

**UNIVERSIDADE ESTADUAL PAULISTA  
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA DE  
BOTUCATU**

**SÊMEN CONGELADO E INSEMINAÇÃO ARTIFICIAL EM CÃES**

**CARMEN CECILIA SICHERLE**

Botucatu, São Paulo  
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**UNIVERSIDADE ESTADUAL PAULISTA  
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BOTUCATU**

## **SÊMEN CONGELADO E INSEMINAÇÃO ARTIFICIAL EM CÃES**

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### RESUMO

O objetivo deste estudo foi o de avaliar os efeitos da criopreservação sobre a qualidade e fertilidade do sêmen de cão. Para isso foram realizados dois experimentos. No primeiro experimento foram colhidos 4 ejaculados de 5 cães (n=20), os quais foram avaliados no sêmen fresco, resfriado e descongelado. A motilidade total (MT), progressiva (MP) e porcentagem de rápidos (RAP) por sistema computadorizado e por citometria de fluxo, a fluidez da membrana plasmática [Yo-Pro 1 (YP) e merocianina 540 (M540)], a translocação da fosfatidil serina (FS) (anexina V e FITC-PSA), integridade da membrana plasmática e acrossomal (iodeto de propídeo (IP) combinado ao FITC-PSA), potencial da membrana mitocondrial (JC1), lipoperoxidação dos lipídeos de membrana (LPO, C11-BODIPY) e apoptose (CellEvent<sup>®</sup>). O sêmen do cão que apresentou melhores resultados numéricos em todas as análises foi escolhido para ser utilizado separadamente para as inseminações (cão 1) e o sêmen dos demais 4 cães foram utilizados em *pool*. Foram inseminadas 20 cadelas (2 IAs/cadela), por via transcervical (TCIA) sendo 10 delas com o cão 1 e 10 com o *pool*. Foram utilizadas 2 concentrações espermáticas (160 e 450 x 10<sup>6</sup> espermatozoides/TCIA). Houve diferença para os índices de motilidade (p < 0,01) entre o sêmen fresco e resfriado quando comparados ao descongelado para todos os índices avaliados, (MT = 87,00 ± 1,24; 88,15 ± 1,38; 72,55 ± 6,26); (MP = 69,95 ± 1,28 ; 71,75 ± 1,91; 56,30 ± 6,00) e (RAP = 83,15 ± 1,94; 81,55 ± 1,53; 64,30 ± 7,68). Em relação a fluidez da membrana espermática houve diferença na porcentagem da população de células íntegras (CI) e de células com aumento de fluidez de membrana (CIMF) (p < 0,01) entre o sêmen fresco e resfriado quando comparados ao descongelado, (CI = 78,29 ± 6,22; 75,76 ± 6,60; 23,02 ± 9,12); (CIMF = 18,33 ± 5,54; 22,55 ± 6,49; 71,78 ± 9,85). Sobre a identificação da translocação da FS houve diferença (p < 0,01) na porcentagem de células íntegras nos 3 momentos avaliados (CI = 79,90 ± 7,95; 63,50 ± 4,66; 27,19 ± 9,22). A porcentagem de células apresentando a membrana plasmática e acrossomal íntegras foi diferente (p < 0,01) também entre o sêmen fresco e resfriado quando comparados ao descongelado (MPAI = 85,71 ± 6,22; 70,37 ± 6,43; 30,87 ± 9,90). Quanto ao potencial da membrana mitocondrial houve diferença (p < 0,01) na porcentagem de células com alto potencial

mitocondrial nos 3 momentos avaliados (APM =  $85,15 \pm 2,76$ ;  $60,52 \pm 3,31$ ;  $38,06 \pm 6,40$ ). A LPO da membrana plasmática foi diferente ( $p < 0,05$ ) no sêmen congelado quando comprado ao resfriado mas não teve diferença entre o sêmen fresco e resfriado e do fresco ao congelado. Não foi observada qualquer diferença, nos momentos estudados, na atividade da caspase. O cão 1 foi significamente melhor para os índices de integridade de membrana plasmática e acrossomal e potencial mitocondrial. Pelo calculo da resposta média do animal e limites mínimos e máximos, apesar de não haver diferença, observamos que o animal 1 apresentou melhores valores pós descongelação para todas os testes realizados. O aumento na concentração espermática foi positivo e observamos 50% de prenhez no grupo inseminado com o *pool* de sêmen utilizando a concentração de  $450 \times 10^6$ . Nenhuma gestação foi observada nas TCIA com o sêmen do cão 1. Em conclusão a criopreservação leva a alterações estruturais e funcionais da célula espermática, o que explica a sua sobrevivência curta após a descongelação. As mudanças semelhantes a crio-capacitação podem estar relacionadas a outros fatores, como as proteínas do plasma seminal que ainda não são totalmente conhecidas nesta espécie. O processo de apoptose pode ser iniciado com a abertura dos poros mitocondriais durante o processo de congelação/descongelação, o que libera fatores pro-apoptóticos no citoplasma celular. O potencial mitocondrial não está relacionado com a motilidade dos espermatozoides, mas sim com a sobrevivência do espermatozoide. Melhores resultados de gestação puderam ser obtidos quando mais de  $100 \times 10^6$  de espermatozoides móveis e morfológicamente íntegros for utilizados por TCIA. Não houve relação entre a qualidade seminal, avaliada pelas técnicas aqui descritas, e a fertilidade.

**Palavras-chave:** cão, espermatozoide, criopreservação, viabilidade espermática, fertilidade.

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#### ABSTRACT

The aims of the study were to assess individual characteristics and sperm concentration of cryopreserved semen characteristics using different structural and functional analysis and dog fertility. Twenty ejaculates were collected from 5 dogs and sperm were cryopreserved by one-step protocol. To better understand dog semen quality after thawing five healthy mature dogs were used to the one-step sperm cryopreservation protocol. Sperm analysis was performed at three time points: after collection, refrigeration, and after thawing, by computer sperm motility analysis (total [TM] and progressive motility [PM], and rapid sperm), membrane damage (membrane fluidity and viability - Yo-Pro 1 and merocianina 540, phosphatidyl serine translocation – annexin – V – propidium iodide - PI, acrosomal and membrane integrity FITC-PSA – PI), mitochondrial potential (JC-1), membrane lipid peroxidation, LPO (BODIPY 581/591 C<sub>11</sub>) and apoptosis (kit CellEvent™ Caspase-3/7- FITC Green Flow Cytometry), evaluated by flow cytometry. Twenty bitches were inseminated by transcervical intrauterine (TCAI), twice using 2 sperm concentration (160 and 450 x 10<sup>6</sup> spermatozoa/TCAI). A decrease on frozen/thawed semen comparing to fresh and cooled semen occurred for the parameters of total and progressive motility (TM 87.00 ± 1.24, 88.15 ± 1.38, 72.55 ± 6.26; PM 69.95 ± 1.28, 71.75 ± 1.91, 56.30 ± 6.00, fresh cooled and frozen/thawed respectively P < 0.01), percentage of rapids (83.15 ± 1.94, 81.55 ± 1.53, 64.30 ± 7.68 P < 0.01), membrane fluidity and cell viability (78.29 ± 6.22, 75.76 ± 6.60, 23.02 ± 9.12 P < 0.01) and acrosomal and membrane integrity (85.71 ± 6.22, 70.37 ± 6.43, 30.87 ± 9.90 P < 0.01). Sperm cells were affect for cooling and freezing process when analyzed for the translocation of phosphatidyl serine (79.90 ± 7.95, 63.50 ± 4.66, 27.19 ± 9.22 P < 0.01) and high mitochondrial potential (85.15 ± 2.76, 60.52 ± 3.31, 38.06 ± 6.40 P < 0.01). For LPO significant difference was observed in semen after thawing only comparing to cooled semen (16.57 ± 5.44, 16.22 ± 2.43, 23.00 ± 4.13 P < 0.05). No difference on caspase activity was observed. Dog 1 was significantly better for the parameters of plasma membrane and acrosome integrity and mitochondrial potential. By calculating the average of the animal response and minimum and maximum limits, although there is no difference, we observed that the semen from Dog

1 displayed better post thaw values for all analyzes. The pregnancy rate was 0% for dog 1 and 50% for pool at concentration  $450 \times 10^6$  spermatozoa. In conclusion, cryopreservation leads to structural and functional changes in sperm cell, which explains their short survival after thawing. The cryo-capacitation like changes can be related to others factors, as the seminal plasma proteins that are not know yet on this species. Apoptosis process can be initiated with the opening of mitochondrial pores during the freezing/thaw process, which releases pro-apoptotic factor on the cytoplasm. The mitochondrial potential is not related to sperm motility but sperm survival.

Although the dog 1 displayed better sperm quality, no pregnancy was observed, which makes us to rethink the prediction of fertility of semen samples by commonly used parameters. The concentration dose used for AI procedures must consider motility and sperm viability to satisfactory pregnancy rates.

**Keywords:** dog, sperm, cryopreservation, viability, fertility, intrauterine insemination.

Capítulo 1

**SÊMEN CONGELADO E INSEMINAÇÃO ARTIFICIAL EM CÃES**

## INTRODUÇÃO

A procura por cães geneticamente superiores de diferentes linhagens é comum entre os criadores e pode trazer benefícios como a diminuição da consanguinidade dentro de um mesmo criatório, o que é interessante no caso de doenças geneticamente transmissíveis. Em programas de melhoramento genético, o uso de biotecnologias reprodutivas torna-se determinante pois propicia o uso de animais geneticamente superiores a distância e a qualquer momento. Neste contexto, a inseminação artificial é uma das ferramentas mais utilizadas por ser de fácil execução e não ter um custo elevado. Atualmente, a inseminação artificial com uso de sêmen refrigerado é bastante utilizada e difundida no meio da cinofilia. Porém, considerando longas distâncias torna-se inviável e o uso do sêmen congelado se faz necessário (1,2).

O processo de criopreservação causa alterações morfológicas e funcionais na célula espermática o que diminui a sua sobrevivência bem como sua interação com o trato reprodutivo feminino. O espermatozoide canino frequentemente, apresenta baixos índices de concepção pós-descongelamento e uma variabilidade individual muito grande (3). Muitos estudos têm sido conduzidos com o intuito de conhecer e de otimizar a criopreservação do sêmen de cães, como diferentes diluidores, crioprotetores e métodos de criopreservação (4–7). Contudo, as taxas de concepção com o uso de sêmen congelado ainda são muito variáveis.

Atualmente, a identificação das diferentes populações espermáticas no sêmen de mamíferos tem se tornado um assunto de interesse para a avaliação da fertilidade de machos. O sêmen de diferentes espécies apresenta distintas populações e estas são afetadas pelo processo de criopreservação (4,8–10).

Desta forma, torna-se determinante o aprofundamento do conhecimento dos efeitos da congelamento do sêmen de cães e pelas análises de motilidade e integridade morfológica e funcional, predizer como um determinado indivíduo responderá a criopreservação e concepção.

### **Hipótese**

A criorresistência espermática é uma característica individual e está relacionada a fertilidade de cães?

Portanto os objetivos do presente trabalho foram:

1. Caracterizar as populações espermáticas no ejaculado de cães;

2. Identificar como a refrigeração e a congelação modificam as populações nos indivíduos;
3. Avaliar se os resultados individuais de criopreservação identificando indivíduos de menor e maior fertilidade, quando utiliza-se técnicas de reprodução assistida com sêmen congelado

## **REVISÃO DE LITERATURA**

Atualmente, a globalização possibilitou uma maior interação entre os criadores de cães pelo mundo, de forma que a procura e o interesse pela inseminação artificial tornou-se importante, pois nem sempre é possível adquirir o reprodutor que se deseja utilizar e nem sempre este animal encontra-se a uma distância que possibilite a cobertura natural ou o uso do sêmen fresco ou refrigerado.

O sêmen congelado apresenta vantagens práticas, pois, não há dependência do tempo e nem da distância, além de manter a genética de animais superiores por tempo indeterminado. Todavia, o sêmen congelado/descongelado apresenta um tempo de vida curto por sofrer perdas na integridade da célula espermática durante o processo de refrigeração, congelação e descongelação. Após a criopreservação ocorre um decréscimo significativo da motilidade espermática em relação ao sêmen fresco e ao refrigerado. A motilidade é de extrema importância pois o espermatozoide precisa chegar até o local da fertilização. Além disso, o fato da cadela ovular oócitos imaturos, que levam de 2 a 3 dias para estarem aptos para a fertilização cria dificuldades na determinação do momento ideal para a inseminação artificial, tornando ainda mais importante o tempo de vida funcional do espermatozoide após a descongelação (3,11–13). Contudo, a viabilidade dos espermatozoides analisada pela motilidade é maior que sua capacidade real de fertilização em estudo realizado por Maxwell e Watson (14), devido as alterações causadas às membranas celulares durante o processo de criopreservação.

