Maes, 2017). In the gloved-hand technique, the gloved-hand may come into contact with the preputial area, previously described as a risk factor for contamination, resulting in higher bacterial load in ejaculates (Martin Rillo, Shokouhi, Boix, & Hernandez-Gil, 1998). According to Goldberg et al. (2013) preputial liquid flowing into the collection container, dirty gloves and collection

lasting more than 7 min increase the number of colony-forming units per milliliter (CFU/mL) in ejaculates.

Results concerning the bacterial load in ejaculates collected in semi-automated systems are scarce, but similar critical factors found in manual collection tend to be present. In semi-automatic semen collection, the structure used for penis fixation in some systems prevents the exposure of the ejaculate to the air during collection. Although an artificial cervix is used, a manual procedure still exists until fixing the penis in the dummy, resulting in a risk of contact between the artificial cervix and preputial region. Even with similar technology, an effect of boar stud has already been shown (Goldberg et al., 2013; Bennemann, Machado, Girardini, Sonálio, & Tonin, 2018), probably due to differences in protocol, routine procedures, and personnel training. Thus, some studs have more challenges, and a different method for penis fixation in semi-automated systems may be useful to reduce bacterial load. Therefore, this study verified the efficacy of the Adjusted method for penis fixation in semi-automatic collection on reducing bacterial contamination of ejaculates, in two different boar stud scenarios.

2. Material and methods

Procedures involving animals were performed according to the Ethics Committee on Use of Animals of the Federal University of Rio Grande do Sul, under protocol number 39447.

2.1. Boar studs and animals

The experiment was conducted on two commercial boar studs in the south of Brazil, between February and April, late summer and early autumn in the southern hemisphere. Boars were individually housed in crates (0.70 × 2.4 m) with slatted floor

and were weekly used for semen collection. Water access was *ad libitum*, and all boars were fed 2.1–2.4 kg per day of the same nutritional corn and soybean meal diet. The collection area consisted of a pre-collection pen, to clean and empty the preputial diverticulum, and a collection pen (1.0 × 2.5 m) with a semi-automated dummy (BoarMatic®, Minitüb GmbH, Tiefenbach, Germany) an adjacent pit (0.9 m height) for the technicians.

The difference concerning bacterial contamination between studs was known. Boar stud A had a controlled bacterial load in raw semen, no additional antibiotic was required; however, boar stud B had historical problems with contaminated semen doses and additional antibiotics were added to the extender.

2.2. Experimental design

Each boar stud was visited twice. On the first visit, 28 healthy mature boars (21.3 ± 7.8 months of age) were allocated to one of the methods of semi-automatic semen collection: 1) Traditional: using a clean glove, the technician performed fixation of the penis directly with the artificial cervix (Minitüb GmbH, Tiefenbach, Germany) and transferred it to the adjustable clamp; or 2) Adjusted: the initial fixation of the penis was performed with one gloved-hand. After exteriorization, the technician griped the penis using the artificial cervix with the other gloved-hand and the penis was transferred to the adjustable clamp. On the second visit, semen was collected using the other method, resulting in two collections per boar and 56 collections per stud.

Disposable collection gloves and an artificial cervix were used for each boar. Semen was collected into a thermal collection container prepared with a disposable bag and filter (BlueBag®, Minitüb, GmbH, Tiefenbach, Germany). At collection, the pre-spermatic fraction was retained with the inner liner and the gel fraction was discarded by

filtration prior to transference to the laboratory. Only appropriate ejaculates (minimum of 75% morphologically normal spermatozoa, at least 80% total sperm motility, and a total number $\geq 30 \times 10^9$ spermatozoa per ejaculate) were extended.

2.3. Critical factors for bacterial contamination

Critical factors for bacterial contamination were adapted from the factors proposed by Goldberg et al. (2013) for the gloved-hand technique (Table 1). The same technician recorded the factors during the whole experimental period.

2.4. Semen evaluation and processing

A total of 112 ejaculates were macroscopically (color, odor, volume, and aspect) and microscopically (sperm concentration, total and progressive motility with the CASA system – Androvision®, Minitüb GmbH, Tiefenbach, Germany) evaluated. Raw semen was isothermally diluted in Androstar® Plus supplemented with 0.3 g/100 mL gentamicin (Minitüb GmbH, Tiefenbach, Germany). Due to the high level of bacterial contamination, boar stud B assumed additional use of 0.02 g/100 mL of enrofloxacin (Iflox, Hipra, Brazil) per liter of extender. Semen doses were held at 21°C for 90 minutes and transported at 17 °C to the laboratory where the subsequent analyses were performed.

2.5. Semen dose analyses

Extended semen samples were evaluated for sperm motility (total and progressive) and pH at 24 h, 72 h, and 120 h of storage at 17 °C. One dose per boar was used for each day of analysis and was subsequently discarded.

A sample of 1 mL of extended semen was incubated in a metal block (Labnet Accublock HT200, Minitüb, GmbH, Tiefenbach, Germany) at 37 °C for 10 minutes for

sperm motility assessment using the CASA system equipped with a microscope (Axio Scope.A1, Zeiss®, Germany) with a magnification x 200, using chamber slides of 20 µm depth (Leja®, Nieuw-Vennep, The Netherlands) filled with 3.0 µL of sample. Immediately after sampling for motility, pH was determined using a previously calibrated digital pH-meter (Akso® pH Pro, São Leopoldo, RS, Brazil).

2.6. Evaluation of bacterial contamination

A total of 6 mL of raw semen and extended semen at 72 h of storage from each boar was aliquoted in a sterile tube and the pour plate count method in Plate Count Agar (PCA) (Acumedia®, Neogen Corporation, Lansing, Michigan, US) was used to evaluate the presence of colony-forming units. Decimal dilutions were prepared (10^0 to 10^{-5} for raw and 10^0 to 10^{-2} for extended semen) by transferring 1 mL of sample into a sterile tube with 9 mL of saline solution (0.85%) and subsequent dilution of 1 mL of each previous sample into 9 mL of saline solution (0.85%). Each tube was mixed, and 1 mL of each dilution was plated on Petri plates in duplicate. Subsequently, 15–20 mL of PCA were added to each plate, which was gently mixed on a flat surface. After solidifying the agar, a second PCA layer was added to the plates, which were incubated for 48 hours at 37 °C (Goldberg et al., 2013).

The number of colony-forming units was counted, and the average of duplicates was considered. The number of colony-forming units per milliliter (CFU/mL) of semen was calculated by multiplying the average number of the duplicates by the reciprocal of the dilution at which the count was performed.

2.7. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System software (SAS, version 9.3; SAS Inst. Inc., Cary, NC, USA). The number of CFU/mL was analyzed with the GLIMMIX procedure, logarithmic transformation for comparison of boar studs, and the method of penis fixation. Ordinal multinomial models were used to compare the method within each boar stud. For these analyses, five categories of CFU/mL were created: $\leq 10^1$ CFU/mL; $>10^1$–$\leq 10^2$ CFU/mL; $>10^2$–$\leq 10^3$ CFU/mL; $>10^3$–$\leq 10^4$ CFU/mL; $>10^4$–$\leq 10^5$ CFU/mL. Logistic regression models (PROC GLIMMIX) were used to investigate the occurrence of the critical factors for contamination that may contribute to high bacterial contamination in the ejaculates. Depending on the presence of a given critical factor, the methods were compared on the occurrence of high contaminated ejaculates (> 231 CFU/mL), based on the median value of CFU/mL observed in both studs. Repeated measures using PROC GLIMMIX were used for sperm motilities and pH analyses. The fixed effect included the category of contamination, storage time, and the interaction between them. The differences were considered significant if P was ≤ 0.05 and tendency if $P > 0.05$ and $P \leq 0.10$.

3. Results

A total of 99.1% (111/112) of the ejaculates had bacterial growth. Considering only the Traditional method, ejaculates from stud A showed lower bacterial contamination than ejaculates from stud B (Fig. 1; $P < 0.0001$). The medians observed for the Traditional method were 135.5 CFU/mL (range: 4–10,200 CFU/mL) and 1,992.5 CFU/mL (range: 19–39,000 CFU/mL) for raw semen samples from boar studs A and B, respectively. The frequency of samples with bacterial load > 231 CFU/mL was lower ($P = 0.0035$) in stud A (35.7%; 20/56) compared to B (64.3%; 36/56). Moreover, the median of bacterial load ($P = 0.0045$) and the occurrence of ejaculates > 231 CFU/mL (P =

187 0.0101) were reduced in the Adjusted method (79.5 CFU/mL and 37.5%, respectively),
188 compared to the Traditional (875 CFU/mL and 62.5%, respectively).

189 Regarding each boar stud, in stud A the use of the Adjusted method did not reduce
190 ($P = 0.3545$; Fig. 2) bacterial load (median: 70.5 CFU/mL), but in stud B, the Adjusted
191 method was efficient in reducing ($P = 0.0011$; Fig. 2) the bacterial load in ejaculates
192 (median: 170.5 CFU/mL).

193 The average bacterial load in extended boar semen at 72 h of storage was 1.7
194 CFU/mL (median: 0.5 CFU/mL; range: 0–15.0 CFU/mL), with no effect of the method
195 used for penis fixation at semen collection in studs A ($P = 0.8874$) or B ($P = 0.6355$).

196 The occurrence of critical factors in each boar stud is presented in Table 2. A
197 higher frequency of the penis escaping during semen collection was observed in boar stud
198 A ($P = 0.0008$). However, boar stud B showed a higher occurrence ($P \leq 0.0034$) of dirty
199 dummies, boars with dirt on the body, boars with a dirty preputial ostium, preputial liquid
200 removed in the collection pen, and from the tenth boar onward collected on the same
201 dummy than stud A.

202 Considering both studs (Table 3), the use of the Adjusted method for semen
203 collection reduced the occurrence of ejaculates with > 231 CFU/mL when the preputial
204 ostium was dirty ($P = 0.016$), and the duration of semen collection was > 7 min ($P =$
205 0.022) and tended to reduce from the tenth boar onwards collected on the same dummy
206 ($P = 0.076$), compared to the Traditional method.

207 Total and progressive motilities of ejaculates were no different between methods
208 (92.7% and 85.7%, respectively in stud A, and 91.8% and 83.7%, respectively in stud B).
209 In boar stud A, total and progressive motilities were reduced at 120 h of storage compared
210 to 24 h ($P \leq 0.042$; Table 4), with no influence observed by method nor by interaction
211 between method and storage time ($P \geq 0.846$). In boar stud B, the 2-way interaction

affected total (P = 0.021) and progressive motility (P = 0.039). In the Traditional method, the sperm motilities reduced over storage time, while in the Adjusted method, the sperm motilities reduced at 72 h, remaining constant at 120 h of storage. The pH in samples from boar stud A and B increased (P < 0.001) during storage.

4. Discussion

The main particularity of the semi-automated system used in our study is that the penis tip and collection cup remain protected against air contaminants during ejaculation, reducing the risk of bacterial contamination. Another feature in this device is the removal of an inner liner after the pre-sperm fraction ejaculation (Kuster and Althouse, 2016). The use of the gloved-hand method results in greater exposure to bacterial contaminants. In order to maximize semen collection efficiency and reduce bacterial contaminants, boar studs have been choosing to use semi-automatic semen collection rather than the gloved-hand method (Aneas et al., 2008; Terlouw et al., 2008). Although an artificial cervix is used, it is important to emphasize that manual assistance precedes the fixation of the penis in the dummy of the automated device. Thus, the risk of the artificial cervix coming into contact with the preputial region persists. Differences in contamination levels among boar studs have already been reported (Goldberg et al., 2013). In this study, we documented a reduction in bacterial load using the Adjusted method of penis fixation in semi-automated collection if the boar stud had high contamination challenge.

More than 99% of the ejaculates were positive for bacterial load in our study, similar to studies performed with the gloved-hand method (Bennemann et al., 2018; Martín et al., 2010). In fact, the expected and claimed reduction of ejaculate contamination in semi-automated systems is not well established. No evidence for difference was found (Terlouw et al., 2008) between gloved-hand and semi-automatic

methods (28.5 vs. 19.6 CFU/mL, respectively; P = 0.158). Thus, using a semi-automated system does not prevent bacterial contamination by itself, i.e., some factors may be still present influencing the bacterial load.

The critical factors for bacterial contamination during semen collection are well established for the gloved-hand method (Goldberg et al., 2013); however, for semi-automatic semen collection, the information is scarce. Based on the critical factors previously described for manual collection, we observed a variation in occurrence between studs. The higher contamination in ejaculates from stud B may be explained by the presence of dirt on the dummies, boars, and preputial ostium. Moreover, removing preputial liquid in the collection pen and collection from the tenth boar onwards on the same dummy, also contribute to contamination. The lack of boar hygiene, mainly the preputial area, seems to lead to high contamination since the preputial liquid is one of the primary sources of contamination during semen collection (Martin Rillo et al., 1998). Furthermore, the association of two or more risk factors has been shown to enhance bacterioal load in ejaculates (Goldberg et al. 2013). Therefore, sanitary and hygienic measures during semen collection should involve several procedures to reduce bacterial load.

There was no significant evidence that the Adjusted method reduced the occurrence of ejaculates > 231 CFU/mL when the boars were dirty or got down from the dummy during collection. However, if dirt was present in the preputial region, the Adjusted method prevented the occurrence of samples > 231 CFU/mL. In fact, boars considered as dirty and the penis escaping during the manual semen collection have little impact on the occurrence of highly contaminated ejaculates (Goldberg et al., 2013). Boars getting down from the dummy and the penis escaping during semen collection are frequent events related to personnel training. For this study, the technicians were

previously trained to use the method proposed, and it was expected that using a new approach could bring difficulty for penis fixation. However, the new method proved to be simple to consolidate in practice.

Semen collection techniques have some recommended practices for minimum contamination (Althouse et al., 2000) that have been adapted for use worldwide. Simple modifications such as grasping the penis with the thumb and extending the forefinger, prevents the trickling of preputial liquid into the collection container and reduces the proportion of contaminated ejaculates (Goldberg et al., 2013). As previously discussed, the preputial liquid represents a significant source of contamination (Martin Rillo et al., 1998); therefore, the lower contamination in the Adjusted method could be attributed to the fewer contact between the artificial cervix and prepuce. As the semi-automatic method starts with a manual procedure, it is possible to assume that the penis should not be first grasped by the artificial cervix, but after exteriorization, since the penis tip is away from the preputial region, and the artificial cervix can be safely used. A similar technique has been described for manual collection as the three-glove collection method (Reicks, Kuster, & Rossow, 2015), consisting of one glove to evacuate the prepuce and two gloves to grasp and re-grasp the penis. A reduction in bacterial load is expected, although no information is available in the literature to the best of our knowledge.

The tendency of reducing the frequency of samples with > 231 CFU/mL in the Adjusted method, from the tenth boar onwards on the same dummy, evidenced the importance of using clean dummies to reduce contamination throughout the collection routine. The dirt of the ventral region, including the prepuce, favors cumulative dummy contamination after successive collections. It is commonly recommended that the semen collection area and any collection equipment should be thoroughly cleaned and disinfected at the end of each collection day (Althouse & Lu, 2005). Our findings suggest

that dry cleaning could be performed along with the collection routine to reduce contamination in the last ejaculates collected. Wet products or water must be avoided during the routine but can be used at the final cleaning.

A long duration of semen collection has been reported as a risk factor for highly contaminated ejaculates (Goldberg et al., 2013). The Adjusted method reduced contamination when the semen collections lasted > 7 min. This finding must be interpreted with caution as the penis tip and container are protected from air contaminants and the preputial liquid, regardless of the method used. One possible explanation for the reduced contamination is that the artificial cervix is likely to be more contaminated in the Traditional method; the longer the collection process, the longer the contact of ejaculate with bacteria present in that material.

Bacteriospermia is detrimental to semen quality as sperm agglutination and reduced motility are commonly observed (Althouse et al., 2000). The type and ratio of sperm:bacteria, bacteriospermia may also decrease the longevity of the sperm during storage (Sepúlveda, Bussalleu, Yeste, Torner, & Bonet, 2013; Sepúlveda, Bussalleu, Yeste, & Bonet, 2016) and impair fertility, causing vulvar discharges, reduced litter size, and higher regular return to estrus rate (Althouse et al., 2000; Martín et al., 2010). It is essential to consider that not only the negative effect of bacteriospermia is worrying but also the increase of bacteria isolated from extended semen showing antibiotic resistance (Kuster & Althouse, 2016). Antimicrobials, most commonly gentamicin, are added to semen extender to control disease transmission and bacterial growth; however, bacterial resistance to this drug has been reported (Schulze et al., 2015). In our study, the reduced bacterial load of extended semen during storage in both studs suggests that the bacterial contaminants were sensitive to the antibiotics. However, the inclusion of additional antibiotics can alter the resistant bacteria profile and should not be encouraged as a long-

term solution (Kuster and Althouse, 2016), as adopted by stud B. Thus, reducing the bacterial load with procedures for minimum contaminations is beneficial. Controlling the risk factors for bacterial contamination and improving personnel training have been proven to decrease contaminated extended semen samples from 19% to 5.5% after consecutive audits (Nitsche-Melkus, Bortfeldt, Jung, & Schulze, 2020).

