UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

AVALIAÇÃO DA ADIÇÃO DE CISTEÍNA NO SÊMEN RESFRIADO PARA INSEMINAÇÃO EM SUÍNOS

DISSERTAÇÃO DE MESTRADO

Carolina Klein Severo

Santa Maria, RS, Brasil

2009

AVALIAÇÃO DA ADIÇÃO DE CISTEÍNA NO SÊMEN RESFRIADO PARA INSEMINAÇÃO EM SUÍNOS

Por

Carolina Klein Severo

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria, RS, como requisito parcial para obtenção do grau de **Mestre em Medicina Veterinária.**

Orientador: Prof. Paulo Bayard Dias Gonçalves
Co-orientador: Prof. Marcelo Soares

Santa Maria, RS, Brasil

2009

Severo, Carolina Klein, 1982-

S498a

Avaliação da adição de cisteína no sêmen resfriado para inseminação em suínos / por Carolina Klein Severo ; orientador Paulo Bayard Dias Gonçalves ; co-orientador Marcelo Soares. - Santa Maria, 2009.

93 f. ; il.

Dissertação (mestrado) – Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós-Graduação em Medicina Veterinária, RS, 2009.

1. Medicina veterinária 2. Cisteína 3. Centrifugação do sêmen 4. Sêmen resfriado 5. *Beltsville Thawing Solution* 6. Suínos I. Gonçalves, Paulo Bayard Dias, orient. II. Soares, Marcelo, co-orient. III. Título

CDU: 619:636.4.082

Ficha catalográfica elaborada por
Luiz Marchiotti Fernandes – CRB 10/1160
Biblioteca Setorial do Centro de Ciências Rurais/UFSM

UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

Avaliação da adição de cisteína no sêmen resfriado para inseminação em suínos

elaborada por
Carolina Klein Severo

como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

Comissão Examinadora:

João Francisco Coelho de Oliveira, Dr.
(Presidente)

Marcelo Soares, Dr. (UFSM)

João Carlos Deschamps, PhD, (UFPel)

Santa Maria, 07 de agosto de 2009.

AGRADECIMENTOS

Gostaria de agradecer a minha família por ter me dado à oportunidade, as condições e o apoio necessário para concluir mais esta etapa.

Ao meu namorado Ronaldo Rafael Roque por sempre me incentivar a crescer científica, ética, profissional e pessoalmente.

A minha amiga Daiana Calefi Lima por sempre me apoiar e a todos que incentivaram e apoiaram a pesquisa.

Aos colegas do Laboratório de Biotecnologia e Reprodução Animal e do Setor de Clínica e Reprodução de Suínos, principalmente os estagiários Andressa Minussi Pereira, Vitor Sales Truzzi, Lucas Oberherr e ao Medico Veterinário Gustavo Freitas Ilha por me auxiliarem no desenvolvimento da pesquisa.

Ao meu orientador professor Doutor Paulo Bayard Dias Gonçalves, ao meu co-orientador professor Doutor Marcelo Soares e aos professores Doutores João Francisco Coelho de Oliveira e Gabriel Ribas Pereira pela paciência, dedicação e conhecimentos transmitidos.

A Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Medicina Veterinária por promover ambiente adequado para o desenvolvimento a pesquisa.

Ao Laboratório de Virologia da Universidade Federal de Santa Maria pela concessão do microscópio para realização das análises.

E por fim, mas não menos importante ao Conselho Nacional de Pesquisa (CNPq) pela concessão da bolsa.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

Avaliação da adição de cisteína no sêmen resfriado para inseminação em suíno

AUTORA: CAROLINA KLEIN SEVERO
ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria, 07 de agosto de 2009.

O crescimento da suinocultura se deve a diversos avanços na área tecnológica. Buscando obter maior eficiência reprodutiva, foi analisado o efeito da cisteína ao diluente *Beltsville Thawing Solution* (BTS) e do processo de centrifugação sobre qualidade espermática. No primeiro experimento, foram avaliadas diferentes concentrações de cisteína no diluente BTS, conforme os seguintes tratamentos: BTS (grupo controle); CIS0,1 (BTS + 0,1 mM de cisteína); CIS0,5 (BTS + 0,5mM de cisteína); CIS1,0 (BTS + 1,0 mM de cisteína); CIS2,5 (BTS + 2,5 mM de cisteína); CIS5,0 (BTS + 5,0 mM de cisteína); CIS10,0 (BTS + 10,0 mM de cisteína) e CIS20,0 (BTS + 20,0 mM de cisteína). No segundo experimento, o sêmen foi dividido em: sêmen não centrifugado diluído em BTS/NC (grupo controle), sêmen não centrifugado diluído em BTS + 5,0 mM de cisteína (BTSCIS/NC), sêmen centrifugado diluído em BTS (BTS/CENT) e sêmen centrifugado diluído em BTS + 5,0 mM de cisteína (BTSCIS/CENT). Ambos os experimentos foram realizados para avaliar a influência dos diferentes tratamentos sobre a qualidade espermática quando o sêmen é armazenado a 17°C por até 72 horas. Para avaliar o efeito da cisteína e da centrifugação sobre fertilidade, 136 fêmeas foram selecionadas e inseminadas nos seguintes tratamentos: BTS/NC, BTSCIS/NC e BTSCIS/CENT. Após a inseminação artificial, as fêmeas foram avaliadas quanto a taxa de retorno e o tamanho da leitegada. A qualidade espermática no primeiro experimento foi determinada pelos testes de motilidade e vigor, alterações morfológicas e viabilidade espermática (membrana plasmática e acrossomal intactas e célula com potencial de mitocôndria). No entanto, no segundo experimento além dos testes citados anteriormente, foram realizados os testes de compactação de DNA e funcionalidade de membrana plasmática. As avaliações dos tratamentos foram realizadas 0, 24, 48 e 72 horas após a diluição do sêmen. Em ambos experimentos, o efeito dos tratamentos em relação ao período de armazenamento foi determinado através da análise para dados repetidos (PROC MIXED). O efeito dos tratamentos em relação a taxa de retorno e o número de nascidos foi analisado usando o PROC GLM, do programa estatístico SAS e aplicado o teste de Tukey quando o modelo foi significativo. A percentagem de alterações morfológicas não excedeu a 20% durante o armazenamento por 72 horas e nem diferiu entre os tratamentos, nos dois experimentos. Porém, no primeiro experimento a motilidade, o vigor, a integridade de membrana plasmática e acrossomal bem como o potencial de mitocôndria foram reduzidas ao longo do período de armazenamento. A motilidade, o vigor e a viabilidade diminuíram a níveis abaixo de 10% nos tratamentos CIS10,0 e CIS20,0 nas primeiras 24 horas de armazenamento a 17ºC. Ao final do período de armazenamento todos os grupos apresentavam média abaixo de 65% de espermatozóides com a membrana plasmática intacta. No segundo experimento, no entanto, o tratamento BTSCIS/CENT apresentou motilidade e vigor inferiores ao demais tratamentos, sendo a motilidade espermática inferior a 60% a partir de 24 horas de armazenamento. A integridade das membranas plasmática e acrossomal e o potencial de mitocôndria foram inferiores a 60% nos tratamentos BTSCIS e BTSCIS/CENT. No entanto na parte a campo, o grupo BTSCIS apresentou menor média (8,83 ± 3,38) de nascidos e maior taxa de retorno (86,05 ± 0,39) quando comparados aos outros grupos. Portanto, a cisteína em baixas concentrações mantém tão bem quanto o grupo controle a qualidade espermática. Mas, apesar do tratamento BTSCIS/NC ter atingido os mesmos índices que o grupo controle em relação qualidade espermática, foi o tratamento que obteve maior taxa de retorno e menor número de leitões nascidos.
Palavras-chaves: Cisteína, centrifugação, sêmen resfriado, *Beltsville Thawing Solution*, suínos.

ABSTRACT

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

Evaluation of cysteine adition in cooled semen for insemination in swine

AUTORA: CAROLINA KLEIN SEVERO
ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria 07 de agosto de 2009.

The improvement of pig industry is a consequence of the technological advances. The aim of the present study is to evaluate the effect of cysteine in BTS (Beltsville Thawing Solution) and centrifugation on swine semen to increase the sperm quality and fertility. In the first experiment, different concentrations of cysteine in *Beltsville Thawing Solution*: BTS (control group); CYS0.1 (BTS added 0.1 mM cysteine); CYS0.5 (BTS added 0.5 mM cysteine); CYS1.0 (BTS added 1.0 mM cysteine); CYS2.5 (BTS added 2.5 mM cysteine); CYS5.0 (BTS added 5.0 mM cysteine); CYS10.0 (BTS added 10.0 mM cysteine) and CYS20.0 (BTS added 20.0 mM cysteine) were evaluated. In the second experiment, semen added to BTS were not centrifuged without (control group) or with 5.0 mM of cysteine (BTSCYS/NC). In other treatment groups, semen were centrifuged with (BTSCYS/CENT) or without (BTS/CENT) 5.0 mM cysteine. Semen were stored at 17 ºC for 72 h. To assess the effect of cysteine and centrifugation on fertility, 136 females were randomly allotted in the following groups for artificial insemination with semen diluted in BTS and a) without centrifugation and cysteine (BTS/NC); b) without centrifugation and with 5.0 mM cysteine (BTSCYS/NC) or c) with centrifugation and with 5.0 mM cysteine (BTSCYS/CENT). After artificial insemination, the return to estrus rate and litter size were evaluated. In the first experiment, the quality of semen was determined by tests of sperm motility, vigor, morphology and viability (plasma and acrossomal membrane integrity and mitochondrial potential). In the second experiment, the semen were evaluated by the tests above, DNA compactation and function of plasma membrane. The treatments were evaluated at 0, 24, 48 and 72 h after dilution. In both experiments, the effect of treatments on the storage period was determined by analysis for repeated data (PROC MIXED) and the effect of treatments on the return to estrus rate and the number of piglets were analyzed by using PROC GLM of SAS software and applied the Tukey test for significant models. The percentage of morphological changes did not exceed 20% during storage for 72 h and do not differ between treatments in both experiments. However, the motility in the first experiment, vigor, integrity of plasma membrane and acrossomal well as the potential of mitochondria reduced the period of storage. The motility, vigor and viability decreased to levels below 10% in treatments CYS10.0, CYS20.0 in the first 24 hours of storage at 17 ºC. At the end of the storage period all groups had average below 65% of sperm with intact plasma membrane while the second experiment, treatment BTSCYS/CENT showed lower motility and force the other treatments, and the sperm motility below 60% from 24 hours of storage. The integrity of plasma membranes and acrossomal and the potential of mitochondria was less than 60% in treatments BTSCYS and BTSCYS/CENT. However in the field to the group BTSCYS showed lower average (8.83 ± 3.38) for infants and more return rate (86.05 ± 0.39) when compared to other groups. Therefore, the cysteine at low concentrations as well as maintains the control group the sperm quality. But despite treatment BTSCYS/NC have reached the same rates as the control group for sperm quality, was the treatment that received higher rates of return and lower number of piglets born.

Key Words: Cysteine, centrifugation, cooled semen, *Beltsville Thawing Solution*, swine

LISTA DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE TABELAS

CAPÍTULO 1

LISTA DE ABREVIATURAS

ABIPECS – Associação da Indústria Produtora e Exportadora de Carne Suína
AI – Membrana acrossomal íntegra
ART – Biotecnologia de reprodução assistida
ATP – Adenosina trifosfato
BSA – Soro albumina bovina.
BTS – Beltsville Thawing Solution
CP – Espermatozóide com potencial de mitocôndria
CYS – Cysteine
DMSO – Dimethyl sulphoxide
DPBS – Dulbecco´s phosphate-buffered saline solution
DPDAH – Damaged plasma membrane, damaged acrosomal membrane and enhanced mitochondrial function
DPDAL – Damaged plasma membrane, damaged acrosomal membrane and decreas mitochondrial function
DPIAH – Damaged plasma membrane, intact acrosomal membrane and high mitochondrial function
DPIAL – Damaged plasma membrane, intact acrosomal membrane and decreased mitochondrial function
EDTA – Ethylenediamine-tetra-acetic acid
FITC-PSA – Isotiocinato conjugado ao Pisum Sativum
GR – Glutationa redutase
GPx – Glutationa peroxidase
GSH – Glutationa reduzida
GSSG – Glutationa oxidada
IA – Inseminação artificial

IPDAH – Intact plasma membrane, damaged acrosomal membrane and enhanced mitochondrial function

IPDAL – Intact plasma membrane, damaged acrosomal membrane and decreased mitochondrial function

IPIAH – Intact plasma membrane, intact acrosomal membrane and high mitochondrial function

IPIAL – Intact plasma membrane, intact acrosomal membrane and low mitochondrial function

JC-1 – Iodeto de 5, 5', 6, 6'-tetracloro 1, 1', 3, 3'- tetraetilbenzimidazolocarbocianina

KCL – Cloreto de potássio

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

NAC – N-acetil-L-cisteína

$NaHCO_3$ – Bicarbonato de sódio

PI – Membrana plasmática íntegra

ROS – Espécies reativas ao oxigênio

TALP – Modified Tyrode's Medium

TRIS – Tris-hidroximetil

UPL – Unidade Produtora de Leitões

SUMÁRIO

1 INTRODUÇÃO

A suinocultura é um setor que está em contínua ascensão, o rebanho de suínos no Brasil cresce aproximadamente 2% ao ano desde 2002. Há um aumento gradual no rebanho de suínos da região centro - oeste, mas sem desencadear a redução no crescimento da região sul, responsável por maior concentração do rebanho nacional (Ministério da Agricultura, Pecuária e Abastecimento, MAPA, www.agricultura.gov.br). O Brasil é o quarto maior exportador de carne suína, ficando atrás somente da União Européia, Estados Unidos e Canadá (Associação da Indústria Produtora e Exportadora de Carne Suína, ABIPECS, www.abipecs.org.br) e, ao melhorar as condições sanitárias, deverá ascender no ranking mundial. Há uma ampla área de terra que ainda pode ser aproveitada para o desenvolvimento do setor suinícola. Esse fator preocupa a concorrência, por isso as barreiras não tarifárias são cada vez mais rígidas.

Para o país se tornar mais competitivo no mercado externo, deve-se incentivar pesquisas envolvendo tanto o manejo nutricional quanto reprodutivo, para obter os melhores índices por animal e, conseqüentemente, um aumento na produção suinícola Nacional. Uma alternativa que já está consolidada é a técnica de inseminação artificial (IA), que além de reduzir problemas sanitários, aumenta a eficiência do macho suíno. Com o uso da IA, se pode evitar o trânsito de animais e a necessidade de um grande número de machos para suprir o plantel, assim reduzindo custos na produção suinícola.

A técnica de IA tornou-se consagrada mundialmente, com isso, foi dada maior importância ao papel do macho na produção. Um mesmo macho pode ser usado para inseminar em média 100 fêmeas por mês. Portanto se faz necessário um controle mais rigoroso quanto à qualidade genética deste animal para garantir ejaculados com padrões elevados de fertilidade (BORTOLOZZO et al., 2005a). Atualmente, devido à importância da redução de custos, várias técnicas estão sendo desenvolvidas para garantir a qualidade espermática. Entre os fatores que exercem grande impacto sobre a

reprodução e qualidade das doses inseminantes estão o diluente escolhido, a temperatura e o tempo de armazenamento (JOHNSON et al., 2000).

Atualmente, o sêmen resfriado apresenta altos índices de produtividade (ANTUNES, 2007), e uso de sêmen resfriado mantém um maior número de espermatozóides viáveis por um período de tempo maior, enquanto que a concentração de espermatozóides e o tempo de processamento do sêmen são inferiores quando comparado a utilização de sêmen congelado (ANTUNES, 2007). Porém, a viabilidade espermática começa a diminuir após 72 horas de armazenamento independente do diluente utilizado (MURGAS et al., 2002; WEITZE, 1991).

Outro fator que determina o sucesso da IA é a temperatura de processamento e armazenamento do sêmen. Segundo Bortolozzo e colaboradores (2005a), a temperatura entre o diluente e o sêmen não deve exceder dois graus de variação no momento da diluição para evitar o choque térmico e consequente perda da viabilidade espermática. De acordo com De Leeuw e colaboradores (1991), o armazenamento do sêmen sob temperaturas inferiores a 15°C resultam em diminuição da sobrevivência espermática, isto porque ocorrem modificações estruturais e bioquímicas que levam a ruptura da membrana plasmática, degeneração do acrossoma e alterações na permeabilidade da membrana. A membrana plasmática do espermatozóide torna-se mais permeável após o resfriamento (ORTMAN; RODRIGUEZ-MARTINEZ, 1994), levando a perda de íons e enzimas (DE LEEUW et al., 1991), e capacitação espermática é induzida pelo aumento da permeabilidade da membrana devido à entrada de íons cálcio para dentro da célula (WATSON, 1995a).

Tendo em vista os possíveis problemas relacionados ao resfriamento do sêmen, o presente trabalho teve por objetivo avaliar a influência da adição de um antioxidante na prevenção dos danos causados ao espermatozóide pela armazenagem a 17°C por 72 horas. Além disso, foram avaliados o papel deste antioxidante juntamente ao processo de centrifugação sobre a qualidade espermática e o efeito da interação entre os mesmos como resultado a IA em suínos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Tecnologia do Sêmen Suíno

A necessidade de tecnificação do processo produtivo na cadeia suinícola desencadeou o uso e a disseminação da técnica de IA. Com isto, também tem sido intensificada a busca por novas tecnologias, com o objetivo de diminuir custos de produção e aumentar a produtividade (DIEHL et al., 2006). Uma forma de diminuir os custos seria através da redução do número de espermatozóides/fêmea/ano que pode ser obtido pela utilização da técnica de inseminação intra-uterina (WOLKEN et al., 2002; WATSON; BEHAN, 2002), já que, a inseminação tradicional utiliza duas a três doses inseminantes por estro sendo que cada dose contém em média dois a quatro bilhões de espermatozóides em 80-100ml (MARTINEZ et al., 2001).

Os primeiros experimentos de IA em suínos foram feitos em 1926-1927 por Ivanov e foram continuados por Milanov e colaboradores entre 1930 e 1936 (JOHNSON et al., 2000). A difusão da IA deve-se as exigências impostas pelo mercado, que busca uma carne de melhor qualidade, a melhora do sistema de transporte e das técnicas de processamento de sêmen (BORTOLOZZO et al., 2005; JOHNSON et al, 2000).