De fato, além de móvel é necessário que as estruturas celulares importantes para a interação do espermatozoide com o oócito estejam funcionais. A relação entre as propriedades funcionais e fertilidade é muito mais complexa e pode ser afetada por fatores variados, originados na espermatogênese, na maturação no epidídimo, na

ejaculação e na interação com o trato reprodutivo feminino. Como não é possível a identificação de todas as características que garantam o sucesso da fertilização, torna-se mais viável, avaliar propriedades celulares funcionais e fundamentais pela identificação de células anormais, do que tentar identificar aquelas células consideradas normais (15).

#### Membrana plasmática e acrossomal

A estrutura celular mais afetada durante o processo de congelação e descongelação é a membrana plasmática. No sêmen de cães, Alhaider e Watson (3) observaram que após a descongelação houve uma diminuição significativa da integridade de membrana plasmática, de 81% no sêmen fresco para 35% no descongelado, utilizando o fluorocromo iodeto de propídeo (IP). No mesmo estudo os autores também observaram um aumento significativo da fluidez da membrana plasmática em células íntegras (de 11% para 90,5%) utilizando as sondas Yo-Pro1 e Merocianina 540 (M540). A sonda Yo-Pro1 penetra na célula quando ocorre a desestabilização da membrana plasmática pelo aumento da permeabilidade dos canais panexina, antes mesmo da perda total da integridade (4). A sonda merocianina 540 (M540) por sua vez detecta mudanças no arranjo de lipídeos contidos na membrana, uma modificação inicial do processo de capacitação, conforme aumenta a permeabilidade mais o corante consegue se ligar a membrana agindo como marcador da desestabilização (16,17).

Steckler et al. (18) observaram que a combinação das sondas Yo-Pro1 e M540 é capaz de detectar alterações iniciais da membrana plasmática no sêmen fresco de cães após incubação em meio contendo ou não bicabornato como indutor da capacitação. A membrana plasmática relaciona-se a muitas das funções espermáticas, garantindo a capacidade da célula em manter a homeostase, manutenção da motilidade e a capacidade de interação com o meio, incluindo o epitélio do trato genital feminino e as células do complexo *cumulus*-oócito (8).

A membrana plasmática recobre toda a célula espermática, mas não é uma peça única. É composta por compartimentos distintos, como o que envolve a parte externa da membrana acrossomal; o compartimento recobre a porção pós-acrossomal da cabeça do espermatozoide até o *annulus* e um compartimento cobrindo a peça principal e o restante da cauda. A combinação das sondas fluorescentes iodeto de propídeo (IP) e

diacetato de carboxifluoresceína (DIC) são utilizadas e há muito tempo para a avaliação quantitativa das células com membrana plasmática íntegra e lesada (19), porém, a análise pela citometria de fluxo necessita de uma terceira sonda capaz de marcar e identificar as células de debris, partículas da gema de ovo e outros artefatos (11). Neste contexto a sonda Hoechst 33342 pode ser associada ao IP e DIC completando a análise (20).

### Subpopulações espermáticas

Apesar das diferentes técnicas já bem estabelecidas, capazes de identificar aspectos individuais da função celular, isso não implica necessariamente numa relação causal com a capacidade do espermatozoide em fertilizar o oócito (15). Apesar do ejaculado de mamíferos ser considerado uma mistura homogênea e bem constituída, na verdade trata-se de um composto heterogêneo que contém diferentes populações espermáticas que apresentam distintos padrões fisiológicos, químicos, cinéticos e morfológicos (21,22).

A maioria dos estudos sobre subpopulações espermáticas têm sido realizados baseados na análise das características da cinética espermática, porém outros aspectos como a morfologia e integridade espermática também identificam e caracterizam as diferentes subpopulações (21–23). Em suínos nas diferentes frações do sêmen fresco e congelado/descongelado, observou-se a presença de quatro diferentes subpopulações espermáticas, onde pequenas diferenças morfológicas puderam ser identificadas pela análise computadorizada, utilizando a anexina-V conjugada ao isotiocionato de fluoresceína – FITC e o IP (24).

Em estudo realizado em suínos onde o sêmen de dois ou mais cachos foram misturados para a inseminação artificial, demonstrou-se que as ninhadas resultantes favoreceram um determinado macho (25). Entre as subpopulações espermáticas e entre machos a sensibilidade responsiva ao bicarbonato variou, e uma proteína específica presente nas tubas uterinas modulam esta resposta. Este mecanismo também selecionou a subpopulação que atingiu o oócito para a fertilização. Estes fatos conduzem a uma possível explicação para a baixa correlação entre a fertilidade e os testes para determinação espermática quando não se considera a estrutura das subpopulações espermáticas (8,9,22). Uma das funções das populações espermáticas estaria

relacionada a um mecanismo para auxiliar o espermatozoide fértil a evitar a resposta imune do trato reprodutivo feminino, onde apenas os mais imunoresistentes teriam prioridade na fertilização (21,22). As características seminais individuais são amplamente estudadas, para o entendimento da fertilidade e da infertilidade de machos.

As secreções epiteliais do trato reprodutivo feminino também podem influenciar as mudanças que ocorrem no espermatozoide ao longo de seu trajeto, até o momento da fertilização indicando um diálogo entre o trato reprodutivo feminino e o espermatozoide, como demonstrado em estudo onde verificou-se a mudança nas secreções das tubas uterinas na presença do espermatozoide (26–28).

#### Potencial mitocondrial, apoptose celular e translocação da fosfatidil serina

Em humanos o uso de sonda fluorescente (mito tracker green) associado a citometria de fluxo permitiu concluir que o potencial da membrana mitocondrial apresenta uma correlação com outros padrões de análise espermática como a capacitação e a fragmentação do DNA. Ademais, a subpopulação com alto potencial mitocondrial foi a que caracterizou melhor a função espermática em humanos (21). A mitocôndria é responsável pela maior parte da energia celular, a qual ocorre por meio do processo de fosforilação oxidativa (FO). Também é responsável pela maior parte da produção endógena de espécies reativas ao oxigênio (ROS) como biometabólitos, sendo considerada a reguladora central da apoptose celular (29).

A perda do potencial de membrana mitocondrial é um fenômeno detectado no estágio inicial da apoptose sendo um marcador importante no avanço das alterações semelhantes a apoptose causadas pela criopreservação (30). A apoptose, morte celular programada, delimita uma sequência de eventos que levam a destruição celular, também é parte importante do desenvolvimento e manutenção da saúde pela eliminação de células velhas ou doentes (31). Os espermatozoides com defeitos são eliminados nos túbulos seminíferos durante a espermatogênese pelo processo da apoptose, o qual é um evento fisiológico (30). As caspases são componentes sinalizadores da cascata apoptótica sendo que outros marcadores pró-apoptóticos como as Bax, citocromo c, fator indutor da apoptose e a caspase-9 também já foram identificados no ejaculado de diversas espécies (17,20,31).

Sokolowska et al. (32) detectaram caspases ativas no sêmen

congelado/descongelado de cães utilizando o marcador *in situ* da caspase FITC-VAD-FMK, um peptídeo permeável inibidor da caspase conjugado ao FITC que se liga a caspase ativada. Todavia, apesar de presente na célula espermática, o fato das caspases estarem localizadas na peça intermediária pode representar que, mesmo após a fase de maturação da espermatogênese, estas células demonstrando atividades das caspases, apresentem citoplasma remanescente ou que ainda estejam presentes no espermatozoide ejaculado por uma falha no processo da maturação espermática (33).

Durante a ejaculação o espermatozoide é secretado juntamente com substâncias oriundas das glândulas acessórias masculina variando em quantidade e composição. O modo como as substâncias contidas nestas secreções modificam a fisiologia espermática ainda não foi totalmente elucidada, mas o fato é que nem todos os espermatozoides contidos em um ejaculado estão na mesma condição fisiológica (22). Um estudo sugere que espermatozoides que apresentam uma translocação da enzima fosfatidil serina são preferencialmente fagocitados aumentando a chance de outros espermatozoides alcançarem o oócito para a fertilização (34). Assim, acredita-se que apenas uma pequena porcentagem de espermatozoides são competentes para realização da fertilização (9,35).

Quando a membrana plasmática é rompida os fosfolipídios, fosfatidil serina (FS), nela contidos se deslocam de dentro para fora da célula. Esta translocação da FS é um dano primário da membrana plasmática (36). A anexina V é uma sonda cálcio dependente utilizada para detectar a externalização da FS (31). Apesar da externalização da FS também ser um indicativo de apoptose, ainda não há evidências suficientes de que todos os componentes apoptóticos, como a caspase-3 e 8, são produzidos pelo espermatozoide ejaculado (20). Kim et al. (36) utilizaram a sonda fluorescente anexina V combinada ao IP e observaram que a criopreservação provoca uma translocação da FS no sêmen de cães.

#### Peroxidação lipídica

O plasma seminal contém uma série de antioxidantes enzimáticos ou não, os quais são importantes para a proteção da célula espermática contra os radicais produzidos pelo metabolismo do oxigênio (ROS). A atividade destes antioxidantes varia de acordo com a espécie e também com a fertilidade individual (37,38).

As ROS apresentam também um papel fisiológico importante para a célula

espermática, porém quando produzidos em alta quantidade causam efeitos nocivos que aceleram a morte celular. Os lipídeos contidos na membrana celular são alvo principal das ROS, que quando produzidas em quantidade fisiológicas levam a capacitação espermática de forma que o espermatozoide esteja pronto para a fertilização do oócito. A oxidação dos lipídeos da membrana (lipoperoxidação) libera radicais livres peróxil e alkoxil que para se estabilizarem retiram um átomo de hidrogênio do lipídeo mais próximo criando um novo radical, que combinado ao dióxigênio cria um novo peróxido lipídico e assim torna-se um ciclo de produção de radicais oxidativos (39). Altos níveis de ROS são nocivos a célula espermática e podem ser a causa de infertilidade em 20 a 40% dos casos de infertilidade em humanos (40).

Em cães, Kim et al. (36) notaram um aumento significativo na produção de peróxido de hidrogênio intracelular no sêmen congelado. Este fato também pode ser observado no sêmen congelado de equinos e bovinos (41,42). A sonda membrana fluorescente BODIPY-C11 foi utilizada recentemente para avaliar a peroxidação dos lipídeos de membrana no sêmen fresco e congelado de cães (6) onde percebeu-se que o aumento na lipoperoxidação esta relacionado a alguns indivíduos apenas.

Muitos são os fatores que podem influenciar e até mesmo determinar o sucesso da célula espermática em realizar a fertilização reforçando o conceito que o sêmen é uma mistura complexa e que apenas uma avaliação ou um teste funcional são insuficientes para prever a fertilidades em machos.

#### Inseminação artificial com sêmen congelado

O primeiro relato de uso de sêmen congelado de cão foi a 46 anos atrás. Desde então, muitas inovações no processo de congelação de sêmen, entendimento do ciclo estral da cadela e técnicas de inseminação artificial foram desenvolvidos. Apesar de muitos estudos, as taxas de gestação ainda são bastante variáveis quando o sêmen congelado é usado para IA nesta espécie (43–46).

A criopreservação causa alterações estruturais e funcionais no espermatozoide o que leva a diminuição do tempo de vida útil e capacidade fertilizante da célula (36,47). Desta forma, torna-se fundamental que a inseminação artificial com sêmen congelado seja feita no momento mais próximo a possibilidade de fertilização (45). Na cadela a determinação deste momento é mais complexa, pois além de ovular oócitos imaturos, a

determinação do momento ovulatório também requer conhecimento e acompanhamento adequado (48). Nesta espécie, os óvulos liberados são oócitos primários e requerem 24 a 72 horas de maturação antes que possam ser fertilizados. Uma vez maduros, a vida fértil de cada oócito pode durar de dois a quatro dias (49).