In boar stud A, sperm motility was only affected by storage time. The aging process is determined by the conditions and length of storage, even when long-term extenders are used (Johnson, Weitze, Fiser, & Maxwell, 2000). On the contrary, in boar stud B, an interaction between method and storage time was observed. Although the antibiotic agent in the extender was effective against the bacterial load in semen doses, this interaction may indicate that the initial contamination might have influenced the storage environment. We speculate that initial bacterial contamination, even if controlled with antibiotics, can play a role in sperm metabolism during storage due to the release of metabolites in the ejaculate. It is also important to emphasize that sperm motility had a slight decrease, but this effect may not be biologically relevant. However, further studies are required to elucidate the impact of the contamination of ejaculate on extended semen with controlled bacterial growth.

The pH rise during storage may occur due to the loss of CO_2 from the air in the semen dose, which could be explained by the presence of a buffering system in the extender (Vyt, Maes, Sys, Rijsselaere, & Van Soom, 2007). In highly contaminated samples, a decrease in pH and toxic effect in sperm would be expected, as bacterial growth leads to pH acidification (Althouse et al., 2000; Althouse & Lu, 2005; Prieto-Martínez, Bussalleu, Garcia-Bonavila, Bonet, & Yeste, 2014). It is possible to infer that, as the bacteria were susceptible to the antimicrobials used, the low bacterial load in extended semen doses prevented acidification during storage.

5. Conclusion

In boar studs with a high contamination challenge, using the Adjusted method of penis fixation for semi-automatic semen collection efficiently reduces bacterial contamination of ejaculates. This new method is also useful when the preputial region is dirty or in long semen collections.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Authors Contribution

Paschoal AFL performed experiment ‘on field’, laboratory and bacteriological analysis, collaborated on data analysis and wrote the manuscript; Mellagi APG collaborated on study design, data analysis and manuscript revision; Ferrari CV, collaborated on experiment ‘on field’; Takeuti KL and Oliveira GS contributed with bacteriological analysis, Bernardi ML, Ulguim RR and Bortolozzo FP collaborated with experimental design, data analysis and manuscript review.

Data availability statement

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

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Table 1. Critical factors for contamination of boar ejaculates evaluated on two studs during semi-automatic semen collection

Penis escaped from the hand of the technician or the clamp during collection
Presence of dirt and/or urine on the dummy during semen collection
Presence of perceptible dirt such as feces on the boar's body
Presence of perceptible dirt on the preputial region of the boar
Preputial hair length longer than 1.0 cm
Preputial liquid was removed in the collection pen, instead of in the pre-collection pen
Boar got down from the dummy at least once during semen collection
Incomplete removal of the pre-spermatic fraction of the ejaculate, which remains in the inner liner
From the tenth boar onwards collected on the same dummy
Semen collection lasted more than 7 min

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Table 2. Frequency of critical factors for contamination during semi-automatic semen collection in boar studs A (n = 56 ejaculates) and B (n = 56 ejaculates)

Critical fator	Stud A	Stud B	P-value
Penis escaped from the hand of the technician or the clamp during collection (%) (n/n)	41.1 (23/56)	10.7 (6/56)	0.0008
Presence of dirt and/or urine on the dummy during semen collection (%) (n/n)	17.9 (10/56)	100 (56/56)	0.0002
Presence of perceptible dirt such as feces on the boar's body (%) (n/n)	41.1 (23/56)	69.6 (39/56)	0.0034
Presence of perceptible dirt on the preputial region of the boar (%) (n/n)	10.7 (6/56)	62.5 (35/56)	<0.0001
Preputial hair length longer than 1.0 cm (%) (n/n)	0.0 (0/56)	25.0 (14/56)	<0.0001
Preputial liquid removed in the collection pen, instead of in the pre-collection pen (%) (n/n)	0.0 (0/56)	28.6 (16/56)	<0.0001
Boar got down from the dummy at least once during collection (%) (n/n)	30.4 (17/56)	35.7 (20/56)	0.5483
Incomplete removal of the pre-spermatic fraction of the ejaculate, which remains in the inner liner (%) (n/n)	1.8 (1/56)	0.0 (0/56)	0.3151
From the tenth boar onward collected on the same dummy (%) (n/n)	12.5 (7/56)	44.6 (25/56)	0.0005
Semen collection lasted more than 7 min (%) (n/n)	33.9 (19/56)	41.1 (23/56)	0.4372

Table 3. Occurrence of ejaculates > 231 CFU/mL of aerobic mesophiles, according to the method of penis fixation in the semi-automatic semen collection system in the presence of some critical factors for high contamination

	Method	>231 CFU/mL (%) (n/n)	95% CI	P-value
Presence of perceptible dirt such as feces on the boar's body (n = 62)	T	67.7 (21/31)	0.156–1.279	0.131
	A	48.4 (15/31)		
Presence of perceptible dirt on the preputial region of the boar (n = 41)	T	90.9 (20/22)	0.019–0.649	0.016
	A	52.6 (10/19)		
Boar got down from the dummy at least once during collection (n = 37)	T	57.1 (8/14)	0.172–2.748	0.587
	A	47.8 (11/23)		
From the tenth boar onward collected on the same dummy (n = 71)	T	68.6 (24/35)	0.153–1.099	0.076
	A	47.2 (17/36)		
Semen collection lasted more than 7 min (n = 42)	T	72.7 (16/22)	0.052–0.782	0.022
	A	47.2 (7/20)		

T – Traditional method

A – Adjusted method

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Table 4. Characteristics of extended semen according to the method of penis fixation during semen collection (LSMeans ± SEM)

		Boar stud A		P-value			Boar stud B		P-value		
		Traditional (n=28)	Adjusted (n=28)	Method	Time	Method × Time	Traditional (n=28)	Adjusted (n=28)	Method	Time	Method × Time
Total motility (%)	24 h	84.8±1.7A	84.7±1.7A	0.766	0.042	0.846	90.0±0.8A	89.7±0.8A	0.545	<0.001	0.021
	72 h	84.0±1.7AB	82.9±1.8AB				88.6±0.9B	86.4±1.1B			
	120 h	82.4±1.9B	81.5±1.9B				86.0±1.1C	86.3±1.1B			
Progressive motility (%)	24 h	76.4±2.3A	75.4±2.4A	0.654	0.022	0.945	83.7±1.3A	83.1±1.3A	0.646	<0.001	0.039
	72 h	74.8±2.5AB	73.0±2.6AB				80.1±1.5B	77.4±1.6B			
	120 h	72.6±2.2B	76.4±2.3B				76.8±1.7C	77.5±1.6B			
pH	24 h	7.14±0.01A	7.14±0.01A	0.833	<0.001	0.870	6.99±0.01A	7.00±0.01A	0.393	<0.001	0.508
	72 h	7.25±0.01B	7.26±0.01B				7.10±0.03B	7.14±0.03B			
	120 h	7.33±0.01C	7.34±0.01C				7.21±0.02C	7.22±0.02C			

Traditional: fixation of the penis directly with the artificial cervix; Adjusted: first fixation performed with one hand and the second, with the artificial cervix; A, B, C: different letters mean difference among the Time within each Method in a boar stud

Figure 1. Bacterial load in ejaculates of each boar stud (A and B), considering the Traditional (fixation of the penis directly with the artificial cervix) method of penis fixation (n = 56 samples).

435 **Figure 2.** Degree of bacterial contamination in ejaculates (n = 112 samples),
436 according to boar stud (A and B) and method of penis fixation. Traditional: fixation of
437 the penis directly with the artificial cervix; Adjusted: first fixation performed with one
438 hand and the second, with the artificial cervix.

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Preputial hair length longer than 1.0 cm
Preputial liquid was removed in the collection pen, instead of in the pre-collection pen
Boar got down from the dummy at least once during semen collection
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Presence of perceptible dirt such as feces on the boar's body (n = 62)	T	67.7 (21/31)	0.156–1.279	0.131
	A	48.4 (15/31)		
Presence of perceptible dirt on the preputial region of the boar (n = 41)	T	90.9 (20/22)	0.019–0.649	0.016
	A	52.6 (10/19)		
Boar got down from the dummy at least once during collection (n = 37)	T	57.1 (8/14)	0.172–2.748	0.587
	A	47.8 (11/23)		
From the tenth boar onward collected on the same dummy (n = 71)	T	68.6 (24/35)	0.153–1.099	0.076
	A	47.2 (17/36)		
Semen collection lasted more than 7 min (n = 42)	T	72.7 (16/22)	0.052–0.782	0.022
	A	47.2 (7/20)		

T – Traditional method

A – Adjusted method

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Table 4. Characteristics of extended semen according to the method of penis fixation during semen collection (LSMeans ± SEM)

		Boar stud A			P-value		Boar stud B			P-value	
		Traditional (n=28)	Adjusted (n=28)	Method	Time	Method × Time	Traditional (n=28)	Adjusted (n=28)	Method	Time	Method × Time
Total motility (%)	24 h	84.8±1.7A	84.7±1.7A	0.766	0.042	0.846	90.0±0.8A	89.7±0.8A	0.545	<0.001	0.021
	72 h	84.0±1.7AB	82.9±1.8AB				88.6±0.9B	86.4±1.1B			
	120 h	82.4±1.9B	81.5±1.9B				86.0±1.1C	86.3±1.1B			
Progressive motility (%)	24 h	76.4±2.3A	75.4±2.4A	0.654	0.022	0.945	83.7±1.3A	83.1±1.3A	0.646	<0.001	0.039
	72 h	74.8±2.5AB	73.0±2.6AB				80.1±1.5B	77.4±1.6B			
	120 h	72.6±2.2B	76.4±2.3B				76.8±1.7C	77.5±1.6B			
pH	24 h	7.14±0.01A	7.14±0.01A	0.833	<0.001	0.870	6.99±0.01A	7.00±0.01A	0.393	<0.001	0.508
	72 h	7.25±0.01B	7.26±0.01B				7.10±0.03B	7.14±0.03B			
	120 h	7.33±0.01C	7.34±0.01C				7.21±0.02C	7.22±0.02C			

Traditional: fixation of the penis directly with the artificial cervix; Adjusted: first fixation performed with one hand and the second, with the artificial cervix; A, B, C: different letters mean difference among the Time within each Method in a boar stud

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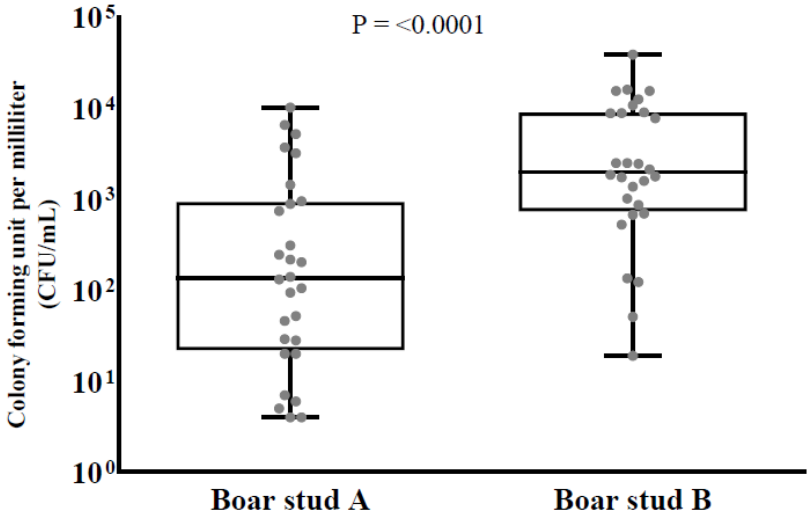

Figure 1. Bacterial load in ejaculates of each boar stud (A and B), considering the Traditional (fixation of the penis directly with the artificial cervix) method of penis fixation (n = 56 samples).

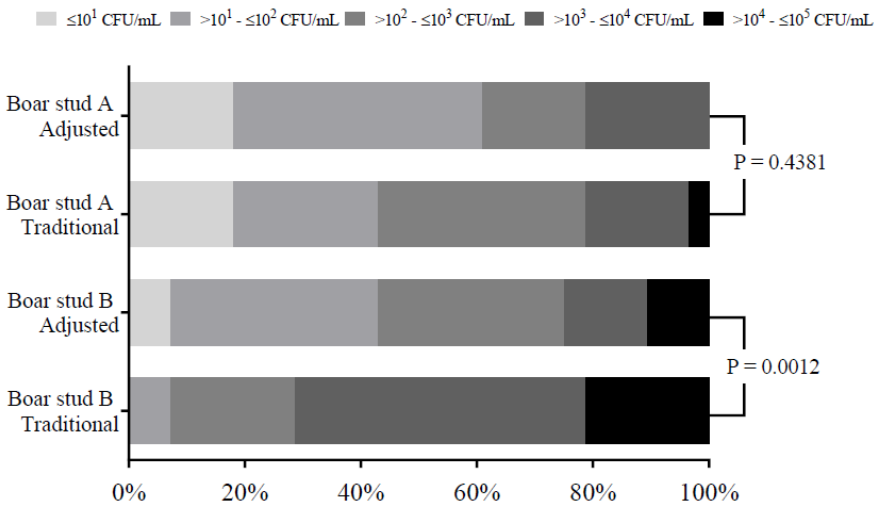

Figure 2. Degree of bacterial contamination in ejaculates (n = 112 samples), according to boar stud (A and B) and method of penis fixation. Traditional: fixation of the penis directly with the artificial cervix; Adjusted: first fixation performed with one hand and the second, with the artificial cervix.

CAPÍTULO III – SEGUNDO MANUSCRITO

DETERMINATION OF A COOLING-RATE FRAME FOR BOAR SEMEN PRESERVED AT 5 °C IN AN ANTIBIOTIC-FREE EXTENDER

MANUSCRITO PUBLICADO NO PERÍODICO

PLOSONE

(de acordo com as normas do periódico)

Determination of a cooling-rate frame for boar semen preserved at 5 °C in an antibiotic-free extender

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Short title

Cooling-rates for boar semen preserved at 5 °C in an antibiotic-free extender

Abstract

Hypothermic storage of boar semen provides the possibility to omit antibiotics from semen extenders so long as sperm quality is maintained and bacterial growth prevented. The objective of this study was to determine an optimal cooling-rate frame for boar semen preserved at 5 °C in an antibiotic-free extender. Semen from eight boars extended in AndroStar® Premium was cooled from 30 °C to 5 °C using seven different cooling rates, ranging initially from 0.01 to 0.36 °C min^{-1} and reaching 5 °C between 2 h and 24 h after dilution. Sperm motility, membrane integrity, membrane fluidity, mitochondrial membrane potential and the response to the capacitation stimulus bicarbonate remained at a high level for 144 h at 5 °C when the semen was initially cooled in a cooling-rate frame ranging from 0.01 to 0.09 °C min^{-1} in the temperature zone from 30 to 25°C, followed by 0.02 to 0.06 °C min^{-1} to 10 °C and 0.01 to 0.02 °C min^{-1} to the final storage temperature. A cooling rate of 0.07 °C min^{-1} in the temperature zone from 30 to 10 °C led to a reduced response to bicarbonate ($P < 0.01$) and fast cooling to 5 °C within 1 h with a cooling rate of 0.31 °C min^{-1} resulted in lower values ($P > 0.05$) of all sperm parameters. In a further experiment, slow cooling with a holding time of 6 h at 22 °C induced after 6 h storage a temporary increase in *Escherichia coli* of 0.5×10^3 to 2.4×10^3 CFU mL^{-1} in the sperm-free inoculated extender. Overall, the load of mesophilic bacteria in the stored semen was below 6×10^3 CFU mL^{-1}, a level that was not regarded as critical for sperm quality. In conclusion, appropriate cooling protocols were established for the antibiotic-free storage of boar semen at 5 °C, allowing the application of hypothermic preservation in research and in artificial insemination.

Keywords: Bacteria, chilling, hypothermic storage, semen preservation, boar spermatozoa

Introduction

Artificial insemination (AI) in pigs is a highly efficient breeding technology used worldwide and currently applied in more than 90% of the sows in the main pork-producing countries. Despite the merits of AI in promoting herd health and genetic progress, there is an increasing concern about the role of antibiotics in boar semen extenders in the development of antimicrobial-resistant bacteria and the global spread of resistance genes [1]. Currently, the addition of antibiotics is mandatory in many countries, but antimicrobial alternatives are under research [2,3]. To counteract increasing antimicrobial resistance, efforts have been developed to reduce bacterial load during semen collection, processing and storage [4,5]. The reduction of the conventional storage temperature of boar spermatozoa from 17 °C to 5 °C was recently proposed as a novel concept to abolish the use of antibiotics in semen extenders [6]. Beside the bacteriostatic effect of low-temperature storage, there is an increasing interest in the preservation of boar semen at 5 °C to facilitate temperature stabilization during the transport of semen from the AI center to the sow farm, especially when environmental temperatures are unstable or extreme. However, cold damage to boar spermatozoa is an important concern during storage at such low temperatures [7,8], and it also precludes the large-scale use of cryopreserved boar semen. Compared to other species, boar sperm's sensibility to chilling injury is associated with a low sterol/phospholipid ratio and a thermotropic phase transition in membrane lipids between 30 °C and 10 °C [9, 10].