Segundo BORTOLOZZO et al. (2005), aproximadamente 51% do plantel das granjas tecnificadas estão sendo submetidas à utilização da técnica de IA. Do total de 19 milhões de inseminações realizadas em todo mundo, acredita-se que 99% delas são feitas com sêmen resfriado e usado no mesmo dia, ou armazenado a uma temperatura entre 15-20°C por no máximo cinco dias (HUO et al., 2002; JOHNSON et al., 2000). Como os espermatozóides são sensíveis ao choque térmico, temperaturas inferiores a 15°C levam a uma perda significativa no número de espermatozóides viáveis (ALTHOUSE et al., 1998). Acredita-se que a sensibilidade ao frio está relacionada à

composição lipídica da membrana plasmática por afetar sua fluidez. Com a queda da temperatura, ocorre uma restrição nos movimentos dos fosfolipídios da membrana podendo resultar na transição da fase de fluido para a fase gel (JOHNSON et al., 2000; ALTHOUSE et al., 1998). Assim, o aperfeiçoamento de diluentes e a utilização de técnicas modernas de avaliação do sêmen podem proporcionar um aumento significativo na eficácia do processo de IA em suínos.

O uso de sêmen suíno congelado em nível comercial ainda não é uma realidade (ANTUNES, 2007), apesar desta técnica estar disponível desde 1975 (SARAIVA et al., 2005). Devido aos baixos índices de produtividade o congelamento está restrito a empresas de melhoramento genético (SCHEID; SILVEIRA, 2002). Porém, alguns pesquisadores acreditam que a técnica possui um futuro promissor com os avanços das novas tecnologias e equipamentos (WOLDERS; TEN NAPEL, 2005).

2.2 Resfriamento do sêmen suíno

A utilização do sêmen suíno resfriado ocorre na maioria das inseminações realizadas atualmente, como citado no tópico anterior. O resfriamento do sêmen diminui a atividade metabólica do espermatozóide equino, reduz o crescimento microbiano e consequentemente mantém a viabilidade do sêmen diluído por um maior período de tempo (KATILA, 1997). O uso de sêmen suíno resfriado se dá pela facilidade de armazenamento, pela proximidade das centrais de inseminação com as granjas produtoras de leitões e pela alta produtividade obtida com este processo juntamente com a utilização da técnica de IA (BORTOLOZZO et al., 2005a). Existem diversos protocolos de resfriamento, isto porque há diversas temperaturas de armazenamento, apesar de Johnson et al. (2000) afirmar que temperaturas inferiores a 15°C provocam danos irreversíveis às membranas do espermatozóide. Tanto o sêmen suíno fresco quanto o diluído em *Androhep*, quando armazenados sob temperatura entre 15 e 20°C apresentam maior motilidade espermática (ZOU;YANG 1999; ALTHOUSE et al., 1998).

2.2.1 Sistema de refrigeração

Existem diversos protocolos de resfriamento utilizados para minimizar o efeito do choque térmico na integridade das membranas espermáticas em suínos, provocado pela queda brusca da temperatura de resfriamento (WEBER, 1989). Visto que o ejaculado suíno tem uma temperatura média de 36°C, o diluente preparado deve ser mantido a uma temperatura em torno de 34°C e o sêmen armazenado a uma temperatura inferior a esta, como já citado anteriormente.

A fim de controlar os danos pela queda de temperatura Funahashi e Sano (2005) utilizaram um banho-maria programado para reduzir lentamente a temperatura de resfriamento até obter 10°C. O resfriamento ocorria da seguinte forma: o sêmen era mantido no banho-maria por 4 horas, onde o sêmen reduzia a temperatura lentamente até 15°C e então mantido nesta temperatura por 12 horas. Após este período, o sêmen era mantido por 6 horas até alcançar a temperatura de 10°C e então resfriado a 10°C. Enquanto que, Huo et al. (2002) apenas mantinha o sêmen a temperatura ambiente (22°C) por 1 hora. De acordo com Funahashi; Sano (2005), Kotzias-Bandeira (1999) a refrigeração lenta apresenta resultados de motilidade e morfologia espermática melhores que a refrigeração rápida.

2.2.2 Centrifugação

A técnica de centrifugação é utilizada no processo de criopreservação do sêmen suíno (CARVAJAL et al., 2004) e de outras espécies, tais como, bovinos (PICKET et al, 1975), humanos (SHEKARRIZ et al., 1995) e eqüinos (PADILLA; FOOTE, 1991). A centrifugação tem o papel de remover o plasma seminal e concentrar os espermatozóides (CARVAJAL et al., 2004). Porém, algumas espécies parecem ser mais sensíveis ao processo de centrifugação, como é o caso de ratos (CARDULLO;

CONE, 1986), humanos (NG et al., 1990) e camundongos (KATKOV; MAZUR, 1998), enquanto que, bovinos (KATKOV; OSTASHKO, 1996) e equinos (CROCKETT et al, 2001) são mais resistentes (PICKET et al, 1975). Isto indica que há uma especificidade entre as espécies. A remoção do sobrenadante e o dano físico provocado a célula são alguns dos problemas relacionados à centrifugação (KATKOV; MAZUR, 1998; ALVAREZ et al, 1993). A formação de espécies reativas ao oxigênio parece exercer um efeito nocivo às células espermáticas, e está diretamente ligado ao tempo e a força *g* utilizados durante a centrifugação. A utilização de uma força *g* maior por um curto período de tempo minimiza este efeito (CARVAJAL et al., 2004; SHEKARRIZ et al., 1995).

A formação destas espécies reativas ao oxigênio (ROS) influencia a peroxidação lipídica, provocando perdas na motilidade e viabilidade espermática e, consequentemente, reduzindo os índices de fertilidade (CARVAJAL et al., 2004; SHEKARRIZ et al., 1995). Uma forma de manter a motilidade é através do uso de antioxidantes (PARINAUD et al., 1997; GRIVEAU; LE LANNOU, 1994). A percentagem de células com o DNA íntegro é maior na presença de antioxidantes quando o sêmen é submetido a centrifugação (DONNELLY et al., 2000).

2.2.3 Diluentes

O diluente é de fundamental importância pois ele é responsável por proporcionar um ambiente adequado ao sêmen durante o armazenamento (GACZARZEWICZ et al., 2003; ROCA et al., 2000). O pH, a força iônica, o tipo de íon e a pressão osmótica do meio são fatores que influenciam na manutenção deste meio adequado. O pH do sêmen suíno varia entre 7.2 e 7.5. A maioria dos diluentes possui altas quantidades de glicose que levam a redução do pH intracelular e a conseqüente queda na motilidade (JOHNSON et al., 2000). Além disto, a glicose mantém a osmolaridade do meio, portanto a força iônica parece não ter grande importância para diluentes de sêmen suíno (WATSON, 1995). A introdução de íons tem a função de conter a exaustão dos

íons de potássio e redução da motilidade, além de iniciar a desestabilização da membrana plasmática (HARRISON, 1996; ALVAREZ; STOREY, 1982).

Os diluentes podem ser classificados conforme seu tempo de armazenamento. Existem diluentes de longa-duração como é o caso do *Androhep* e *Zorlesco*, e de curta-duração como é o caso do *Beltsville Thawing Solution* (BTS) e do KIEV (PURSEL; JOHNSON, 1975; PLISKO, 1965). O diluente *Androhep* contém glicose, *ethylenediamine-tetra-acetic acid* (EDTA), citrato de sódio, bicarbonato de sódio, HEPES e soro albumina bovina (BSA) (WEITZE, 1990), e é considerado sensível a temperaturas de armazenamento inferiores a 12ºC, em estudo realizado em suínos, sendo observado queda da motilidade (ALTHOUSE et al., 1998). Já, o diluente *Zorlesco* é um meio mais complexo que contém TRIS, ácido cítrico, cisteína, BSA, além de glicose e EDTA (GOTTARDI et al., 1980). Tanto o *Androhep* quanto o *Zorlesco* contém soro albumina bovina (BSA) que parece neutralizar os produtos do metabolismo dos espermatozóides e das bactérias e também podem exercer efeito sobre a atividade peroxidativa (HUO et al., 2002).

Modificações estão sendo feitas para aumentar o tempo de armazenamento e os índices de fertilidade (GADEA, 2003). O diluente KIEV é composto de glicose, EDTA, citrato de sódio e bicarbonato de sódio (PLISKO, 1965). Enquanto que, o BTS foi desenvolvido por Pursel; Johnson (1975) para sêmen congelado na forma de *pellet* e adaptado para sêmen armazenado na fase liquida por Johnson et al. (1988). Este diluente contém baixas concentrações de potássio e parece manter a concentração intracelular deste íon em níveis fisiológicos durante o armazenamento. Tanto o BTS quanto o KIEV possuem EDTA na sua composição e tem função de capturar íons divalentes, principalmente Ca** para prevenir a capacitação e o início da reação acrossomal (JOHNSON et al., 2000).

2.2.4 Cisteína

A cisteína, aminoácido de baixo peso molecular contendo tiol, é um antioxidante não enzimático que previne a peroxidação lipídica (BILODEAU et al., 2001), que ocorre devido a formação de radicais livres como, peróxido de hidrogênio, anion superóxido e radicais hidroxil (SINHA et al., 1996). Os espermatozóides são altamente susceptíveis ao estresse oxidativo devido a grande quantidade de ácidos graxos poliinsaturados na membrana plasmática. Estes ácidos graxos são essenciais para a manutenção funcional das células germinativas masculinas (HENKEL, 2005). A incubação aeróbica, a contaminação por leucócitos e o excesso de resíduo citoplasmático do espermatozóide levam a formação de espécies reativas ao oxigênio que provocam efeitos deletérios as membranas espermáticas, diminuição da motilidade e eventualmente queda do potencial de fertilidade (AITKEN; BAKER, 2004; ALVAREZ et al., 1987).

Estes radicais livres são bloqueados pelos sistemas antioxidantes. Os antioxidantes têm a função de recolher os radicais livres que podem causar a peroxidação lipídica da membrana plasmática dos espermatozóides (BAUMBER et al., 2000). Tanto a cisteína quanto a N-acetil-L-cisteína (NAC), forma conhecida da cisteína que inibe a apoptose das células espermáticas nos túbulos seminíferos humanos (ERKKILA et al., 1998), são precursoras da biossíntese da glutationa e aumentam os níveis de glutationa reduzida (GSH) (BILODEAU et al., 2001). A glutationa reduzida é capaz de agir diretamente em muitas ROS (MEISTER, 1994), além de ser um co-fator para a glutationa peroxidase (GPx) que catalisa a redução do peróxido de hidrogênio e hidroperóxidos. A forma oxidada da glutationa (GSSG) é regenerada in vivo pela glutationa redutase (GR) e NADPH (BILODEAU et al., 2001). A glutationa peroxidase é uma selenocisteína contendo uma enzima antioxidante, que além de auxiliar na eliminação de peróxidos, também age na desintoxicação de lipídios reativos (FRIDOVICH, 1983).

2.2.5 Testes específicos para análise espermática

A qualidade espermática pode ser analisada tanto por testes que avaliem a integridade estrutural quanto à funcionalidade do espermatozóide. A motilidade tem sido utilizada como principal parâmetro para avaliar a diminuição da capacidade fecundante, porém é uma estimativa subjetiva (HIRAI et al., 2001) e há muitas amostras de sêmen com motilidade elevada, porém resultam em altos índices de falhas de concepção (BRAUNDMEIER; MILLER, 2001; JOHNSON et al., 2000). O sêmen deve ser avaliado diariamente, sendo a motilidade de aproximadamente 60% considerada satisfatória. Outro teste comum é a avaliação das alterações morfológicas, que não permite estabelecer a fertilidade do ejaculado (BRAUNDMEIER; MILLER, 2001), apesar de existir uma correlação negativa entre percentagem de gota citoplasmática proximal no ejaculado com taxa de nascimento e tamanho de leitegada (WABERSKI et al., 1994). Recomenda-se que a percentagem total de gota citoplasmática proximal, intermediária e distal não exceda 15% e a percentagem total de alterações morfológicas não seja superior a 20% (JOHNSON et al., 2000; KUSTER; ALTHOUSE, 1999).

Técnicas modernas de avaliação da qualidade espermática vêm sendo utilizadas a fim de diminuir a subjetividade dos testes e melhorar a predição da fertilidade do sêmen. Há vários parâmetros que determinam a capacidade fecundante do espermatozóide, dentre eles à avaliação da membrana plasmática, verificando sua funcionalidade e integridade. O teste hipoosmótico avalia a habilidade da célula de aumentar de volume em uma solução com baixa osmolaridade, indicando se a membrana plasmática intacta é também bioquimicamente ativa, e funcionalmente íntegra (CABRITA et al., 1999; VAZQUEZ et al., 1997). O teste hipoosmótico é simples, barato e facilmente aplicável, apresentando uma correlação positiva entre membrana plasmática funcional e capacidade fecundante (PETRUNKINA et al., 2001; ROTA et al., 2000). O teste é considerado como uma ferramenta bastante útil para identificação de espermatozóides pouco viáveis quando utilizado com outra técnica de avaliação de integridade de membrana (VAZQUEZ et al., 1997).

A integridade da membrana plasmática é de fundamental importância para função espermática. Somente uma célula intacta é capaz de provocar uma série de alterações no trato reprodutivo da fêmea, o que permite a capacitação e reação do acrossomo, e finalmente a aquisição da capacidade de fecundar um oócito (ANDRADE et al., 2007; PETRUNKINA et al., 2001). Para avaliação da integridade de membrana plasmática, existem diversas sondas fluorescentes que funcionam como marcadores. O iodeto de propídio é uma das sondas especificas utilizadas, por se ligar ao DNA quando a membrana está danificada, emitindo uma fluorescência vermelha-alaranjada em microscopia de epifluorescência sob excitação de 536 mm e emissão de 617 mm (CELEGHINI et al., 2008; ANDRADE et al., 2007; CELEGHINI et al., 2007).

Além da membrana plasmática, outros fatores são essenciais para determinar o sucesso da fecundação. Um deles é a integridade da membrana acrossomal, a qual é indispensável para que ocorra a reação do acrossoma e, outro exemplo é o potencial mitocondrial, o qual influencia diretamente a motilidade. A integridade da membrana acrossomal é importante para que ocorra a fecundação, já que a reação acrossomal é caracterizada pela liberação de enzimas acrossomais, e é um evento essencial para que o espermatozóide penetre na zona pelúcida e ocorra a fusão com a membrana plasmática do oócito (CELEGHINI et al., 2007; FLESCH; GADELLA, 2000). Sua integridade pode ser identificada por diversas técnicas de fluorescência, sendo comumente usada a marcação de uma glicoproteína, como é o caso das lecitinas. Entre essas técnicas, a mais utilizada é a lecitina *Pisum Sativum* (PSA) marcada com o conjugado de isotiocinato de fluoresceína (FITC). A PSA é uma aglutinina de ervilha que se liga a glicoconjugados na matriz acrossomal, especificamente ao açúcar α-manose (ANDRADE et al., 2007; CELEGHINI et al., 2007; GILLAN et al., 2005). Quando o PSA se ligada ao FITC, o acrossoma danificado é marcado de amarelo-esverdeado (CELEGHINI et al., 2007).

A percentagem de reação acrossomal espontânea não é significativamente diferente em machos suínos férteis e subférteis. No entanto, a incidência da reação induzida por progesterona é significativamente menor em animais subférteis comparada com os cachaços férteis, sugerindo que a reação acrossomal pode ser um parâmetro para testar fertilidade (HERRERA et al., 2002). As células espermáticas possuem

receptores para progesterona na membrana plasmática (FLESCH; GADELLA, 2000). A progesterona é liberada no fluido folicular no momento da ovulação e acredita-se que doses de progesterona induzam a reação acrossomal em muitas espécies de mamíferos (MELENDREZ et al., 1994; OSMAN et al., 1989).

A principal função da mitocôndria nas células vivas é realizar a fosforilação oxidativa e produzir adenosina trifosfato (ATP), fonte energética indispensável para a motilidade espermática (CELECHINI et al., 2007). A avaliação da função mitocondrial é uma das mais acuradas. O iodeto de 5, 5', 6, 6'-tetracloro 1, 1', 3, 3'-tetraetilbenzimidazolocarbocianina (JC-1) tem sido uma das sondas mais comumente utilizada para determinar alteração funcional de mitocôndria (CELEGHINI et al., 2007; COSSARIZZA et al., 1993). Esta sonda é uma carbocianina catiônica lipofílica de baixa toxicidade e boa solubilidade (ANDRADE et al., 2007; ARRUDA et al., 2007; GILLAN et al., 2005). Essa molécula existe na forma de monômeros e dependendo do potencial da mitocôndria é capaz de formar agregados (COSSARIZZA et al., 1993). Quando o espermatozóide possui alto potencial de mitocôndria, o JC-1 forma agregados e cora-se de vermelho-alaranjado (ANDRADE et al., 2007; GILLAN et al., 2005). As avaliações de integridade de membrana plasmática, membrana acrossomal e potencial mitocondrial podem ser visualizados através da utilização de um citômetro (GILLAN et al., 2005; COSSARIZZA et al., 1993) ou um microscópio de epifluorescência (ANDRADE et al., 2007; CELEGHINI et al., 2007).

Além da integridade das membranas acrossomal e plasmática e do potencial de mitocôndria, a compactação do DNA é outro fator que pode alterar os índices de fertilidade. Alterações no complexo DNA-proteína têm sido apontadas, ultimamente, como importante causa de subfertilidade em humanos (COSTA et al., 1996). Alta quantidade de plasma seminal (>20%) em garanhões férteis pode levar a um declínio na integridade do DNA, sem se observar queda na motilidade espermática (ARRUDA et al., 2007; KATILA, 1997). Há várias técnicas que podem determinar a condensação do DNA, entre elas laranja de acridina (CELEGHINI et al, 2008), TUNEL (GILLAN et al., 2005), COMET e a metacromasia induzida que é realizada através da utilização de azul de toluidina (MACHADO et al., 2003). A técnica laranja de acridina se dá através desta coloração para determinar anormalidade do DNA das células, que para tal se utiliza

microscopia de epifluerescência ou citometria de fluxo (ROCHA et al., 2002), enquanto que os testes TUNEL e COMET determinam à fragmentação do DNA (DONNELLY et al., 2000a). O método de metacromazia induzida é baseado no tratamento ácido seguido da coloração de azul de toluidina. Tonalidades são obtidas em função da disponibilidade e proximidade dos grupos fosfato do DNA não ligados à proteína (BRANDÃO et al., 2006; NAVES et al., 2004). Alterações na condensação de cromatina podem ocorrer tanto na ausência de alterações morfológicas quanto no aumento das mesmas, além de serem causa relevante de morte no primeiro trimestre de gestação (NAVES et al., 2004).

Portanto, com a tecnificação do processo produtivo esta ocorrendo a propagação de diversas técnicas, dentre elas a Inseminação artificial. Com isso diversos avanços estão sendo realizados sobre a tecnologia do sêmen. O sêmen pode ser resfriado ou congelado, sendo o sêmen resfriado mais utilizado devido aos índices de produtividade. O sistema de resfriamento e a escolha do diluidor são fatores que influenciam na manutenção da viabilidade espermática. Buscando otimizar o processo de resfriamento do sêmen foi avaliado o efeito da centrifugação e da adição de cisteína. Para tal, foram utilizadas técnicas conhecidas, motilidade, vigor e alterações morfológicas, além de técnicas modernas como o teste hipoosmótico, DNA-proteína e avaliação da integridade de membranas acrossomal e plasmática e potencial de mitocôndria através de sondas fluorescentes.