Vários parâmetros são utilizados para determinação do período ovulatório, entre estes os aspectos clínicos e reprodutivos, citologia vaginal, endoscopia vaginal ou vaginoscopia, dosagens hormonais e, mais recentemente, a ultrassonografia ovariana (12,50,51). Parâmetros clínicos como a data de início do edema de vulva, quantidade e aspecto da secreção vulvar, lateralização da cauda ou aceitação da monta, não são suficientemente precisos para detectar a ocorrência e o período da ovulação, pois o aparecimento dessas alterações varia muito entre as cadelas, (50–52). A mudança para o comportamento receptivo característico do estro também não é confiável para detectar a ovulação, pois essa mudança comportamental normalmente ocorre um dia após o pico de LH, mas pode ocorrer desde três dias antes, até cinco dias depois do pico, ou ainda pode não ocorrer (50). A vaginoscopia para o manejo reprodutivo consiste na observação das mudanças da mucosa vaginal durante proestro e estro. No início do proestro, as pregas vaginais encontram-se edemaciadas e túrgidas, e a mucosa rósea sob o efeito do estrógeno. Com a progressão para o estro, as pregas vaginais perdem a turgidez, tornando-se “enrugadas” e anguladas, enquanto a mucosa torna-se pálida e menos rósea. Esse fenômeno é chamado de crenulação das pregas vaginais (50,53–55). Contudo, apesar das mudanças observadas no aspecto da mucosa vaginal serem progressivas, as mesmas não ocorrem em um momento determinado do proestro e do estro e portanto não determina o dia exato da ovulação, podendo ainda variar entre os animais (50). Macedo (56) não encontrou correlação entre a crenulação vaginal e a dosagem de progesterona ( $P_4$ ).

A data da ovulação pode ser determinada pela dosagem hormonal de LH ou da  $P_4$ , sendo que a ovulação ocorrerá após 36 a 50 horas após o pico de LH (49). Apesar da dosagem de LH ser mais determinante para avaliação do momento ovulatório, não é comumente utilizada pois além do alto custo necessita de dosagem diária. De forma que a dosagem da  $P_4$  é o método mais utilizado para determinação do pico de LH que de forma geral se apresenta ao redor de 2,0 ng/mL neste período (48) a depender do método de análise utilizado. Os métodos mais comuns para dosagem desse hormônio são radioimunoensaio (57) e quimioluminescência (58). Existem também os testes semi-quantitativos como o ELISA, que apesar de rápidos apresentam acurácia reduzida

(48,57). A concentração plasmática de progesterona começa a aumentar rapidamente, além dos níveis basais, aproximadamente dois dias antes da ovulação, durante o pico de LH (Figura 1), por isso a realização de dosagens seriadas permite a antecipação da ovulação em um ou dois dias e, se continuada, permite a confirmação da ovulação e retrospectiva detecção do período ovulatório (50,59). A concentração plasmática de progesterona no dia do pico de LH varia de acordo com os autores, encontrando-se em torno de 2 e 4 ng/ml (12,60). E durante o período ovulatório, 4,7 e 6,2 ng/ml (43), 4 a 7 ng/mL (1) e 4 a 10 ng/mL (48). Estas diferenças entre os autores se deve provavelmente ao método utilizado para quantificação da P4.

Recomenda-se a realização de dosagens diárias de progesterona ou em dias alternados até que um valor indique a ocorrência da ovulação (12,57). Como o período ótimo para fertilização ocorre entre 2 a 4 dias após a ovulação, quando os óocitos estão completamente maduros e ainda não sofreram degeneração (12,51), considera-se que o período fértil da cadela seja do 4<sup>o</sup> ao 7<sup>o</sup> dia após o pico de LH (61).

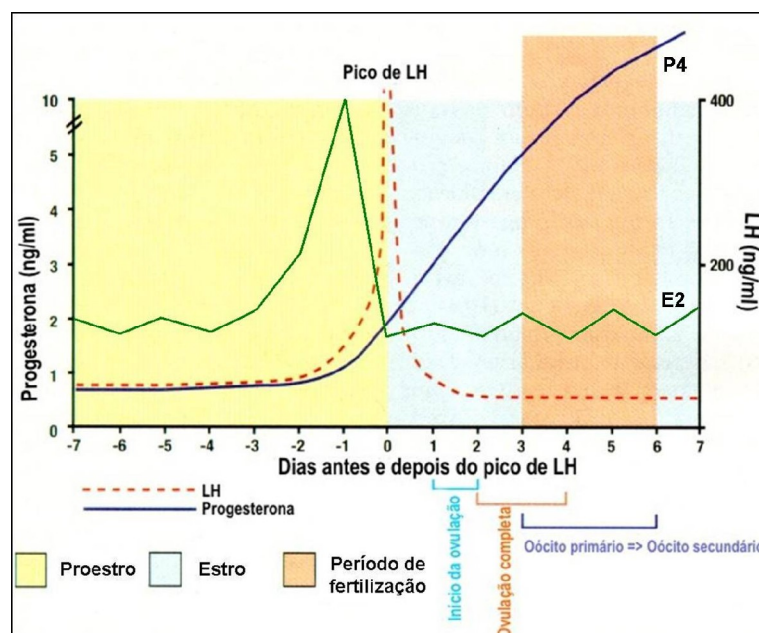


FIGURA 1: Variações hormonais na cadela durante o proestro, estro e período ovulatório P4: progesterona; E2: estrogênio. Fonte: Macedo, 2009.

Além do momento ideal para que a IA com sêmen congelado seja bem sucedida outros fatores devem ser levados em consideração, como a técnica de inseminação e a concentração espermática inseminada. Em cadelas, melhores taxas de gestação foram observadas quando mais de  $100 \times 10^6$  de espermatozoides morfologicamente normais e

móveis foram utilizados (46,62). Baixas concentrações espermáticas foram correlacionadas com alta variação nos resultados de prenhes em vacas de leite (63). Uma menor taxa de gestação também foi observada em humanos, quando baixas concentrações espermáticas foram utilizadas em programas de reprodução assistida (64). Nizanski et al. (65) obtiveram 60% de gestação utilizando uma dose total de  $500 \times 10^6$  / mL de espermatozoides para uma viabilidade espermática em torno de 60%.

O método de IA com sêmen congelado em pequenos animais, utilizado há 25 anos, para deposição do sêmen dentro do útero foi a inseminação cirúrgica por laparotomia (43). As perdas sofridas pela célula espermática durante o processo de criopreservação faz necessária que a deposição do sêmen na IA seja feita dentro do útero (43,45,46). A cateterização cervical (TC) começa a ser possível quando pesquisadores escandinavos relataram a adaptação bem sucedida de um cateter rígido usado em raposas para cadelas (43,44). Atualmente a TC foi melhorada pela adaptação de endoscópios utilizados em humanos, para cães, o que permite uma manipulação do colo uterino e cateterismo mais rápido. A técnica de inseminação transcervical assistida por endoscopia foi desenvolvida na Nova Zelândia, tendo como grande vantagem a visualização direta do óstio cervical (59). Mas ainda sim requer prática e destreza do operador pois envolve a manipulação do endoscópio e do cateter simultaneamente. Além disso, algumas particularidades da anatomia da cadela que dificultam a cateterização da cérvix devem ser consideradas para a realização dessa técnica. O acesso ao orifício cervical é restrito pela prega mediana dorsal, pelo lúmen estreito da porção vaginal da paracérvix e o direcionamento ventral do óstio cervical externo (Figura 2) (46,59). A posição do clitóris no interior da comissura ventral da vulva e do óstio uretral ventralmente no vestíbulo devem ser evitados; e a presença das pregas vaginais pode dificultar a passagem do endoscópio. A prega mediana dorsal é uma prega mais firme localizada longitudinalmente na parede dorsal da vagina, reduzindo significativamente o lúmen vaginal até que se possa visualizar a cérvix (Figura 2). Um ponto de referência importante é o tubérculo cervical, que consiste na porção vaginal da cérvix, incluindo o óstio e o canal cervical (Figura 2). O tubérculo cervical está localizado na extremidade cranial da prega mediana dorsal e está separada da mesma por uma prega transversal evidente. A cérvix encontra-se diagonalmente na junção útero-vaginal com o óstio cervical posicionado ventralmente na direção do assoalho vaginal e o canal cervical direcionado crânio-dorsalmente da vagina para o útero (Figura 2) (66,67). O canal cervical apresenta cinco a dez milímetros de comprimento, e

nem sempre é reto, podendo ser sinuoso (68).

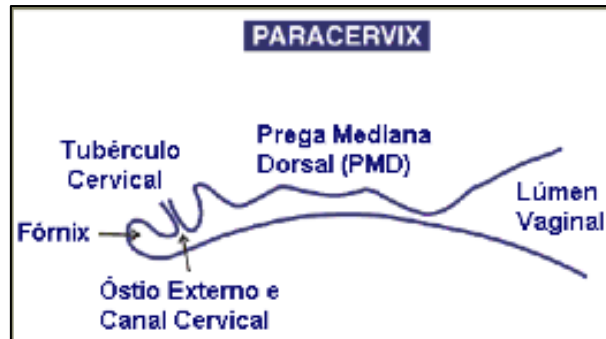


FIGURA 2: Anatomia da porção cranial da vagina da cadela e pontos de referência para realização da cateterização do óstio cervical. Fonte: Feldman e Nelson (69).

Uma vantagem da IA TC é a não necessidade de anestesia ou sedação do animal, devendo ser realizada com o animal em estação (1,70).

O procedimento da TC está se tornando uma técnica de rotina para inseminação artificial em cães, pois proporciona uma melhor fertilidade, especialmente quando se utiliza sêmen congelado (70).

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## Capítulo 2

**Viability of cooled and frozen canine semen using combination of fluorescent probes by flow cytometry**

### **ARTIGO 1**

Artigo enviado a revista Theriogenology de acordo com as normas da revista.

1 **Title: Viability of cooled and frozen canine semen using combination of fluorescent**  
2 **probes by flow cytometry**

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22

23 **Abstract**

24 Sperm cryopreservation has become an indispensable tool in biology. However,  
25 frozen/thawed semen has a short life span due to losses in the integrity of sperm cells  
26 during the process of cooling, freezing and thawing. To better understand dog semen  
27 quality after thawing five healthy mature dogs were used to the one-step sperm  
28 cryopreservation protocol. Sperm analysis was performed at three time points: after  
29 collection, refrigeration, and after thawing, by computer sperm motility analysis (total  
30 [TM] and progressive motility [PM], and rapid sperm), membrane damage (membrane  
31 fluidity and viability - Yo-Pro 1 and merocianina 540, phosphatidyl serine translocation  
32 – annexin – V – propidium iodide - PI, acrosomal and membrane integrity FITC-PSA –  
33 PI), mitochondrial potential (JC-1), membrane lipid peroxidation, LPO (BODIPY  
34 581/591 C<sub>11</sub>) and apoptosis (kit CelleEvent<sup>TM</sup> Caspase-3/7- FITC Green Flow  
35 Cytometry), evaluated by flow cytometry. A decrease on frozen/thawed semen  
36 comparing to fresh and cooled semen occurred for the parameters of total and  
37 progressive motility (TM 87.00 ± 1.24, 88.15 ± 1.38, 72.55 ± 6.26; PM 69.95 ± 1.28,

1 71.75 ± 1.91, 56.30 ± 6.00, fresh cooled and frozen/thawed respectively P < 0.01),  
2 percentage of rapids (83.15 ± 1.94, 81.55 ± 1.53, 64.30 ± 7.68 P < 0.01), membrane  
3 fluidity and cell viability (78.29 ± 6.22, 75.76 ± 6.60, 23.02 ± 9.12 P < 0.01) and  
4 acrosomal and membrane integrity (85.71 ± 6.22, 70.37 ± 6.43, 30.87 ± 9.90 P < 0.01).  
5 Sperm cells were affect for cooling and freezing process when analyzed for the  
6 translocation of phosphatidyl serine (79.90 ± 7.95, 63.50 ± 4.66, 27.19 ± 9.22 P < 0.01)  
7 and high mitochondrial potential (85.15 ± 2.76, 60.52 ± 3.31, 38.06 ± 6.40 P < 0.01).  
8 For LPO significant difference was observed in semen after thawing only comparing to  
9 cooled semen (16.57 ± 5.44, 16.22 ± 2.43, 23.00 ± 4.13 P < 0.05). No difference on  
10 caspase activity was observed. In conclusion cryopreservation leads to structural and  
11 functional changes in sperm cell, which explains their short survival after thawing. The  
12 cryo-capacitation like changes can be related to others factors, as the seminal plasma  
13 proteins that are not know yet on this species. Apoptosis process can be initiated with  
14 the opening of mitochondrial pores during the freezing/thaw process, which releases  
15 pro-apoptotic factor on the cytoplasm. The mitochondrial potential is not related to  
16 sperm motility but sperm survival.

17 Keywords: dog, sperm, cryopreservation, viability.

## 18 **1. Introduction**

19 Currently, globalization made possible a greater interaction between dog breeders  
20 around the world, so that the demand and interest in artificial insemination has become  
21 important. It is not always that the male of interest is in a distance that allows the  
22 natural mating or the use of fresh or cooled semen.