It is well established that delayed cooling increases the resistance of sperm to cooling stress [11], although the underlying mechanisms are only partially known. Holding the diluted semen for up to 24 h at a temperature above 15 °C stabilizes the lipid architecture of the plasma membrane [12] and increases the phosphorylation levels of serine residues of several sperm proteins, including heat-shock protein 70 [13]. Holding times have therefore been implemented in boar sperm cryopreservation protocols and have also been recommended for sperm protection in 5 °C storage [12]. Extended holding times above 15 °C, however, may favor bacterial growth, and thus frustrate antibiotic-free storage strategies at supra-zero temperatures. Hence, ideally, cooling rates would balance the risks of chilling injury and bacterial growth. From this perspective, the optimal cooling rates for boar semen to be stored at 5 °C have not yet been reported. For the further development and incorporation of the hypothermic storage concept into AI practice, determining a cooling-rate frame that concurrently is tolerated by spermatozoa and inhibits bacterial growth is required. Therefore, the objective of this study was to determine upper and lower cooling-rate limits for chilling boar spermatozoa in a protective antibiotic-free extender to the desired storage

temperature of 5 °C. Sperm functionality was assessed with sensitive flow cytometric assays and bacterial growth during storage up to 144 h was considered.

Materials and Methods

Reagents and media

Chemicals were of analytical grade and, unless otherwise stated, purchased from Sigma-Aldrich (Steinheim, Germany). Fluorochromes were obtained from Life Technologies (Darmstadt, Germany), and semen extender media were obtained from Minitüb (Tiefenbach, Germany).

Animals and semen processing

A total of eight mature, fertile boars of different breeds (Landrace and Pietrain), under routine semen collection for the production of semen doses, were used. The boars were housed in individual pens with free access to water. All procedures involving animals were performed according to the European Commission Directive for Pig Welfare and were approved by the Institutional Animal Welfare Committee of the University of Veterinary Medicine, Hannover. Semen was collected by the gloved-hand method by trained personnel from the University service dedicated to animal care. At collection, the pre-spermatic phase was discarded and the gel fraction removed by gauze filtration, so that the sperm rich and the sperm poor fractions were collected. Normospermic ejaculates only (a minimum of 75% morphologically normal spermatozoa, at least 70% total sperm motility, and total number ≥30 × 10^9 spermatozoa per ejaculate) were isothermally (32 °C) diluted in one step with antibiotic-free AndroStar® Premium extender (Minitüb, Germany) to 2.0 × 10^6 spermatozoa mL^{-1} in a final volume of 90 mL. Semen doses were then cooled to 5 °C as described below and stored at 5 ± 1 °C in the dark in a temperature-controlled refrigerator (14160/0352, Minitüb, Germany).

Semen packaging and cooling

Cooling curves as shown in Figure 1 were obtained by variations of semen packaging and holding times. Freshly extended semen was added to tubes (Quick Tip Flexitubes®, Mintüb) to a volume of 90 mL and placed in the center of cardboard boxes surrounded by water-filled tubes. The temperature of all filled tubes was 30 °C, corresponding to the temperature of the freshly extended semen. Cooling Curves A, Aa, Ab and B were obtained with 35 tubes in a cardboard box (31.5 × 21.5 × 10.0 cm) and the use of different holding times at 22 °C before storage at 5 °C. Holding times: Curve A, 6 h; Curve Aa, 4 h; Curve Ab, 2 h; and Curve B, 0 h. Cooling Curve C was obtained with 13 filled tubes in a cardboard box (21.5

× 15.0 × 10 cm), and cooling Curve D was realized with six filled tubes. To obtain cooling Curve E, a single semen tube without packaging was placed into the 5 °C storage cabinet directly after semen dilution. For cooling Curves B, C, D and E, no holding times were applied.

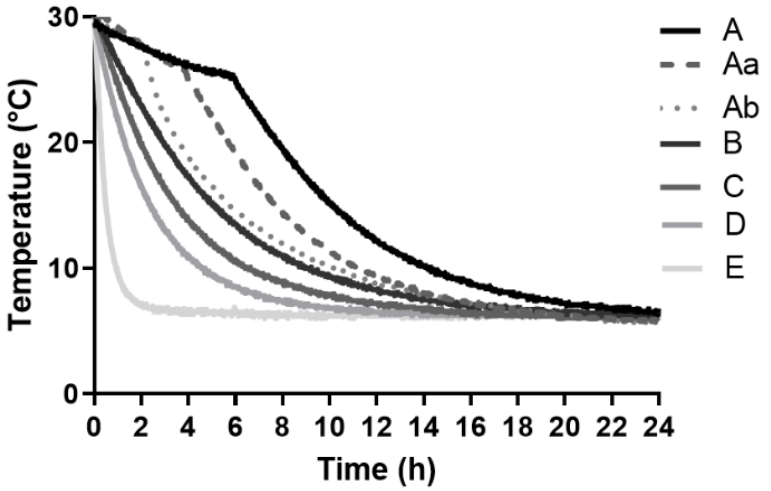

Figure 1. Graphic presentation of the temperature curves used in Experiment 1 (A, B, C, D and E) and Experiment 2 (A, Aa, Ab, B) for cooling semen doses from 30 ± 1 °C to 5 ± 1 °C). Samples cooled by Curve A, B, C, D and E had an initial cooling rate of 0.01, 0.06, 0.09, 0.14 and 0.65 °C min^{-1}, respectively, until reaching 25 °C. After that, samples were cooled to 10 °C at the rates of 0.03, 0.03, 0.05, 0.06 and 0.31 °C min^{-1}, respectively. Finally, samples were cooled to 5 °C at a rate of 0.01 °C min^{-1} (Curves A, B, C and D) and at 0.02 °C min^{-1} (Curve E). Samples cooled by Curves Aa and Ab had an initial cooling rate of 0.04 and 0.02 °C min^{-1}, respectively, until reaching 25 °C. Samples were then cooled to 10 °C at a rate of 0.04 °C min^{-1}. The final cooling from 10 °C to 5 °C was performed with cooling rates of 0.01 °C min^{-1}. The corresponding exponential regressions are described in Table 1.

Temperature recording during cooling was performed with a multiple-channel datalogger (Mikromec® Multisens MLm 424, Technetics GmbH, Freiburg, Germany) equipped with a flexible sensor positioned in the center of a tube filled with water. This tube was placed inside a box in the central position. The temperature was recorded every 1 min for 24 h. An extra-flexible sensor was placed outside the box to record the temperature of the refrigerator. The respective cooling curves are presented in Figure 1, and the exponential regressions are described in Table 1.

Experimental designs

Experiment 1

Semen doses prepared from one ejaculate of each boar (n = 8) were cooled to 5 °C using five (A, B, C, D and E) different cooling rates and stored for 144 h (Figure 1). Samples cooled by Curve A, B, C, D and E had an initial cooling rate of 0.01, 0.06, 0.09, 0.14 and 0.65 °C min^{-1}, respectively, until reaching 25 °C. After that, samples were cooled to 10 °C at the rates of 0.03, 0.03, 0.05, 0.06 and 0.31 °C min^{-1}, respectively. Finally, samples were cooled to 5 °C at a rate of 0.01 °C min^{-1} (Curves A, B, C and D) and at 0.02 °C min^{-1} (Curve E). Sperm analyses were performed after 24, 72 and 144 h of storage at 5 °C. Bacterial species and bacterial counts (colony forming units (CFU) mL^{-1}) were determined in the raw and extended semen of four randomly selected boars after 0, 24, 72 and 144 h storage and aerobic culture on blood agar for 48 h at 37 °C.

Experiment 2

In this experiment using one ejaculate of each boar (n = 8), two intermediate cooling rates, named Aa and Ab (Figure 1), were added between Curves A and B (see Experiment 1) to test the effect of initial slow-to-moderate cooling between 30 °C and 10 °C. Samples cooled with Curves Aa and Ab had an initial cooling rate of 0.02 °C min^{-1}, during 2 and 4 h, respectively. After the holding times the samples reached temperatures of 25 °C and 28 °C, respectively. Samples were then cooled to 10 °C at a rate of 0.04 °C min^{-1}. The final cooling from 10 °C to 5 °C was performed with cooling rates of 0.01 °C min^{-1}, for Curves Aa and Ab, respectively. Samples were cooled using Curves A and B, as previously described. Sperm analyses were performed after 24, 72 and 144 h storage at 5 °C.

Experiment 3

Two aliquots of antibiotic-free AndroStar® Premium extender were inoculated with *Escherichia coli* (*E. coli*; isolated from boar semen) at a concentration of 0.5×10^3 CFU mL^{-1} in a final volume of 30 mL in a sterile Falcon tube. The tubes were placed in a cardboard box and stored in a temperature-controlled refrigerator at 5 ± 1 °C. Samples were split into two groups with different holding times at 22 °C, for 1 h and 6 h, respectively, and then cooled to 5 °C. At 0, 3, 6, 24, 48, 72 and 144 h after bacterial inoculation, aliquots were plated on Luria Broth (LB) agar plates and incubated for 24 h at 37 °C under aerobic conditions for enumeration of bacterial cells. The CFU mL^{-1} count was determined from two dilutions with two replicates per sample.

Table 1. Exponential regressions describing cooling curves used in Experiment 1 (A, B, C and D, and E) and Experiment 2 (A, Aa, Ab and B) for freshly extended semen. Samples were cooled from 30 ± 1 °C to 25 °C; from 25 to 10 °C and from 10 °C to the desired storage temperature of 5 ± 1 °C. Corresponding curves are presented in Figure 1.

Cooling	Temperature zone used to calculate each exponential regression		
Rate	30 °C to 25 °C	25 °C to 10 °C	10 °C to 5 °C
A*	$y = 29.2\ e^{-4E-04x}$	$y = 24.5e^{-0,002x}$	$y = 9.6e^{-7E-04x}$
Aa*	$y = 30.5e^{-8E-04x}$	$y = 24.9e^{-0,002x}$	$y = 9.7e^{-1E-03x}$
Ab*	$y = 30.4e^{-9E-04x}$	$y = 22.7e^{-0,002x}$	$y = 9.8e^{-8E-04x}$
B	$y = 30.3e^{-0,002x}$	$y = 24.1e^{-0,002x}$	$y = 9.5e^{-7E-04x}$
C	$y = 30.6e^{-0,004x}$	$y = 23.9e^{-0,003x}$	$y = 9.6e^{-8E-04x}$
D	$y = 29.8e^{-0,005x}$	$y = 23.6e^{-0,004x}$	$y = 9.3e^{-1E-03x}$
E	$y = 31.1e^{-0,023x}$	$y = 24.3e^{-0,02x}$	$y = 8.7e^{-0,003x}$

* Semen doses cooled with the Curves A, Aa and Ab had holding times of 6, 4 and 2 h at 22 °C. All other samples (B, C, D and E) were cooled from 30 °C to 5 °C directly after filling, with no holding time.

Evaluation of sperm motility

To assess sperm motility, 2.0 mL aliquots were incubated at 38 °C in a water bath under air contact. After 30 min of incubation, total sperm motility was determined by the computer-assisted semen analysis (CASA) system, AndroVision®, version 1.1 (Minitüb, Germany), using four-chamber slides (Leja, Nieuw Vennep, The Netherlands) with a depth of 20 µm. For each sample, six consecutive fields in the central axis of the chamber were recorded at a rate of 60 frames s^{-1} for each field using 100× magnification. At least 500 spermatozoa per sample were analyzed in all samples. Spermatozoa were evaluated as "motile" when their amplitude of lateral head displacement was greater than 1 µm and their curve line velocity was greater than 24.0 µm s^{-1}.

Flow cytometric assessment

Analyses were performed using a flow cytometer (CytoFLEX, Beckman Coulter, Krefeld, Germany) equipped with 405 nm and 488 nm solid-state lasers. Fluorescence signals of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), Yo-Pro1 and Fluo3-AM were gathered via a 525/40 nm band-pass filter. Hoechst 33342 signals were gathered via a Pb 450/45 nm band-pass filter, propidium iodide (PI) signals via a PC 5.5 690/50 nm band-pass filter, and Merocyanin 540 and JC-1 signals via a PE 585/42 nm long-pass filter. Non-DNA (Hoechst negative (neg)) particles were excluded and the sperm population was gated by referring to the expected forward- and side-scatter signals.

Evaluation of sperm membrane integrity

Integrity of sperm plasma membranes and acrosomes was analyzed at 24, 72 and 144 h using PI and FITC-PNA [14]. An aliquot of 50 µL of each diluted semen sample was transferred to 950 µL HEPES-buffered saline medium (HBS: 137 mM NaCl, 20 mM HEPES; 10 mM glucose; 2.5 mM KOH) + Bovine Serum Albumin (BSA: 1 mg mL^{-1}) with adjusted pH (7.4 at 20 °C) and osmolarity (300 ± 5 mOsmol kg^{-1}) containing 5 µL Hoechst 33342 (final concentration 0.45 µg mL^{-1}), 5 µL PI (final concentration 1 µg mL^{-1}) and 5 µL FITC-PNA (final concentration 0.6 µg mL^{-1}). After 5 min of incubation at 38 °C in the dark, 10,000 events were analyzed. Spermatozoa were categorized as: (1) intact plasma membrane and acrosome (PI-neg/FITC-PNA-neg); (2) intact plasma membrane and damaged acrosome (PI-neg/FITC-PNA-positive (pos)); (3) damaged plasma membrane and intact acrosome (PI-pos/FITC-PNA-neg); (4) damaged plasma membrane and acrosome (PI-pos/FITC-PNA-pos). Sperm populations that exhibited intact plasma and acrosomal membranes were reported.

Evaluation of sperm membrane fluidity

The fluidity of the plasma membrane was assessed after staining the sperm samples with Merocyanine 540 and Yo-Pro-1 iodide [10]. An aliquot of 485 µL of each semen dose was incubated for 15 min at 38 °C. After incubation, 5 µL Hoechst 33342 (final concentration 0.45 µg mL^{-1}), 5 µL Yo-Pro-1 (final concentration 0.01 nM) and 5 µL Merocyanine 540 (final concentration 0.27 nM) were added to the semen samples and incubated for 15 min at 38 °C. Thereafter, 50 µL of the sample was added to 950 µL of HBS solution and analyzed immediately. The spermatozoa were categorized as: (1) viable sperm with low plasma membrane fluidity (stable plasma membrane; Yo-Pro-1-neg/M-540-neg); (2) viable sperm with high plasma membrane fluidity (unstable plasma membrane; Yo-Pro-1 neg/M-540-pos); (3) dead sperm (Yo-Pro-1-pos). Viable spermatozoa with low plasma membrane fluidity were reported.

Evaluation of sperm mitochondrial membrane potential

The percentage of cells with a mitochondrial inner transmembrane potential (DCm) above a value of 80–100 mV was estimated after labeling samples with JC-1 and PI [15]. An aliquot of 1 mL of each semen dose was supplemented with 6 µL Hoechst 33342 (final concentration 0.9 µg mL^{-1}), 12 µL PI (final concentration 12 µg mL^{-1}) and 1 µL JC-1 (final concentration 1.53 nmol mL^{-1}) and incubated for 15 min at 38 °C. After incubation, 200 µL of the sample were added to 800 µL of HBS solution and immediately analyzed. Viable spermatozoa with a high mitochondrial membrane potential (hMMP; PI-neg and JC-1 pos) were reported.

Evaluation of sperm calcium influx

The response to capacitating conditions was evaluated by measurement of intracellular calcium [6, 16] in semen samples stored for 72 h. Spermatozoa were exposed to capacitating or non-capacitating conditions using two types of a Tyrode medium [17]. Tyrode capacitating medium (Tyrode A) consisted of 96 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 5 mM glucose, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.3 mM KH_2PO_4, 20 mM Hepes, 100 µg mL^{-1} gentamicin sulfate, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 3 mg BSA mL^{-1}, 100 µg gentamicin mL^{-1} and 20 µg mL^{-1} phenol red. In non-capacitating Tyrode control medium (Tyrode C) $NaHCO_3$ was omitted and the NaCl content was increased to 111 mM. Both media were adjusted to a pH of 7.6 at 20 °C and a final osmolarity of 300 ± 5 mOsmol kg^{-1}. Prior to analysis, 2 µL of PI (final concentration 2 µg mL^{-1}) and 2 µL of Hoechst (final concentration 0.6 µg mL^{-1}) were added to 946 µL of both Tyrode media and incubated at 38 °C under CO_2 (Tyrode A) or under air contact (Tyrode C), in the dark. In parallel, aliquots of 2 mL of extended semen were supplemented with 2.5 µL Hoechst 33342 (final concentration 0.75 µg mL^{-1}) and 2 µL Fluo-3-AM (final concentration 1 µM) and incubated for 30 min at room temperature in the dark. After incubation, 50 µL of each sample was added to 950 µL of Tyrode A and Tyrode C. Samples were assessed after 3 and 60 min of incubation at 38 °C under CO_2 (Tyrode A) or under air contact (control).