3 CAPÍTULO 1

TRABALHO ENVIADO PARA PUBLICAÇÃO:

DETRIMENTAL EFFECTS OF CYSTEINE ON BOAR SPERM CELLS IN SHORT-TERM COOLED SEMEN PRESERVATION

Carolina Klein Severo, Andressa Minussi Pereira, Gustavo Freitas Ilha, Gabriel Ribas Pereira, Marcelo Soares, João Francisco Oliveira, Paulo Bayard Gonçalves

ANIMAL REPRODUCTION SCIENCE, 2009

Detrimental effects of cysteine on boar sperm cells in short-term cooled semen preservation

Carolina Klein Severo[a], Andressa Minussi Pereira[a], Gustavo Freitas Ilha[a], Gabriel Ribas Pereira[a], Marcelo Soares[b], João Francisco Oliveira[a], Paulo Bayard Gonçalves[a*]

[a] Laboratory of Biotechnology and Animal Reproduction - BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil, 97105-900.

[b] Swine Clinic and Reproduction Sector, Federal University of Santa Maria, Santa Maria, RS, Brazil, 97105-900.

Correspondonding author:

Paulo Bayard Gonçalves, Departamento de Clínica de Grandes Animais, Hospital Veterinário, Universidade Federal de Santa Maria, Postal code 97105-900, Santa Maria, RS, Brazil.

Phone: 55-55-3220-8752.

Fax: 55-55-3220-8484.

E-mail: bayard@biorep.ufsm.br

Abstract

Artificial insemination is routinely used in the swine industry to reduce the costs of production through to increase the efficiency of the refrigerated boar semen process. The objective of this study was to evaluate the effect of different levels of cysteine added to the "Beltsville Thawing Solution" (BTS) extender semen during cooling for up to 72 h. Ejaculated

from three boars were collected with the gloved-hand technique and semen aliquots were diluted in BTS as follow: BTS only (BTS), BTS + 0.1 mM cysteine (CYS0.1), BTS + 0.5 mM cysteine (CYS0.5), BTS + 1.0 mM cysteine (CYS1.0), BTS + 2.5 mM cysteine (CYS2.5), BTS + 5.0 mM cysteine (CYS5.0), BTS + 10.0 mM cysteine (CYS10.0), and BTS + 20.0 mM cysteine (CYS20.0). Evaluation of sperm motility, vigor and morphology were analyzed before and after semen dilution. Additionally, we evaluated sperm integrity using 0.5 mg/ml propidium iodide (plasma membrane), 100 µg/ml isothiocynate-conjugated Pisum sativun agglutinin (acrosomal membrane) and 153 µM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (mitochondria potential) after semen dilution at specific times (0, 24, 48 and 72 h). The effect of treatments in each dependent variable was analyzed using repeated measured model. All the parameters studied decreased over time in the control and treatment groups. Cysteine at concentrations of 10.0 mM and 20.0 mM resulted in more pronounced reductions even at the time zero. The motility, vigor and viability decreased to levels below 10% at these high levels of cysteine in the first 24 h of storage at 17 °C. At the end of the storage time, less than 65% of sperm cells had intact plasma membrane in all groups. The number of sperm with acrosome membrane integrity and mitochondrial potential were also lower in CYS20.0 and CYS10.0 than in minor concentrations. Also, the results diminished over time at 2.5 and 5.0 mM when compared with lower concentrations of cysteine. In conclusion, the addition of cysteine in the BTS semen extender did not improve spermatic viability of boar spermatozoa preserved by cooling.

Key words: Cysteine, Swine spermatozoa, Beltsville Thawing Solution, Motility, Functional membrane integrity.

1. Introduction

Artificial insemination (AI) has been an important tool used in the swine industry for genetic improvement and to reduce the costs of production. However, cryopreservation and storage in liquid nitrogen is a highly stressful process for boar semen, resulting in poor cellular survival, differently of the well established technology for freezing bovine semen (Johnson et al., 2000; Celeghini et al., 2008). Low temperatures for boar semen preservation, less than 15 °C, lead to a significant reduction of the sperm cell viability caused by high sensibility to heat shock stress (De Leeuw et al., 1990; Johnson et al., 2000). The high content of unsaturated fatty acid promotes lipid peroxidation of the sperm plasma membrane, which is crucial to decrease sperm metabolism, motility and fertilization potential (Storey, 1997; Funahashi and Sano, 2005). Kumaresan et al. (2009) observed a decrease on sperm function and membrane integrity associated with high levels of lipid peroxidation, which occurs during the semen storage period. Therefore, the alternative to maintain good fertilization rates has been to cool and to use the semen at the same day of being collected or to preserve at temperatures from 15 to 20 ^{o}C for using in 5 days (Johnson et al., 2000).

Semen extended in a liquid state is considered an efficient method to preserve spermatozoa viability in swine, is commonly used to transport semen over long distances and is the single technology used by the industry (Johnson et al., 2000; Huo et al., 2002). The semen extender has to protect the sperm and to produce an adequate environment for short-term storage (Gadea, 2003). The Beltsville Thawing Solution (BTS) is an extender used worldwide to maintain the viability of semen over time and to subsequently store liquid boar semen for up to 72 h at temperatures ranging from 15 to 20 °C (Pursel and Johnson, 1975). The BTS extender is considered to have a short action and is commonly used when low levels of potassium is present to make the intracelullar concentration of this important ion at the physiological levels during storage (Gaczarzewicz et al., 2003).

Extender supplemented with antioxidants has an improvement of swine spermatozoa viability and motility in liquid-stored or cryopreserved semen (Funahashi and Sano, 2005). Thiols, including cysteine, *N*-acetyl cysteine and glutathione, are part of a large class of antioxidants. Cysteine and *N*-acetyl cysteine are important precursors of the intracellular biosynthesis of the glutathione (Bilodeau et al., 2001), and are at low levels in cells (Funahashi and Sano, 2005). The cysteine and the *N*-acetyl cysteine act as a cell membrane protector, maintaining sulphydric groups and interacting with free radical to decrease the oxygen reactions responsible for the lipid peroxidation (Bilodeau et al., 2001). Apparently, both thiols can reduce the toxic effect of the reactive oxygen species (ROS) during the sperm cryopreservation process (Bilodeau et al., 2001; Thuwanut et al., 2008).

Funahashi and Sano (2005) concluded that the addition of 5.0 mM of cysteine in boar ejaculated semen supplemented with Modena extender, which contain 20% of seminal plasma, maintained viability, integrity and penetration of spermatozoa observed by in vitro fertilization technique for 29 days. However, seminal plasma contains active antioxidants enzymes and may obscure the results of cysteine. In bovine, the concentration of 0.5 mM of cysteine was sufficient to maintain sperm motility in the absence of hydrogen peroxide (H_2O_2) in semen diluted in TRIS buffer containing egg yolk (Bilodeau et al., 2001). Therefore, the best concentrations of these thiols have to be shown.

A variety of newer approaches for the assessment of sperm characteristics are becoming increasingly available commercially (Turba et al., 2007). The assessment of viability by fluorescent technique and sperm motility are accurate predictors of fertilize ability and non-return rate after AI in pig (Juonala et al., 1999; Sutkeviciene et al., 2005). Functional integrity of sperm membranes can be assessed using fluorescent stains that rely on plasma membranes damage to stain underlying structures. Such technique has been used in horses (De Arruda et al., 2007),

bovine (Celeghini et al., 2007), swine (De Andrade et al., 2007), and humans (Ozaki et al., 2002), to identify “live” and “dead” sperm.

The best concentration of cysteine in BTS is not well established for short-term preservation of boar semen. Our hypothesis was that cysteine maintain sperm viability after cooling and storage at 17 °C during 72 h. The aim of this study were to evaluate different concentrations of cysteine added to BTS extender for a period of up to 72 h to maintain the spermatozoa integrity assessed by fluorescent methods, motility and vigor.

2. Material and Methods

Unless otherwise stated, all media components were purchased from Sigma (Sigma-Aldrich, São Paulo, SP, Brazil).

2.1 Animals and source of semen

All animal experiments followed a protocol approved by the Committee of Ethics and Animal Welfare in Federal University of Santa Maria and were controlled by the Guidelines for Animal Experiments of the Committee (Protocol #: 23081.006881). Animals were housed individually and were maintained at good corporal condition. Fresh clean water was provided *ad libitum* with automated watering devices, throughout the entire trial period. Three boars at the age of 24 month were submitted to two semen collections during the study. Semen collections were performed using a gloved-hand technique described by Hancock and Hovel (1959). The first ejaculatory portion was discharged and the semen was filtered through four 130-mm^2 gauze at the time of collection. Only the rich fraction was used in this study.

2.2 Assessment of subjective sperm motility and vigor

Sperm progressive motility was the percentage of sperm cells that show normal forward movement (0 to 100%) and vigor described how fast the motile sperm were moving forward

(score from 1 to 5). These parameters were estimated under a bright-field microscope (Olympus BX40, Olympus Optical Co., Ltd., Japan) at 200 x magnification in one aliquot of 5 µl of semen placed on a warmed (37 ^{o}C) slide and covered with 22-mm x 22-mm coverslip.

2.3 Assesment of sperm concentration

Immediately after semen collection and sperm motility and vigor evaluation, sperm concentration was assessed. To evaluate sperm concentration, 10 µl of each ejaculation was diluted (1:100) in saline formaldehyde and sperm were counted using a Neubauer haemocytometer chamber (Boeco, Hamburg, Germany) in duplicate, under 400 x magnification. The final concentration was adjusted to 3 x 10^9 spermatozoa for each 80 ml.

2.4 Assessment of sperm morphology

Aliquots of semen were fixed using a 2.9% formaldehyde-citrate buffered solution and following place between slide and coverslip for morphology evaluation. A total of 200 sperm cells were analyzed for each sample and assessed under a phase contrast microscope (Olympus CHS, Olympus Optical Co., Ltd., Japan) at a magnification of 1000 x. Sperm abnormalities observed in acrosome, midpiece and tail were classified as described by Galli and Bosisio (1988).

2.5 Semen dilution

After semen analysis, eight sperm fractions were submitted to different cysteine treatments. For semen dilution, BTS (206 mM Glucose, 20.4 mM Na_3 citrate, 14.9 mM $NaHCO_3$, 3.4 mM Na_2 EDTA, 10.0 mM KCl, penicillin G Na 0.6 g/l, dihydroestreptomicin 1.0 g/l) was prepared as recommended by the manufacturer (Minitub, Tiefenbach, Germany). The diluted semen was assigned into a control and seven treatment groups. In the control group, semen was diluted in BTS (BTS) without cysteine. In treatment groups, semen was diluted in BTS containing 0.1 mM (CYS0.1), 0.5 mM (CYS0.5), 1.0 mM (CYS1.0), 2.5 mM (CYS2.5), 5.0 mM

(CYS5.0), 10.0 mM (CYS10.0) and 20.0 mM (CYS20.0) of cysteine. Then, samples were cooled and kept into a refrigerated chamber at 17 °C. Sperm motility, vigor, morphology and viability were assessed at 0, 24, 48 and 72 h of storage.

2.6 Spermatozoa staining and assesment

Sperm viability was evaluated by the plasma membrane integrity, acrossomal membrane integrity, and mitochondrial potential, using fluorescent probes. Propidium iodide and isothiocynate-conjugated Pisum sativun agglutinin (FITC-PSA) were prepared in a concentration of 0.5 mg/ml and 100 µg/ml in Dulbecco´s phosphate-buffered saline solution (DPBS), respectively. The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was diluted in a concentration of 153 µM in dimethyl sulphoxide (DMSO). A volume of 3 µl of PI, 50 µl of FITC-PSA and 3 µl of JC-1 was added to 150 µl aliquot of 25 x 10^6 sperm/ml, diluted in Modified Tyrode's Medium (TALP). Samples were mixed and maintained in a dark chamber at room temperature for 8 min. After, one aliquot of 7 µl was evaluated under epifluorescence microscope at 1000 x magnification and a total of 200 cells were counted for each treatment group.

Spermatozoa were classified according to De Andrade et al. (2007) as viable (intact plasma membrane, intact acrosomal membrane and high mitochondrial function, IPIAH) and not viable (intact plasma membrane, intact acrosomal membrane and low mitochondrial function, IPIAL; intact plasma membrane, damaged acrosomal membrane and enhanced mitochondrial function, IPDAH; intact plasma membrane, damaged acrosomal membrane and decreased mitochondrial function, IPDAL; damaged plasma membrane, intact acrosomal membrane and high mitochondrial function, DPIAH; damaged plasma membrane, intact acrosomal membrane and decreased mitochondrial function, DPIAL; damaged plasma membrane, damaged acrosomal membrane and enhanced mitochondrial function, DPDAH; damaged plasma membrane, damaged

acrosomal membrane and decreased mitochondrial function, DPDAL).

2.7 Statistical anaylsis

Repeated measurement analysis was performed to determine the effect of each cysteine treatment on each dependent variable considered in this study. Data was statistically analyzed by PROC MIXED procedure using SAS software (SAS Institute, Cary, NC, USA). Differences were considered to be significant when $P < 0.05$ and results were expressed as mean ± standard error of means (S.E.M.).

3. Results

3.1 Assessment of sperm motility, vigor and morphology

Overall, there was a significant decrease on the sperm motility and vigor during semen storage at 17 ^{o}C. The motility and vigor decreased soundly when the semen was added to high concentrations of cysteine (time "0"; CYS10.0 and CYS20.0; $P < 0.05$). However, the osmolality (348 mOsm) and pH (7.2 - 7.4) of these two groups did not differed from the other groups (data not shown). After 24 h of storage at 17 °C, the motility and vigor decreased roughly 50% at 2.5 mM and 5.0 mM and below 5% at 10.0 mM and 20.0 mM of cysteine, which was higher reduction than in the lower cysteine concentrations ($P < 0.05$; Figure 1 and 2). The morphological defects detected by phase contrast microscope (Table 1) were below 20%, during all periods of semen storage.

3.2 Assessment of plasma membrane integrity using fluorescent probes

The treatments showed a significant decrease on viable sperm over time (Figure 3). As observed in motility and vigor assay, the sperm viability decreased significantly when the semen was added at high concentrations of cysteine (time "0"; CYS10.0 and CYS20.0; $P < 0.05$), which did not occur at the other cysteine concentrations. After 72 h of storage, the sperm viability was

below 60% when the cysteine concentrations were from 0 to 5.0 mM and below 10% at 10.0 and 20.0 mM.

The percentage of sperm with plasma membrane integrity decreased over time and was significant lower in CYS20.0 at 24 and 48 h and in CYS10.0 and CYS20.0 at 72 h ($P < 0.05$). At 72 h, less than 65% of sperm cells had intact plasma membrane in all treatments. The acrosome membrane integrity was also lower in CYS20.0 from 0 to 48 h ($P < 0.05$). The integrity of the acrosomal membrane rate was also lower in CYS10.0 at 0 h ($P < 0.05$). The groups BTS, CYS0.1, CYS0.5, CYS1.0, CYS2.5, CYS5.0 and CYS10.0 yielded higher than 60% of break in the integrity of acrosomal membrane after 72 h when storage at 17 °C. The number of sperm with mitochondrial potential was lower in CYS10.0 and CYS20.0 from 0 to 72 h when compared to other groups *(*$P < 0.05$), demonstrating that cysteine may injure the cells even at low concentrations (Figure 4).

4. Discussion

In the present study, the effect of different concentrations of cysteine on the motility, vigor, morphology and viability (plasma and acrosomal membrane integrity and mitochondrial potential) was investigated in sperm cells after cooling boar semen. It has been clearly demonstrated, to our knowledge for the first time, that cysteine has deleterious effect in a dose dependent manner on swine sperm cells maintained at low temperature for short period of time (72 h). All parameters studied were affected by high concentrations of cysteine (10.0 and 20.0 mM).

The sperm motility and vigor obtained in the control group were similar to others done previously, using BTS extender for semen preservation at 15 to 17 °C (Vasconcelos et al., 2001; Dube et al., 2004). The temperature of 17 °C used in this study is ideal for boar semen storage for

up to three days for this extender (Johnson et al., 2000). It is well known that liquid boar semen is commonly stored at temperatures between 15 and 20 °C for using in artificial insemination at the swine industry. Temperatures below 15 °C may impair sperm cell viability during storage for swine artificial insemination (Johnson et al., 2000).

Low concentrations (0.1 to 1.0 mM) of cysteine added to BTS did not have any positive or negative effect on sperm motility, vigor or viability when boar semen was storage for 72 h at 17 °C. In a very short (6 h) incubation period at 38.5 °C, bull sperm motility was protected with 0.5 mM of cysteine in the absence of an external source of oxidative stress and 1.0 mM was required in the presence of 100 mM of H_2O_2 (Bilodeau et al., 2001). Therefore, cysteine seems to protect the cells when the sperm are in a very high metabolic active but does not increase the protection already done by BTS in a low cellular activity (at 17 °C).

By the other hand, high concentration of cysteine seems to have a detrimental effect on boar sperm cells maintained at low temperature. The sperm motility, vigor and viability are impaired by cysteine at concentrations above 2.5 mM. This deleterious effect was not caused by changes in osmolality or pH. The osmolality was around 348 mOsm and pH between 7.2 and 7.4 in all treatment groups (data not shown). The decrease of sperm motility in presence of thiols has been attributed to the binding of disulphide to protamines (Rousseaux and Rousseaux-Prevost, 1995) and also to the excessive scavenging of ROS at lower physiological levels necessary for normal sperm cell function (Kim et al., 1999; Ali et al., 2003; Bedaiwy et al., 2004). In man, semen with higher concentration of thiols are subfertile (Ebisch et al., 2006).

Sperm with intact plasma and acrosomal membranes are required for normal fertilization (Oura and Toshimori, 1990; Flesch and Gadella, 2000). Then, we focused on the cysteine effect to reduce damages of plasma and acrosomal membranes, during boar semen storage at 17 ^{o}C, by protecting the cells against oxidative damages. However, supplementation of BTS with cysteine

did not decrease sperm membranes injuries. Similar results was observed in acrosome membrane integrity in stallions (Baumber et al., 2000) and rams (Sarlos et al., 2002) spermatozoa. However, in ram frozen semen, the addition of 10.0 mM of cysteine reduced acrosome damages and maintained viability, motility, and plasmatic membrane functionality post-thawing (Uysal and Bucak, 2007). Also, cysteine improved the viability and functional status of boar spermatozoa during liquid preservation when Modena medium, containing 20% of fresh seminal plasma, was used (Funahashi and Sano, 2005). This increment in sperm viability may be in consequence of containing active antioxidants enzymes present in seminal plasma. Further studies urge to determine the effect of seminal plasma on boar sperm integrity and viability when stored at 17 °C for up to 72 h.