23 The frozen semen offers practical advantages because there is no dependence on the  
24 time or the distance, and ensures genetics of higher animals indefinitely. However,  
25 frozen/thawed semen has a short life span due to losses in the integrity of sperm cells  
26 during the process of cooling, freezing and thawing. After cryopreservation a significant  
27 decrease in sperm motility compared to fresh semen and refrigerated is noted [3].

28 Motility is extremely important because the sperm needs to reach the site of  
29 fertilization. Moreover, the fact that the bitch ovulate immature oocytes, which take 2 to  
30 3 days to be read for fertilization, creates difficulties in determining the optimum time  
31 for insemination, making it even more important long functional life of the sperm after  
32 thawing [3–5]. However, the viability of sperm when analyzed by motility is greater  
33 than its actual capacity of fertilization due to changes caused to the cell membranes

1 during the cryopreservation process. [6], In fact, besides motility it is necessary that  
2 cellular structures important for the interaction of sperm and oocytes are functional. The  
3 relationship between functional properties and fertility is much more complex and can  
4 be affected by various factors, originating in spermatogenesis, maturation in the  
5 epididymis, ejaculation and interaction with the female reproductive tract. As it is not  
6 possible to identify all the characteristics that guarantee the success of fertilization, it  
7 becomes more feasible to evaluate the functional and basic cellular properties and  
8 identify the cells that don't have those characteristics [7].

9 In vitro fertilization (IVF), which is widely used in cattle as a way of assessing sperm  
10 quality, is not used in dogs, due to factors related to oocyte maturation. Thus other  
11 parameters may be used in an attempt to determine sperm viability in this species.

12 The most affected cell structure during freezing and thawing is the sperm plasma  
13 membrane. Frozen/thawed canine sperm presents decreased plasma membrane integrity  
14 and increased membrane fluidity [3]. Many different probes have been used to access  
15 cell structures to check its viability and possible fertilizing potential of semen samples.

16 The fluorescent probe Yo-Pro1 (YP) penetrate the cell when there is destabilization of  
17 the plasma membrane and a increase permeability of panexin-gated channels even  
18 before the total loss of membrane integrity occurs [8]. The merocyanine 540 (M540) in  
19 turn detects changes in lipid arrangement contained in the membrane, an initial  
20 modification of the capacitation process, as the permeability increase more the stain can  
21 bind to membrane acting as a marker for membrane destabilization [9,10].

22 Steckler et al. [11] observed that the combination of YP and M540 probes can detect  
23 early changes in the plasma membrane in fresh semen dogs after incubation in medium  
24 containing or not bicarbonate as an capacitation inducer. Plasma membrane is related to  
25 many of sperm function, ensuring the cell's ability to maintain homeostasis, motility and  
26 the ability to interact with the environment including the female genital tract epithelium  
27 and the cumulus-oocyte complex cells [12].

28  
29 When plasma membrane is ruptured the phospholipids, phosphatidyl serine (PS) move  
30 from inside to outside the cell. This translocation of PS identifies a primary damage of  
31 the plasma membrane [13]. The annexin V is a calcium-dependent probe used to detect  
32 the externalization of PS [14]. Despite the translocation of PS be indicative of  
33 apoptosis, there is no sufficient evidence that all apoptotic components, such as caspase-

1 3 and 8, are produced by the ejaculated sperm [15]. Kim et al. [13] combined propidium  
2 iodide (PI) to annexin V and observed that cryopreservation lead to PS translocation in  
3 dogs.

4

5 Another event observed at the initial phase of apoptosis is the decrease of potential of  
6 inner mitochondrial membrane (IMM) this alteration could be a marker for the  
7 alternative pathway of apoptotic-like changes observed after cryopreservation [16].

8 Different probes have been used to evaluate the mitochondrial activity as mitotracker  
9 deep red, red, orange, green and JC-1 [10,17,18]. Unexpectedly, Volpe et al. [19]  
10 observed that sperm with low motility had high IMM what suggest that mitochondrial  
11 activity is not the only source of energy for sperm motility. In a recent study a positive  
12 correlation among static cells and high IMM were found during cooling process of dog  
13 semen [20].

14 Caspases are signaling components in apoptosis cascade and others pro-apoptotic  
15 factors as Bax, cytochrome c, apoptosis inducing factor and caspase 9 was found in  
16 ejaculated spermatozoa [10,14,15]. Although detected on frozen/thawed dog semen  
17 [21], activated caspases are locate on the intermediate piece and could represent a  
18 remaining cytoplasm from maturation phase of spermatogenesis [7,22].

19 Individual resistance to sperm cryopreservation was noted to occur on males of many  
20 species [23–25]. In dogs Neagu et al. [26] observed that only some individuals shows  
21 increased signs of sperm plasma membrane lipid peroxidation (LPO). The LPO is also a  
22 physiological event, which radicals from oxygen metabolism (ROS – reactive oxygen  
23 species) will react with membrane lipids in an oxidative process that prepares the  
24 spermatozoa to be read for fertilization. However, high levels of ROS were claimed to  
25 be the cause of 20 to 40% of infertility in mans [27].

26 Cryopreservation causes different levels of damages to the spermatozoa of all species.  
27 In dogs is still necessary the better understanding of cryo-damages aiming greater  
28 fertility results. Thus, the aim of the present study was to access the effects of  
29 cryopreservation on sperm motility, membrane damage, mitochondrial potential, LPO  
30 and apoptosis evaluated by flow cytometry.

31

## 32 2. Materials and methods

## 1    2.1 *Animals*

2    Five adult dogs ranging from 3 to 5 years from different breed were used in this study.  
3    All dogs were clinic and andrologically examined, and only health dogs with sperm  
4    motility superior to 70% were used in the study. The ethical committee of the university  
5    reviewed and approved the experimental design (number 133/2014-CEUA).

## 6    2.2 *Reagents*

7    All reagents used were from Sigma-Aldrich (São Paulo, Brazil) and Merck SA (São  
8    Paulo, Brazil), unless otherwise cited.

## 9    2.3 *Semen collection and processing*

10   Semen was collected by digital manipulation in a pre-warmed graduated tube. No  
11   female was present on any one of the collections. Four samples from each dog were  
12   collected weekly (20 ejaculates). After collection an aliquot of sperm samples was  
13   evaluated for sperm motility, concentration, morphology and all other flow cytometry  
14   analysis. Thus, semen was centrifuged for 10 min at 800 Xg. The seminal plasma was  
15   then removed and the sperm pellet re-suspended in one step in Tris-egg-yolk extender at  
16   room temperature (200 mM Tris, 67 mM citric acid, 44.4 mM D-fructose v:v egg-yolk  
17   glycerol 8% orvus WA paste [Proctor & Gamble], 0.02% amikacin sulfate and distilled  
18   water qsp) ([28] modified by [29]), resulting in a concentration of  $80 \times 10^6$  sperm per  
19   mL. After dilution semen were loaded in 0.5 mL French straws and cooled to 5°C over  
20   1 hour. Samples were frozen horizontally in racks, placed 6 cm above the surface of  
21   liquid N<sub>2</sub> in a closed Styrofoam box, for 20 min and then plunged directly in liquid N<sub>2</sub>.  
22   At the moment of analysis straws were thawed in a water bath at 40°C for 20 s. All  
23   experimental analysis were performed on fresh, cooled and thawed semen.

24

## 25   2.4 *Motility analysis*

26

27   Motility was measured on fresh, cooled and thawed semen using CASA system (HTMA  
28   – IVOS 10, IMV, Beverly, MA, USA). The percentage of total motility (TM),  
29   progressive motility (PM) and rapid cells (RAP) were evaluated.

30

## 1 *2.5 Membrane fluidity and cell viability*

2 Yo-Pro 1 (YP), merocianina 540 (M540) (Molecular Probes<sup>®</sup>, Invitrogen – Life  
3 Technology Co, Carlsbad, CA, USA) were used in combination to determine cell  
4 viability and changes in plasma membrane fluidity, respectively. The hoescht 33342  
5 probe (H 33342, cod 14533, Sigma-Audrich Co., Saint Louis, Missouri, EUA) was also  
6 used to distinguish sperm cells from others artifacts. These stain distinguish three sperm  
7 subpopulations. The first subpopulation of unstained spermatozoa was considered live  
8 and without membrane alteration (YP and M540 negative). A second subpopulation was  
9 the Yo-Pro-1 negative and positive M540, the cells were considered in early stages of  
10 increased membrane fluidity. And the third subpopulation of cryo-injury-induced  
11 nonviable sperm with increased membrane fluidity (YP and M540 positive).

12 To each 191µL of diluted semen (at  $5 \times 10^6$  sperm/mL), H 33342 (7 µM in distilled  
13 water), M540 (7.5 nM in DMSO) was added and after incubation at 37<sup>0</sup> C in the dark  
14 for 10 min, YP (25 nM in DMSO) and samples were then incubated for more 20 min.

15

## 16 *2.6 Assessment of phosphatidyl serine translocation*

17

18 Annexin V-FITC apoptosis detection kit I (550475, BD Pharmigen, San Diego, CA,  
19 USA) was used according to the manufacturer's instructions. To each 185 µL of semen  
20 ( $2 \times 10^6$  sperm/mL) diluted in TALP–PVA (according to [30] modified by [31], 100  
21 mM NaCl, 3.1 mM KCl, 25.0 mM NaHCO<sub>3</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 21.6 mM DL-sodium  
22 lactate 60%, 2.0 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 10.0 mM Hepes acid free, 1.0 mM sodium  
23 pyruvate, 1,0mg/mL polyvinyl alcohol - PVA and 25µg/mL gentamicin sulfate), H  
24 33342 (7 µM in aqueous solution) and propidium iodide (PI) (1.5 µM in TALP-PVA,  
25 P470 Sigma-Audrich Co., Saint Louis, Missouri, EUA) and 5 µL of annexin V-FITC  
26 (according to manufactures instructions) was added. Samples were then incubated at  
27 37°C in the dark for 15 min. After incubation, an additional 200 µL of the binding  
28 buffer (10 mM Hepes-NaOH pH 7.4, 140 mM NaCl and 2.5 mM CaCl<sub>2</sub>) was added for  
29 flow cytometry analysis. These probe distinguish three sperm subpopulations. The first  
30 subpopulation of unstained spermatozoa was considered live and in the absence of  
31 membrane alteration (Anexin V-FITC and PI negative – AN-/PI-). A second

1 subpopulation was the viable cells but showing PS translocation (AN+/PI-). And third  
2 population of non-viable and PS translocation cells (AN+/PI+).

3

#### 4 *2.7 Acrosomal and plasma-membrane integrity*

5

6 Sperm acrosomal and plasma membrane integrity was assessed using the combination  
7 of FITC-PSA (L-0770, Sigma-Audrich Co., Saint Louis, Missouri, EUA), PI and H  
8 33342. To each 189.5  $\mu\text{L}$  of semen diluted in TALP-PVA ( $2 \times 10^6$  sperm/mL), H 33342  
9 diluted in distilled water (to achieve a final concentration of  $7 \mu\text{M}/200 \mu\text{L}$ ) and PI  
10 diluted in TALP (to achieve a final concentration of  $1.5 \mu\text{M}/200 \mu\text{L}$ ) and FITC-PSA ( $2$   
11  $\text{ng}/400 \mu\text{L}$  in DMSO, according to manufacture's instructions). Samples were then  
12 incubated at  $37^\circ\text{C}$  in the dark for 15 min. After incubation, an additional  $200 \mu\text{L}$  of  
13 TALP-PVA buffer was added for flow cytometry analysis. Four different  
14 subpopulations were found. The first subpopulation was of cells with intact acrosome  
15 and plasma membrane (FITC-PSA-/PI-). Second population was the cells showing  
16 intact acrosome but damage plasma membrane (FITC-PSA-/PI+). Third population of  
17 damage acrosome and intact plasma membrane (FITC-PSA+/PI-) and forth population  
18 of damage plasma membrane and acrosome ((FITC-PSA+/PI+)

19

#### 20 *2.8 Mitochondrial potential*

21 For this analysis was used the lipophilic cationic compound, JC-1 (T3168, Molecular  
22 Probes<sup>®</sup>, Invitrogen – Life Technology Co, Carlsbad, CA, USA). A  $4 \mu\text{L}$  of JC-1  
23 solution ( $50 \mu\text{g}/\text{mL}$  in DMSO) was added to a  $500 \mu\text{L}$  of semen ( $10 \times 10^6$  sperms/mL),  
24 and maintained for 40 min at  $25^\circ\text{C}$ . After that  $100 \mu\text{L}$  of this solution was diluted 1:5 in  
25 TALP-PVA containing aqueous solution  $2 \mu\text{M}$  H 33342, and immediately evaluated by  
26 flow cytometry [31]. Sperm population was divided on two, with high (HMP) and low  
27 mitochondrial potential.