The analysis focused on the evaluation of the specific response to bicarbonate of viable spermatozoa [10, 16]. Therefore, the percentage of viable sperm with low intracellular calcium content (PI-neg/Fluo-3-neg) and viable sperm with high calcium content (PI-neg/Fluo-3-pos), as well as percentages of PI-pos sperm, were determined. The response to Tyrode A was calculated as the difference between the percentage of spermatozoa at 60 min and at the onset of incubation (Tyrode A Δ60-3) in the sperm populations. The same procedure was applied to the sperm populations in the control medium (control Δ60-3). The specific response to the capacitating stimulus (bicarbonate) was then calculated as the difference between the responses in the low intracellular calcium content in viable sperm in capacitating Tyrode and in control medium.

Statistical analyses

Data analysis was performed using Statistical Analysis System software (SAS Enterprise Guide, version 7.1; SAS Inst. Inc., Cary, NC). Experimental data were checked for normal distribution followed by pairwise comparisons using Student's t-test and two-factorial analysis of variance (ANOVA) with repeated measurements. Boars were considered as random effect in the models used. When the ANOVA revealed a significant difference among groups, comparison using post hoc Tukey–Kramer test was performed. Differences among means were considered significant at $P < 0.05$. Data are presented as mean ± standard deviation (SD).

Results

Experiment 1

The cooling rate influenced (P < 0.01) sperm motility, membrane integrity, membrane fluidity and mitochondria membrane potential, whereas storage time did not show an influence. Sperm quality traits were at a high level at 144 h storage time in samples subjected to cooling rates A– D. Mean values of total motility at 144 h for cooling rates A–D were 82.0 ± 5.1%, 82.5 ± 3.8%, 82.4 ± 6.8% and 81.3 ± 7.7%, respectively (P > 0.05). The means of membrane intact spermatozoa, spermatozoa with low membrane fluidity and hMMP are shown in Table 2. In samples submitted to cooling rate E, the percentages of motile spermatozoa (Figure 2) and of membrane intact (PI-neg/FITC-PNA neg) spermatozoa were lower (P < 0.01) compared to samples cooled at the other cooling rates (Table 2) after 24, 72 and 144 h storage. Accordingly, the percentage of viable spermatozoa with low membrane fluidity was lower (P < 0.01) at all time points in doses cooled with Curve E compared to all others. At 72 h of storage, the doses cooled with Curve D had lower (P < 0.01) percentages of viable spermatozoa with low membrane fluidity (73.6 ± 7.7%) compared to B (79.4 ± 4.4% - Table 2). The percentage of spermatozoa with hMMP at 24 h was lower (P < 0.01) when doses were cooled with Curve E compared to those cooled with Curves A, B, C and D. The specific response to the capacitating stimulus bicarbonate was lower (P < 0.01) for the doses cooled with cooling rates D and E compared to cooling rates A, B and C (Figure 3).

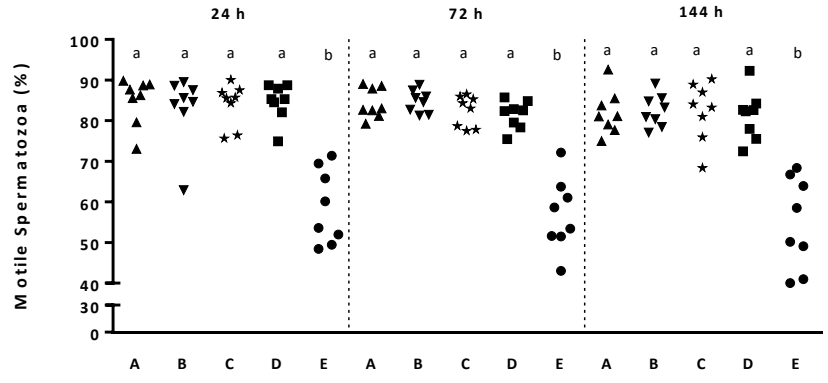

Figure 2. Scatterplots showing sperm motility in semen samples (n = 8) cooled at different cooling rates (A, B, C, D and E – Experiment 1) at 24, 72 and 144 h storage at 5 °C. a,b: values differ significantly (P < 0.01).

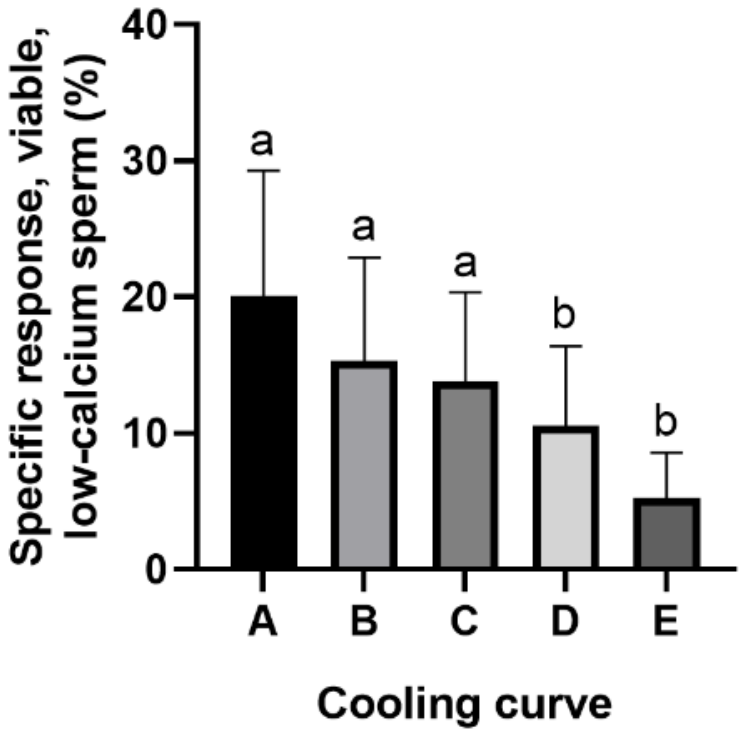

Figure 3. Specific response to capacitating conditions calculated as the difference in the responsiveness to incubation conditions in Tyrode (Tyrode A Δ60 – 3) and control (control Δ60 – 3) medium for the population of viable sperm with low calcium content (PI-neg./Fluo-3-neg.) in semen samples cooled with different cooling rates (A, B, C, D and E – Experiment 1) to 5 °C and stored for 72 h. Data are shown as mean ± SD (n = 8 boars). a,b

Table 2. Spermatozoa with intact plasma membrane (viable) and acrosome (PI-neg and FITC-PNA-neg), viable spermatozoa with low plasma membrane fluidity (Yo-Pro1-neg and M-540-neg) and viable spermatozoa with high mitochondrial membrane potential in semen samples extended in antibiotic-free AndroStar® Premium and submitted to cooling rates A, B, C, D and E (Experiment 1; c.f. Table 1) before storage at 5 °C. Data are presented as mean ± SD (n = 8 boars).

		Storage time		
Parameter	Cooling curve	24 h	72 h	144 h
Viable spermatozoa (%) with intact acrosomes (PI-neg and FITC-neg)	A	81.9 ± 5.6^{a}	82.8 ± 5.3^{a}	83.8 ± 3.3^{a}
	B	82.7 ± 4.6^{a}	84.7 ± 3.0^{a}	85.1 ± 2.9^{a}
	C	81.8 ± 5.2^{a}	82.7 ± 3.5^{a}	83.0 ± 3.2^{a}
	D	79.0 ± 4.7^{a}	80.7 ± 5.0^{a}	80.8 ± 3.8^{a}
	E	58.8 ± 9.6^{b}	59.0 ± 10.1^{b}	55.5 ± 9.4^{b}
Viable spermatozoa with high mitochondrial membrane potential (PI-neg and JC-1-pos) (%)	A	94.2 ± 2.0^{a}	95.2 ± 2.0^{a}	94.2 ± 0.8^{a}
	B	94.1 ± 1.8^{ab}	94.3 ± 1.4^{a}	94.0 ± 1.5^{a}
	C	94.0 ± 2.5^{ab}	94.3 ± 2.2^{a}	93.8 ± 1.4^{a}
	D	93.7 ± 1.5^{ab}	93.1 ± 1.4^{a}	92.8 ± 1.6^{a}
	E	92.0 ± .2.6^{b}	90.0 ± 2.5^{b}	88.1 ± 4.2^{b}
	A	75.4 ± 6.2^{a}	76.9 ± 5.8^{ab}	76.3 ± 6.6^{a}

Viable spermatozoa (%) with low membrane fluidity (Yo-Pro-1-neg and M-540-neg)	B	76.2 ± 5.2[a]	79.4 ± 4.4[a]	79.3 ± 6.5[a]
	C	76.1 ± 4.6[a]	76.9 ± 5.9[ab]	77.1 ± 5.1[a]
	D	71.2 ± 5.0[a]	73.6 ± 7.7[b]	75.9 ± 6.1[a]
	E	55.8 ± 12.0[b]	49.3 ± 11.7[c]	46.4 ± 10.4[b]

[a–c] indicates significant differences within a column ($P < 0.05$). FITC-PNA: fluorescein isothiocyanate-conjugated peanut agglutinin; PI: propidium iodide; M-540: Merocyanine 540.

Bacterial counts in raw semen ranged from 1.7×10^4 to 8.5×10^4 CFU mL^{-1} ($4.1 \times 10^4 \pm 2.7 \times 10^4$ CFU mL^{-1}), being identified Gram-negative (*E. coli*, *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp.) bacteria. Microbiology results in extended semen doses immediately after dilution (0 h), at 24, 72 and 144 h storage are presented in Figure 4. The bacterial load in diluted sperm at 0 h storage ranged from 1.0×10^1 to 7.5×10^3 CFU mL^{-1}. At 72 h, bacterial load in two samples, one cooled with Curve A and one cooled with Curve B, increased to 1.1×10^4 and 1.4×10^4 CFU mL^{-1}, respectively (Figure 3). At 144 h, all samples revealed CFU < 6.0×10^3 mL^{-1}.

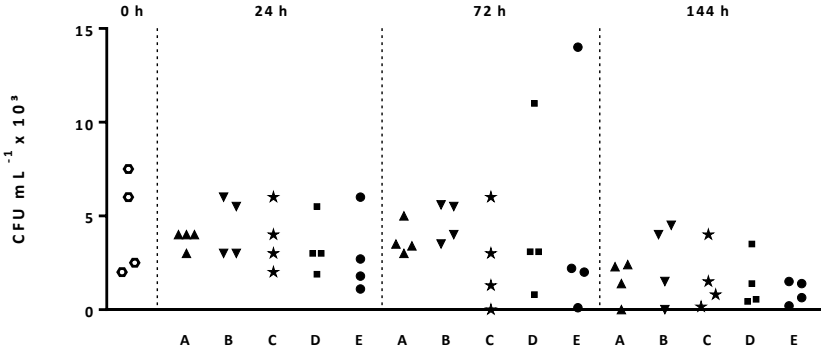

Figure 4. Scatterplots (n = 4 boars) showing bacteria counts (CFU mL^{-1}) in semen extended in antibiotic-free AndroStar® Premium and cooled with different cooling rates (A, B, C, D and E – Experiment 1) before storage at 5 °C.

Experiment 2

Sperm motility (range 91.3 ± 3.0 to 86.1 ± 1.6) remained on high level throughout storage and was not influenced by the cooling rate. Likewise, the cooling rate did not influence membrane integrity and mitochondria membrane potential (Table 3). A significant effect of cooling rate and time of storage was observed for the percentage of sperm with low membrane fluidity ($P = 0.02$; $P = 0.01$, respectively). The percentage of viable spermatozoa with low membrane fluidity was lower ($P < 0.01$) after 24 h storage of spermatozoa cooled by A (75.9 ± 4.2) compared to spermatozoa cooled by B (79.4 ± 3.9%). However, sperm quality traits were at a high level after 144 h storage in samples subjected to cooling rates A, Aa, Ab and B (Table 3). The specific response to the capacitating

stimulus bicarbonate did not differ for the cooling rates A, Aa, Ab and B (range 12.8 ± 5.6 to 15.1 ± 4.9).

Table 3. Spermatozoa with intact plasma membrane (viable) and acrosome (PI-neg and FITC-PNA-neg), viable spermatozoa with low plasma membrane fluidity (Yo-Pro-1-neg and M-540-neg) and viable spermatozoa with high mitochondrial membrane potential in semen samples extended in antibiotic-free AndroStar® Premium and submitted to cooling rates A, Aa, Ab and B (Experiment 2) before storage at 5 °C. Data are presented as mean ± SD (n = 8 boars).

		Storage time		
Parameter	Cooling curve	24 h	72 h	144 h
Viable spermatozoa (%) with intact acrosomes (PI-neg and FITC-neg)	A	86.6 ± 0.7[a]	84.2 ± 2.6[a]	86.1 ± 2.8[a]
	Aa	87.1 ± 1.7[a]	85.4 ± 1.8[a]	85.8 ± 2.1[a]
	Ab	87.2 ± 2.0[a]	86.2 ± 1.6[a]	86.8 ± 2.8[a]
	B	87.0 ± 2.1[a]	85.2 ± 3.2[a]	86.5 ± 3.1[a]
Viable spermatozoa (%) with low membrane fluidity (Yo-Pro-1-neg and M-540-neg)	A	75.9 ± 4.2[a]	77.1 ± 5.8[a]	73.6 ± 11.2[a]
	Aa	76.6 ± 5.0[ab]	77.5 ± 6.2[a]	74.4 ± 10.8[a]
	Ab	78.0 ± 3.9[ab]	79.4 ± 6.0[a]	76.6 ± 9.2[a]
	B	79.4 ± 3.9[b]	79.5 ± 5.8[a]	77.0 ± 7.7[a]
Viable spermatozoa (%) with high mitochondrial membrane potential (PI-neg, JC-1-pos)	A	95.0 ± 1.2[a]	94.5 ± 0.8[a]	94.5 ± 1.5[a]
	Aa	95.6 ± 1.5[a]	94.5 ± 0.9[a]	94.7 ± 1.3[a]
	Ab	95.1 ± 1.0[a]	94.4 ± 1.1[a]	94.8 ± 1.0[a]
	B	94.8 ± 1.1[a]	94.2 ± 0.7[a]	94.9 ± 1.0[a]

[a,b] indicates significant differences within a column ($P < 0.05$). FITC-PNA: fluorescein isothiocyanate-conjugated peanut agglutinin; PI: propidium iodide; M-540: Merocyanine 540.

Experiment 3

Following inoculation of semen-free AndroStar® Premium extender with 0.5×10^3 CFU mL^{-1} (*E. coli*), samples exposed to a short holding time of 1 h at RT (22 °C) prior to cooling contained 1.0×10^3 CFU mL^{-1}, while samples held for 6 h at RT contained 2.4×10^3 CFU mL^{-1} (Table 4). Bacterial counts were reduced in both samples after cooling, with 0.7×10^3 CFU mL^{-1} (1 h holding time) and 2.2×10^3 CFU mL^{-1} (6 h holding time) respectively, after 24 h. Continuous storage at 5 °C for 144 h resulted in an overall decrease of the bacterial load to $<1 \times 10^3$ CFU mL^{-1}. In samples exposed to a long holding time of 6 h before cooling, bacterial counts did not fall below the initial inoculation number.

Table 4. Bacterial counts (CFU mL^{-1}) in antibiotic-free AndroStar® Premium extender inoculated with 0.5×10^3 *E. coli* and held either at 1 h or 6 h at room temperature (RT, 22 °C) before storage at 5 °C. Data are single values and mean of two replicates (n = 2, Experiment 3).

	Time of storage (h) at 5°C									
	0 h		6 h		24 h		72 h		144 h	
Time at RT	RT1	RT6	RT1	RT6	RT1	RT6	RT1	RT6	RT1	RT6
Sample 1	455	773	1045	2682	727	2591	591	1818	409	955
Sample 2	636	364	1000	2045	682	1773	136	2227	182	773
Mean	545	568	1023	2364	705	2182	364	2023	295	864

RT1: Samples were held during 1 hour at RT (22 °C) before storage at 5 °C.

RT6: Samples were held during 6 hours at RT (22 °C) before storage at 5 °C.