Sperm morphological defects have minor effect in swine fertility when are below 20% (Johnson et al., 2000). Our results did not reach this cut-off point when sperm morphology was assessed under a phase contrast microscope. However, structural changes increase significantly when fluorescent probes were applied. These findings support that analysis by phase contrast microscope have low efficiency in detecting morphological defects in sperm cells (Braundmeier and Miller, 2001).

Furthermore, we assessed the effect of cysteine on the mitochondrial function of boar sperm cell storage for three days. It is known that mitochondrial function is crucial to promote oxidative phosphorylation and to produce the energy source of adenosine-tri-phosphate (ATP) that is essential for cell motility (Oura and Toshimori, 1990; Frey and Mannella, 2000). However, cysteine did not improve the mitochondrial potential in our study, which is in agreement with the data observed by Celeghini et al. (2007).

5. Conclusion

In conclusion, addition of cysteine at 2.5 mM or higher concentration in the BTS semen extender reduces the motility and vigor when semen is storage for up to 72 h at 17 ºC. The spermatozoa integrity and viability is also reduced when 10.0 mM or 20.0 mM of cysteine is added to BTS extender. Therefore, the addition of cysteine in the BTS semen extender does not improve spermatic viability of boar spermatozoa preserved by cooling.

Acknowledgements

This study was supported by the Brazilian Federal Council for Higher Education Personnel (CAPES) and the National Council for Scientific Research and Development (CNPq).

References

Ali, A.A., Bilodeau, J.F., Sirard, M.A., 2003. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology 59, 939-949.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 21, 895-902.

Bedaiwy, M.A., Falcone, T., Mohamed, M.S., Aleem, A.A., Sharma, R.K., Worley, S.E., Thornton, J., Agarwal, A., 2004. Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 82, 593-600.

Bilodeau, J.F., Blanchette, S., Gagnon, C., Sirard, M.A., 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 56, 275-286.

Braundmeier, A. G. and Miller, D. J., 2001. Invited Review: The search is on: Finding accurate molecular markers of male fertility. J. Dairy Sci. 84, 1915-1925.

Celeghini, E.C., de Arruda, R.P., de Andrade, A.F., Nascimento, J., Raphael, C.F., 2007. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Domest Anim 42, 479-488.

De Andrade, A.F., de Arruda, R.P., Celeghini, E.C., Nascimento, J., Martins, S.M., Raphael, C.F., Moretti, A.S., 2007. Fluorescent stain method for the simultaneous determination of mitochondrial potential and integrity of plasma and acrosomal membranes in boar sperm. Reprod Domest Anim 42, 190-194.

De Arruda, R.P., de Andrade, A.F., Peres, K.R., Raphael, C.F., Nascimento, J., Celeghini, C., 2007. Biotécnicas aplicadas à avaliação do potencial de fertilidade do sêmen eqüino. Rev Bras Reprod Anim 31, 8-16.

De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology 27, 171-183.

Dube, C., Beaulieu, M., Reyes-Moreno, C., Guillemette, C., Bailey, J.L., 2004. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. Theriogenology 62, 874-886.

Ebisch, I.M., Peters, W.H., Thomas, C.M., Wetzels, A.M., Peer, P.G., Steegers-Theunissen, R.P., 2006. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 21, 1725-1733.

Flesch, F.M., Gadella, B.M., 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469, 197-235.

Frey, T.G., Mannella, C.A., 2000. The internal structure of mitochondria. Trends Biochem Sci 25, 319-324.

Funahashi, H., Sano, T., 2005. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. Theriogenology 63, 1605-1616.

Gaczarzewicz, D., Piasecka, M., Udala, J., Blaszczyk, B., Laszczynska, M., Kram, A., 2003. Oxidoreductive capability of boar sperm mitochondria in fresh semen and during their preservation in BTS extender. Reprod Biol 3, 161-172.

Gadea, J., 2003. Review: Semen extender used in the artificial insemination of swine. Spanish Journal of Agricultural Research 1, 17-27.

Galli, A., Bosisio, M., 1988. Quality of semen stored at +15 degrees 16 degrees C as related to fertility of artificially inseminated swine. Theriogenology 30, 1185-1190.

Hancock, J.L., Hovel, G.J.R., 1959. The collection of boar semen. Vet Rec 71, 664-665.

Huo, L.J., Ma, X.H., Yang, Z.M., 2002. Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. Theriogenology 58, 1349-1360.

Johnson, L.A., Weitze, K.F., Fiser, P., Maxwell, W.M., 2000. Storage of boar semen. Anim Reprod Sci 62, 143-172.

Juonala, T., Salonen, E., Nurttila, T., Andresson, M., 1999. Three fluorescence methods for assessing boar sperm viability. Reprod Domest Anim 34, 83-87.

Kim, I.H., Van Langendonckt, A., Van Soom, A., Vanroose, G., Casi, A.L., Hendriksen, P.J., Bevers, M.M., 1999. Effect of exogenous glutathione on the in vitro fertilization of bovine oocytes. Theriogenology 52, 537-547.

Kumaresan, A., Kadirvel, G., Bujarbaruah, K.M., Bardoloi, R.K., Das, A., Kumar, S., Naskar, S., 2009. Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim Reprod Sci 110, 162-171.

Oura, C., Toshimori, K., 1990. Ultrastructural studies on the fertilization of mammalian gametes. Int Rev Cytol 122, 105-151.

Ozaki, T., Takahashi, K., Kanasaki, H., Miyazaki, K., 2002. Evaluation of acrosome reaction and viability of human sperm with two fluorescent dyes. Arch Gynecol Obstet 266, 114-117.

Rousseaux, J., Rousseaux-Prevost, R., 1995. Molecular localization of free thiols in human sperm chromatin. Biol Reprod 52, 1066-1072.

Sarlos, P., Molnar, A., Kokai, M., Gabor, G., Ratky, J., 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Vet Hung 50, 235-245.

Storey, B.T., 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod 3, 203-213.

Sutkeviciene, N., Andersson, M.A., Zilinskas, H., Andersson, M., 2005. Assessment of boar semen quality in relation to fertility with special reference to methanol stress. Theriogenology 63, 739-747.

Thuwanut, P., Chatdarong, K., Techakumphu, M., Axner, E., 2008. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology 70, 233-240.

Turba, M.E., Fantinati, P., Bernardini, C., Gentilini, F., Bacci, M.L., Forni, M., 2007. Relationships between innovative and traditional parameters to investigate semen quality in pigs. Anim Reprod Sci 99, 72-81.

Uysal, O., Bucak, M.N., 2007. Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. Acta Vet Brno 76, 383-390.

Vasconcelos, A.M.M.A., Moraes, G.V., Moreira, I., Rigolon, L.P., Martins, E.N., 2001. Características Espermáticas de Sêmen Resfriado de Suíno e Conservado em Diferentes Diluentes. Rev Bras Zootec 30, 394-401.

List of Figures

List of Tables

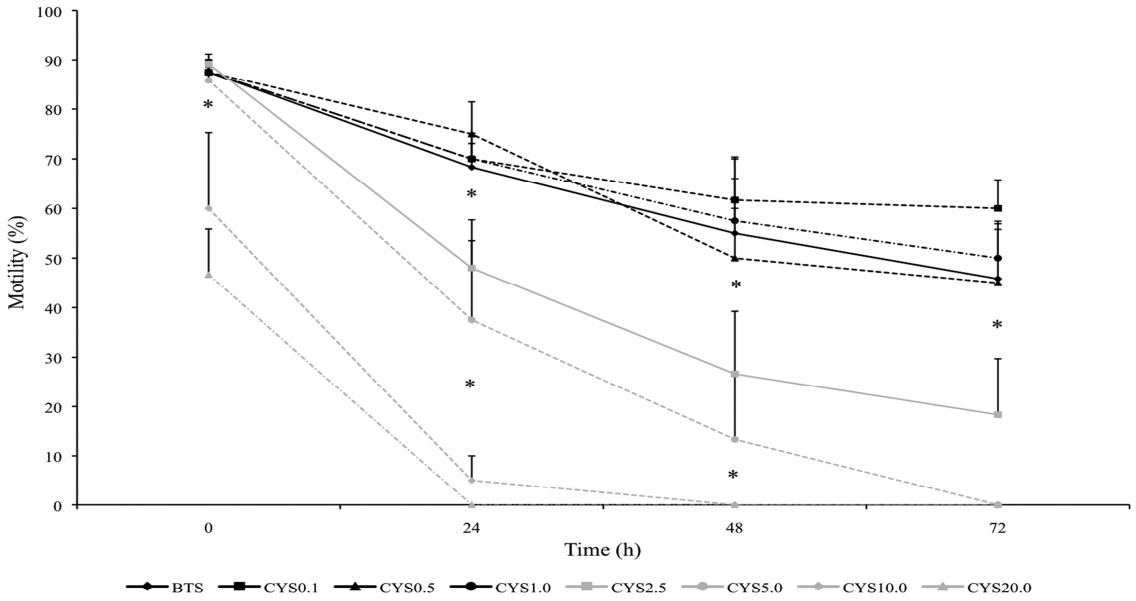

Figure 1. The effect of different concentration of cysteine added to BTS extender on motility of swine semen storage at 17^{0}C for 72 h. * Difference between treatments ($P < 0.05$).

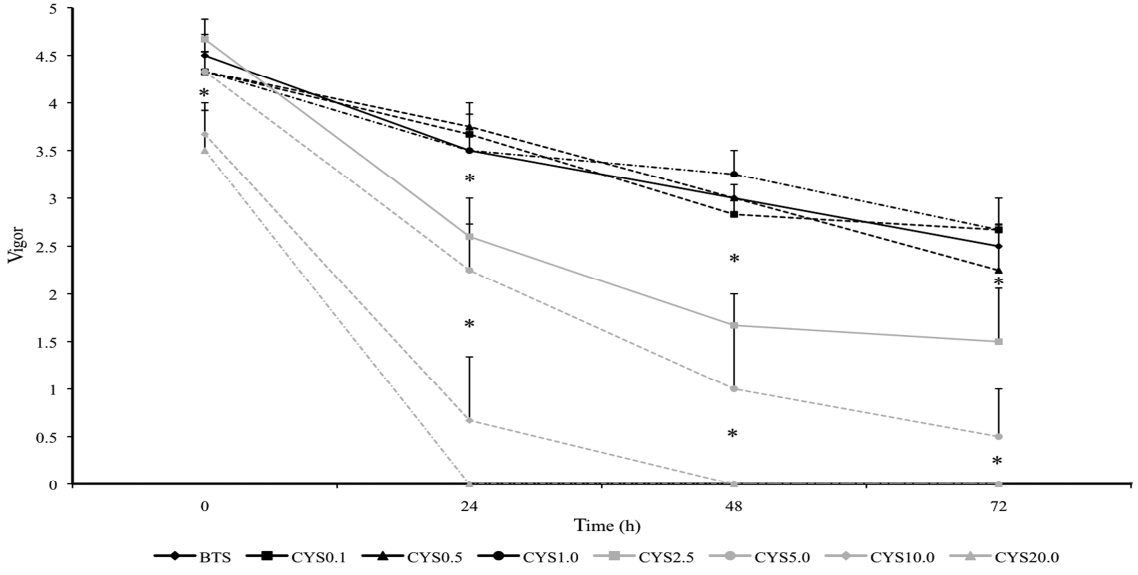

Figure 2. The effect of different concentration of cysteine added to BTS extender on vigor of swine semen storage at 17^{0}C for 72 h. * Difference between treatments ($P < 0.05$).

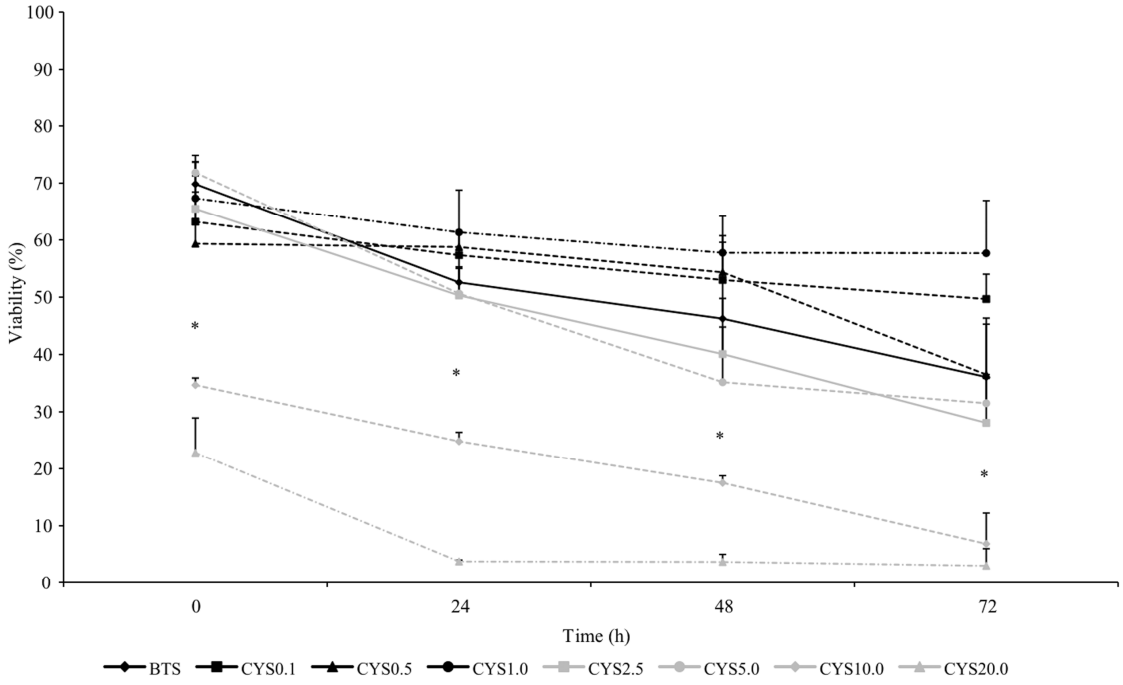

Figure 3. The effect of different concentration of cysteine added to BTS extender on spermatozoa viability of swine semen storage at 17^0C for 72 h.

* Difference between treatments ($P < 0.05$).

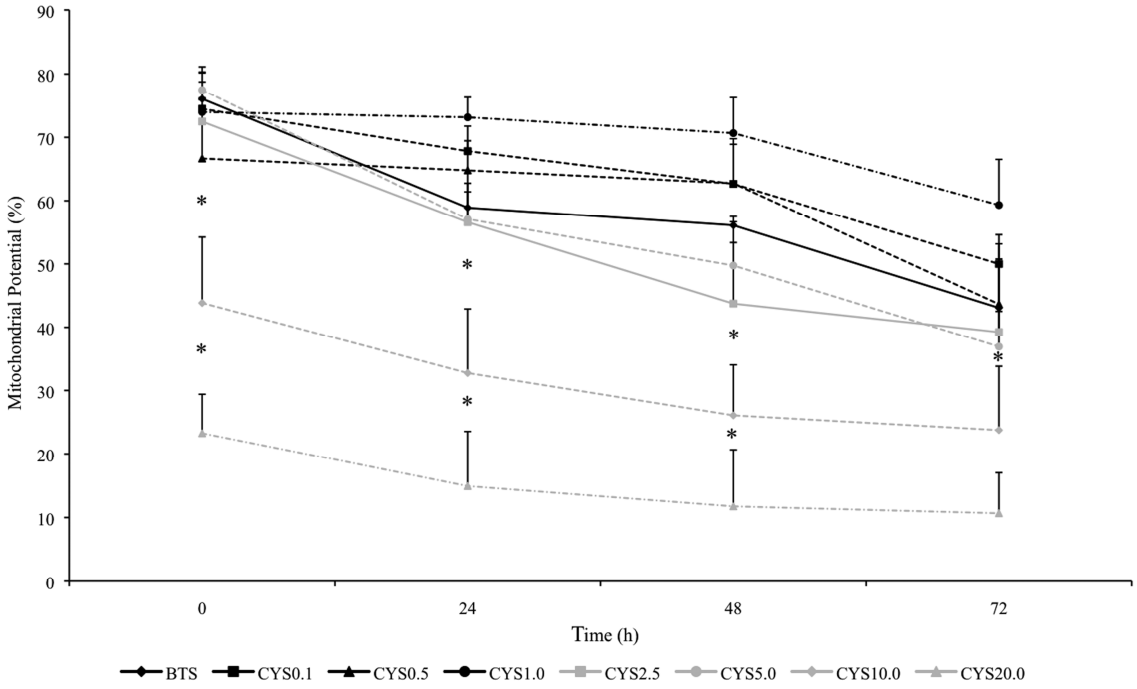

Figure 4. The effect of different concentration of cysteine added to BTS extender on mitochondrial potential of swine semen storage at 17ºC for 72 h.

* Difference between treatments ($P < 0.05$).

Table 1. Morphological defects in boar sperm cells submitted to Beltsville Thawing Solution extender (BTS; control group) or BTS and different concentrations of cysteine (CYS; 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 mM) storage for 0, 24, 48 and 72 h at 17 °C.

	Morfology (%)			
	Hour			
Treatment	0	24	48	72
BTS	10.58 ± 2.98^{abc}	11.58 ± 2.03^{a}	13.00 ± 1.00^{a}	13.67 ± 1.50^{ab}
CYS0.1	8.33 ± 1.81^{ac}	8.83 ± 1.48^{a}	11.83 ± 2.35^{a}	15.67 ± 4.76^{ab}
CYS0.5	7.00 ± 1.69^{a}	10.37 ± 1.95^{a}	11.17 ± 3.22^{a}	15.37 ± 2.66^{ab}
CYS1.0	7.58 ± 1.57^{ac}	8.58 ± 1.10^{a}	13.62 ± 1.97^{a}	15.33 ± 3.22^{ab}
CYS2.5	7.08 ± 1.43^{a}	10.50 ± 2.42^{a}	10.50 ± 2.03^{a}	11.08 ± 2.64^{a}
CYS5.0	12.33 ± 3.35^{abc}	12.50 ± 2.68^{a}	14.17 ± 3.88^{a}	19.75 ± 6.25^{b}
CYS10.0	15.17 ± 1.88^{bc}	14.33 ± 2.89^{a}	15.50 ± 3.00^{a}	14.17 ± 2.45^{ab}
CYS20.0	12.50 ± 1.89^{c}	13.00 ± 2.00^{a}	13.17 ± 4.81^{a}	14.25 ± 6.25^{ab}

Different superscript letters in the same column indicate statistical differences ($P < 0.05$).