#### 28 *2.9 Lipid peroxidation*

29 Membrane lipid peroxidation (LPO) was measured using the probe BODIPY 581/591  
30  $\text{C}_{11}$  (D-3861; Molecular Probes, Leiden, The Netherlands). To this test were used  $489.5$   
31  $\mu\text{L}$  containing  $1 \times 10^6$  sperm cells/mL diluted in TALP-PVA and added PI ( $1.5 \mu\text{M}/200$   
32  $\mu\text{L}$  in TALP-PVA), H33342 ( $7 \mu\text{M}/200 \mu\text{L}$  in distilled water) and BODIPY (in a final

1 concentration of 1  $\mu\text{M}$  in DMSO). The samples were then incubated at 37<sup>0</sup> C for 30  
2 min. After incubation, to remove egg yolk, 2 consecutive washes by centrifugation  
3 were performed, (300 Xg for 5 min) with TALP-PVA and the pellet re-suspended in  
4 TALP-PVA.

#### 5 *2.10 Assessment of activated caspases*

6 For the assessment of activated caspases, the caspase kit CellEvent<sup>TM</sup> Caspase-3/7-  
7 FITC Green Flow Cytometry (C10427, Molecular Probes<sup>®</sup>, Invitrogen – Life  
8 Technology Co, Carlsbad, CA, USA) was used to detect active caspases 3 and 7  
9 according to manufacturer instructions in association with H 33342 and PI. To 1  $\mu\text{L}$  of  
10 semen diluted in TALP-PVA ( $1 \times 10^6$  sperm cells/mL) were added 1  $\mu\text{L}$  of CellEvent<sup>®</sup>  
11 (diluted as manufacture's instructions) and H 33342 (7  $\mu\text{M}$ /200  $\mu\text{L}$  in distilled water)  
12 and incubated at 37<sup>0</sup> C for 25 min and after PI (1.5  $\mu\text{M}$ /200  $\mu\text{L}$  in TALP) was added  
13 and incubated for more 5 min before analyses. Sperm population was divided on two,  
14 displaying or not activated caspases.

#### 15 *2.11 Flow cytometry*

16 Flow cytometry analysis were carried out on a BD LSR Fortessa (Becton Dickinson,  
17 Mountain View, CA, USA) equipped with the lasers: blue performing 100 mW at 488  
18 nm, red performing 40 mW at 640 nm, and violet performed in 100 mW at 405 nm. H  
19 33342 was used in combination of all stains used for all flow cytometry analysis to  
20 enable clear distinction of sperm cells from others artifacts as egg yolk particles. Data  
21 were analyzed using FACSDiva<sup>tm</sup> software V6.2 (Becton Dickinson, Mountain View,  
22 CA, USA). For all analysis 10.000 sperm cells were evaluated.

#### 23 *2.12 Statistical analysis*

24 The results were presented as mean  $\pm$  standard error. Statistical analysis of data was  
25 performed by variance analysis for repeated measures complemented followed by  
26 multiple comparisons of Bonferroni [32].

### 27 **3. Results**

28 As expected, a decrease on frozen/thawed semen comparing to fresh and cooled semen  
29 occurred for the parameters of total and progressive motility, percentage of rapids,

1 membrane fluidity and cell viability and acrosomal and membrane integrity. Sperm  
 2 cells were affect for cooling and freezing process when analyzed for the translocation of  
 3 PS and mitochondrial potential. For LPO significant difference was observed on dog  
 4 semen after thawing only comparing to cooled semen. No difference on caspase activity  
 5 was observed (Table 1).

6

7 **Table 1**

8 Mean  $\pm$  standard error of the percentage of sperm cells for all parameters analyzed on  
 9 fresh, cooled and frozen/thawed semen.

10

Parameter	Moment		
	Fresh	Cooled	Frozen/Thawed
TM	87,00 $\pm$ 1,24 <sup>a</sup>	88,15 $\pm$ 1,38 <sup>a</sup>	72,55 $\pm$ 6,26 <sup>b</sup>
PM	69,95 $\pm$ 1,28 <sup>a</sup>	71,75 $\pm$ 1,91 <sup>a</sup>	56,30 $\pm$ 6,00 <sup>b</sup>
RAP	83,15 $\pm$ 1,94 <sup>a</sup>	81,55 $\pm$ 1,53 <sup>a</sup>	64,30 $\pm$ 7,68 <sup>b</sup>
YP-/M540-	78,29 $\pm$ 6,22 <sup>a</sup>	75,76 $\pm$ 6,60 <sup>a</sup>	23,02 $\pm$ 9,12 <sup>b</sup>
YP-/M540+	18,33 $\pm$ 5,54 <sup>a</sup>	22,55 $\pm$ 6,49 <sup>a</sup>	71,78 $\pm$ 9,85 <sup>b</sup>
AN-/PI-	79,90 $\pm$ 7,95 <sup>a</sup>	63,50 $\pm$ 4,66 <sup>b</sup>	27,19 $\pm$ 9,22 <sup>c</sup>
AN+/PI-	2,23 $\pm$ 2,06	6,88 $\pm$ 2,12	8,25 $\pm$ 10,16
FITC-PSA-/PI-	85,71 $\pm$ 6,22 <sup>a</sup>	70,37 $\pm$ 6,43 <sup>a</sup>	30,87 $\pm$ 9,90 <sup>b</sup>
HMP	85,15 $\pm$ 2,76 <sup>a</sup>	60,52 $\pm$ 3,31 <sup>b</sup>	38,06 $\pm$ 6,40 <sup>c</sup>
LPO	16,57 $\pm$ 5,44 <sup>ab*</sup>	16,22 $\pm$ 2,43 <sup>a*</sup>	23,00 $\pm$ 4,13 <sup>b*</sup>
CASP+	3,15 $\pm$ 2,38	8,65 $\pm$ 4,13	10,25 $\pm$ 6,75

11 Within a column, values with different superscripts differ significantly:  $P < 0.01$  \*  $P <$   
 12  $0.05$  (n = 20).

13 **4. Discussion**

14

15 In the present study we investigated the effects of cooling and cryopreservation on the

1 canine sperm subpopulation. As expected a decline in motility population occurred after  
2 cryopreservation as well as of the membrane integrity [33,34](Kim et al., 2010; Rota et  
3 al., 2010). However, besides motility the sperm membrane integrity is also extremely  
4 important for the fertilization success [35]. Despite capacitation to be a physiological  
5 event that able sperm to be ready to interact with the oocyte it should not happen  
6 prematurely. Changes in temperature during the cryopreservation process shortens the  
7 capacitation timing causing changes to the membrane lipid architecture and therefore on  
8 membrane permeability reducing the enzymatic activity necessary for the extrusion of  
9 calcium ions [10]. These alterations can also be explained by the presence of seminal  
10 plasma proteins, which could be noted in other species as boars, stallions and rams [36].  
11 In bovine, the BSPs (bovine seminal proteins) are initially considered as a  
12 decapacitation factor. However after the ejaculation and contact time and amount of  
13 these macromolecules in the seminal plasma, the capacitation process can be started  
14 with displacement of phospholipids and cholesterol of sperm membrane. These  
15 alterations depend on the time of semen dilution, according to Bergeron & Manjunath  
16 [37], the LDLs egg yolk, present in semen extenders, are capable of sequestering these  
17 proteins and minimize the effects of cryo-capacitation induced like changes. However,  
18 the technique used for the cryopreservation of canine semen involves a centrifugation  
19 and subsequent dilution, which prolongs the contact time of the seminal plasma proteins  
20 and sperm cells, as in the present study. Such effects are mentioned to occur in other  
21 species besides bovines as boars, stallions and rams [36,38], which could also occur in  
22 canines. These proteins are heparin-binding proteins, that have already been identified  
23 in dogs [39], despite not having been identified in dogs similar proteins as the BSPs.  
24 Cryopreservation process, including cooling, affects sperm cells in either lethal or sub-  
25 lethal aspect and sub-lethal alterations accelerates sperm function, which impair cell  
26 life. Burgess et al. [40] observed that after the freeze/thaw the sperm lose the capacity  
27 of adhesion on epithelial cell of the oviduct; the authors comment that this fact is  
28 probably due to an increase in the number of sperm with capacitation like-changes and  
29 acrosome reacted.

30 Therefore, subtle changes on membrane permeability, as detected by YP and M540 on  
31 the present study, permits that the sperm still mobile but could make the cell more  
32 readily to undergo to acrosome reaction and die [3]. As YP can penetrate in an early  
33 stage damaged plasma membrane it is a useful indicator in predict cell death in dog  
34 sperm making the combination of YP and M540 a sensitive indicator of cell viability

1 [11].  
2 Recently it has been shown that cryopreservation process also induces membrane PS  
3 translocation on dog's spermatozoa [13]. This fact was confirmed in the present study in  
4 which there was significant decreasing in intact cells of cooled and frozen/thawed  
5 semen. However, cryopreservation process didn't influence the population of live sperm  
6 showing PS translocation in our study and as showed in dog sperm before [13].  
7 Although it is a necrotic process, still controversial whether PS translocation is a marker  
8 for apoptosis or not, thus annexin -V can be useful in detect the response of the sperm  
9 cell in stress conditions [15] and could be one more tool in determine bad and good  
10 freezers. These necrotic alterations caused by cryopreservation process could be  
11 observed in the frozen/thawed semen in humans, boars, bulls and dogs [33,41-43]  
12 indicating that the freezing process decrease cell viability and its fertilizing ability post-  
13 thaw.  
14 Cryopreservation of spermatozoa also had an effect on sperm mitochondria. On the  
15 present study a decreased mitochondrial activity potential occurred after cooling and  
16 freezing/thawing. This fact we could think that would influence directly on sperm  
17 motility but the ATPs from glycolysis instead of oxidative phosphorylation that is  
18 essential for the motility hyper activation, tyrosine phosphorylation, binding to the zona  
19 pellucida and acrosomal reaction [15]. On the other hand apoptosis lead to opening of  
20 mitochondrial pores, which would cause a decreased mitochondrial activity by the  
21 liberation of pro-apoptotic factors on the cytoplasm [10].  
22 About membrane lipid peroxidation in our study a significant change were observed  
23 comparing cooled to frozen/thawed semen. The efficiency of BODIPY-C11 staining in  
24 mark cells that undergone to membrane LPO was confirmed on human sperm after an  
25 induced reaction with iron [44]. An individual difference on LPO were observed in dogs  
26 on fresh semen by Neagu et al. [26] and the difference remained for one of the tested  
27 dogs after semen cryopreservation, showing that analysis of LPO could be another  
28 interesting tool to determine good and bad semen freezers.  
29 Differences in caspase activity was not seen in our study on fresh, cooled or thawed  
30 semen. Although previously reported in dog sperm [21] the activity of caspase was  
31 noted on the intermediate piece and sperm tail and that could represent a remaining  
32 cytoplasm from sperm maturation instead of a apoptosis like-changes [7,10,14,15,22].  
33 Based on our results we conclude that cryopreservation leads to structural and  
34 functional changes in sperm cell, which explains their short survival after thawing. The

1 cryo-capacitation like changes can be related to others factors, as the seminal plasma  
 2 proteins that are not know yet on this specie. Apoptosis process can be initiated with the  
 3 opening of mitochondrial pores during the freezing/thaw process, which releases pre  
 4 apoptic factor on the cytoplasm. The mitochondrial potential is not related to sperm  
 5 motility but sperm survival.

6

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9

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4

## Capítulo 3

### **Fertility and seminal quality in dogs**

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1 **Title: Fertility and seminal quality in dogs**

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19

20 **Abstract**

21 The aims of the study were to assess individual characteristics and sperm concentration  
22 of cryopreserved semen using different structural and functional analysis and dog  
23 fertility. Twenty ejaculates were collected from 5 dogs and sperm were cryopreserved  
24 one-step protocol. Sperm analysis was performed in fresh, cooled and thawed semen, by  
25 computer sperm motility analysis (total [TM] and progressive motility [PM], and rapid

1 sperm), membrane fluidity and viability, phosphatidyl serine translocation, acrosomal  
2 and membrane integrity, mitochondrial potential and membrane lipid peroxidation  
3 (LPO). The dog semen that displayed better numerical results in all analysis was chose  
4 to inseminate separately (dog 1) and to compare with semen pool from others 4 dogs.  
5 Twenty bitches were inseminated by transcervical intrauterine (TCAI), twice using 2  
6 sperm concentration ( $160$  and  $450 \times 10^6$  spermatozoa/TCAI). There was a great total  
7 and progressive motility in fresh and cooled than frozen/thawed semen, rapid  
8 percentage, membrane fluidity and cell viability and plasma- and acrosome-membrane  
9 integrity. Sperm cells were affected for cooling and freezing process when analyzed for  
10 the translocation of phosphatidyl serine and mitochondrial potential. The LPO was  
11 greater in thawed than cooled samples. Dog 1 was significantly better for the parameters  
12 of plasma membrane and acrosome integrity and mitochondrial potential. By calculating  
13 the average of the animal response and minimum and maximum limits, although there is  
14 no difference, we observed that the semen from Dog 1 displayed better post thaw values  
15 for all analyzes. The pregnancy rate was 0% for dog 1 and 50% for pool at  
16 concentration  $450 \times 10^6$  spermatozoa. In conclusion, although the dog 1 displayed better  
17 sperm quality, no pregnancy was observed which makes us to rethink the prediction of  
18 fertility of semen samples by commonly used parameters. Considering motility and  
19 sperm viability to satisfactory pregnancy rates more than  $100 \times 10^6$  spermatozoa/TCAI  
20 are necessary.