Discussion

In the present study, an appropriate cooling-rate frame for extended boar semen designed for antibiotic-free storage was established, from 0.01 to 0.09 °C min^{-1} in the temperature zone from 30 to 25°C, followed by 0.02 to 0.06 °C min^{-1} to 10 °C and 0.01 to 0.02 °C min^{-1} to the final storage temperature. The respective temperature graphs are presented by the cooling rates Aa, Ab, B and C in this article. In cryopreservation protocols, cooling rates ranging from 0.08 to 1.5 °C min^{-1} within a temperature zone of 15 °C to 5 °C are considered effective for chilling boar spermatozoa prior to freezing [18, 19]. Recommendations of cooling rates between 30 °C and 5 °C and subsequent liquid storage at this temperature have been lacking until now. Freshly ejaculated sperm react sensitively to fast temperature decrease, as demonstrated by the lipid phase transition and fluidity decrease of plasma head membranes occurring between 30 °C and 10 °C in boar spermatozoa [10, 20]. Loss of motility and membrane integrity caused by rapid initial cooling becomes increasingly apparent during conventional semen storage at 17 °C [21] and might be even more pronounced at 5 °C. Consequently, slow cooling with holding times at RT were recommended before liquid storage at 17°C [21]. Here we provide the first evidence for an optimal cooling rate from 30 °C to 5 °C for boar spermatozoa, considering both sperm quality traits and bacterial growth. The moderate and slow cooling rates between Curves A and C yielded consistently high sperm quality throughout long-term semen storage. Notably, a protecting extender was used, which had previously proved to preserve sperm characteristics and fertility during storage at 5 °C [6]. Fast cooling by Curve E yielded highly variable motility values already at 24 h of storage indicating that boars might differently respond to chilling stress, similarly as described for the inter-boar variability in the response to sperm cryopreservation [22].

Loss of sperm motility indicates chilling and storage damage in spermatozoa, and thus motility is the most important indicator used in boar AI centers, with a minimum threshold of 65% for usable semen [1]. In our study, only those semen doses cooled rapidly (cooling Curve E) did not reach threshold values of sperm motility and had already displayed a significant loss of membrane integrity after 24 h storage. Chilling injury at a sublethal level was evaluated in plasma membrane intact (“viable”) spermatozoa by flow cytometric assessment of plasma membrane fluidity, MMP, and the response to the capacitation stimulant bicarbonate. Confirming earlier studies that reported an influence of hypothermic liquid storage on membrane fluidity [10, 12], we observed a clear decrease in viable sperm with low membrane fluidity in semen samples with the fastest cooling rate (Curve E) compared to samples cooled more slowly (A, B and C). The MMP was also affected by this cooling rate, thus confirming a previous

study that reported a decrease in MMP and oxidoreductive capability of boar sperm stored at 5 °C [23]. The chilling-induced loss of mitochondrial function may contribute to the lower ATP concentration reported in chilled boar spermatozoa [24], which could cause metabolic disruption; specifically, the decrease of motility [25] as observed here. Perturbance of mitochondrial function is probably connected to a loss of coordinated transfer of ions and other solutes through the plasma membrane. This may also explain a chilling-induced increase in cytosolic calcium ions leading to premature cell destabilization and a reduced specific response to the capacitation stimulus bicarbonate [26].

Assessment of calcium influx under capacitation conditions, particularly the response to bicarbonate, is an established measure for chilling injury in hypothermically stored boar semen [10, 26] and proved to be most sensitive in the present study for mirroring the effects of cooling velocity on sperm function. Rapid cooling, according to cooling Curves D and E, resulted in a lower response to bicarbonate, indicating that the loss of essential sperm function may occur before sperm motility or viability are affected, moreover, it is important to consider that plasma membrane alterations could lead to a higher permeability to calcium due to the disruption of the ability to achieve normal capacitation [27]. The ability to respond to capacitation signals depends on complex signal transduction pathways [28], and a decreased *in vitro* responsiveness is associated with subfertility in normospermic boars [29].

As outlined above, from the perspective of sperm function, slow cooling seems to be advantageous; however, the risk of bacterial growth during delayed cooling needs to be considered. This general notion is supported by the results of the present study, which showed higher bacterial counts of *E. coli* in the inoculated sperm-free semen extender held for 6 h at 22 °C compared to samples held for only 1 h (Experiment 3). The presence of microbial contaminants, mostly identified as Gram-negative bacteria belonging to the *Enterobacteriaceae* family [4], may impair sperm viability and fertility in a dose-dependent manner. However, the experimental inoculation of semen with 10^4 CFU mL^{-1} of *E. coli* has been found not to influence the quality of human spermatozoa [30], and adverse effects on sperm traits were reported for boar semen starting with minimum values of 10^7 or 10^8 CFU mL^{-1} of *E. coli* [31, 32]. In previously studies [33], it was reported that *E. coli* counts of 3.5×10^3 CFU mL^{-1} affected litter size, several dose-response studies showed that the detrimental effects of bacteria (*E. coli, Cl. perfringens, Ps. aeruginosa*) were only apparent at the highest concentrations, ranging between 10^6 and 10^8 CFU mL^{-1} [31, 34 -36]. Some authors propose that the sperm/bacteria ratio of 1:1 presents the tolerance limit for sperm injury [30, 37, 38], which would translate into an upper limit of $2–3 \times 10^7$ CFU mL^{-1} in extended boar semen. In our previous study [6], in which slow cooling according to Curve A was applied to

antibiotic-free semen doses, fertility was not affected in the presence of up to 7.6 × 10^3 CFU mL^{-1}. In agreement with previous reports [37, 39], all ejaculates included in the present study were positive for the presence of bacteria, confirming that the collection of sterile semen is virtually impossible. More bacteria were found in semen samples with slower cooling rates (Curves A, B), but bacterial counts (up to 10^4 CFU mL^{-1}) did not exceed the proposed limits of sperm/bacteria ratios as described above. Due to the mesophilic character of the typical semen contaminants, the bacteriostatic effect of hypothermic storage increased with ongoing storage time, thus favoring the use of stored semen rather than freshly diluted ejaculates.

It is important to consider that semen doses prepared for transport to the research laboratory or to the farm might differ in their cooling velocity depending on whether they were placed in the center or at the margin of tube bundles. The volumes of semen doses (commonly in tubes or bags with 30– 100 mL of extended semen), packing units and types of packaging may vary, thus pointing to the necessity to control cooling rates at representative positions of a given semen package. For transfer of the current findings into practice, it must be ensured that all semen doses in a package reach a cooling velocity that lies within the recommended frame reported in this study.

Conclusions

In conclusion, moderate cooling rates within the frame presented by cooling-rate Curves Aa and C are recommended for antibiotic-free storage of boar semen at 5 °C. With the cooling protocols described here, an appropriate cooling regime can easily be implemented into research laboratories or AI centers, thus allowing instant use of the hypothermic storage concept. Further studies are encouraged to assess the potential of hypothermic semen preservation with the aim of reducing the use of antibiotics in artificial insemination of pigs.

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Competing interests

The authors have no competing interests to declare.

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CAPÍTULO IV – QUARTO MANUSCRITO

FACTORS INFLUENCING THE RESPONSE OF SPERMATOZOA TO AGITATION STRESS: IMPLICATIONS FOR TRANSPORT OF EXTENDED BOAR SEMEN

MANUSCRITO PUBLICADO EM PERIÓDICO

THERIOGENOLOGY

(de acordo com as normas do periódico)

Factors influencing the response of spermatozoa to agitation stress: Implications for transport of extended boar semen

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Abstract

The shipping of liquid preserved semen is common practice in animal breeding and prior to cryopreservation for gene banking. Vibration emissions during transport may be harmful to spermatozoa. Therefore, strategies to minimize agitation-induced sperm injury are needed. The aim was to examine whether the type of semen extender, time after semen processing and the temperature in simulated transport conditions influence the response of boar spermatozoa to agitation stress. In Experiment 1, boar semen samples (n = 16) extended in Beltsville Thawing Solution (BTS) or Androstar Plus (APL) medium were filled in 90 mL tubes and shaken for 4 h at 200 rpm either at 22 °C or 17 °C. Samples were then stored at 17 °C for 144 h. In Experiment 2, semen samples (n = 11) extended in Androstar Premium were shaken either directly after filling at 22 °C or 20 h later after cooling to 5 °C. Samples were stored at 5 °C for 144 h. In Experiment 1, sperm motility and viability were lower ($p < 0.05$) in the shaken samples compared to the controls. The temperature, extender and the storage length had no effect on the agitation-induced loss of sperm quality. Sperm quality traits were higher in samples stored in APL compared to BTS. In Experiment 2, sperm motility at 24 h was reduced ($p < 0.05$) in those samples shaken at 22 °C but not at 5 °C. Sperm viability, membrane fluidity and mitochondria membrane potential were not affected in either of the treatment groups. Extended boar semen designed for 17 °C storage and shipped on the day of collection is sensitive to agitation stress. In contrast, spermatozoa slowly cooled to 5 °C and shaken 20 h after processing are more resistant to agitation-induced shear forces and interfacial phenomena.

1 INTRODUCTION

Transport of preserved semen in the liquid stage is common for artificial insemination (AI) in pigs but is also realized in equine and bovine breeding. Typically, semen is shipped a few hours after collection and processing. Semen transport from the AI center can take hours or even days before reaching the breeder. Preserved semen is a vulnerable product to be used in the short term that should maintain its full fertilizing capacity when delivered on the farm and a few days beyond. Additionally, overnight shipping of pre-diluted boar semen in a liquid stage to a central laboratory is common before cryopreservation for gene banking. Usually, semen is shipped in insulated packages to protect the temperature-sensitive cells from cold shock and heat stress. Recently, it was shown that in addition to temperature imbalances[1], boar spermatozoa can be affected by vibration emission during shipping, as reflected by reduced motility, mitochondrial activity, membrane integrity and thermo-resistance during subsequent long-term storage[2]. Fluid motion alongside the cell surface together with bubble formation cause shear and interfacial phenomena, which might induce sperm damage as described for cultured somatic cells[3,4].

Transport-induced sperm damage by agitation is particularly relevant in pig reproduction, given that 99 % of all AI worldwide are performed with liquid preserved semen[5], and centralization of semen production units resulting in longer transport distances is increasing. Moreover, boar spermatozoa react especially sensitively to all different kinds of cell stressors associated with semen handling, which leads to reduced survival rate and sublethal cell damage[6]. Motion-induced cell stress might act additively to temperature stress when cooling boar sperm below 12 °C, this being the critical temperature at which cold shock becomes evident[7].

Recently, hypothermic storage of boar semen at 5 °C was proposed instead of the conventional storage temperature at 17 °C to reduce the risk of bacterial growth and concomitantly the use of antibiotics in semen extenders[8]. Although chilling stress is relatively low when using the novel preservation concept[9], additional motion stress could result in lower survival rates. Resistance to cold injury is achieved by using a protective extender and protracted cooling in the temperature range between 30 °C and 10 °C[10], where phase transitions of sperm

membrane lipids occur[11]. In this scenario, the time and temperature of transport-associated vibration stress might be decisive for the quality of hypothermic preserved semen. During transport, temperature might be higher than the final storage temperature if the semen is transported within a few hours after collection and processing.

The objective of the present study was to elucidate whether the type of extender and the temperature during simulated transport stress influence the damaging effect of passive sperm motion. For this, in the first part of the study, the effect of semen agitation on sperm quality traits was compared between a long-term extender and the control extender BTS during semen storage at 17 °C. In the second part, the effect of temperature during simulated shipping on boar spermatozoa stored at 5 °C was examined. Overall, this study was designed to stimulate future strategies for sperm protection during prolonged shipping periods.

2 MATERIAL AND METHODS

2.1 Chemicals and media

If not otherwise stated, chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). The fluorescent dyes Hoechst 333442 and Yo-Pro-1 were purchased from Thermo Fisher Scientific (Waltham, MA, USA), FITC-PNA from Biozol (Eching, Germany) and JC-1 from Enzo Life Science (Lörrach, Germany). Semen extenders were obtained from Minitüb (Tiefenbach, Germany).

2.2 Animals, facility and semen collection

Fertile, sexually mature boars (Landrace, Piétrain, Large White) under routine semen collection for the production of AI doses were used. The experiment was approved by the Animal Welfare Officer of the University of Veterinary Medicine Hannover, Foundation, Germany. The boars received commercial feed for AI and were housed in individual pens and treated in accordance with the European Commission Directive for Pig Welfare. Only normospermic ejaculates (a minimum of 75 % morphologically normal spermatozoa, at least 70 % total sperm motility, and a total number $\geq 30 \times 10^9$ spermatozoa per ejaculate) were used. The ejaculates were collected by the “gloved-hand” method, the pre-spermatic phase being discarded and the gel fraction retained by gauze filtration during the semen collection.

2.3 Semen processing and treatment

One ejaculate from each boar was extended in a split-sample design according to treatments to 20 $\times 10^6$ spermatozoa/mL and filled in 90 mL QuickTip Flexitubes® (Minitüb GmbH, Tiefenbach, Germany). The filling volume was 85 mL extended semen and 5 mL residual air. Dilution was isothermally performed (32 °C) in one step. Semen prepared for storage at 17 °C (Experiment 1) was held for 1.5 h to 4 h at 22 °C, having an initial cooling rate of 0.04 to 0.02°C min–1, and then placed in a cabinet at 17 °C. Semen doses designed for storage at 5 °C (Experiment 2) were slowly cooled following the ideal time/temperature frame as previously reported [10]: Samples had an initial cooling rate of 0.02 min^{-1} until reaching 25 °C and were then cooled to 10 °C at a rate of 0.04 °C min^{-1}. The final cooling from 10 °C to 5 °C was performed with a cooling rate of 0.01 °C min^{-1}. For each treatment group, one semen tube was prepared for each day of analysis.

Agitation during transport was simulated through mechanical vibration emission with an orbital shaker as described by Schulze et al. (2018) with slight modification. Semen doses were placed in a horizontal position on a shaker (Swip Shaker, Bühler KL-2, Edmund Bühler GmbH, Bodelshausen, Germany) and moved for four hours in the dark (Fig. 1 and Supplemental Information: Video). In Experiment 1, two shakers placed at different environmental temperatures were used to allow timely parallel treatments. In contrast to Schulze et al. (2018), who described a harmful effect on sperm quality at 300 rpm for 6 h, the effect of a milder vibration stress was studied. For this, the shakers were configurated to perform circular horizontal movements with a rotation speed (frequency) of 200 rpm and 1 cm of amplitude.

[Figure 1]

2.4 Experimental design

2.4.1 Experiment 1

Single ejaculates from sixteen boars were extended on a split-sample basis in BTS or Androstar Plus (APL) extenders (Minitüb GmbH) with gentamicin sulfate (0.25 g/L). After filling, subsamples were subjected to three treatments

before storage at 17 °C (Table 1): 1.) semen doses were held motionless at 22 °C for 90 min (controls: BTS-C; APL-C), 2.) semen doses were shaken at 22 °C for 4 h (BTS-22; APL-22), 3.) semen doses were held motionless at 22 °C for 90 min and thereafter at 17 °C for 60 min, and then shaken at 17 °C for 4 h (BTS-17; APL-17). After 24, 72 and 144 h of storage, semen doses were submitted to analyses of total and progressive motility, plasma membrane and acrosome integrity.

2.4.2 Experiment 2

Single ejaculates from eleven boars were extended on split-sample basis in Androstar Premium extender (Minitüb GmbH). After filling, subsamples were subjected to three treatments before storage at 17 °C (Table 1): 1) semen doses were held motionless at 22 °C for 90 min (control: APR-C), 2.) semen doses were shaken at 22 °C for 4 h (APR-22), 3.) semen doses were held motionless at 22 °C for 4 h and thereafter at 5 °C for 16 h, and then shaken at 5 °C for 4 h (APR-5). After 24, 72 and 144 h of storage, semen doses were submitted to the analyses of total and progressive motility, plasma membrane and acrosome integrity, membrane fluidity and mitochondria membrane potential.

2.5 Semen assessment

2.5.1 Evaluation of sperm motility

Sperm motility was determined with the computer-assisted semen analysis (CASA) system, AndroVision®, Version 1.1 (Minitüb GmbH), using four-chamber slides (Leja®, Lena Products B.V., Nieuw-Vennep, the Netherlands) with a depth of 20 µm. To assess sperm motility, 2.0 mL aliquots were incubated for 30 min at 38 °C in a water bath with air contact. Spermatozoa were classified as “motile” when their amplitude of lateral head displacement was higher than 1 µm and their curve line velocity higher than 24.0 µm/sec. A spermatozoon was considered “progressively motile" when its curved-line velocity was higher than 42 µm/s or its straight-line velocity was higher than 15 µm/s. For each sample, six successive fields in the central axis of the chamber were recorded at a rate of 60 frames per 1 s for each field using a 100 × magnification. At least 500 spermatozoa per sample were recorded in all samples.

2.5.2 Flow cytometry

Analyses were performed using a flow cytometer (CytoFLEX, Beckman Coulter GmbH, Krefeld, Germany) equipped with 405 and 488 nm solid state lasers as previously described[10]. Fluorescence signals of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), Yo-Pro1 gathered via 525/40 nm band-pass filter, Hoechst 33342 gathered via Pb 450/45 nm band-pass filter and propidium iodide (PI), gathered via PC 5.5 690/50 nm band-pass filter, merocyanin (M) 540 and JC-1 gathered via PE 585/42 nm long-pass filter. Non-DNA (Hoechst negative - neg) particles were excluded and the sperm population was gated, referring to the expected forward and side-scatter signals.