4 CAPÍTULO 2

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

EFFECTS OF CENTRIFUGATION AND THE ADDITION OF CYSTEINE IN COOLED BOAR SEMEN

Carolina Klein Severo, Lucas Oberherr, Andressa Minussi Pereira, Vitor Sales Truzzi, Rosinara da Silva Costa, Carolini Machado Landarin, Gabriel Ribas Pereira, João Francisco Oliveira, Marcelo Soares, Paulo Bayard Gonçalves

THERIOGENOLOGY, 2009

Effects of centrifugation and the addition of cysteine in cooled boar semen

C. K. Severo[a], L. Oberherr[b], A. M. Pereira[a], V. S. Truzzi[a], R. S. Costa[b], C. M. Landarin[b], G. R. Pereira[a], J. F. Oliveira[a], M. Soares[b], P. B. Gonçalves[a*]

[a] Laboratory of Biotechnology and Animal Reproduction - BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil, 97105-900.

[b] Swine Clinic and Reproduction Sector, Federal University of Santa Maria, Santa Maria, RS, Brazil, 97105-900.

Correspondonding author:

Paulo Bayard Gonçalves, Departamento de Clínica de Grandes Animais, Hospital Veterinário, Universidade Federal de Santa Maria, Postal code 97105-900, Santa Maria, RS, Brazil.

Phone: 55-55-3220-8752.

Fax: 55-55-3220-8484.

E-mail: bayard@biorep.ufsm.br

Abstract

The Brazilian pig industry development has been occurred as a consequence of the technological advances in the last years. The aim of the present study is to evaluate the effects of cysteine addition in the *Beltsville Thawing Solution* (BTS) extender and semen centrifugation to enhance sperm quality and fertility in swine production. Sperm-rich fractions were collected from three boars, using five ejaculates from each animal. The samples were subdivide as follow: a) semen diluted in BTS without centrifugation (BTS/NC), b) semen diluted in BTS with 5.0 mM cysteine without centrifugation (BTSCYS/NC), c) semen diluted in BTS and centrifuged (BTS/CENT) and d) semen diluted in BTS with 5.0 mM cysteine and centrifuged (BTSCYS/CENT). The integrity and functionality of plasma membrane (PI), integrity of acrosomal membrane (AI), mitochondria potential (MP), chromatin integrity, sperm motility and morphology were assessed at specific times (0, 24, 48 and 72 h). A second experiment was designed to determine fertility rates using 136 females. Ejaculated semen was divided in three treatments, as follow: BTS/NC (n = 49), BTSCYS/NC (n = 42) and BTSCYS/CENT (n = 45). After artificial insemination, the rate of estrous return and the number of piglets from all animals used in the experiment were recorded. In the first experiment, the effect of treatments on each dependent variable was analyzed using repeated measured model and in the second, the difference between the treatments on the rate of estrous return and on the number of piglets was analyzed using PROC GLM model both by SAS Software. The BTSCYS/CENT treated semen after 24 h of storage at 17 ℃ showed a decrease in the motility compared to others ($P < 0.05$). The BTS/NC and BTSCYS/NC groups have a higher percentage of cells with intact membranes and potential of

mitochondria than BTS/CENT and BTSCYS/CENT when storage at 17 ℃. Based on our study, we concluded that ejaculated semen diluted with BTS extender and not centrifuged showed the lowest rate of estrous return and also the highest mean of piglets borned per female after artificial insemination

Keywords: Semen, Beltsville Thawing Solution, Cysteine, Centrifugation, Swine Production.

1. Introduction

Artificial insemination is a widespread technique used in the swine industry to increase the production of piglets. According to BORTOLOZZO et al. **[1]**, 51% of the breeding farms have been using this biotechnology to reduce production costs and to achieve a better use of the male gametes into swine industry **[1 - 3]**. The use of assisted reproduction technologies (ART) in domestic animals aims to improve the reproductive efficiency. However, the success of artificial insemination programs in swine depends on the farm and semen management **[4]**. During the artificial insemination, several steps are crucial to obtain an insemination dose containing a good number of viable spermatozoa, such as the addition of extenders into ejaculated semen to provide energy and substrates to spermatozoa survival **[5]**.

The centrifugation is a process that removes seminal plasma from ejaculated semen to decrease the deleterious effects of oxidants compounds on the spermatozoa **[6]**. However, the removal of seminal plasma, the mechanical damage and the formation of reactive oxygen species (ROS) appear to have negative effect on spermatozoa

viability **[6 - 9]**. Some species are more sensitive to the process of centrifugation, such as rats **[10]**, human **[11]** and mice **[8]**, whereas horses **[12]** and cattle **[13]** are more resistant. This indicates that there is specificity between species according to the centrifugation steps used to separate spermatozoa from seminal plasma.

The use of extenders is of fundamental importance to maintain ejaculated-sperm viability **[5]**. The BTS is a world widely extender used in the swine industry that contains glucose as the mainly source of semen energy **[14]**. It also contains low levels of potassium to maintain the intracellular concentration of ions at physiological levels during semen storage **[15]**. Despite considerable progress in knowledge, there still a little academic research being conducted to establish appropriate protocols in order to benefits the commercial swine industry.

The temperature of storage is another factor that influences the maintenance of sperm viability. The swine sperm is sensitive to cold shock due to the lipid composition of the plasma membrane **[5]** and to the temperatures below 10 °C that cause irreversible damage to the spermatozoa membrane leading to decrease its fertilization potential **[2]**. According to ALTHOUSE et al. **[16]**, temperatures lower than 15 °C are detrimental to the movement of membrane phospholipids that can result in the transition from a fluid to a gel phase.

The content of unsaturated fatty acid in spermatozoa results in high sensibility to oxidative stress **[17]**. The thiol is part of a large class of antioxidants, including cysteine, N-acetyl cysteine and glutathione. Both, the cysteine and N-acetyl cysteine, are precursors of the biosynthesis of intracellular glutathione **[18]**, and they consist as an important molecule observed at lower levels in spermatozoa **[19]**. The cysteine and the N-acetyl cysteine act as a cell membrane protector through the maintenance of

sulphydric groups and they are also known to interact with free radical to decrease the oxygen reactions responsible for the lipid peroxidation and consequently to decrease its spermatozoa function **[18]**. Apparently, both, can reduce the toxic effect of the reactive oxygen species during the sperm cryopreservation process **[18, 20]**. Therefore, the aim of the study is to investigate the effect of the addition of 5.0 mM cysteine in combination with centrifugation on the maintenance of sperm quality and their influence on fertility.

2. Materials and methods

Unless otherwise stated, all media components were purchased from Sigma (Sigma-Aldrich, São Paulo, SP, Brazil). Two experiments were conducted to elucidate the potential role of the cysteine and the centrifugation process on the piglet's production. In experiment 1 it was evaluated the sperm integrity using different cysteine and centrifugation treatments. The second experiment was conducted in a Producing Unit of Piglets (UPL) at The Cooperative Languiru, Teutonia, RS, Brazil to evaluate the effect of the treatments over the reproductive performance of sows.

2.1. *Experiment 1*

2.1.1. Animals and source of semen

Three boars at the age of 36 month were allocated in individual cages and collected five times during this study. Animals were maintained at good corporal condition and fresh clean water was available *ad libitum* throughout the entire trial

period. Semen collection were performed using a gloved-hand technique **[21]**. The first ejaculatory portion was discharged and the semen was filtered through four 130-mm^2 gauze at the time of collection. Only the rich fraction was used during this study.

2.1.2. Semen preparation and assessment

Semen was divided in four sperm fractions and only two fractions were centrifuged at 800 g for 10 min **[22]**. The BTS extender used in this study contained 206 mM Glucose, 20.4 mM Na_3citrate, 14.9 mM $NaHCO_3$, 3.4 mM Na_2EDTA, 10 mM KCl, penicillinGNa 0.6 g/l, dihydroestreptomicin 1.0 g/l (Minitub, Tiefenbach, Germany) and it was diluted in warmed tridestilated water at 34 ºC at proportion of 50 g/l. To evaluate sperm concentration, 10 µl of each ejaculated was diluted (1:100) in formaldehyde-citrate buffered solution and the number of spermatozoa/ml were assessed using a Neubauer haemocytometer chamber (Boeco, Hamburg, Germany). The final concentration of extended semen was adjusted to 3 x 10^9 spermatozoa for each 80 ml. After that the semen aliquots were submitted to different treatments groups as follow: 1) semen diluted in BTS (BTS/NC); 2) semen centrifuged and diluted in BTS (BTS/CENT); 3) semen diluted in BTS containing 5.0 mM cysteine (BTSCYS/NC) and 4) semen centrifuged diluted in BTS containing 5.0 mM cysteine (BTSCYS/CENT).

After dilution samples were stored into a refrigerated chamber at 17 ºC and evaluated at hour 0, 24, 48 and 72. One aliquot of 7 µl of semen was placed in a warmed (37 ˚C) slide covered with 22-mm x 22-mm coverslip to evaluate semen motility and vigor. Sperm progressive motility (0 to 100%) and vigor (1 to 5) were estimated

under a bright-field microscope (Olympus BX40, Olympus Optical Co. Ltd., Tokyo) at 200 x magnification. For evaluation of sperm morphology aliquots of semen were fixed using a 2.9% formaldehyde-citrate buffered solution and placed in a slide covered with 22-mm x 22-mm coverslip. A total of 200 spermatozoa were analyzed for each sample and assessed under a phase contrast microscope (Olympus CHS, Olympus Optical Co. Ltd., Tokyo) at a magnification of 1000 x. Spermatozoa abnormalities (nuclear pouches, defective acrosomes, non-intact acrosomes, loose heads, proximal cytoplasmic droplets, midpieces, and abnormal tails, i.e., double-folded, single-bent, and coiled tails) were classified according to a system developed by Galli and Bosisio **[23]**. The relative percentage of morphologically normal spermatozoa in the ejaculate was taken as the mean percentage of spermatozoa without defects for each evaluated aliquots.

A hipoosmotic test was performed using Aliquots of semen (0.1 ml) were added to 0.9 ml solution of 150 mOsm/ml containing 50% fructose and 50% sodium citrate (Nuclear CAQ Ind. e Com. Ltda, São Paulo, SP, Brazil) and incubated for 30 min at 37 ℃. After, the total of 200 cells was counted in a Neubauer chamber under phase contrast microscope (Olympus CHS) at a magnification of 400 x. The percentage of swelling and/or coiling spermatozoa was determined as the functional membrane according to Correa-Perez et al. **[24]** and Neild et al. **[25]**.

For the chromatin structure assay, swabs of semen were fixed in acetic acid glacial (Nuclear CAQ Ind. e Com. Ltda) and ethanol 70% (F Maia Ind. e Com. Ltda, Cotia, SP, Brasil) using a dilution rate of (3:1) for 1 min and subsequently washed with ethanol 70% for 3 min. After fixation, samples were hydrolysed with 4 N acid cloridric (Quimibras Indústrias Químicas S/A, Rio de Janeiro, RJ, Brasil) for 8 min **[26]**. After this period, one aliquot of 7 µl of 0.025% toluidine blue in 0.1 M citric acid (Nuclear CAQ Ind.

e Com. Ltda) and 0.2 M phosphate dibasic of sodium (Nuclear CAQ Ind. e Com. Ltda) was placed in a slide covered with 22-mm x 22-mm coverslip. The total number of 400 cells were counted under bright-field microscope (Olympus BX40) at 200 x magnification and classified according to the presence and absence of color.

2.1.3. Spermatozoa staining and fluorescence assessment

Spermatozoa viability was evaluated by the plasma membrane integrity (PI), acrossomal membrane integrity (AI), and mitochondrial potential (CP), using fluorescent probes. One aliquot of semen was diluted into Modified Tyrode's Médium (TALP) in order to obtain 25 x 10^6 espermatozoa/ml. Then, a 150 µl aliquot was added into an tube of 2.5ml and 3 µl of propidium iodide diluted in 5 mg/l of Dulbecco´s phosphate-buffered saline solution (DPBS), 50 µl of fluorescein isothiocynate-conjugated Pisum sativun agglutinin (FITC - PSA) in 5 mg/l of DPBS, and 3 µl of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) in 153 µM of dimethyl sulphoxide (DMSO) were added and mixed within the aliquot. Samples were maintained in a dark chamber at room temperature for 8 min. After, one aliquot of 7 µl was evaluated under epifluorescence microscope at 1000 x magnification and a total of 200 cells were counted for each treatment group.

2.2. *Experiment 2*

2.2.1. Animal population and experimental design

Five hybrid males aged over 24 months, four Agroceres and one Geneticpork genetics, maintained at good corporal condition were used throughout the entire trial period. Animals were allocated in the Insemination Center within the UPL Cooperative Languiru. After the semen collection, fresh semen was evaluated for concentration, motility and vigor using the same parameters as the experiment one. Semen was divided between treatments, BTS/NC, BTSCYS/NC and BTSCYS/CENT. The BTS extender was prepared in advance and kept at 34 °C before semen dilution. Then, semen was divided in two aliquots and diluted to obtain 3.5 x 10^9 sperm in 90 ml, in each. One aliquot was used to inseminate the animals as explained above and the other was sent to the laboratory to evaluate motility, morphological abnormalities and sperm viability.

A 136 females were previously selected on the basis of genetics (Debrecht 90 and Cambrough 25), the parity date (between three and six) and the weaning estrus interval (between three and five days). Then, females were subdivided into three insemination treatments as follow: semen diluted in BTS/NC (control, n = 49); semen diluted in BTS added 5.0 mM of cysteine (BTSCYS/NC, n = 42) and centrifuged semen diluted in BTS added 5.0 mM of cysteine (BTSCYS/CENT, n = 45). Three cervical inseminations per animal were performed from each treatment group after detection of estrus. Semen used for insemination was stored from 0 to 24 h at 17 °C. After the inseminations all females were assessed for estrus return. Females that returned were not inseminated again. Therefore, females do not return estrus were assessed for the total number of piglets born.

2.3. Statistical analysis

In the experiment 1, data was statistically analyzed using a repeated measurement analysis approach to determine the effect of each treatment on each dependent variable, using PROC MIXED of SAS software (SAS Institute, Cary, NC, USA). In the experiment 2, the effect of treatment on each dependent variable was analyzed using a model of analysis for unbalanced data (PROC GLM - SAS software) to determine the effects of treatment and genetics. The means for the effect of different treatments and genetics in relation to the order of birth, rate of return and number of piglets were compared using the Tukey test. Differences were considered to be significant when $P < 0.05$ and results were expressed as mean ± standard error of means (S.E.M.).

3. Results

3.1. Experiment 1

The motility (**Fig. 1**) and vigor presented a similar parameters in all treatments. After 24 h of storage BTSCYS/CENT had less than 40% motility when compared to BTS/NC, BTSCYS/NC and BTS/CENT ($P < 0.05$). After 24 h of storage at 17 °C, the BTSCIYS/CENT showed lower vigor mean when compared to BTS/NC and BTS/CENT ($P < 0.05$). The motility rate of the BTSCYS/NC (48.13 ± 9.75) and BTSCYS/CENT (29.47 ± 7.68) groups were lower than BTS/NC (65.0 ± 7.86) and BTS/CENT (60.77 ± 4.59) at 48 h of storage ($P < 0.05$). All treatments showed means less than 60% of

sperm motility after 72 h of storage, however BTSCYS/CENT presented the lowest motility rate (12.13 ± 4.68) and vigor (0.60 ± 0.25) after 72 h of storage at 17 °C.

Sperm morphology and function of plasma membrane did not differ between treatments during storage at 17 °C ($P > 0.05$). The percentage of sperm that showed morphological alterations, such as changes in head, tail and acrosomes, do not to exceed 20% in any of the treatments assessed during storage. The functionality of plasma membrane at 72 h of storage did not exceed 30% (BTS/NC; 22.37 ± 3.41, BTS/CENT; 21.79 ± 1.94, BTSCYS/NC; 25.71 ± 2.22 and BTSCYS/CENT; 21.03 ± 1.74). During semen storage, the all treatments do not differ of compacted DNA.

All treatments showed a reduction in the percentage of sperm with intact membranes and mitochondrial potential during the period of storage at 17 °C. In the first 48 h of storage, the BTS/NC and BTSCYS/NC treatments showed higher mean of the plasma and acrosomal membrane integrity and mitochondrial potential when compared to BTS/CENT and BTSCYS/CENT (**Fig. 2, 3 and 4**). However, the BTSCYS/CENT present lower mean of mitochondria potential at 72 h when compared to others treatments (**Fig. 4**).

3.2. Experiment 2

The mean of motility after 24 h were 64.64% BTS/NC, 7.29% BTSCYS/CENT and 11.25% BTSCYS/CENT. The mean of morphological alterations were 14.50% BTS/NC, 20.07% BTSCYS/NC and 22.63% BTSCYS/CENT. The mean semen viability was 17.61% BTS/NC, 10.86% BTSCYS/NC and 15.69% BTSCYS/CENT.

The genetic did not influence the rate of estrus return neither the number of piglets' borned. The order of parturition and the interval of estrus return did not differ between treatments. The BTS/NC (10.20 ± 0.39) treated group showed a lower rate of estrus return (**Fig. 5**) when compared to others (BTSCYS/NC; 86.05 ± 0.39 and BTSCYS/CENT; 58.70 ± 0.39), and the highest total number of piglets born per treatment (12.71 ± 3.38 vs 9.00 ± 3.38 and 8.83 ± 3.38, respectively) (**Fig. 6**).

4. Discussion

In the present study, the effect of cysteine in combination with centrifugation on the motility and vigor showed a similar pattern. The motility and vigor decreased during semen storage at 17 ℃ independent of the treatment used **[27, 28]**. In agreement with Carvajal et al. **[6]** and Shekarriz et al. **[9]**, the negative effect of centrifugation on motility and vigor was observed when was centrifuged and BTS added cysteine. However, studies have been shown that the addition of antioxidants to centrifuged semen minimizes the decrease on sperm motility **[29, 30]**.

The number of spermatozoa showing functional plasma membrane is less observed than the number of sperm showing motility **[31, 32]**. In our study, neither centrifugation nor the addition of cysteine into BTS extender appears to influence the maintenance the plasma membrane functionality and the appearance of abnormalities in sperm morphology. Johnson et al. **[2]** reported to have no importance on morphology parameters when less than 20% alteration are detected during swine semen analysis.

According to Donelly et al. **[33]** the addition of antioxidant in centrifuged semen showed that the spermatozoa DNA remains compressed. However, in the current study

we observed a lower mean of compacted DNA at 48 h of storage on the BTSCYS/CENT group compared to BTS/CENT. Although, when only, centrifuge or addition of cysteine was used, the sample showed no difference. This finding indicates that the presence of cysteine on the process of centrifugation influences negatively the number of cells with compacted DNA.

In this study, we also evaluated the integrity of plasma and acrossomal membranes and the mitochondria potential using fluorescence probes as a parameter for integrity analyses. The centrifugation process reduced sperm viability as described in pigs **[6]** and humans **[9]**. In contrast, treatment BTSCYS/NC has behaved similarly to the control treatment (BTS/NC) in all parameters. These observations suggest that centrifugation is detrimental to increase damage on the plasma and acrosomal membranes and to decrease the mitochondria potential of spermatozoa.