21

22 Keywords: dog, sperm cryopreservation, intrauterine insemination, fertility

23

## 24 **1. Introduction**

25 The first report of the use of dog frozen semen was 46 years ago, since many

1 innovations in semen freezing process, in the understanding the estrous cycle of the  
2 bitch and in the artificial insemination (AI) techniques have been developed. Despite  
3 many research on canine reproduction, pregnancy rates still are inconsistent when  
4 frozen semen is used for AI (Linde-Forsberg et al., 1999; Steckler et al., 2013;  
5 Thomassen et al., 2006).

6 Cryopreservation causes structural and functional changes in spermatozoa,  
7 which leads to decrease in their lifetime and fertilizing capacity (Burgess et al., 2012;  
8 Kim et al., 2010). Thus it is essential that artificial insemination with frozen semen to be  
9 made in the nearest time of fertilization (Steckler et al., 2013). In bitch determining this  
10 moment is more complex, because in addition to ovulate immature oocytes, the  
11 determination of ovulatory time also requires knowledge and proper monitoring (Root  
12 Kustritz, 2012). In this species the released eggs are primary oocytes and require 24 to  
13 72 hours of maturation before they can be fertilized, making it even more important  
14 long functional life of the sperm after thawing (Alhaider and Watson, 2009; Farstad,  
15 2012; Peña et al., 1999). In that manner the optimal fertilization period is between two  
16 to four days after ovulation, when the oocytes are fully mature, and have not undergone  
17 degeneration (Fontbonne, 2007), it is considered that the fertile period in the bitches is  
18 the fourth to the seventh day after the LH peak (Tsutsui et al., 2000).

19 Besides the ideal time, for the success of AI with frozen semen other factors as  
20 the insemination site and sperm concentration used must be taken into account. Because  
21 of losses in sperm functionality during cryopreservation process is necessary that the AI  
22 with frozen/thawed semen must be done intrauterine (Linde-Forsberg et al., 1999;  
23 Mason and Rous, 2014; Steckler et al., 2013). About sperm concentration, better  
24 pregnancy rates were observed when more than  $100 \times 10^6$  and mobile morphologically  
25 normal sperm were used for AI in canines (Mason and Rous, 2014; Mickelsen et al.,

1 1993). Low sperm concentrations were correlated with high variation in the results of  
2 pregnancy in milk cows (Andersson et al., 2004). A lower pregnancy rates were also  
3 observed in humans, when low sperm concentrations were used in assisted reproduction  
4 programs (Miller et al., 2002). Nizański,( 2006) found 60% of pregnancy using a total  
5 dose of  $500 \times 10^6$ /mL sperm with viability around 60%. Although there is some  
6 successful AI using low doses of frozen semen, as  $50 \times 10^6$  of motile sperm, with good  
7 pregnancy rates (Hye et al., 2007).

8         Despite individual characteristics, the relationship between functional properties  
9 and fertility is much more complex and can be affected by various factors, originating in  
10 spermatogenesis, maturation in the epididymis, ejaculation and interaction with the  
11 female reproductive tract. Sperm plasma membrane is the most affected cell structure  
12 during freezing and thawing. Frozen/thawed canine sperm presents decreased plasma  
13 membrane integrity and increased membrane fluidity (Alhaider and Watson, 2009). In  
14 this regard, different probes have been used to access cell structures to check its  
15 viability and possible fertilizing potential of semen samples (Nizański et al., 2012;  
16 Petrunkina and Harrison, 2013).

17         Cryopreservation causes different levels of damages to the spermatozoa of all  
18 species. In dogs is still necessary the better understanding of cryo-damages aiming  
19 greater fertility results. Thus, the aim of the present study was to access the effects of  
20 cryopreservation and spermatozoa concentration on fertility after AI in dogs.

21

## 22         **2. Materials and methods**

23

### 24         2.13 *Animals*

25         Five adult dogs ranging from 3 to 5 years from different breed were used in this

1 study. Four ejaculates were obtained from each dog and were collected every four days.  
2 All dogs were clinic and andrologically examined, and only health dogs with sperm  
3 motility superior to 70% were used in the study. For the AIs 20 bitches, ranging from 1  
4 to 5 years old, of mixed breed that were assisted at the university ambulatory clinic for  
5 castration were used with the agreement of they owners. Ten bitches were inseminated  
6 with the male 1 (5 females for each sperm concentration) and 10 bitches were  
7 inseminated with a pool of semen of the others 4 dogs (5 females for each sperm  
8 concentration). The ethical committee of the university reviewed and approved the  
9 experimental design (number 133/2014-CEUA).

10

#### 11 2.14 *Reagents*

12 All reagents used were from Sigma-Aldrich (São Paulo, Brazil) and Merck SA  
13 (São Paulo, Brazil), unless otherwise cited.

14

#### 15 2.15 *Semen collection and processing*

16 Semen was collected by digital manipulation in a pre-warmed graduated tube.  
17 No female was present on any one of the collections. Four samples from each dog were  
18 collected weekly (20 ejaculates). After collection an aliquot of sperm samples was  
19 evaluated for sperm motility, concentration, morphology and all other flow cytometry  
20 analysis. Thus, semen was centrifuged for 10 min at 800 xg. The seminal plasma was  
21 then removed and the sperm pellet re-suspended in one step in Tris-egg-yolk extender at  
22 room temperature (200 mM Tris, 67 mM citric acid, 44.4 mM D-fructose v:v egg-yolk  
23 glycerol 8% orvus WA paste [Proctor & Gamble], 0.02% amikacin sulfata and distilled  
24 water qsp) (MORTON, 1998 modified by Chirinéa et al., 2006;), resulting in a  
25 concentration of  $80 \times 10^6$  sperm per mL. After dilution semen were loaded in 0.5 mL

1 French straws and cooled to 5°C over 1 hour. Samples were frozen horizontally in  
2 racks, placed 6 cm above the surface of liquid N<sub>2</sub> in a closed Styrofoam box, for 20 min  
3 and then plunged directly in liquid N<sub>2</sub>. At the moment of analysis straws were thawed in  
4 a water bath at 40°C for 20 s. All experimental analysis was performed on fresh, cooled  
5 and thawed semen.

6

### 7 *2.16 Motility analysis*

8 Motility was measured on fresh, cooled and thawed semen using CASA system  
9 (HTMA – IVOS 10, IMV, Beverly, MA, USA). The percentage of total motility (TM),  
10 progressive motility (PM) and rapid cells (RAP) were evaluated.

11

### 12 *2.17 Membrane fluidity and cell viability*

13 Yo-Pro 1 (YP), merocianina 540 (M540) (Molecular Probes<sup>®</sup>, Invitrogen – Life  
14 Technology Co, Carlsbad, CA, USA) were used in combination to determine cell  
15 viability and changes in plasma membrane fluidity, respectively. The hoescht 33342  
16 probe (H 33342, cod 14533, Sigma-Audrich Co., Saint Louis, Missouri, EUA) was also  
17 used to distinguish sperm cells from others artifacts. These stain distinguish three sperm  
18 subpopulations. The first subpopulation of unstained spermatozoa was considered live  
19 and without membrane alteration (YP and M540 negative). A second subpopulation was  
20 the Yo-Pro-1 negative and positive M540, the cells were considered in early stages of  
21 increased membrane fluidity. And the third subpopulation of cryo-injury-induced  
22 nonviable sperm with increased membrane fluidity (YP and M540 positive).

23 To each 191µL of diluted semen (at 5 x 10<sup>6</sup> sperm/mL), H 33342 (7 µM in  
24 distilled water), M540 (7.5 nM in DMSO) was added and after incubation at 37<sup>0</sup> C in  
25 the dark for 10 min, YP (25 nM in DMSO) and samples were then incubated for more

1 20 min.

2

3 *2.18 Assessment of phosphatidyl serine translocation*

4 Annexin V-FITC apoptosis detection kit I (550475, BD Pharmigen, San Diego,  
5 CA, USA) was used according to the manufacturer's instructions. To each 185  $\mu$ L of  
6 semen ( $2 \times 10^6$  sperm/mL) diluted in TALP-PVA (according to Parrish et al. (1988)  
7 modified by Freitas-Dell'Aqua (2011), 100 mM NaCl, 3.1 mM KCl, 25.0 mM  
8  $\text{NaHCO}_3$ , 0.3 mM  $\text{NaH}_2\text{PO}_4$ , 21.6 mM DL-sodium lactate 60%, 2.0 mM  $\text{CaCl}_2$ , 0.4 mM  
9  $\text{MgCl}_2$ , 10.0 mM HEPES acid free, 1.0 mM sodium pyruvate, 1.0 mg/mL polyvinyl  
10 alcohol - PVA and 25  $\mu$ g/mL gentamicin sulfate), H 33342 (7  $\mu$ M in aqueous solution)  
11 and propidium iodide (PI) (1.5  $\mu$ M in TALP-PVA, P470 Sigma-Audrich Co., Saint  
12 Louis, Missouri, EUA) and 5  $\mu$ L of annexin V-FITC (according to manufactures  
13 instructions) was added. Samples were then incubated at 37°C in the dark for 15 min.  
14 After incubation, an additional 200  $\mu$ L of the binding buffer (10 mM HEPES-NaOH pH  
15 7.4, 140 mM NaCl and 2.5 mM  $\text{CaCl}_2$ ) was added for flow cytometry analysis. These  
16 probe distinguish three sperm subpopulations. The first subpopulation of unstained  
17 spermatozoa was considered live and in the absence of membrane alteration (Annexin V-  
18 FITC and PI negative – AN-/PI-). A second subpopulation was the viable cells but  
19 showing PS translocation (AN+/PI-). And third population of non-viable and PS  
20 translocation cells (AN+/PI+).

21

22 *2.19 Acrosomal and plasma-membrane integrity*

23 Sperm acrosomal and plasma membrane integrity was assessed using the  
24 combination of FITC-PSA (L-0770, Sigma-Audrich Co., Saint Louis, Missouri, EUA),  
25 PI and H 33342. To each 189.5  $\mu$ L of semen diluted in TALP-PVA ( $2 \times 10^6$  sperm/mL),

1 H 33342 diluted in distilled water (to achieve a final concentration of 7  $\mu\text{M}/200 \mu\text{L}$ ) and  
2 PI diluted in TALP (to achieve a final concentration of 1.5  $\mu\text{M}/200 \mu\text{L}$ ) and FITC-PSA  
3 (2 ng/400  $\mu\text{L}$  in DMSO, according to manufacture's instructions). Samples were then  
4 incubated at 37°C in the dark for 15 min. After incubation, an additional 200  $\mu\text{L}$  of  
5 TALP-PVA buffer was added for flow cytometry analysis. Four different  
6 subpopulations were found. The first subpopulation was of cells with intact acrosome  
7 and plasma membrane (FITC-PSA-/PI-). Second population was the cells showing  
8 intact acrosome but damage plasma membrane (FITC-PSA-/PI+). Third population of  
9 damage acrosome and intact plasma membrane (FITC-PSA+/PI-) and forth population  
10 of damage plasma membrane and acrosome ((FITC-PSA+/PI+)

11

## 12 2.20 *Mitochondrial potential*

13 For this analysis was used the lipophilic cationic compound, JC-1 (T3168,  
14 Molecular Probes<sup>®</sup>, Invitrogen – Life Technology Co, Carlsbad, CA, USA). A 4  $\mu\text{L}$  of  
15 JC-1 solution (50  $\mu\text{g}/\text{mL}$  in DMSO) was added to a 500  $\mu\text{L}$  of semen ( $10 \times 10^6$   
16 sperms/mL), and maintained for 40 min at 25°C. After that 100  $\mu\text{L}$  of this solution was  
17 diluted 1:5 in TALP-PVA containing aqueous solution 2 $\mu\text{M}$  H 33342, and immediately  
18 evaluated by flow cytometry (Freitas-Dell'Aqua, 2011). Sperm population was divided  
19 on two, with high (HMP) and low mitochondrial potential.