Evaluation of sperm membrane integrity

The integrity of plasma membranes and acrosomes was analyzed at 24 h, 72 h and 144 h by double staining with PI and FITC-PNA as previously described[10]. Briefly, an aliquot of 50 µL of each diluted semen sample was transferred to 950 µL HEPES-buffered saline medium (HBS: 137 mM NaCl, 20mM HEPES; 10mM glucose; 2.5 mM KOH) + bovine serum albumin (BSA: 1 mg/mL) with adjusted pH (7.4 at 20°C) and osmolarity (300 ± 5 mOsmol/kg) containing 5 µL Hoechst 33342 (final concentration 0.45µg/mL), 5 µL PI (final concentration 1 µg/mL) and 5 µL FITC-PNA (final concentration 0.6 µg/mL). Immediately after 5 min of incubation at 38 °C in the dark, 10,000 events were analyzed. Spermatozoa were categorized as membrane intact when they were PI-negative, indicating an intact plasma membrane, and FITC-PNA-negative, indicating an intact acrosome.

Evaluation of sperm membrane fluidity

The fluidity of the plasma membrane was assessed using the fluorescence probes M 540 and YoPro-1 iodide as previously described[10]. Briefly, 5 µL Hoechst 33342 (final concentration 0.45 µg/mL), 5 µL YoPro-1 (final concentration 0.01 nM) and 5 µL merocyanine 540 (final concentration 0.27 nM) were added to 485 µL semen samples and incubated for 15 min at 38 °C. After incubation, 50 µL of the labeled sample was added to 950 µL of HBS solution and then analyzed. The spermatozoa were categorized as viable (plasma membrane intact) sperm with low plasma membrane fluidity if they were Yo-Pro-1 negative and M-540 negative.

Evaluation of sperm mitochondrial membrane potential

The percentage of viable spermatozoa with high mitochondrial inner transmembrane potential was estimated after staining with JC-1 and PI as

previously described[10]. Briefly, 1 mL semen aliquot was incubated with 6 µL Hoechst 33342 (final concentration 0.9 µg/mL), 12 µL PI (final concentration 12 µg/mL) and 1 µL JC-1 (final concentration 1.53 nM /mL) for 15 min at 38 °C. Then, 200 µL of the sample was added to 800 µL of HBS solution and immediately analyzed. Viable (PI negative) spermatozoa with a high mitochondrial membrane potential (JC-1 positive) were reported.

2.5.3 Monitoring of pH

The pH value was determined in boar semen doses at 24 h, 72 h and 144 h of storage using a pH meter (BApMX3000/ION, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

2.6 Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). To analyze the data of the repeated measures of the experimental design, a generalized mixed linear model (GMLM) was performed. In Experiment 1, the fixed effects were time, agitation (yes/no), extender and their interactions. In Experiment 2, the fixed effects were time, agitation (yes/no) and their interaction. To show the pairwise differences between the groups concerning the effect of vibration, a pairwise comparison with the Wilcoxon Test corrected by Holm Bonferroni was performed. Values were considered as statistically significantly different with $p< 0.05$. Data are presented as means ± standard deviation (SD) or as box-plots including the median and 25^{th} and 75^{th} percentiles.

3 RESULTS

3.1 Experiment 1

Analysis with the GMLM showed that there was no interaction between shaking effects, extender and semen storage length for total and progressive motility, plasma membrane and acrosome integrity and pH ($P > 0.05$ for all combinations of the fixed effects). A significant effect of shaking and/or extender was observed for sperm motility (Fig. 2), plasma membrane and acrosome integrity and pH (Table 2). The temperature at shaking did not influence sperm parameters. Total motility was reduced on average by between 3.4 % and 6.3 % in shaken

samples compared to the controls, and progressive motility was reduced in a similar range (Supplemental Table 1). In individual males, agitation stress reduced total and progressive sperm motility by between 0 and 13.7 % regardless of the semen extender and storage time. The percentage of sperm with intact plasma membranes was reduced on average by between 4.0 % and 5.9 % in shaken samples compared to controls (Table 2). In individual males, agitation stress reduced the percentage of sperm with intact plasma membranes and acrosomes by between 0 % and 13.5 % regardless of the semen extender and storage time. In the treatment and control groups, sperm motility and membrane integrity were higher in samples stored in APL compared to BTS at all storage times. The pH values differed between extenders and were not influenced by treatment (Table 2). After 144 h, pH 7.32 was the maximum level observed.

[Fig. 2]

3.2 Experiment 2

Analysis showed that there was no interaction found between time of storage and shaking for total and progressive motility ($P = 0.116$, $P = 0.331$, respectively) membrane integrity ($P = 0.673$), membrane fluidity ($P = 0.924$) and mitochondria membrane potential ($P = 0.746$ – Tab. 3). Sperm motility data of semen stored at 5 °C are presented in Figure 3 and Supplemental Table 2. Sperm data obtained by flow cytometry are shown in Table 3. An effect of agitation stress was observed for the total motility ($p = 0.027$), but not for progressive motility ($p = 0.179$), membrane integrity ($p = 0.309$), membrane fluidity ($p = 0.946$) and mitochondria membrane potential ($p = 0.755$). At 24 h of storage, motility values differed significantly ($p < 0.05$) between controls and semen doses shaken immediately after processing; sperm motility in semen doses shaken 20 h after processing at 5 °C did not differ from controls. No significant differences between treatments and control were observed at 72 h and 144 h of semen storage.

[Figure 3]

4 DISCUSSION

The present study showed that agitating semen doses within the first hours after processing reduces motility, viability (plasma membrane integrity) and

acrosome integrity of sperm stored at 17 °C, whereas semen cooled and then subjected to vibration stress at 5 °C on the following day is not affected.

The higher sensitivity of freshly processed semen to agitation might be caused by an additive effect of hydrodynamic forces on the sperm surface resulting from immediate dilution stress[6] just prior to agitation. In addition, exposure to vibration emission at a higher temperature (22 °C) compared to 5 °C could induce a higher level of oxidative stress, leading to an increased production of reactive oxygen species as a possible cause for agitation-induced loss of motility[2]. Alternatively, the reduced susceptibility of cooled sperm could result from the extended holding time for 20 h before shaking, which would allow a higher uptake of membrane stabilizing seminal plasma and extender components in the absence of shearing forces. A similar effect of membrane protection through holding time for 24 h between 17 °C and 24 °C prior to chilling stress has been described for boar sperm[12,13].

The severity of cellular damage depends on both the magnitude and the duration of the shear stress[3]. In the present study, simulated transport of semen doses for 4 h at 22 °C before cooling to 5 °C revealed only a transient reduction of sperm motility, indicating that shear force-induced sperm injury can be reversible. Shear forces damage cells and alter their behavior by a slight disruption of the membrane bilayer structure or through mechanical effects on the functionality of membrane-bound receptors, surface molecules or ion channels. Besides, the external forces could change the structure and function of the cytoskeleton[3]. For detecting sublethal alteration arising from cold-storage and/or agitation, the phospholipid disorder of plasma membranes and the mitochondria membrane potential in viable sperm were evaluated in the present study. These quality traits were not affected by agitation, thus supporting the notion that sperm surviving chilling stress are especially resistant to vibration stress, similar to their high resistance against stressors resulting from long-term storage or thermic incubation under capacitating conditions[9]. It cannot be ruled out, however, that a higher intensity of agitation for a longer time might also affect mitochondria as previously shown for boar sperm exposed to 300 rpm for 6 h[2]. Together, our results underline the option to use 5 °C in pig AI without affecting fertility as long as a sufficient number of viable sperm are present in the semen dose. All the more, it is more

feasible to provide stable transport conditions at 5 °C compared to 16-18 °C as is currently common practice.

In the original study by Schulze et al. (2018) describing the effect of vibration emission on sperm quality stored at 17°C, the agitation-induced alkalinization of the BTS extender medium was suggested to contribute to the loss of motility. This notion was supported by the observation that pH values above 7.5 arising from loss of CO_2 in the air compartment of the semen tube are detrimental to boar spermatozoa extended in BTS medium[14]. In the present study, the pH level remained below critical values in both semen extenders, indicating that pH-shifts play a minor role in agitation injury. It is to emphasize that the extent of cell damage as expressed in reduced motility and membrane integrity is relatively small but it could still be relevant for fertility if cell stressors from other sources are present or low sperm numbers per semen dose are used for AI. Moreover, our data indicate that some males could show a higher susceptibility to this sperm stressor. Noteworthy, agitation under the described standardized laboratory conditions did not affect the long-term storage capacity of sperm neither when stored at 17 ° C nor at 5 °C. Regardless of agitation stress, sperm quality in Androstar Plus was consistently higher compared to BTS, these results confirming several previous studies[15-17] and thus offering a higher sperm protection.

Besides shearing forces, interfacial phenomena have been identified as a significant source of cell damage in agitated suspensions. Sperm encounter gas-liquid interfaces due to bubble formation in shaken semen doses. The dynamic forces associated with bubble formation and bubble bursting appear to be the main source of cell injury and are related to the bubble amounts and sizes[3]. A loss of mitochondria potential in endothelial cells after contact with air bubbles[18] was not observed for sperm in the present study despite the agitation-induced formation of bubbles with different sizes. The damaging cell-interfacial effects could be enhanced if foam layers were present[3]. This was not the case in our study but does occur if foam production is stimulated, for example, by alternate dilution techniques[19] or extender media containing albumin.

In conclusion, extended semen designed for 17 °C storage and shipped on the day of collection is sensitive to vibration emission, whereas semen slowly cooled to 5 °C and shaken 20 h after processing is more resistant. Strategies to counteract agitation stress on liquid preserved semen during shipping should be

developed. These might include reduction of shear sensitivity in spermatozoa through membrane protection and prevention of bubble formation by adapted extender formula, as well as reduced air inclusion in semen tubes and the reduction of vibration emission by shock absorbing semen boxes. A combination of these measures will be most promising to maintain high quality in transported semen.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

DW, AL and MS designed the research study; AP and AL performed the experiments; AP, UJ and AL analyzed and visualized the data. AP wrote the original draft of the manuscript. DW critically revised the manuscript with contributions from AL, MS, UJ and FB. DW, FB and MS were involved in the funding acquisition. All authors have read and approved the submitted version of the manuscript.

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TABLES

Table 1. Treatment of semen extended in Beltsville Thawing Solution (BTS), Androstar Plus (APL) before storage at 17 °C (Experiment 1) or Androstar Premium (APR) before storage at 5 °C (Experiment 2). Samples were held motionless (controls, C), or shaken (200 rpm, 4 h) either at 22 °C or 17 °C (Experiment 1) or at 22 °C or 5 °C (Experiment 2) to simulate agitation stress during transport.

Extender / treatment groups (Experiment 1)	**Control, held at 22 °C, 90 min**	**Shaken at 22 °C, 4 h**	**Shaken at 17 °C, 4 h**
BTS-C	X	-	-
BTS-22	-	X	-
BTS-17	X	-	X[1)]
APL-C	X	-	-
APL-22	-	X	-
APL-17	X	-	X[1)]
Extender / treatment groups (Experiment 2)	**Control, held at 22 °C, 4 h**	**Shaken at 22 °C, 4 h**	**Shaken at 5 °C, 4 h**
APR-C	X	-	-
APR-22	-	X	-
APR-5	X	-	X[2)]

[1)]Samples were held motionless at 17 °C for 60 min to achieve temperature adaptation before the onset of shaking.

[2)]Samples were held motionless at 17 °C for 4 h, then motionless at 5 °C for 16 h to achieve temperature adaptation before the onset of shaking.

Table 2. Effect of agitation stress on the membrane integrity (plasma membrane and acrosome) of sperm extended in Beltsville Thawing Solution (BTS) or Androstar Plus (APL); n = 16 boars; Experiment 1. Agitation during transport was simulated by shaking 90 mL semen doses at 200 rpm for 4 h immediately after semen processing at 22 °C or after a holding time (22°C, 90 min,) at 17°C. The effect of shaking was examined during subsequent semen storage at 17 °C. Data are shown as mean ± standard deviation.

			Time of storage at 17 °C		
Parameter	Treatment	Extender	24 h	72 h	144 h
Membrane intact sperm (PI and FITC negative; %)	Control	BTS	86.8 ± 4.9^{aA}	87.7 ± 3.6^{a}	85.4 ± 3.1^{aA}
		APL	88.4 ± 4.0^{aB}	88.8 ± 3.3^{a}	87.3 ± 3.7^{aB}
	Shaken at 17°C	BTS	81.8 ± 4.8^{bA}	82.6 ± 4.0^{b}	79.5 ± 4.4^{bA}
		APL	83.6 ± 4.2^{bB}	83.6 ± 3.8^{b}	83.2 ± 3.5^{aB}
	Shaken at 22°C	BTS	81.8 ± 4.2^{bA}	82.4 ± 2.5^{b}	80.3 ± 3.5^{bA}
		APL	83.6 ± 4.5^{bB}	83.5 ± 2.9^{b}	83.3 ± 3.0^{bB}
pH value	Control	BTS	7.18 ± 0.07^{aA}	7.25 ± 0.06^{aA}	7.30 ± 0.08^{aA}
		APL	7.09 ± 0.06^{aB}	7.15 ± 0.04^{aB}	7.19 ± 0.07^{aB}
	Shaken at 17°C	BTS	7.18 ± 0.06^{aA}	7.25 ± 0.06^{aA}	7.30 ± 0.07^{abA}
		APL	7.09 ± 0.5^{aB}	7.16 ± 0.05^{aB}	7.20 ± 0.08^{aB}
	Shaken at 22°C	BTS	7.18 ± 0.06^{aA}	7.25 ± 0.06^{aA}	7.32 ± 0.08^{bA}
		APL	7.09 ± 0.05^{aB}	7.15 ± 0.05^{aB}	7.20 ± 0.06^{aB}

FITC-PNA: Fluorescein isothiocyanate-conjugated peanut agglutinin; PI: Propidium iodide.

[a,b] Values with different superscripts in a column indicate statistical difference among treatments within each extender ($p < 0.05$). A,B values with different superscript capital letters in a column indicate statistical difference among extenders within the treatments ($p < 0.05$).

Table 3. Effect of agitation stress immediately after semen processing at 22 °C or 20 h later at 5 °C on quality traits of sperm extended in Androstar Premium (APR); n = 11 boars, Experiment 2. Agitation during transport was simulated by shaking 90 mL semen doses at 200 rpm for 4 h immediately after semen processing at 22 °C (APR-22) or after a holding time (22°C, 90 min,) at 5 °C (APR-5). The effect of shaking was examined during subsequent semen storage at 5 °C. Data are shown as mean ± standard deviation.

		Time of storage at 5° C		
Parameter	Treatment	24 h	72 h	144 h
Spermatozoa with intact plasma membrane and acrosome (PI and FITC negative; %)	Control	82.6 ± 3.2	80.4 ± 3.0	81.3 ± 2.9
	APR-22	79.4 ± 3.3	79.7 ± 2.9	80.8 ± 2.3
	APR-5	79.8 ± 3.8	80.3 ± 2.9	79.4 ± 3.4
Viable spermatozoa with low membrane fluidity (Yo-Pro1 and M-540 negative; %)	Control	73.1 ± 15.2	73.5 ± 13.2	71.2 ± 15.7
	APR-22	70.5 ± 14.2	73.5 ± 14.9	70.2 ± 16.4
	APR-5	72.3 ± 15.8	74.8 ± 14.1	71.4 ± 15.6
Viable spermatozoa with high mitochondrial membrane potential (PI neg, JC-1 positive; %)	Control	95.1 ± 1.0	94.9 ± 0.9	93.0 ± 1.6
	APR-22	95.1 ± 1.1	94.7 ± 1.1	92.9 ± 1.8
	APR-5	94.8 ± 1.7	94.3 ± 1.1	93.1 ± 1.9

FITC-PNA: Fluorescein isothiocyanate-conjugated peanut agglutinin; PI: Propidium iodide; M-540: Merocyanine 540. Values between treatments did not differ ($p > 0.05$).

FIGURE LEGENDS

Figure 1. Experimental setting: Semen tubes (width: 4.5 cm, length: 13.5 cm, filling volume 90 ml) shaken on an orbital shaker with 200 rpm and 1 cm amplitude. The 90 mL tubes were filled with 85 mL extended semen and 5 mL residual air. Shaking was performed for 4 h at different times after filling and at different temperatures.

Figure 2. Effect of agitation stress on motility (total) of sperm extended in Beltsville Thawing Solution (BTS) or Androstar Plus (APL); n = 16 boars, Experiment 1. Agitation during transport was simulated by shaking semen doses at 200 rpm for 4 h immediately after semen processing at 22 °C or after a holding time (22 °C, 90 min,) at 17°C. The effect of shaking was examined during subsequent semen storage at 17 °C. Data are shown as box-plots with the highest and lowest values represented by whiskers, inter-quartiles between quartiles 1 and 3 shown as boxes, and median values. a,b: Significant differences ($p< 0.05$) between treatment within extender group. A,B: Significant differences ($p < 0.05$) between extenders within treatment group.

Figure 3. Effect of agitation stress immediately after semen processing at 22 °C or 20 h later at 5 °C on motility (total) of sperm extended in Androstar Premium (APR); n = 11 boars, Experiment 2. Agitation during transport was simulated by shaking semen doses at 200 rpm for 4 h. The effect of shaking was examined during subsequent semen storage at 5 °C. Data are shown as box-plots with the highest and lowest values represented by whiskers, inter-quartiles between quartiles 1 and 3 shown as boxes, and median values. a,b: Significant differences ($p < 0.05$) between treatments.