The addition of 5.0 mM cysteine to the BTS extender did not influence the acrossomal membrane integrity contradicting the findings of Funahashi and Sano **[19]** who claim that antioxidants reduce the percentage of defects in acrosomes. Although in horses **[34]** and ram **[35]** there was no effect of cysteine on the acrossomal membrane integrity.

Beside several organelles in the spermatozoa organization, mitochondria is known to promote oxidative phosphorylation and also to produce energy from adenosine tri-phosphate (ATP) that is critical for cell motility **[36, 37]**. In the current study, the presence of mitochondrial potential showed similar parameters on the motility and vigor as observed on studies in swine **[38]** and bovine **[39]** semen. Of this, the potential of mitochondria membrane viability can be an important indicator of functional integrity **[40]**. According Peña et al. **[41]**, the maintenance of mitochondria function of boar

spermatozoa depends of the protector effect of the antioxidants. Several studies were realized to evaluate the addition of antioxidants in semen swine **[38]**, stallion **[42]**, bovine **[43]**, ovine **[44]**, birds **[45]** and human **[46]**.

The deleterious effects, decrease of plasma and acrosomal membrane integrity and mitochondria potential, produced by centrifugation observed in our study is possible explained by the presence of active antioxidant enzymes in the seminal plasma **[19]**. Moreover, the antioxidants have an important function of scavenging the reactive oxygen species that are found on spermatozoa and leukocyte in bovine and boar semen **[47, 48]**. However, according to studies by Ebisch et al. **[49]** men subfertile have high concentrations of thiol in semen. Therefore, the excessive scavenging of ROS at lower physiological levels are necessary for normal spermatozoa function.

In this study, we evaluated the effect of different cysteine and/or centrifugation treatment on rate return estrous to estrus and the total number of piglets born. These reproductive parameters were also used to assess the influence of diluents BTS, Merk III and coconut water on swine productivity **[50]**. In our study we observed that the removal of seminal plasma lead to an increase on the rate return estrous and to a decrease to the total number of piglet's born compared to the control (BTS/NC). In contrast, previous studies have suggested no difference in the absence or presence of seminal plasma on the rate of cleavage and blastocyst formation in swine **[51]**. The current study demonstrates that insemination performed with BTSCYS/NC showed a greater negative effect than BTSCYS/CENT. Beside, Funahashi and Sano **[19]** reported no difference on the penetration of sperm in oocytes between diluted semen using modified Modena extender in the presence or absence of cysteine up to eight days of storage at 10 ℃. However, the same study showed that until 29 days of semen storage

diluted in modified Modena extender and added 5 mM cysteine showed the highest rates of sperm penetration.

The number of piglets born was higher in BTS compared to others and BTSCYS/CENT when compared to BTSCYS/NC. This finding indicates that the absence of cysteine is important to maintain the number of piglets' borned. The reduction in the number of piglets using centrifuged semen is possible explained by fact that insemination has occurred a long time before ovulation due to the absence of seminal plasma that promotes sperm capacitation changes **[52]**. Although this negative effect of cysteine on the number of piglets were not expected, when the rate of return was superior over 10 % we pass to consider it.

In conclusion, the current study demonstrates that cysteine and centrifugation negatively affect the sperm viability resulting in a higher percentage of return to estrus and lower number of piglets borned.

Acknowledgements

This study was supported by the Brazilian Federal Council for Higher Education Personnel (CAPES) and the National Council for Scientific Research and Development (CNPq).

References

[1] Bortolozzo FP. et al. Situação atual da inseminação artificial em suínos. Acta Sci Vet 2005;33:17-32.

[2] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. Anim Reprod Sci 2000;62:143-172.

[3] DIehl GN et al. Nova pipeta para inseminação intra-uterina em suínos. Ciência Rural 2006:36:179-185.

[4] Lamberson W, Safranski T. A model for economic comparison of swine insemination programs. Theriogenology 2000;54:799-807.

[5] Gadea J. Review: Semen extender used in the artificial insemination of swine. Span J Agricultural Res 2003;1:17-27.

[6] Carvajal G, Cuello C, Ruiz M, Vasquez JM, Martinez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389-396.

[7] Alvarez JG et al. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile life. Hum Reprod 1993**:**8:1087-1092.

[8] Katkov II, Mazur P. Influence of centrifugation regimes on motility, yield and cell associations of mouse spermatozoa. J Androl 1998;19:232–241.

[9] Shekarriz M et al. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. Eur Urology 1995;28:31-35.

[10] Cardullo RA, Cone RA. Mechanical immobilization of rat sperm does not exchange their oxygen consumption rate. Biol Reprod 1986;34:820–830.

[11] Ng SC et al. Microcentrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. Hum Reprod 1990**;**5:209–211.

[12] Katkov II, Ostashko FI. Correlation between electromechanical stability of cytoplasm membrane and cryoresistivity of bovine spermatozoa. Cryobiology 1996;33:680–687.

[13] Crockett EC et al. Effect of cooling on equine spermatozoa before freezing on post-thaw motility: preliminary results. Theriogenology 2001;55:793–803.

[14] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871-891.

[15] Gaczarzewicz D, Piasecka M, Udala J, Blaszczyk B, Laszczynska M, Kram A. Oxidoreductive capability of boar sperm mitochondria in fresh semen and during their preservation in BTS extender. Reprod Biol 2003;3:161-172.

[16] Althouse GC et al. Characterization of lower temperature storage limitations of fresh-extended porcine semen. Theriogenology 1998;50:535-543.

[17] Henkel R. The impact of oxidants on sperm function. Androl 2005;37:205–206.

[18] Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 2001;56:275-286.

[19] Funahashi H, Sano T. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. Theriogenology 2005;63:1605-1616.

[20] Thuwanut P, Chatdarong K, Techakumphu M, Axner E. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology 2008;70:233-240.

[21] Hancock JL, Hovel GJR. The collection of boar semen. Vet Rec 1959;71:664-665.

[22] Ohata PM, Wentz I, Bernardi ML, Castagna C, Bortolozzo FP. Viabilidade do sêmen suíno congelado submetido a um período de equilíbrio pré-congelamento com ou sem presença de plasma seminal. Arquiv Fac Vet-UFRGS 2001;29:123-129.

[23] Galli A, Bosisio M. Quality of semen stored at +15 degrees 16 degrees C as related to fertility of artificially inseminated swine. Theriogenology 1988;30:1185-1190.

[24] Correa-Pérez JR et al. The effect of colloid osmotic pressure in human spermatozoa exposed to hypoosmotic conditions. Androl 2003;35:117-120.

[25] Neild DM, Chaves MG, Flores M, Miragaya MH, Gonzalez E, Agüero A. The HOST test and its relationship to fertility in the stallion. Androl 2000;32:351-355.

[26] Machado ER et al. Diagnóstico de alterações na compactação da cromatina em espermatozóides de suíno através de Azul de Toluidina e Laranja de Acridina. Rev Bras Rep Anim 2003;27:381-382.

[27] Vasconcelos AMMA, Moraes GV, Moreira I, Rigolon LP, Martins EN. Características espermáticas de sêmen resfriado de suíno e conservado em diferentes diluentes. Rev Bras Zootec 2001;30:394-401.

[28] Dube C, Beaulieu M, Reyes-Moreno C, Guillemette C, Bailey JL. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. Theriogenology 2004;62:874-886.

[29] Griveau JF, Le Lannou D. Effects of antioxidants on human sperm preparation techniques. Int J Androl 1994;17:225–231.

[30] Parinaud J et al. Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit®) following ejaculation. Hum Reprod 1997;12:2434–2436.

[31] Zou CX, Yang ZM. Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. Theriogenology 2000;53:1477-1488.

[32] Vazquez JM et al. Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology 1997;47:913-922.

[33] Donnelly ET et al. Glutathione and hipotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. Mutagenesis 2000;15:61-68.

[34] Baumber J, Ball, BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 2000;21:895-902.

[35] Sarlos P, Molnar A, Kokai M, Gabor G, Ratky J. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Vet Hung 2002;50:235-245.

[36] Oura C, Toshimori K. Ultrastructural studies on the fertilization of mammalian gametes. Int Rev Cytol 1990;122:105-151.

[37] Frey TG, Mannella CA. The internal structure of mitochondria. Trends Biochem Sci 2000;25:319-324.

[38] Cerolini S, Maldjian A, Surai P, Boble R. Viability susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim Reprod Sci 2000;58:99-111.

[39] Celeghini ECC, de ARruda PR, de Andrade AFC, Nascimento J, Raphael CF, Rodrigues PHM. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Anim Reprod Sci 2008;104:119-131.

[40] Cummins JM, Jequier AM, Kan R. Molecular biology of the human male infertility: links with aging, mitochondrial genetics and oxidative stress. Mol Reprod 1994;37:345-362.

[41] Peña FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. Assessment of fresh and frozen thawed boar semen using an Annexin V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677-689.

[42] Ball BA, Medina V, Gravance CG, Baumber J. Effects of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5ºC. Theriogenology 2001;56:577-589.

[43] Beeoni MT, Francia CR, Mora NG, Afranchino MA. Effect of natural antioxidants on frozen on bovine semen preservation. Theriogenology 1993;40:841-851.

[44] Maxwell WMC, Stojanov T. Liquid storage of ram semen in the absence or presence of some antioxidants. Reprod Fertil Dev 1996;8:1013-1020.

[45] Donoghue AM, Donoghue DJ. Effects of water and lipid-soluble antioxidants on turkey sperm viability, membrane integrity and motility during liquid storage. Poult Sci 1997;76:1440-1455.

[46] Askari HA, Check JH, Peymer N, Bollendorf A. Effect of Natural Antioxidants Tocopherol and Ascorbic Acids in Maintenance of Sperm Activity During Freeze-Thaw Process. Systems Biology in Reproductive Medicine 1994;33:11-15.

[47] Bilodeu JF, Blanchete S, Cormier N, Sirad MA. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris exyender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology 2002;57:1105-1122.

[48] Brezezinska-Stevbodzinska F, Slebodzinski AB, Prietas B, Wieezorek G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. Biol Trace Elem Res 1995;47:69-74.

[49] Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006;21:1725-1733.

[50] Kotzias-Bandeira, E. Influência de diferentes diluentes e temperaturas de refrigeração sobre a qualidade de sêmen suíno. Braz J Vet Res Anim Sci 1999;36:[online] ISSN 1413-9596.

[51] Maxwell WMC, Long CR, Johnson LA, Dobrinskyet JR, Welch GR. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 1998;10:433-440.

[52] Maxwell WMC, Johnson LA. Physiology of spermatozoa at high dilution rates: the influence seminal plasma. Theriogenology 1999;52:1353-1362.

List of Figures:

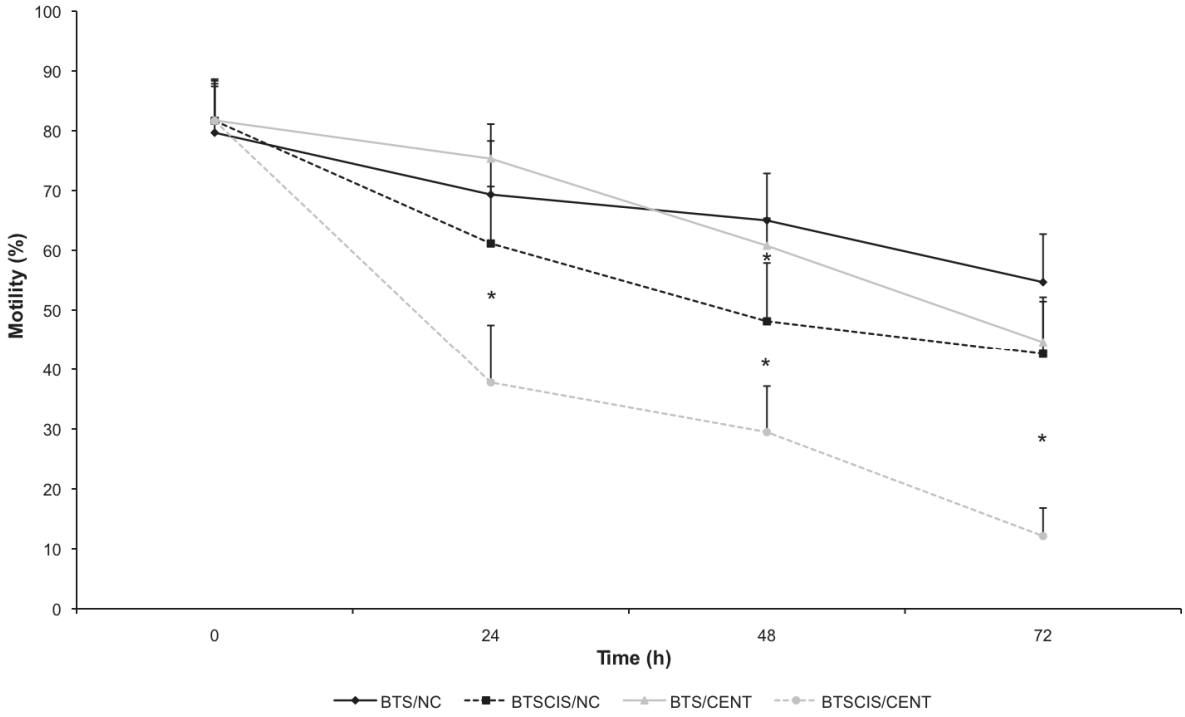

Fig. 1. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the motility of swine semen storage at 17 ˚C for 72 h. *Difference between treatments ($P < 0.05$).

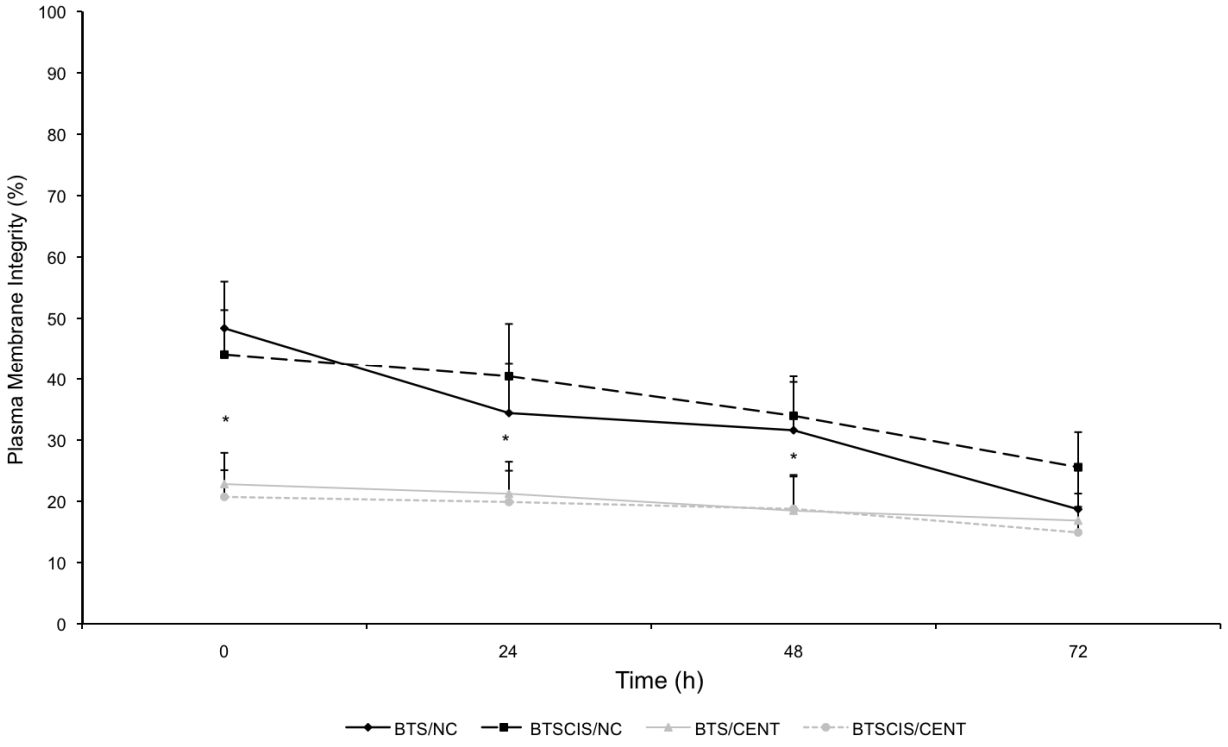

Fig. 2. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the integrity of plasma membrane (PI) of swine semen storage at 17 °C for 72 h. *Difference between treatments ($P < 0.05$).

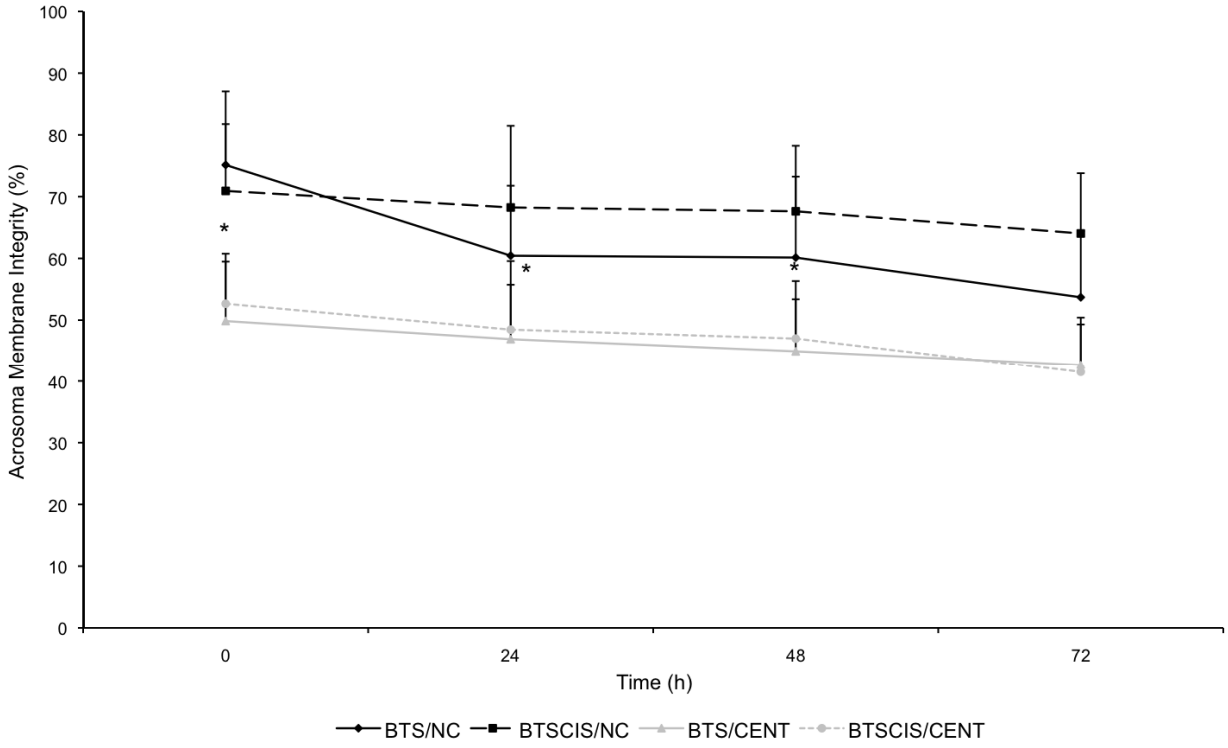

Fig. 3. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the integrity of acrosomal membrane (AI) of swine semen storage at 17 °C for 72 h. *Difference between treatments ($P < 0.05$).