20

## 21 2.21 *Lipid peroxidation*

22 Membrane lipid peroxidation (LPO) was measured using the probe BODIPY  
23 581/591 C<sub>11</sub> (D-3861; Molecular Probes, Leiden, The Netherlands). To this test were  
24 used 489.5  $\mu\text{L}$  containing  $1 \times 10^6$  sperm cells/mL diluted in TALP-PVA and added PI  
25 (1.5  $\mu\text{M}/200 \mu\text{L}$  in TALP-PVA), H33342 (7  $\mu\text{M}/200 \mu\text{L}$  in distilled water) and

1 BODIPY (in a final concentration of 1  $\mu$ M in DMSO). The samples were then incubated  
2 at 37<sup>0</sup> C for 30 min. After incubation, to remove egg yolk, 2 consecutives washes by  
3 centrifugation were performed, (300 Xg for 5 min) with TALP-PVA and the pellet re-  
4 suspended in TALP-PVA.

5

## 6 2.22 *Flow cytometry*

7 Flow cytometry analysis were carried out on a BD LSR Fortessa (Becton  
8 Dickinson, Mountain View, CA, USA) equipped with the lasers: blue performing 100  
9 mW at 488 nm, red performing 40 mW at 640 nm, and violet performed in 100 mW at  
10 405 nm. H 33342 was used in combination of all stains used for all flow cytometry  
11 analysis to enable clear distinction of sperm cells from others artifacts as egg yolk  
12 particles. Data were analyzed using FACSDiva<sup>tm</sup> software V6.2 (Becton Dickinson,  
13 Mountain View, CA, USA). For all analysis 10.000 sperm cells were evaluated.

14

## 15 2.23 *Artificial insemination*

16 After semen analysis one superior dog was selected to compose a group to  
17 insemination, and others were used as pool.

18 To determine the ideal time to perform the AI, females estrous cycles were  
19 monitored by vaginal cytology and progesterone (P4) concentration. When bitches were  
20 presented in heat, blood samples were collected every other day, by venipuncture and  
21 centrifuged (600 xg for 15 minutes) and serum stored at -20°C to P4 determination. The  
22 P4 analyzes were performed using commercial kits for enzyme chemiluminescence  
23 immunoassay (IMMULITE<sup>®</sup>, Siemens Healthcare Diagnostics, Los Angeles, CA,  
24 USA), with an intra- and inter-assay variation coefficient at 20 nmol/L of 6.3 and 5.8%,  
25 respectively, and a detection limit of 0.6 nmol/L. The intra- and inter-assay coefficients

1 of variation at 5 nmol/L were 7.9 and 10%, and at 65 nmol/L, 5.0 and 8.6%,  
2 respectively. Values around 2.0 ng/mL were considered as corresponding to LH peak  
3 (considered Day 0 of cycle) (Fontbonne, 2008). Progesterone determination was  
4 performed from the beginning of heat until the confirmation of ovulations (more than  
5 10 ng/mL), and the estimated LH surge day was confirmed for AI. Two inseminations  
6 were performed on days 5 and 6 after the LH peak (Table 1).

7 AIs were performed with a rigid endoscope (TCI - Minitub/Karl-Storz,  
8 Tuttlingen, Germany) with 43 cm long, suitable for transcervical insemination in bitches  
9 attached to a video camera with a light source. This equipment has working channel to  
10 insertion of the insemination catheter (catheter TCI - 70 cm – Minitub, Tiefenbach,  
11 Germany). After the identification of cervical tubercle the catheter was inserted into the  
12 uterus and thawed semen was deposited (Pretzer et al., 2006). Two sperm concentration  
13 were tested, 160 and 450 x 10<sup>6</sup> sperm cells/TCAI, in a total volume of ~2.0 mL and 4.5  
14 mL, respectively.

15 Females were submitted to ovariectomy around 30 days after the LH  
16 peak to confirm pregnancy.

17

#### 18 2.24 *Statistical analysis*

19 The results were presented as mean ± standard error.

20 Statistical analysis of data was performed by variance analysis for repeated  
21 measures followed by multiple comparisons of Bonferroni (Johnson, R A, Wichern,  
22 1988).

23 To select the dog with superior sperm parameters was evaluated the drop from  
24 fresh to frozen/thawed of values from each analysis, using Kruskal-Wallis test (TM,  
25 PM, RAP, YP/M540, HMP) or ANOVA (FITC PSA/PI, AN/PI, LPO) and Fisher LSD

1 method to compare multiple pairwise. Moreover, for calculating the average response of  
2 the animal and minimum and maximum limits (with 95% confidence interval), we used  
3 the nonparametric Friedman complemented by the multiple comparison test of Dunn  
4 (ZAR, 2009). For the pregnancy rate the association test of Goodman were performed.

5

### 6 **3. Results**

7 A decrease on analyzed parameters for thawed semen comparing to fresh semen  
8 were noted (Table 2). When all dogs were analyzed together a significant decrease on  
9 frozen/thawed semen comparing to fresh and cooled semen occurred for the parameters  
10 of total and progressive motility, percentage of rapids, membrane fluidity and acrosomal  
11 and plasma membrane integrity. Sperm cells were affect for cooling and freezing  
12 process when analyzed for the translocation of PS and mitochondrial potential. For LPO  
13 significant difference was observed in semen after thawing only comparing to cooled  
14 semen (Table 2).

15 Dog 1 was significantly better for the parameters of plasma membrane and  
16 acrosome integrity and mitochondrial potential (Table 3) considering the drop of values  
17 from fresh to thawed semen, meaning that this dog loose less in semen quality after  
18 thaw when comparing to the others dogs. By calculating the average of the animal  
19 response and minimum and maximum limits, although there was no difference, we  
20 observed that the semen from dog 1 displayed better values and minor variation post-  
21 thaw for most analysis (Fig. 1 and 2). With the observation of this fact this dog was  
22 used separately from the others for the AI. However, no pregnancy were observed when  
23 AI were performed using the dog 1, even with the increase of the sperm  
24 concentration/AI. For the AIs using the pool of semen we observed a result of 50% of  
25 pregnancy in the group of females inseminated with the sperm concentration of 450 x

1  $10^6$ . While the group inseminated with  $160 \times 10^6$  sperm concentration no pregnancy  
 2 were observed (Table 4).

3

4 **Table 1**

5 Progesterone concentrations on the AI days sperm concentration and pregnancy results.

Bitch	Day of 1 <sup>st</sup> AI after LH surge	P4 ng/mL	Day of 2 <sup>st</sup> AI after LH surge	P4 ng/mL	Dog	Total sperm concentration/ AI	Result
1	5	15.3	6	17.2	1	$160 \times 10^6$	negative
2	6	21.5	7	26.4	1	$160 \times 10^6$	negative
3	5	20.2	6	22.0	1	$160 \times 10^6$	negative
4	5	16.0	6	19.0	1	$160 \times 10^6$	negative
5	6	20.5	7	22.6	1	$160 \times 10^6$	negative
6	5	15.6	6	15.3	1	$160 \times 10^6$	negative
7	5	8.04	6	7.51	Pool	$160 \times 10^6$	negative
8	5	29.6	6	37.7	Pool	$160 \times 10^6$	negative
9	5	18.0	6	20.0	Pool	$160 \times 10^6$	negative
10	5	13.6	6	20.9	Pool	$160 \times 10^6$	negative
11	5	17.8	6	21.5	1	$450 \times 10^6$	negative
12	5	16.0	6	19.0	1	$450 \times 10^6$	negative
13	5	15.4	6	21.5	1	$450 \times 10^6$	negative
14	5	7.0	6	6.94	1	$450 \times 10^6$	negative
15	5	14.0	6	16.5	Pool	$450 \times 10^6$	negative
16	5	14.8	6	17.1	Pool	$450 \times 10^6$	negative
17	5	18.2	6	14.9	Pool	$450 \times 10^6$	negative
18	5	19.2	6	26.2	Pool	$450 \times 10^6$	Positive – 4 embryonic vesicles
19	5	16.1	6	22.2	Pool	$450 \times 10^6$	Positive – 3 embryonic vesicles
20	5	17.2	6	24.1	Pool	$450 \times 10^6$	Positive – 3 embryonic vesicles

1 **Table 2**

2 Mean  $\pm$  standard error of the percentage of sperm cells for all parameters analyzed on  
 3 fresh, cooled and frozen/thawed semen from all dogs

4

Parameter	Moment		
	Fresh	Cooled	Frozen/Thawed
TM	87.00 $\pm$ 1.24 <sup>b</sup>	88.15 $\pm$ 1.38 <sup>b</sup>	72.55 $\pm$ 6.26 <sup>a</sup>
PM	69.95 $\pm$ 1.28 <sup>b</sup>	71.75 $\pm$ 1.91 <sup>b</sup>	56.30 $\pm$ 6.00 <sup>a</sup>
RAP	83.15 $\pm$ 1.94 <sup>b</sup>	81.55 $\pm$ 1.53 <sup>b</sup>	64.30 $\pm$ 7.68 <sup>a</sup>
YP-/M540-	78.29 $\pm$ 6.22 <sup>b</sup>	75.76 $\pm$ 6.60 <sup>b</sup>	23.02 $\pm$ 9.12 <sup>a</sup>
YP-/M540+	18.33 $\pm$ 5.54 <sup>a</sup>	22.55 $\pm$ 6.49 <sup>a</sup>	71.78 $\pm$ 9.85 <sup>b</sup>
AN-/PI-	79.90 $\pm$ 7.95 <sup>c</sup>	63.50 $\pm$ 4.66 <sup>b</sup>	27.19 $\pm$ 9.22 <sup>a</sup>
AN+/PI-	2.23 $\pm$ 2.06	6.88 $\pm$ 2.12	8.25 $\pm$ 10.16
FITC PSA-/PI-	85.71 $\pm$ 6.22 <sup>b</sup>	70.37 $\pm$ 6.43 <sup>b</sup>	30.87 $\pm$ 9.90 <sup>a</sup>
HMP	85.15 $\pm$ 2.76 <sup>c</sup>	60.52 $\pm$ 3.31 <sup>b</sup>	38.06 $\pm$ 6.40 <sup>a</sup>
LPO	16.57 $\pm$ 5.44 <sup>ab*</sup>	16.22 $\pm$ 2.43 <sup>a*</sup>	23.00 $\pm$ 4.13 <sup>b*</sup>

5 Within a column, values with different superscripts differ significantly:  $P < 0.01$  \* $P <$

6 0.05 (n = 20)

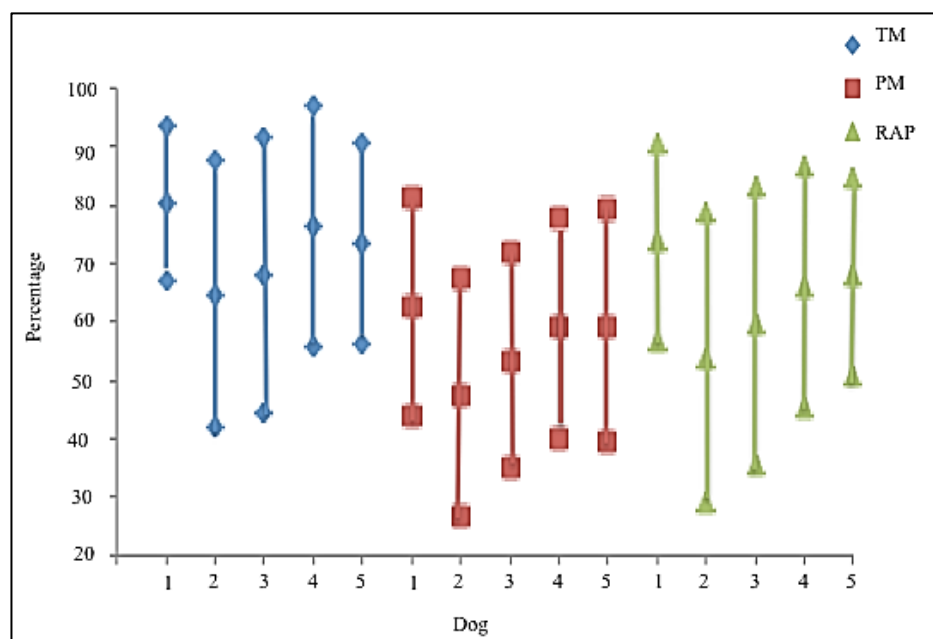
7

8 **Table 3**

9 Mean  $\pm$  standard error of the drop (frozen minus fresh data) in viable sperm cells  
 10 percentage for the parameters of MT e MP e RAP sperm membrane fluidity  
 11 (YP/M540), phosphatidyl serine translocation (AN/PI), membrane and acrosomal  
 12 integrity (FITC PSA/PI), mitochondrial potential (HMP) and membrane  
 13 lipoperoxidation (LPO) from each dog, analyzed on frozen/thawed semen.