Figure 1

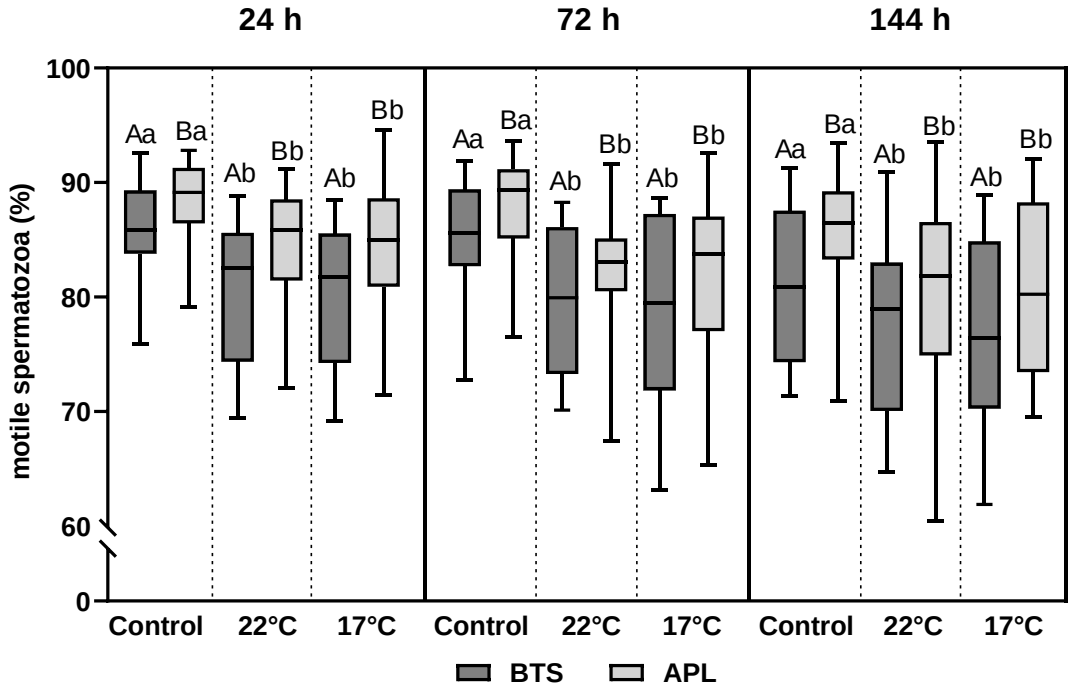
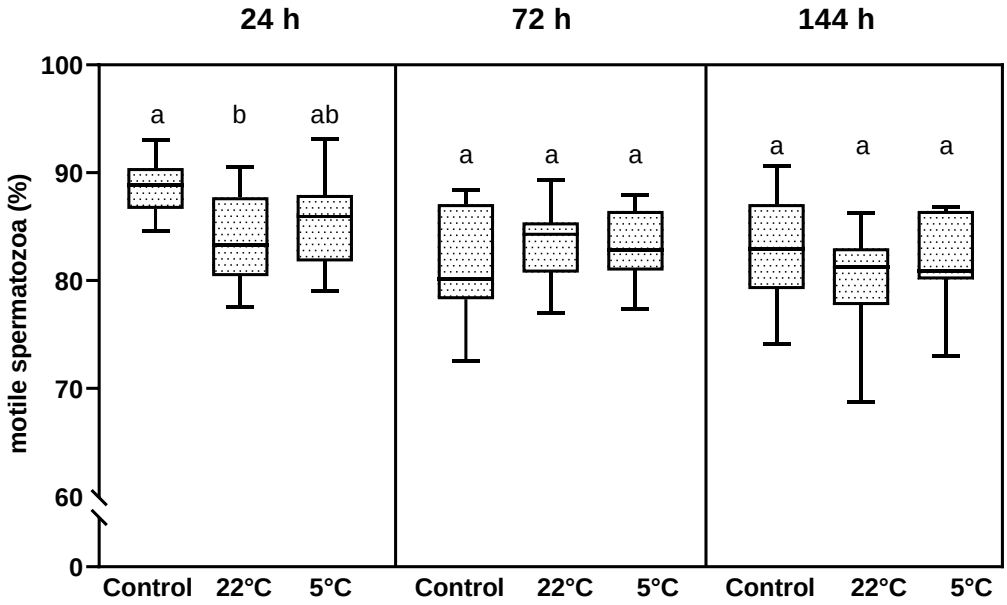

24 h
72 h
144 h
100
90
80
70
60
0
motile spermatozoa (%)
Aa
Ba
Ab
Bb
Ab
Bb
Aa
Ba
Ab
Bb
Ab
Bb
Aa
Ba
Ab
Bb
Ab
Bb
Control
22°C
17°C
Control
22°C
17°C
Control
22°C
17°C
BTS
APL

Figure 2


24 h
72 h
144 h
motile spermatozoa (%)
100
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22°C
5°C
Control
22°C
5°C
Control
22°C
5°C

Figure 3

SUPPLEMENTAL MATERIAL

STable 1. Effect of agitation stress on total and progressive motility of sperm extended in Beltsville Thawing Solution (BTS) or Androstar Plus (APL); n = 16 boars; Experiment 1. Agitation during transport was simulated by shaking of 90 mL semen doses at 200 rpm for 4 h immediately after semen processing at 22 °C or after a holding time (22 °C, 90 min) at 17°C. The effect of shaking was examined during subsequent semen storage at 17 °C. Data are shown as mean ± standard deviation.

			Time of storage		
Parameter	Treatment	Extender	24 h	72 h	144 h
Total motility (%)	Control	BTS	85.7 ± 4.5^{aA}	85.3 ± 4.7^{aA}	81.1 ± 6.5^{aA}
		APL	88.1 ± 3.8^{aB}	87.7 ± 5.1^{aB}	85.3 ± 5.7^{aB}
	Shaked at 17°C	BTS	80.4 ± 6.2^{bA}	79.0 ± 7.9^{bA}	76.7 ± 8.2^{bA}
		APL	84.7 ± 6.3^{bB}	82.2 ± 7.8^{bB}	80.6 ± 7.5^{aB}
	Shaked at 22°C	BTS	80.8 ± 6.3^{bA}	79.5 ± 6.3^{bA}	77.2 ± 7.5^{bA}
		APL	83.9 ± 6.1^{bB}	81.6 ± 7.2^{bB}	80.1 ± 9.1^{bB}
Progressive motility (%)	Control	BTS	83.0 ± 5.7^{aA}	82.3 ± 5.5^{aA}	76.6 ± 8.9^{aA}
		APL	88.5 ± 4.8^{aB}	84.9 ± 6.0^{aB}	81.8 ± 6.4^{aB}
	Shaked at 17°C	BTS	77.4 ± 7.1^{bA}	75.5 ± 8.6^{bA}	72.3 ± 10.0^{bA}
		APL	81.5 ± 7.3^{bB}	78.7 ± 9.0^{bB}	76.6 ± 8.7^{bA}
	Shaked at 22°C	BTS	77.3 ± 7.4^{bA}	76.1 ± 6.7^{bA}	72.2 ± 10.1^{bA}
		APL	80.3 ± 7.4^{bB}	78.3 ± 8.2^{bB}	76.3 ± 10.6^{bB}

[a,b] Values with different superscripts in a column indicate statistical difference among treatments within each extender ($P < 0.05$). A,B Values with different superscript capital letters in a column indicate statistical difference among extenders within the treatments ($P < 0.05$

STable 2. Effect of agitation stress immediately after semen processing at 22 °C or 20 h later at 5 °C on total and progressive motility of sperm extended in Androstar Premium (APR); n = 11 boars, Experiment 2. Agitation during transport was simulated by shaking of 90 mL semen doses at 200 rpm for 4 h immediately after semen processing at 22 °C (APR-22) or after a holding time (22 °C, 90 min) at 5 °C (APR-5). The effect of shaking was examined during subsequent semen storage at 5 °C. Data are shown as mean ± standard deviation.

			Time of storage		
Parameter	Treatment	Extender	24 h	72 h	144 h
Total motility (%)	Control	APR	88.5 ± 2.5[a]	81.2 ± 4.9[a]	83.0 ± 4.6[a]
	Shaked at 5°C	APR	85.1 ± 4.0[ab]	83.2 ± 3.4[a]	81.6 ± 3.9[a]
	Shaked at 22°C	APR	83.9 ± 4.0[b]	83.5 ± 3.1[a]	79.9 ± 4.5[a]
Progressive motility (%)	Control	APR	84.6 ± 2.7[a]	78.5 ± 5.6[a]	78.0 ± 4.8[a]
	Shaked at 5°C	APR	82.3 ± 4.4[ab]	80.0 ± 3.3[a]	76.8 ± 4.7[a]
	Shaked at 22°C	APR	80.5 ± 3.7[b]	79.4 ± 3.6[a]	74.9 ± 4.9[a]

[a,b] Values in a column indicate statistical difference within the treatments ($P < 0.05$)

CAPÍTULO V – QUARTO MANUSCRITO

REPRODUCTIVE PERFORMANCE OF SOWS INSEMINATED WITH ANTIBIOTIC-FREE SEMEN DOSES PRESERVED AT 5°C

MANUSCRITO A SER SUBMETIDO EM PERIÓDICO CIENTÍFICO

REPRODUCTIVE PERFORMANCE OF SOWS INSEMINATED WITH ANTIBIOTIC-FREE SEMEN DOSES PRESERVED AT 5°C

Short running title: Fertility after antibiotic-free insemination

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Abstract

The concerning about worldwide bacterial resistance to conventional antimicrobials lead to the development of strategies to reduce the usage of antibiotics in extended boar semen as a precaution measure against bacterial resistance. The antibiotic-free storage of semen doses at 5 °C, instead of storage at 17 °C with antibiotics, is a promising alternative. The aims were to access the *in vitro* sperm quality and fertility of sows inseminated with semen doses without antibiotic and stored at 5°C. Ejaculates of four boars were extended in one step to 1.7 $\times 10^9$ spermatozoa in a total of 50 mL. Semen was split into 2 groups: control group (17C), extended with Androstar® Premium with antibiotics, stored at 17°C; hypothermic storage group (5C), semen was extended with antibiotic-free Androstar® Premium, stored at 5°C. For the fertility trial, 256 sows were randomly selected and assigned into the 2 groups: 17C vs 5C. Total motility from semen doses remained > 80% during storage until 168 h, regardless the group (P> 0.05). The pregnancy rate was 98.0 (± 0.71) in both groups (P=1.00) while the farrowing rates were 97.0 (± 0.50) and 98.0 (± 0.50) in group 17C and 5C, respectively (P= 0.70). No difference was found (P≥ 0.59) for the total piglets born (16.2 ± 0.3 vs 16.4 ± 0.3) and total born alive (15.0 ± 0.3 vs 15.1 ± 0.2) for groups 17C and 5C, respectively. The present study clearly shows that antibiotic-free semen doses stored at 5°C provided an applicable alternative for boar semen storage.

Key words: Fertility; Hypothermic storage, Reproduction, Swine

1. INTRODUCTION

The use of artificial insemination (AI), an efficient method to optimize the use of genetically superior boars, minimizes the risk of transmitting venereal diseases and accelerates the genetic progress in swine industry. Due to the success of its introduction in swine industry more than 90% of breeding farm worldwide have being using the method for breeding (Waberski et al., 2019). Two main challenges to promote greater results have been discussed: the first is the control of bacteria in semen without antibiotics, since there is a worldwide concerning of multi-drug resistant bacterial (Aslam et al., 2018), including the conventional antimicrobials used in boar semen extenders (Schulze et al., 2015); the second is reduction of sperm cells in semen doses, in order to optimize and disseminate genetic material from high index boars (García-Vázquez et al., 2019).

Boar ejaculates usually contain bacteria originated from the semen collection (Althouse & Lu, 2005; Goldberg et al., 2013) that may impair *in vitro* quality (motility, agglutination, membrane integrity), and fertility rates (Martín et al., 2010). For this reason, the use of antimicrobials is mandatory in the composition of semen extenders (Knox, 2016). However, there is an increasing worldwide bacterial resistance to conventional antimicrobials used in boar semen extenders (Schulze et al., 2015).

In this sense, some efforts have been developed to determine critical factors for contamination to reduce bacterial load in extended boar semen (Schulze et al., 2015). The storage of semen doses at 5 °C in absence of antibiotics is a promising alternative to the common storage at 17°C with antibiotics (Waberski et al., 2019; Menezes et al., 2020; Jäkel 2021). The reduction of the temperature of storage leads to a bacteriostatically effect, maintaining a reduced bacterial load, and preventing

detrimental effects on sperm quality. However, it is well known that boar sperm is very sensible to the negative effect of chilling injury (Waberski et al., 2019; Menezes et al., 2020). The high sensibility of sperm to chilling injury, leads to lethal damage to a minor percentage of sperm but high percentage suffer a sub-lethal damage (Schmid et al., 2013). The sub-lethal damage compromises the functionality of sperm and remains undetected by the routinely and conventional semen analysis (Waberski et al., 2016). Promising results *in vivo* and *in vitro* pointed the antibiotic-free 5 °C preservation as an alternative to conventional storage (17 °C with antibiotics). According to previous studies, it is mandatory that cooling rates remain in an optimal frame (Paschoal et al., 2020) to guarantee great result of sperm quality. For *in vivo* results, only a high amount of sperm was tested (Jäkel et al., 2021), but satisfactory results os sperm quality were found.

Regarding the reduction of sperm cells in semen doses, it is very important pointing that the interest in maximizing efficiency, is leading to the replacement of the Cervical Artificial Insemination - CAI by the Post Cervical Artificial Insemination - PCAI (García-Vázquez et al., 2019). The advantages of using PCAI include a lower number of spermatozoa per dose, an increased number of insemination doses produced per male, causing a reduction on the number of boars allocated in studs (Watson et al., 2002; Mezalira et al., 2005; Hernandez-Caravaca, 2012).

Taking all the background information together, the aims of this study were to access *in vitro* sperm parameters and the reproductive performance of weaned sows submitted to PCAI with semen doses without antibiotic and stored at 5°C.

2. MATERIAL AND METHODS

All procedures involving animals were performed according to the Ethics Committee on Use of Animals of the Federal University of Rio Grande do Sul, under the protocol number 39447.

2.1 Animals, housing and feeding

The study was conducted in a sow farm with an inventory of 5,600 females, located in the Santa Catarina state (26°22′13″S, 50°08′40″W), southern Brazil. The study was conducted between August and December with average, minimum, and maximum temperatures of 23.0, 9.1, and 33.6°C; average relative humidity: 85%.

A total of eighteen healthy boars (Agroceres AG337 PIC), housed in individual crates of 0.70 m width and 2.4 m length with slatted floor equipped with nipple drinkers were used. Water access was *ad libitum* and boars were feed under the same nutritional based corn and soybean diet (2.1 to 2.4 kg per day, 3.30 Mcal ME per kg, 15 % crude protein, 0.55 % standardized ileal digestible Lysine). All boars were under weekly routine of semen collection. As standard, pre spermatic fraction was discarded and the ejaculates (rich and poor sperm fraction) were collected in a pre-warmed thermal cup. Only normospermic ejaculates (minimum of 75 % morphologically normal spermatozoa, at least 80% total sperm motility, and total number $\geq 30 \times 10^9$ spermatozoa per ejaculate) from each boar were extended.

A total of 256 sows (PIC Camborough®) housed in individual crates of 0.60 m width and 2.2 m length during gestation and of 0.70 m width and 2.4 m length during weaning were used. Water access was *ad libitum* and sows were feed under the same nutritional based corn and soybean diet during gestation (3.2 Mcal ME per kg, 12.4 % crude protein, 0.50 % standardized ileal digestible Lysine) receiving 1.7 to kg from the first to the 85^{th} day after insemination; 2.2 kg from the 86^{th} day on.

During lactation sows were feed *ad libitum* (3.3 Mcal ME per kg, 18.7 % crude protein, 1.10 % standardized ileal digestible Lysine).

2.2 Experimental design

Two experiments were performed, the first aimed to access *in vitro* quality for selection of the best 4 boars to proceed with experiment 2. The criteria used to select the boars was the maintenance of high sperm quality during storage at 5°C. The second experiment aimed to access *in vitro* quality and fertility rates of sows inseminated with antibiotic-free doses stored at 5 °C.