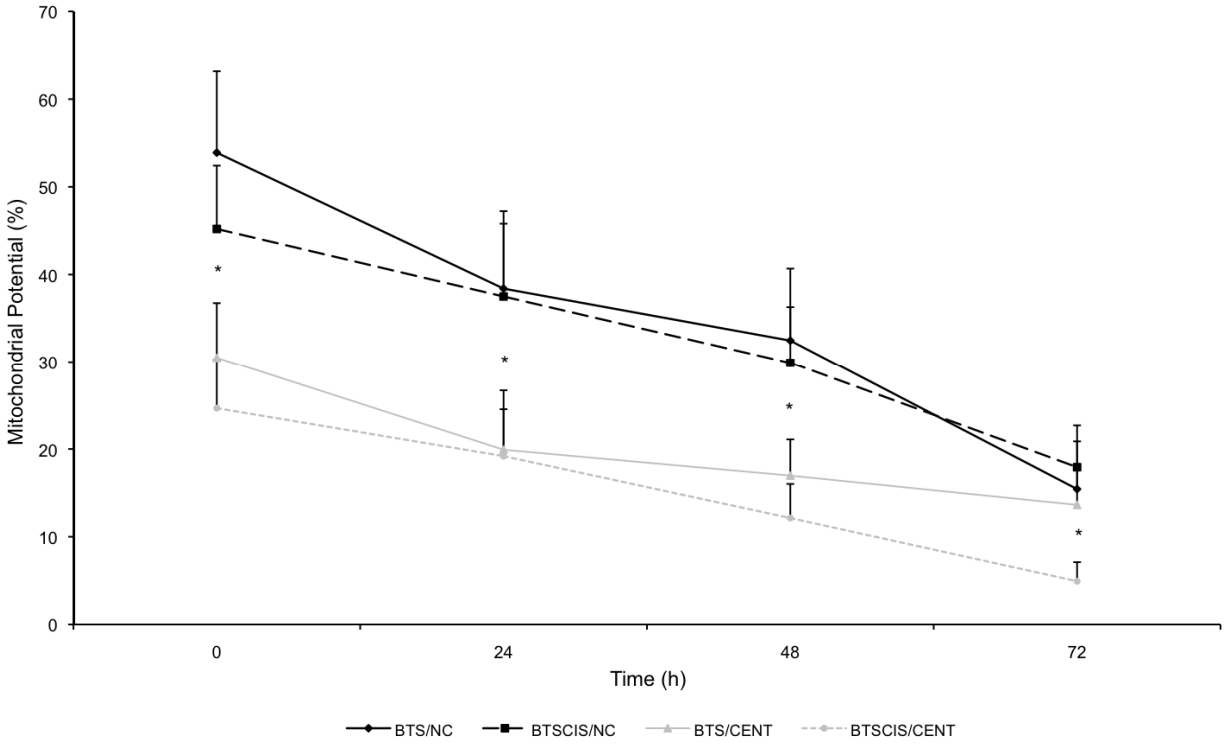

Fig. 4. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the mitochondria potential (MP) of swine semen storage at 17 ºC for 72 h. *Difference between treatments ($P < 0.05$).

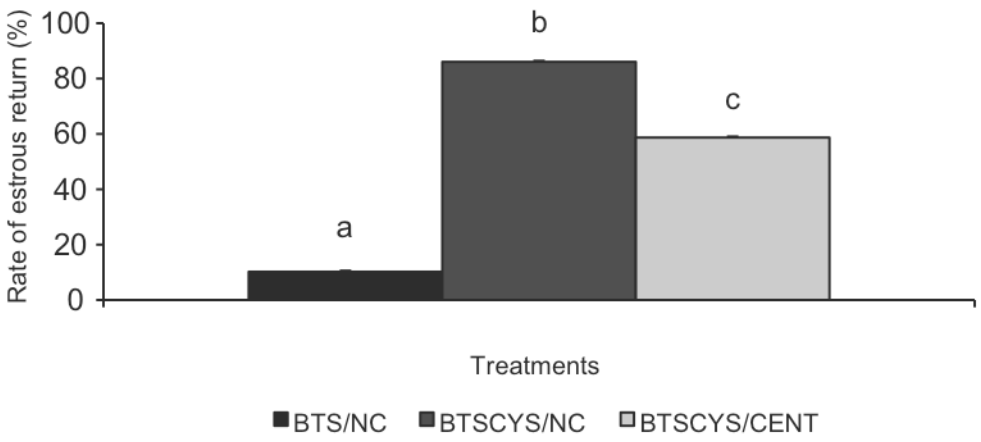

Fig. 5. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the rate of estrous return. Different letters between treatments ($P < 0.01$).

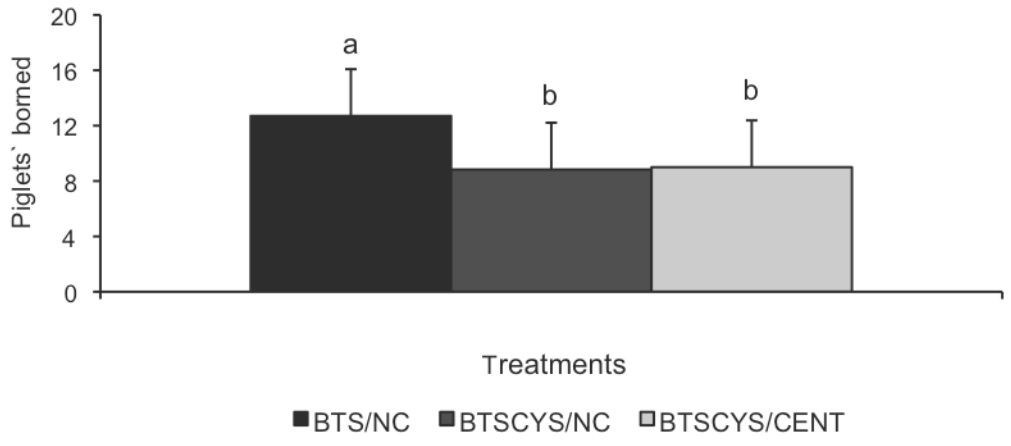

Fig. 6. The effect of cysteine addition into the *Beltsville Thawing Solution* (BTS) extender and centrifugation on the number of piglets' born. Different letters between treatments ($P < 0.01$).

5 DISCUSSÃO

No primeiro experimento, buscou-se através da análise da motilidade, vigor, alterações morfológicas, integridade de membrana acrossomal e plasmática e potencial de mitocôndria do espermatozóide determinar o efeito de diferentes concentrações de cisteína quando o sêmen for armazenado a 17 °C por um período de até 72 horas. No segundo experimento, foram avaliadas a motilidade, vigor, alterações morfológicas, compactação de DNA, funcionalidade e integridade de membrana plasmática, integridade de membrana acrossomal e potencial de mitocôndria. Além da avaliação das características seminais, durante a realização do segundo experimento, foi desenvolvido também um experimento a campo onde avaliou-se a taxa de retorno ao cio e o tamanho da leitegada para determinar o efeito da adição de 5 mM de cisteína ao diluente BTS e da centrifugação do sêmen.

Em ambos os experimentos os resultados de motilidade e vigor foram semelhantes aqueles relatados por Vasconcelos et al. (2001) e Dube et al. (2004) utilizando o diluente BTS para conservação do sêmen a temperaturas entre 15 e 17 °C. A temperatura de 17 °C utilizada foi similar à Johnson et al. (2000), o qual relata que temperaturas inferiores a 15 °C resultam em danos irreversíveis a membrana dos espermatozóides. No segundo experimento, o sêmen centrifugado apresentou a maior queda na motilidade e vigor concordando com estudos prévios realizados em humanos (SCHEKARRIZ et al. 1995) e suínos (CARVAJAL et al. 2004). Porém a adição de 5 mM de cisteína não promoveu a manutenção da motilidade e vigor, contrariando resultados obtidos por Griveau e Le Lannou (1994) e Parinaud et al. (1997) que concluíram que a adição de antioxidantes auxilia na manutenção da motilidade e vigor espermático em humanos quando o sêmen é submetido ao processo de centrifugação.

As alterações na morfologia espermática também foram avaliadas nos dois experimentos, sendo que nem a adição de cisteína nem a centrifugação exerceram

efeito sobre o aumento das alterações morfológicas. Nenhuma das amostras de ambos os testes apresentou morfologia espermática superior a 20% de acordo com resultados obtidos por Johnson et al. (2000). Para complementar os resultados, durante o segundo experimento, foi avaliada a funcionalidade de membrana plasmática, bem como a compactação do DNA.

Os resultados foram similares aos obtidos por Zou e Yang (1999) e Vasquez et al. (1997) que observaram que apesar das membranas plasmáticas não estarem funcionais os espermatozóides ainda apresentavam motilidade. A centrifugação parece exercer um efeito negativo sobre a compactação do DNA que segundo Donelly et al. (2000) pode ser evitada pela adição de um antioxidante, porém no presente estudo o tratamento onde o sêmen foi centrifugado e 5 mM de cisteína adicionada ao BTS após 48 horas de armazenamento mostrou menor média com relação ao grupos onde o sêmen foi somente centrifugado ou somente adicionado cisteína. Portanto, foi observado um efeito negativo da centrifugação e da adição de 5 mM de cisteína ao diluente BTS ao longo do período de armazenamento a 17 ˚C.

A integridade da membrana acrossomal e plasmática, bem como, o potencial de mitocôndria da célula espermática foram avaliadas através da utilização de sondas fluorescentes. Segundo Bilodeau et al. (2001), concentrações de 0,5 e 1 mM de cisteína mantém a motilidade e o vigor concordando com os resultado obtidos em nosso primeiro estudo o qual observou-se que doses inferiores de cisteína mantém a motilidade, o vigor, a integridade de membrana acrossomal e plasmática e o potencial de mitocôndria. Uysal e Bucack (2007), também mostraram que o sêmen de carneiro criopreservado quando suplementado com 10 mM de cisteína mantém a viabilidade, a motilidade e a funcionalidade da membrana plasmática, além de reduzir os danos ao acrossoma.

Apesar dos resultados obtidos no primeiro estudo, o segundo foi baseado no desenho experimental de Funahashi e Sano (2005), mas, foi observado que a adição de 5mM de cisteína não promoveu nenhum efeito positivo sobre a manutenção da viabilidade espermática. Quando o sêmen também foi submetido ao processo de centrifugação houve um efeito negativo sobre a manutenção da qualidade espermática , contrariando os resultados obtidos por Funahashi e Sano (2005). Este achado, pode ser

explicado devido ao diluente utilizado ser acrescido de plasma seminal que pode exercer um papel de proteção ao espermatozóide durante o período de armazenamento e quando utilizados para IA (BORTOLOZZO et al., 2005a).

A reação acrossomal é necessária para que ocorra a fusão do espermatozóide a zona pelucida do oócito (OURA; TOSHIMORI, 1990; FLESCH; GADELLA, 2000). Para tanto, a membrana acrossomal do espermatozóide deve estar intacta para promover a conexão e a interação entre os gametas. Conforme Baumber et al. (2000) e Sarlos et al. (2002), a adição de cisteína não exerce nenhum efeito sobre a integridade acrossomal em equinos e carneiros, respectivamente. Concordando com o primeiro experimento, o que mostra que com baixas concentrações de cisteína foram obtidos os mesmos resultados quando utilizando somente BTS. A função mitocondrial foi outro parâmetro avaliado, visto que é crucial para promover a fosforilação oxidativa e produzir energia (ATP) essencial para a motilidade celular (OURA; TOSHIMORI, 1990; FREY; MANNELLA, 2000). Da mesma forma que Celeghini et al. (2007), foi observado que o potencial de mitocôndria se comporta de forma semelhante a motilidade e o vigor.

Os efeitos deletérios produzidos pela centrifugação observados no segundo estudo podem ser explicados possivelmente porque as enzimas antioxidantes ativas estão no plasma seminal (FUNAHASHI; SANO et al., 2005). A adição de 5 mM de cisteína ao meio só acentua o problema porque possivelmente promova um desequilíbrio entre agentes oxidantes e antioxidantes o que também poderia explicar o porquê em nosso primeiro estudo com concentrações elevadas de cisteína (10 e 20 mM) obtivemos índices baixos de manutenção da qualidade espermática. Esta hipótese foi confirmada por Ebisch et al. (2006) que concluiu que homens subférteis apresentavam altas concentrações de tiol no sêmen,indicando que a remoção excessiva de ROS, abaixo dos níveis fisiológicos necessários, altera a função normal do espermatozóide.

A fim de poder predizer melhor os dados obtidos em nosso laboratório, foi realizada uma segunda parte experimental a campo. Corroborando com os resultados de laboratório, as fêmeas que foram inseminadas com sêmen centrifugado no qual diluente foi acrescido de 5 mM de cisteína mostraram maior taxa de retorno e menor número de leitões nascidos quando comparado ao grupo inseminado com sêmen

diluído somente em BTS, discordando de Maxwell et al. (1998) que não observou diferença na ausência ou presença de plasma seminal ao avaliar a taxa de clivagem e a formação de blastocisto. Por outro lado, quando somente são adicionados 5 mM de cisteína ao diluente há um aumento na taxa de retorno e uma diminuição no número total de nascidos quando comparado a centrifugação e a diluição em BTS somente. Este efeito não era esperado, já que Funahashi e Sano (2005) avaliando a penetrabilidade do espermatozóide no oócito relataram que não houve diferença entre o sêmen diluído em meio Modena modificado acrescido ou não de cisteína até oito dias de armazenamento a 10 ˚C. Após este período e antes de completar 29 dias de diluído em meio Modena modificado acrescido de 5 mM de cisteína mostrou maiores índices de penetrabilidade.

6 CONCLUSÕES

A adição de cisteína ao BTS e a centrifugação afetam negativamente a qualidade espermática do sêmen suíno refrigerado, resultando em aumento da taxa de retorno e redução no número de leitões nascidos após inseminação artificial tradicional.

REFERÊNCIAS BIBLIOGRÁFICAS

AITKEN, R. J.; BAKER, M. A. Oxidative stress and male reproductive biology**. Reprod. Fertil. Dev.**, Collingwood, v. 16, n. 5, p. 581–588, May 2004.

ALI, A.. A.; BILODEAU, J. F.; SIRARD, M. .A.. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. **Theriogenology,** Amsterdam, v. 59, n. 3, p. 939-949, Feb. 2003.

ALTHOUSE, G. C. et al. Characterization of lower temperature storage limitations of fresh-extended porcine semen. **Theriogenology**, Amsterdam, v. 50, n. 4, p. 535-543, Sept. 1998.

ALVAREZ, J. G.; STOREY, B. T. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa: its effect on sperm motility. **Biol. Reprod.**, Pittsburgh, v. 27, n. 5, p. 1102-1108, Dec. 1982.

ALVAREZ, J. G. et al . Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. **J. Androl.**, Schaumburg, v.8, n. 5, p. 338-348, Sept./Oct. 1987.

ALVAREZ, J. G. et al. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile life. **Hum. Reprod.**, Oxford, v. 8, n. 8, p. 1087–1092, July 1993.

ANTUNES R. C. Avanço tecnológico e aplicabilidade da técnica de congelamento de sêmen suíno. **Rev. Bras. Reprod. Anim.,** Belo Horizonte, v.31, n. 1, p.60-63, Jan./Mar. 2007.

ASKARI, H. A. et al. Effect of Natural Antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. **Sys. Biol. Reprod. Med.**, London, v. 33, n. 1, p. 11-15, July 1994.

ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA PRODUTORA E EXPORTADORA DE CARNE SUÍNA-ABIPECS. Disponível em: <http://www.abipecs.org.br>. Acesso em: 05. out. 2007.

BALL, B. A. et al. Effects of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5ºC. **Theriogenology**, Amsterdam, v.56, n. 4, p. 577-589, Sept. 2001.

BAUMBER, J. et al. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. **J. Androl.**, Schaumburg, v. 21, n. 6, p. 895–902, Nov./Dec. 2000.

BEDAIWY, M. .A. et al. Differential growth of human embryos in vitro: role of reactive oxygen species**. Fertil. Steril.,** Birminghan, v. 82, n. 3, p. 593-600, Sept. 2004.

BEEONI, M. T. et al. Effect of natural antioxidants on frozen on bovine semen preservation. **Theriogenology**, Amsterdam, v. 40, n. 4, p. 841-851, Oct. 1993.

BILODEAU, J. F. et al. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen**. Theriogenology**, Amsterdam, v. 56, n. 2, p. 275–286, July 2001.

BILODEU, J. F. et al. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris exyender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. **Theriogenology**, Amsterdam, v. 57, n. 3, p. 1105-1122, Feb. 2002.

BORTOLOZZO, F. P. et al. Situação atual da inseminação artificial em suínos. **Acta Sci. Vet.**, Porto Alegre, v. 33, n. 1, p. 17- 32, Jan./Abr. 2005.

BRANDÃO, A. C. et al. Influência do glicerol e etilenoglicol e da criopreservação sobre o complexo DNA-proteína de espermatozóides em garanhões. **Braz. J. vet.Res. anim. sci.**, São Paulo, v. 43, p. 68-73, 2006, suplemento.

BRASIL, Ministério da Agricultura, Pecuária e Abastecimento. Disponível em: <http://www.agricultura.gov.br>. Acesso em: 06. out. 2007.

BRAUNDMEIER, A. G.; MILLER, D. J. The search is on: finding accurate molecular markers of male fertility**. J. Dairy Sci.**, Champaign, v. 84, n. 9, p. 1915-1925, Sept. 2001.

BREZEZINSKA-STEVBODZINSKA, F. et al. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. **Biol. Trace Elem. Res.**, New York, v. 47, n. 1, p. 69-74, Jan.1995.

CABRITA, E. et al. The hipoosmotic swelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout. **Anim. Reprod. Sci.**, Amsterdam, v. 55, n. 3, p. 279-287, Apr. 1999.

CARDULLO, R. A.; CONE, R. A. Mechanical immobilization of rat sperm does not exchange their oxygen consumption rate. **Biol. Reprod.**, Pittsburgh, v. 34, n. 5, p. 820–830, June 1986.

CARVAJAL, G. et al. Effects of centrifugation before freezing on boar sperm cryosurvival**. J. Androl.**, Schaumburg, v. 25, n. 3, p. 389-396, May/June 2004.

CELEGHINI, E. C. C. et al. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes**. Reprod. Dom. Anim.**, Uppsala, v. 42, n. 5, p. 479-488, Oct. 2007.

CELEGHINI, E. C. C. et al. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. **Anim. Reprod. Sci.,** Amsterdam, v. 104, n. 2, p. 119-131, Mar. 2008.

CEROLINI, S et al. Viability susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. **Anim. Reprod. Sci**., Amsterdam, v. 58, n. 2, p. 99-111, Feb. 2000.

CORREA-PÉREZ, J. R. et al. The effect of colloid osmotic pressure in human spermatozoa exposed to hypoosmotic conditions**. Andrologia**, Giessen, v. 35, n. 2, p. 117-120, Apr. 2003.

COSSARIZZA, A. et al. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the j-aggregate formimg lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethilbenzimidazolcarbocyanine iodide (JC-1). **Biochem. Biophys. Res. Commun.**, Amsterdam, v. 197, n. 1, p. 40-45, Nov. 1993.

COSTA, E. N. et al. The anomalous DNA protein complex in human spermatozoa of varicocele cases. **Braz. J. Gen.**, Ribeirão Preto, v. 19, p. 147, 1996. Supplement.

CROCKETT, E. C. et al. Effect of cooling on equine spermatozoa before freezing on post-thaw motility: preliminary results. **Theriogenology**, Amsterdam, v. 55, n. 3, p. 793–803, Feb. 2001.

CUMMINS, J. M.; JEQUIER, A. M.; KAN, R. Molecular biology of the human male infertility: links with aging, mitochondrial genetics and oxidative stress. **Mol. Reprod. Dev.**, Providence, v. 37, n. 3, p. 345-362, Mar. 1994.

De ANDRADE, A. F. C. de et al. Fluorescent stain method for the simultaneous determination of mitochondrial potential and integrity of plasma and acrosomal membranes in boar sperm. **Reprod. Dom. Anim.**, Uppsala, v. 42, n. 2, p. 190-194, Apr. 2007.

De ARRUDA, R. P. de et al. Biotécnicas aplicadas à avaliação do potencial de fertilidade do sêmen eqüino. **Rev. Bras. Reprod. Anim.**, Belo Horizonte, v. 31, p. 8-16, Jan./Mar. 2007.

DE LEEUW, F.E. et al. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. **Cryobiology,** York, v. 27, n. 2, p. 171-183, Apr. 1990.

DIEHL, G. N. et al. Nova pipeta para inseminação intra-uterina em suínos. **Ciênc. Rural**, Santa Maria, v. 36, n. 1, p. 179-185, Jan./Fev. 2006.

DONNELLY, E. T. et al. Glutathione and hipotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. **Mutagenesis**, Edinburgh, v. 15, n. 1, p. 61-68, Jan. 2000.

DONNELLY, E.T. et al. Differences in nuclear Dna fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. **Hum. Reprod.**, Oxford, v. 15, n. 7, p. 1552-1561, July 2000a.

DONOGHUE, A. M.; DONOGHUE, D. J. Effects of water and lipid-soluble antioxidants on turkey sperm viability, membrane integrity and motility during liquid storage. **Poult. Sci.**, Champaign, v. 76,n. 10, p. 1440-1455, Oct. 1997.

DUBE, C. et al. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. **Theriogenology**, Amsterdam, v. 62, n. 5, p. 874-886, Sept. 2004.

EBISCH, I.M. et al.Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. **Hum. Reprod.**, Oxford, v. 21, n. 7, p. 1725-1733, July 2006.

ERKKILA, K. et al. Nacetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. **J. Clin. Endocrinol. Metab.**, Jersey City, v. 83, n. 7, p. 2523–2531, July 1998.

FLESCH, F. M.; GADELLA, B. M. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. **Biochem. Biophys. Acta**, Amsterdam, v. 1469, n. 3, p. 197-235, Nov. 2000.

FREY, T.G.; MANNELLA, C.A. The internal structure of mitochondria. **Trends Biochem. Sci**., London, v. 25, n. 7, p. 319-324, July 2000.

FRIDOVICH, I. Superoxido radical: an exogenous toxicant. **Annu. Rev. Pharmacol. Toxicol.**, Palo Alto, v. 23, p. 239-257, Apr. 1983.

FUNAHASHI, H.; SANO, T. Select antioxidants improve the function of extended boar sêmen stored at 10℃. **Theriogenology**, Amsterdam, v. 63, n. 6, p. 1605-1616, Apr. 2005.

GACZARZEWICZ, D. et al. Oxidoreductive capability of boar sperm mitochondria in fresh semen and during their preservation in BTS extender. **Reprod. Biol.**, Olsztyn, v. 3, n. 2, p. 161-172, July 2003.

GADEA, J. Review: Semen extender used in the artificial insemination of swine. **Span. J. Agric. Res.**, Madrid, v. 1, n. 2, p. 17-27, Jan./Mar. 2003.

GALLI, A.; BOSISIO, M. Quality of semen stored at +15 degrees 16 degrees C as related to fertility of artificially inseminated swine. **Theriogenology**, Amsterdam, v. 30, n. 6, p. 1185-1190, Dec. 1988.

GILLAN, L. et al. Flow cytometric evaluation of sperm parameters in relation to fertility potential. **Theriogenology**, Amsterdam, v. 63, n.2, p. 445-447, Jan. 2005.

GOTTARDI, L. et al. **New dilution media for artificial insemination in the pig.** 9th Intern. Congr. Anim. Reprod., Madrid, Espanha, 1980.

GRIVEAU, J. F.; LE LANNOU, D. Effects of antioxidants on human sperm preparation techniques. **Int. J. Androl.**, Copenhagen, v. 17, n. 5, p. 225–231, Oct. 1994.

HANCOCK, J.L.; HOVEL, G.J.R. The collection of boar semen. **Vet. Rec.**, London, v. 71, p. 664-665, 1959.

HARRISON, R. A. P. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. **Reprod. Fertil. Dev.**, Adelaide, v. 8, n. 4, p. 581-594, Apr. 1996.

HENKEL, R. The impact of oxidants on sperm function. **Andrololog**ia, Giessen, v. 37, n. 6, p. 205–206, Dec. 2005.

HERRERA, J. et al. Acrossome reaction infertile and subfertile boar sperm. **Arch. Androl.**, London, v. 48, n. 2, p. 133-1339, Mar./Apr. 2002.

HIRAI, M. et al. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. **J. Androl.**, Schaumburg, v. 22, n. 1, p. 104-110, Jan./Feb. 2001.

HUO, L. J. et al. Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. **Theriogenology**, Amsterdam, v. 58, n. 7, p. 1349-1360, Oct. 2002.

JOHNSON, L. A. et al. Artificial insemination of swine: Fecundiy of boar semen stored in Beltsville TS BTS., Modified Modena (MM), or MR-A and inseminated on one three and four days after collection. **Zuchthygiene**, New York, v. 23, n. 2, p. 49-55, Mar./Apr. 1988.

JOHNSON, L. A. et al. Storage of boar semen. **Anim. Reprod. Sci.**, Amsterdam, v. 62, n. 3, p. 143-172, Aug. 2000.

JUONALA, T. et al. Three fluorescence methods for assessing boar sperm viability. **Reprod. Domest. Anim.**, Uppsala, v. 34, n. 2, p. 83-87, May 1999.

JULIANO, F. et al. **Choque hipoosmótico em espermatozóides suínos comparado aos testes convencionais de avaliação da qualidade espermática.** Congresso Latino Americano de Suinocultura, Foz do Iguaçu, Paraná, 2002.

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

KATKOV, I. I.; MAZUR, P. Influence of centrifugation regimes on motility, yield and cell associations of mouse spermatozoa. **J. Androl.**, Schaumburg, v. 19, n. 2, p. 232–241, Mar./Apr. 1998.

KATKOV, I. I.; OSTASHKO, F. I. Correlation between electromechanical stability of cytoplasm membrane and cryoresistivity of bovine spermatozoa. **Cryobiology**, York, v. 33, n. 6, p. 680–687, Dec.1996.

KIM, I.H. et al. Effect of exogenous glutathione on the in vitro fertilization of bovine oocytes. **Theriogenology**, Amsterdam, v. 52, n. 3, p. 537-547, Aug. 1999.

KOTZIAS-BANDEIRA, E. Influência de diferentes diluentes e temperaturas de refrigeração sobre a qualidade de sêmen suíno. **Braz. J. Vet. Res. Anim. Sci.**, São Paulo, v. 36, n. 3, [online] DOI: 101590/51413-95961999000300008, 1999.

KUMARESAN, A. et al. Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. **Anim. Reprod. Sci.**, Amsterdam, v. 110, n. 1, p. 162-171, Jan. 2009.

KUSTER, C. E.; ALTHOUSE, G. C. The fecundity of porcine semen stored for 2 to 6 days in androhep® and x-cell^{tm} extenders. **Theriogenology**, Amsterdam, v. 52, n.3, p. 365-376, Aug. 1999.

LAMBERSON, W.; SAFRANSKI, T. A model for economic comparison of swine insemination programs. **Theriogenology**, Amsterdam, v. 54, n. 5, p. 799-807, Sept. 2000.

MACHADO, E.R. et al. Diagnóstico de alterações na compactação da cromatina em espermatozóides de suíno através de Azul de Toluidina e Laranja de Acridina. **Rev. Bras. Reprod. Anim.**, Belo Horizonte, v. 27, n. 3, p. 381-382, Julho/Set. 2003.

MARTINEZ, E.A. et al. Deep intrauterine insemination and embryo transfer in pigs. **Reprod Suppl.**, Cambridge, v. 58, p. 301-311, 2001.

MAXWELL, W. M. C. et al. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. **Reprod. Fertil. Dev.**, Adelaide, v. 10, n. 6, p. 433-440, Oct.1998.

MAXWELL, W. M. C.; JOHNSON, L. A. Physiology of spermatozoa at high dilution rates: the influence seminal plasma. **Theriogenology**, Amsterdam, v. 52, n. 8, p. 1353-1362, Dec. 1999.

MAXWELL, W. M. C.; STOJANOV, T. Liquid storage of ram semen in the absence or presence of some antioxidants. **Reprod. Fertil. Dev.**, Adelaide, v. 8, n. 6, p. 1013-1020, Oct. 1996.

MEISTER A. Glutathione, ascorbate, and cellular protection. **Canc. Res.**, Philadelphia, v. 54, p. 1969-1975, Apr. 1994, supplement.

MELENDREZ, C. S. et al. Comparasion of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction in vitro. **Mol. Reprod. Dev.**, Providence, v. 39, n. 3, p. 433-438, Nov. 1994.

MELLO, M. L. S. Induced metachromasia in bull spermatozoa. **Histochem. Cell Biol.**, Heidelberg, v. 74, n. 3, p. 387-392, Sept. 1982.

MURGAS, L. D. S. et al. Oxitocina no sêmen suíno heterospérmico resfriado a 15 ºC. **Ciênc. Anim. Bras.**, Goiânia, v. 3, n. 2, p. 33-40, Julho/Dec. 2002.

NAVES, C. S. et al. Avaliação da cromatina espermática em equinos com azul de toluidina e “acridine orange”. **Biosci. J.**, Uberlândia, v.20, n. 3, p. 117-124, May/June 2004.

NG, S. C. et al. Microcentrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. **Hum. Reprod.**, Oxford, v. 5, n. 2, p.209–211, Feb. 1990.

OHATA, P. M. et al. Viabilidade do sêmen suíno congelado submetido a um período de equilíbrio pré-congelamento com ou sem presença de plasma seminal. **Arquiv. Fac. Vet.-UFRGS**, Porto Alegre, v. 29, n. 2, p. 123-129, Nov. 2001.

OSMAN, R. A. et al. Steroid induced exocytosis: the human sperm acrosome reaction. **Biochem. Biophys. Res. Commun.,** Amsterdam, v. 160, n. 2, p. 828-833, Apr. 1989.

OURA, C.; TOSHIMORI, K. Ultrastructural studies on the fertilization of mammalian gametes. **Int. Rev. Cytol.**, Knoxville, v. 122, p. 105-151, 1990.

OZAKI, T. et al. Evaluation of acrosome reaction and viability of human sperm with two fluorescent dyes. **Arch. Gynecol. Obstet.**, Heidelberg, v. 266, n. 2, p. 114-117, Apr. 2002.

PADILLA A. W. & FOOTE, R. H. Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. **J. Anim. Sci.**, Champaign, v. 69, n.8, p. 3308-3313, Aug. 1991.

PARINAUD, J. et al. Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit®) following ejaculation. **Hum. Reprod.**, Oxford, v. 12, n. 11, p. 2434–2436, Nov. 1997.

PEÑA, F. J. et al. Assessment of fresh and frozen thawed boar semen using an Annexin V assay: a new method of evaluating sperm membrane integrity. **Theriogenology**, Amsterdam, v. 60, n. 4, p. 677-689, Sept. 2003.

PETRUNKINA, A. M. et al. Sperm-cell volumetric measurements as parameters in bull semen function evaluation: correlation with nonreturn rate. **Andrologia**, Giessen, v. 33, n. 6, p. 360-367, Nov. 2001.

PICKETT, B. W. et al Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. **Fertil. Steril.**, Birminghan, v. 26, n. 2, p. 167–173, Feb. 1975.

PLISKO, N. T. Method of prolonging the viability and fertilizing capacity of boar spermatozoa. **Svinovodstvo**, v. 9, p. 37-41, 1965.

PURSEL, V. G.; JOHNSON, L. A. Freezing of boar spermatozoa: Fertilizing capacity with concentrated semen and new thawing procedure. **J. Anim. Sci.,** Champaign, v. 40, n.1, p. 99-102, Jan.1975.

ROCA, J. et al. Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15ºC. **Anim. Reprod. Sci.**, Amsterdam, v. 64, n. 2, p. 103-112, Dec. 2000.

ROCHA, H. L. O. G. et al. Uso de laranja de acridina e azul de toluidina na avaliação da fertilidade masculina. **Biosci. J.**, Uberlândia, v. 8, n. 1, p. 65-77, June 2002.

ROTA, A. et al. Hypoosmotic swelling (HOS) as a screening assay for testing in vitro fertility of bovine spermatozoa. **Theriogenology**, Amsterdam, v. 53, n. 7, p. 1415-1420, Apr. 2000.

ROUSSEAUX, J.;, ROUSSEAUX-PREVOST, R. Molecular localization of free thiols in human sperm chromatin. **Biol. Reprod**., Pittsburgh, v. 52, n. 5, p. 1066-1072, May 1995.

SARAVIA, F. et al. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. **Theriogenology**, Amsterdam, v.63, n. 5, p.1320-1333, Mar. 2005.

SARLOS, P. et al. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. **Acta Vet. Hung.**, Budapest, v. 50, n. 2, p. 235-245, May 2002.

SAS INSTITUTE. Sas user's guides: Statistics. Version 6.4 ed. Cary/North Carolina, v. 2. 400p, 1996.

SCHEID, I. R.; SILVEIRA, P. R. S. Uma análise da IA na suinocultura brasileira. **Suín. Cia**., São Paulo, v.1, n. 1, p.25-28, Jan. 2002.

SHEKARRIZ, M. et al. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. **Eur. Urol.**, Switzerland, v. 28, n. 1, p. 31-35, Jan. 1995.

SINHA M. P. et al. The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. **Theriogenology**, Amsterdam, v. 41, n.1, p. 237-243, Jan. 1996.

STOREY, B.T. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. **Mol. Hum. Reprod.**, Cambridge, v. 3, n. 3, p. 203-213, Mar. 1997.

SUTKEVICIENE, N. et al. Assessment of boar semen quality in relation to fertility with special reference to methanol stress. **Theriogenology**, Amsterdam, v. 63, n. 3, p. 739-747, Feb. 2005.

THUWANUT, P. et al. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. **Theriogenology**, Amsterdam, v. 70, n. 2, p. 233-240, July 2008.

TURBA, M. E. et al. Relationships between innovative and traditional parameters to investigate semen quality in pigs. **Anim. Reprod. Sci.**, Amsterdam, v. 99, n. 1, p. 72-81, May 2007.

UYSAL, O.; BUCAK, M.N. Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. **Acta Vet. Brno.**, Brno, v. 76, n. 5, p. 383-390, June 2007.

VASCONCELOS, A.M.M.A. et al. Características Espermáticas de Sêmen Resfriado de Suíno e Conservado em Diferentes Diluentes. **Rev. Bras. Zootec.**, Viçosa, v. 30, n. 2, p. 394-401, Mar./Abr. 2001.

VAZQUEZ, J. M. et al. Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. **Theriogenology**, Amsterdam, v. 47, n. 4, p. 913-922, Mar. 1997.

ZOU, C. X.; YANG, Z. M. Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. **Theriogenology**, Amsterdam, v. 53, n. 7, p. 1477-1488, Apr. 2000.

WABERSKI, D. et al. Fertility of long term-stored boar semen: influence of extender (Androhep and Kiev), storage time ande plasma droplets in the semen. **Anim. Reprod. Sci.**, Amsterdam, v. 36, n. 2, p. 145-151, July 1994.

WATSON, P. F. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. **Reprod. Fertil. Dev.**, Adelaide, v. 7, n. 4. p. 871-891, Apr. 1995.

WATSON, P. F. Cooling of spermatozoa and fertilizing capacity. **Reprod. Dom. Anim.**, Uppsala, v. 31, n. 1, p. 135-140, Aug. 1995a.

WATSON, P.F.; BEHAN, J.R. Intrauterine insemination of sows with reduced sperm numbers: results of a based field trial. **Theriogenology**, Amsterdam, v. 57, n. 6, p. 1683-1693, Apr. 2002.

WEITZE, K. F. The use of long-term extender in pig AI – a view of the international situation. **Pig news inform.**, Oxfordshire, v. 11, n. 1, p. 23-26, Jan. 1990.

WESTENDORF, P. et al. Zur Tiefgefrierung von Ebersperma. Labor-und Besamungsergebnisse mit dem Hu¨lsenberger Paillettenverfahren. **Dtsch.Tiera.¨rztl. Wschr.**, Berlin, v.82, p. 261–267, 1975.

WOLDERS, H.; TEN NAPEL, J. Semen in straws. **Pig Int.**, Laciengo, v.35, n.1, p.10-14, Jan. 2005.

WOLKEN, A. et al. Sows can successfully be inseminated non-surgically into the distal uterine horn with a highly reduced number of sperm cells. **Theriogenology**, Amsterdam, v.57, n. 1, p.392, Jan. 2002.