2.2.1 Experiment 1

Semen from 18 boars was collected and the dilution was isothermally performed (34°C) in one step to 1.5 $\times 10^9$ spermatozoa in a total of 50 mL in transparent QuickTip Flexitubes® (Minitüb, Germany), according to 2 groups: control group (17C), semen was extended with Androstar® Premium (Minitüb, Germany) and stored at 17°C after being held during 90 minutes at 22 °C; hypothermic storage group (5C), semen was extended with antibiotic-free Androstar® Premium and stored at 5°C. To avoid the negative effect of chilling injury, semen doses were cooled into controlled regime as proposed by Paschoal et al. (2020), adapted to the laboratory conditions. Briefly, semen doses were held during 120 min at room temperature (22 °C) and stored together in a closed cardboard box filled with 35 tubes and stored at 5°C, having an initial cooling rate of 0.01 °C min^{-1} before 5 °C storage. Sperm motility was assessed after 24, 72, 120 and 168 h of storage. Samples incubated during 20 min at 37 °C were evaluated with the computer-assisted semen analysis system, AndroVision®, version 1.1 (Minitüb, Germany), using four-chamber slides (Leja, Nieuw Vennep, Nederland) with depth of 20 μm. For each sample, five successive fields in the central axis of the chamber were recorded at a rate of 60 frames per 1 s for each field using a 100

× magnification. At least 500 spermatozoa per sample were analyzed. Spermatozoa were evaluated as "motile" when their amplitude of lateral head displacement was higher than 1 µm and their curve line velocity was higher than 24.0 µm/sec.

Acrosome integrity was assessed after 24, 72, 120 and 168 h of storage. Aliquots of extended semen were fixed in a formalin-citrate solution (2.94%). All samples were analyzed under a phase contrast microscope and 1000× magnification with immersion oil. A total of 200 spermatozoa per sample were analyzed and classified as intact or not intact (Pursel et al., 1972).

2.2.1 Experiment 2

In this experiment, from the eighteen boars used in experiment 1, only 4 proceed to semen doses production. Boars were ranked according to motility descriptive statistics and the selected boars were the ones who maintained higher values of progressive motility during storage at 5°C. Ejaculates of four boars were collected by the "gloved-hand" method and extended isothermically (34 °C) in one step to 1.7 $\times 10^9$ spermatozoa in a total of 50 mL in transparent QuickTip Flexitubes® (Minitüb, Germany). Different from experiment 1, in this experiment a pool of the ejaculates from the 4 boars was used, producing heterospermic semen doses. Semen was split into 2 groups: control group (17C), extended with Androstar® Premium with antibiotics (0.25 g/L gentamicin) and stored at 17°C; hypothermic storage group (5C), semen was extended with antibiotic-free Androstar® Premium, stored at 5°C. The holding regime used for both groups were in accordance with regimes used in Experiment 1.

Sperm motility was assessed after 24, 72, 120 and 168 h of storage using the CASA system, AndroVision®, version 1.1 (Minitüb, Germany), with the identical method and setting described in Experiment 1. Acrossome integrity was assessed

after 24, 72 and 168 h using a phase contrast microscope (Olympus, Tokyo, Japan) as descried in Experiment 1.

For the fertility trial, 256 sows were randomly selected according to the following parameters: parity 1 to 7; number of previous total born (TB) piglets (>10); lactation length of 17-23 days and body condition score (BCS) of 2 – 3.5. No differences ($P \geq 0.52$) were found for sow parity (2.94 ± 0.04), previous TB (14.7 ± 0.22), lactation length (19.9 ± 0.10) and BCS (3.17 ± 0.15) at the onset of the experiment among the evaluation groups. Only sows with a weaning-to-estrus interval of 4 days were considered.

Estrus detection was performed once a day (07:00 AM) using fence-line boar contact and back-pressure test. Sows were post-cervically inseminated at detection of estrus (0 h) and then in 24 h interval while in standing estrus (maximum 3 inseminations per sow). In both groups, sows were inseminated with semen doses produced from the same pool and stored for 24, 48 and 72 h for the first, second and third insemination, respectively.

Return-to-estrus detection started 17 days after the first insemination and pregnancy detection with transabdominal ultrasound (A6V, Sonoscape® Co. Ltda, Shenzhen, China, 5.5-8 MHz) was performed on day 24 after AI. Pregnancy rate (PR), farrowing rate (FR), the total number of piglets born (TB), total number of piglets born alive (TBA), stillborn (SB) and mummified (MM) piglets were recorded.

2.3. Statistical analysis

For data analysis, the Statistical Analysis System for Windows SAS, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used. Data were analyzed by the GLIMMIX procedure: Sperm data were analyzed assuming a normal distribution using repeated measures with groups, time of storage and their

interaction as fixed effect. The week of semen collection was considered as random effect. For fertility data, the group was considered as fixed effect while week as random effect in the models. The PR and FR were analyzed by logistic regression, assuming a binary distribution. The TB and TBA were analyzed assuming continuous distribution, while MM and SB were analyzed assuming binomial distribution with the GLIMMIX procedure.

When the ANOVA revealed a significant difference among groups, comparison using Tukey–Kramer test was performed. Differences were considered significant at $P < 0.05$. Data are presented as mean ± standard error (SE).

3. RESULTS

3.1 Experiment 1

The mean of total motility from the 17C group was 88.6% (± 0.26) while for the group 5C the mean of total motility was 81.2% (± 0.55). The mean progressive motilities were 86.5 % (± 0.30) and 78.2 % (± 0.59) for the 17C and 5C groups, respectively. An interaction between time of storage and group was observed in total ($P<0.0001$) and progressive motility ($P<0.0001$ – Figure 2). Only at 168 h of storage the sperm motilities were lower compared to 24 h in the 17C group, while in 5C group motility reduced in all times of storage.

3.2 Experiment 2

The total motility for pooled semen used for AI remained > 80% during storage until 168 h, regardless the temperature groups. The mean total motility of samples stored at 17 °C ranged 85.0% (± 1.0) to 83.4% (± 1.8) at 168 h of storage, while total motility of samples stored at 5 °C ranged from 87.1% (± 0.6) to 82.9% (± 2.7). The mean progressive motility of samples stored at 17 °C ranged from 82.0% (± 1.1) at 24 h of storage to 78.6% (± 1.5) at 168 h. Doses stored at 5 °C ranged from 88.3 (± 0.9) to 77.0 (± 2.8) at 24 h and 168 h of storage, respectively.

No differences among groups, time of storage or their interactions were found for total (P≥0.41; Table 3) and progressive motility (P≥0.19). Spermatozoa with defective acrosomes were fewer than 10% in all samples at any time of storage. The PR was 98.0 (± 0.71) in both groups (P=1.00) while the FR was 97.0 (± 0.50) and 98.0 (± 0.50) in group 17C and 5C, respectively (Table 4). No difference between groups was found for the total piglets born (16.2 ± 0.3 vs 16.4 ± 0.3) and total born alive (15.0 ± 0.3 vs 15.1 ± 0.2) for group 17C and 5C, respectively. The mean of stillborn piglet was 5.0% (± 0.01) and 5% (± 0.01) in group 17C and 5C, respectively (P=0.99), while the mean of mummified fetuses was 5.0 % (± 0.01) in the 17C group and 5.0 % (± 0.01) in 5C group (P=0.23).

Table 1. Mean (± SE) motility of semen doses extended with Androstar® Premium (n = 18 boras) with and without antibiotics, stored at 17 °C and 5 °C in different times of storage.

Motility	**Group**	**Time of storage, h**			
		24	**72**	**120**	**168**
Total (%)	17C	90.1 (± 0.5) ^{aA}	88.9 (± 0.5) ^{abA}	88.3 (± 0.5) ^{abA}	87.3 (± 0.5) ^{bA}
	5C	86.7 (± 0.5) ^{aB}	83.5 (± 1.0) ^{bB}	78.8 (± 1.1) ^{cB}	75.7 (± 1.1) ^{dB}
Progressive (%)	17C	88.2 (± 0.6) ^{aA}	86.8 (± 0.6) ^{abA}	86.1 (± 0.6) ^{abA}	84.8 (± 0.6) ^{bA}
	5C	84.3 (± 0.5) ^{aB}	80.9 (± 1.0) ^{bB}	75.7 (± 1.2) ^{cB}	71.9 (± 1.1) ^{dB}

17C – Semen diluted with Androstar® Premium with antibiotics (0.25 g/L gentamicin sulphate) and stored at 17°C.

5C – Semen diluted with Androstar® Premium without antibiotics and stored at 5°C. Different capital letters mean statistical difference ($P<0.05$) among groups. Different lower case letters mean statistical difference ($P<0.05$) among the time of storage.

Table 2. Mean (± SE) motility of semen doses extended with Androstar® Premium with and without antibiotics, stored at 17 °C and 5 °C, C in different times of storage.

Motility	**Group**	**Time of storage**			
		24 h	**72 h**	**120 h**	**168 h**
Total (%)	17C	85.0 (± 1.0)	83.6 (± 0.8)	86.2 (± 1.6)	83.4 (± 1.8)
	5C	87.1 (± 0.6)	83.8 (± 2.6)	84.3 (± 1.6)	82.9 (± 2.7)
Progressive (%)	17C	82. 0 (± 1.1)	80.9 (± 0.6)	81.8 (± 1.4)	78.6 (± 1.5)
	5C	83.3 (± 0.9)	79.9 (± 3.4)	78.5 (± 2.1)	77.7 (± 2.8)

17C – Semen diluted with Androstar® Premium with antibiotics (0.25 g/L gentamicin sulphate), stored at 17°C.

5C – Semen diluted with Androstar® Premium without antibiotics and stored at 5°C.

Different capital letters mean statistical difference ($P<0.05$) among groups. Different lower case letters mean statistical difference ($P<0.05$) among the time of storage.

Table 3. Fertility data (Mean ± SE) of sows submitted to artificial insemination with semen doses stored at 17 °C with antibiotics and 5 °C without antibiotics (Experiment 2).

	Group		P-value
	17C	5C	
Sows (n)	128	128	-
Pregnancy rate at day 24, n/n (%)	125/128 (98.0 ± 0.71)	125/128 (98.0 ± 0.71)	1.00
Farrowing rate, n/n (%)	124/128 (97.0 ± 0.50)	125/128 (98.0 ± 0.50)	0.70
Total number of piglets born	16.2 (± 0.3)	16.4 (± 0.3)	0.59
Total number of piglets born alive	15.0 (± 0.3)	15.1 (± 0.2)	0.78
Stillborn piglet, %	5.0 ± 1.0	5.0 ± 1.0	0.99
Mummified piglet, %	5.0 ± 1.0	5.0 ± 1.0	0.23

17C – Semen diluted with Androstar® Premium with antibiotics (0.25 g/L gentamicin sulphate), stored at 17°C.

5C – Semen diluted with Androstar® Premium without antibiotics and stored at 5°C.

4. DISCUSSION

The present study provides evidence that, despite the minor sublethal alterations induced by chilling injury, the hypothermic preservation of boar semen without antibiotics is a promising strategy to replace the antibiotics without impairing fertility rates at field conditions.

Compared to the conventional preservation at 17 °C, storage at 5 °C was expected to reduce sperm quality during storage. The sensibility of sperm against the cold shock would support this hypothesis. However, the lethal sperm damage expressed as loss of motility was low in stored semen samples extended with Androstar® Premium after controlled cooling to 5 °C. Our results are in agreement with recent studies (Paschoal et al., 2020; Jäkel et al., 2021).

The resistance of semen during cooling stress differs between boars (Parilla et al., 2012; Holt et al., 2005) that may be classified as “good” and “bad” coolers, according to their freezing tolerance. In the present study, we selected the best 4 boars to perform the fertility trial, however, the screening of semen quality performed in Experiment 1 revealed that the ejaculates of only 3 from the 18 boars did not fulfilled minimum standards for usable semen, i.e. at least 65% motility and less than 25% abnormal sperm at 72 h of storage at 5 °C. From the perspective of individual sensibility of boars to chilling injury, sperm tolerance to 5 °C cooling and storage seems to be a possibility. Our results are in accordance with Jäkel et al. (2021) that found a high percentage of ejaculates (88.2%) from 34 different boars fulfilling the minimum standards for usable semen, based on the same criteria cited before. The prediction of expected quality of semen submitted to cooling would be an interesting tool to improve the usage of “good” coolers. In this sense some markers and proteomic characteristics have been discussed (Menezes et al., 2020), aiming to make available to identify the hypothermic storage ability of semen from

individual boars before introducing them to the AI centre, leading to even higher security at AI performance.

The high *in vitro* performance found in our study confirmed that cooled semen even with a sub-lethal damage, can be used on field, since our trial revealed high fertility results with semen stored at 5 °C without antibiotics compared to those conventionally stored at 17 °C with antibiotics. Consistent studies have already been performed to assess the effects of semen cooling (Waberski et al., 2020; Helen et al., 2021) and the main difference in the current study is that here we brought the number of sperm used in semen doses closer to the reality of post cervical artificial insemination, we also included one single estrus detection and insemination instead of twice daily, the most common practice in many herds worldwide (Bortolozzo et al., 2015; Waberski et al., 2019). According to Waberski et al. (1994), the cryopreservation of boar semen decreases sperm survival in the female reproductive tract to approximately 4 h compared to approximately 12 h in liquid semen stored at 17 °C. In this sense, it is possible to infer that the chilling and storage at 5 °C will also reduce the functional life span and quality of spermatozoa. This reduction of quality is associated to the sub-lethal chilling effects which might remained undetected with the assays used here. Taking into account this critical scenario, and that a sufficient number of fully competent sperm in the semen doses are needed for maximum fertility (Amman et al., 2018), we can affirm that we promoted a challenge that could drastically reduce fertility rates. In a previous study (Helen et al., 2021) a higher sperm number per dose (2.5×10^9) was chosen, in order to compensate additional challenges of once-daily estrus detection and the employed insemination protocol; however, in our study we used a sperm number of only 1.7×10^9 and fertility rates were not impaired.

5. CONCLUSION

Taking all our results together, the present study clearly shows that with the extender and the cooling rates to 5 °C used here, we provided an applicable alternative to storage at 17 °C, either to prevent or to minimize the misuse of antibiotic and consequent bacterial resistance problems.

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CONSIDERAÇÕES FINAIS

Com a utilização da IA, a pressão pela produção de doses inseminantes de qualidade ótima, tornou-se eminente, recebendo grande importância no ciclo reprodutivo de suínos. No entanto, elementos básicos de qualidade seminal são necessários para que a técnica seja cada vez mais utilizada de forma otimizada. Levando em consideração que o sêmen contém contaminação desde a coleta, com alguns agravantes no ambiente laboratorial, o uso de antimicrobianos na formulação dos diluentes é essencial para evitar os efeitos nocivos da bacteriospermia. No entanto, é importante considerar a preocupação mundial a respeito do desenvolvimento de bactérias multirresistentes aos antimicrobianos. Essa preocupação mobilizou diversos pesquisadores a desenvolverem estratégias para reduzir ou eliminar o uso de antimicrobianos nas doses.

O desenvolvimento desse projeto deixou evidente que o primeiro passo a ser seguido, trata-se de reduzir a contaminação desde a coleta, o que pode ser realizado de forma simples, com uma coleta mais higiênica e com a definição de pontos críticos que podem levar a contaminação, de forma a evitá-los nas rotinas das centrais.

Quando ponderamos a viabilidade de uma estratégia bastante promissora, o armazenamento hipotérmico das doses a 5 °C e não à 17 °C, o que é atualmente consolidado na indústria, levamos em consideração que não apenas a manutenção das doses a 5 °C representa um desafio para os espermatozoides, mas todo o processo de resfriamento que pode levar ao choque térmico, quando muito rápido, ou à proliferação bacteriana, quando muito lento. Nesse sentido, o desenvolvimento das taxas ótimas de resfriamento foi, sem dúvidas um passo crucial para que vislumbrar uma possível consolidação do uso desse armazenamento livre de antimicrobianos.

No entanto, considerando todo o contexto de centralização da produção em centrais bastante grandes, as quais distribuem doses a largas distâncias, o processo de transporte torna-se um desafio complementar, pois além do estresse gerado pelo resfriamento, as células passam ainda pelo choque mecânico e troca de gases gerados durante a emissão de vibrações do transporte. Nesse sentido, buscar definir a melhor temperatura na qual se pode realizar transporte das doses também foi um

passo bastante importante na mobilização pela viabilização de usar as doses armazenadas a 5°C.

Após definir todos esses pontos, foi de extrema importância, ainda verificar se os resultados a campo em condições realistas levaria a uma redução nos índices zootécnicos dentro das granjas. Nesse sentido, em nosso último estudo, de extrema importância, demonstramos que a tecnologia é de fato promissora e factível para ser aplicada na prática. Um cuidado deve ser tomado apenas quando pensamos em aplicar esse resfriamento a todos os machos, pois foi possível verificar durante o desenvolvimento desse projeto, que as características individuais dos machos devem ser consideradas, pois nem todos conseguem manter a qualidade espermática após a redução da temperatura.

Dessa forma, com o presente trabalho concluímos que é possível afirmar que existem no mercado diluentes que mantém a qualidade espermática durante o armazenamento a 5 °C, que essa doses podem ser utilizadas, contando que alguns cuidados sejam tomando com relação a contaminação inicial do sêmen, à taxa de resfriamento e ao transporte. Vale lembrar ainda que essa não é uma tecnologia aplicável ao sêmen de todos os machos suínos, devido às suas características individuais quanto a capacidade de resfriamento.

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