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
MT	7.00 ± 1.87	23.25 ± 10.22	18.75 ± 6.98	11.50 ± 4.57	11.75 ± 3.20
MP	8.25 ± 2.43	24.25 ± 11.00	17.00 ± 4.74	11.00 ± 4.56	10.50 ± 5.49
RAP	9.00 ± 1.47	29.75 ± 12.44	25.50 ± 6.99	18.00 ± 2.86	12.00 ± 5.49
YP-/M540-	3.20 ± 0.48	4.43 ± 0.75	2.45 ± 2.15	3.20 ± 0.01	6.83 ± 0.67
MPI	44.20 ± 4.53a	55.30 ± 8.34abc	63.75 ± 3.02c	38.70 ± 7.90a	61.83 ± 3.78c
HMP	33.97 ± 3.59a	47.15 ± 0.52b	51.20 ± 2.43bc	57.40 ± 3.02c	45.48 ± 4.15b
FITC PSA-/PI-	28.18 ± 2.24a	58.03 ± 2.46bc	65.93 ± 5.43b	66.95 ± 2.26b	53.80 ± 3.48c
AN-/PI-	27.68 ± 5.23	61.30 ± 3.75	67.43 ± 6.17	51.80 ± 17.31	55.33 ± 2.89
LPO	14.05 ± 6.35	13.67 ± 11.98	24.70 ± 12.10	18.35 ± 12.75	11.60 ± 6.20

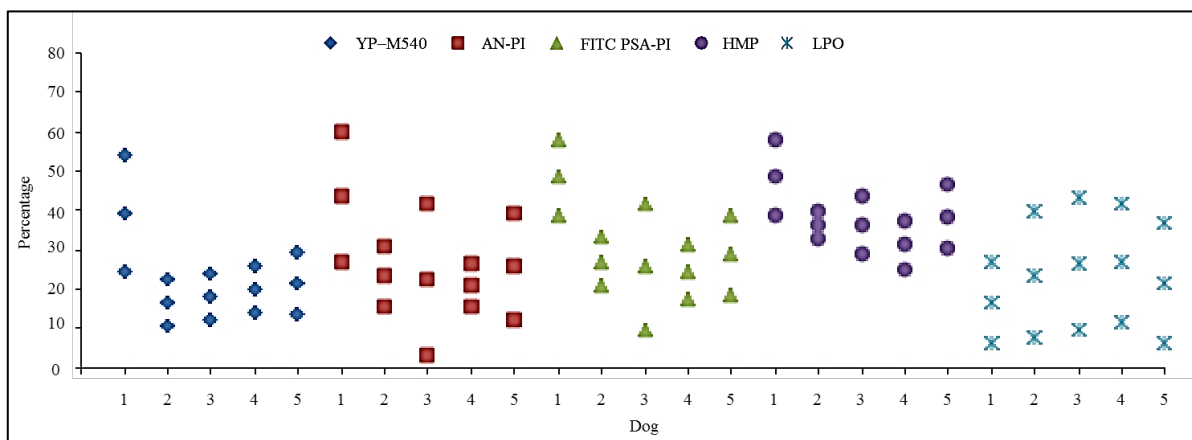
- 1 Within a column, values with different superscripts for each parameter differ
- 2 significantly:  $P < 0.05$ .
- 3



- 4
- 5 **Fig. 1.** Average response of the animal and minimum and maximum limits (with 95%
- 6 confidence interval) for the parameters of total motility (TM), progressive motility (PM)

1 and rapid cells (RAP) from each dog, analyzed on frozen/thawed semen.

2



3

4 **Fig. 2.** Average response of the animal and minimum and maximum limits (with 95%  
5 confidence interval) for the parameters of sperm membrane fluidity (YP/M540),  
6 phosphatidyl serine translocation (AN/PI), membrane and acrosomal integrity (FITC  
7 PSA/PI), mitochondrial potential (HMP) and membrane lipoperoxidation (LPO) from  
8 each dog, analyzed on frozen/thawed semen.

9

#### 10 **Table 4**

11 Pregnancy rate according to sperm concentration

Dog	Total sperm concentration/AI	Number of bitches inseminated	Pregnancy rate %
1	160x10 <sup>6</sup>	6	0% <sup>b</sup>
1	450x10 <sup>6</sup>	4	0% <sup>b</sup>
Pool	160x10 <sup>6</sup>	4	0% <sup>b</sup>
Pool	450x10 <sup>6</sup>	6	50% <sup>a</sup>

12 Within a row values with different superscripts differ significantly:  $P < 0.01$

13

#### 14 **4. Discussion**

1           In the present study we investigated the effects of cryopreservation on the canine  
2 sperm and the relation of the individual results of freezability, sperm concentration in  
3 AI and fertility. A decline in motility population occurred after cryopreservation as well  
4 as of the membrane integrity (Kim et al., 2010; Rota et al., 2010). We hypothesized that  
5 the dog that displayed better semen quality will result also in a better fertility, which  
6 was not confirmed, even when a greater sperm concentration was used. We thought that  
7 this fact could be due to infertility or sperm DNA fragmentation after freeze/thaw. As  
8 describe for other species as humans, bovine and equine, semen displaying high number  
9 of sperm with DNA fragmentation lead to low or no negative fertility rates (Aurich et  
10 al., n.d.; Fraczek et al., 2013; Simões et al., 2013). In dogs no correlation was found  
11 between the cryopreservation and DNA fragmentation (Koderle et al., 2009; Urbano et  
12 al., 2013). Trying to explain the negative fertility results from Dog 1 we investigated  
13 DNA fragmentation using acridine orange staining test but no difference from fresh to  
14 thawed semen were observed. Also, to exclude infertility, two bitches (that were not  
15 include on methodology) were inseminated with fresh semen from dog 1 and one of  
16 then became pregnant, what were confirmed on ovariohysterectomy 30 days after AI. In  
17 boars semen freezing/thawing facilitates destabilization of sperm chromatin structures,  
18 making the sperm more vulnerable to DNA fragmentation (Fraser and Strzeżek, 2007).  
19 Urbano et al. (2013) found that after 24h of incubation, on body temperature, sperm  
20 undergo sperm DNA fragmentation. The authors comment that more time of incubation  
21 may be necessary to proper evaluation of DNA fragmentation. The time of AI in the  
22 present study was comparable with that described in previous studies (Hye et al., 2007;  
23 Mason and Rous, 2014; Steckler et al., 2013) and although some authors comment that  
24 when the right time is used a second TCAI is not necessary. We decided to perform two  
25 AIs within 24 h to guaranty that the oocytes were ready for fertilization at the time of

1 TCAI. In that manner we speculate that other sperm functional problem may happen  
2 with the sperm of this dog after freeze/thawing. Cryopreservation process, including  
3 cooling, affects sperm cells in either lethal or sub-lethal aspect and sub-lethal alterations  
4 accelerates sperm function, which impair cell lifetime. Burgess et al., (2012) observed  
5 that after thawing the sperm lose the capacity of adhesion on epithelial cell of the  
6 oviduct; the authors comment that this fact is due to an increase in the number of sperm  
7 with capacitation like-changes and acrosome reacted. Increased sperm velocity was  
8 observed after dog sperm cryopreservation and this fact could be due to sperm  
9 capacitation like-changes as well (Urbano et al., 2013).

10 Therefore, subtle changes on membrane permeability, as detected by YP and  
11 M540 on the present study, permits that the sperm still mobile but could make the cell  
12 more readily to undergo to acrosome reaction and die (Alhaider and Watson, 2009).  
13 Despite capacitation to be a physiological event that able sperm to be ready to interact  
14 with the oocyte it should not happen prematurely.

15 Despite acceptable motility rates observed after thaw, cryopreservation of  
16 spermatozoa also had an effect on sperm mitochondria. On the present study a  
17 decreased mitochondrial activity potential occurred after cooling and thawing, which  
18 could influence directly sperm motility. However, currently, the ATPs from glycolysis  
19 instead of oxidative phosphorylation that are essentials for the motility hyperactivation,  
20 tyrosine phosphorylation, binding to the zona pellucida and acrosomal reaction (Câmara  
21 and Guerra, 2008; Petrunkina and Harrison, 2011). On the other hand decreased  
22 mitochondrial activity could be due to apoptosis, that lead to opening of mitochondrial  
23 pores, and liberates pro-apoptotic factors on the cytoplasm (Nizański et al., 2012).

24 In our study, the LPO suffered a significant change observed comparing cooled to  
25 thawed semen. An individual difference on LPO were observed in dogs in the fresh

1 semen and the difference remained for one of the tested dogs after semen  
2 cryopreservation, showing that analysis of LPO could be an interesting tool to  
3 determine good and bad semen freezers (Neagu et al., 2011).

4         The increase of sperm concentration from  $160 \times 10^6$  to  $450 \times 10^6$  sperm cells/AI  
5 enhance the number of viable cells used (from  $37 \times 10^6$  to  $104 \times 10^6$  sperm cells/AI,  
6 respectively), considering the mean of sperm PM (55%) and plasma membrane integrity  
7 (42%). This increase was positive and 50% of pregnancy was observed in the group of  
8 bitches inseminated with the pool of semen when  $450 \times 10^6$  sperm cells was used for  
9 AI. These results are in agreement with other authors that use more than  $100 \times 10^6$  of  
10 motile and viable sperm cells/AI and also observed better pregnancy results (Mason and  
11 Rous, 2014; Mickelsen et al., 1993). Negative individual pregnancy rates were before  
12 observed in dogs (Suwa et al., 2009) that displayed high sperm motility after thaw,  
13 although  $200 \times 10^6$  used for TCAI. However the author didn't evaluated sperm  
14 membrane integrity. In bovine, fertility was significantly correlated only with the  
15 percentage of morphologically normal spermatozoa, which confirm the importance to  
16 have high numbers of morphologically normal spermatozoa in AI-doses (Nagy et al.,  
17 2013). According to Kastelic (2013), fertility varies substantially among individuals. In  
18 that manner, methods to predict fertility are better for identifying individuals with low  
19 than for good fertility. Also, males differ in the numbers of sperm required to reach their  
20 maximum fertilization rate (Saacke et al., 2000). There are some males that require  
21 more sperm to have good fertility and those are considered as compensable. Some  
22 sperm abnormalities can be overcome by increasing the dose used for artificial  
23 insemination. In contrast, increasing the insemination dose does not improve fertility for  
24 uncompensable defects, as morphological ones, implying that the sperm are able to  
25 cause fertilization and initiate development, but they do not sustain embryogenesis

1 (Kastelic, 2013; Saacke et al., 2000). We believe that this is the fact of dog1, that  
2 despite greater semen quality, the fertility rate was negative, even with enhanced sperm  
3 concentration for AI.

4 Dog 1 presented the least variation and drop in the results of sperm analysis  
5 from fresh for thawed semen. Nevertheless, the fertility of this individual male was  
6 negative, thus we believe that fertility can be attributed to a number of factors and  
7 analysis, but we were not able to determine them in the present study. Moreover, this  
8 animal had lower drop on the values of plasma and acrosomal integrity, which is  
9 generally one of the most important parameters to determine the freezeability of semen  
10 (Alhaider and Watson, 2009). However, in this situation there was no relationship  
11 between good freezeability and fertility, which let us rethink what analyzes are really  
12 important to determine that. As there is no effort aimed to genetical improvement in  
13 dogs, each male must be assessed individually.

14 Based on our results we conclude that cryopreservation leads to structural and  
15 functional changes in sperm cell, which explains their short survival after thawing. The  
16 negative fertility observed in dog 1 could be due to cryo-capacitation like changes that  
17 affected sperm transport and the capacity of adhesion on epithelial cell of the oviduct.  
18 Greater pregnancy rates were obtained when more than  $100 \times 10^6$  of motile and viable  
19 thawed sperm cells/AI was used. Dog fertility is not related to semen parameters  
20 evaluated by the analysis described in the present study.

21

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24

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## Capítulo 4

### **Considerações finais**

### **Conclusões finais**

1. Concluímos que o ejaculado de cães possui diferentes populações espermáticas, o que pôde ser confirmado pelas análises feitas pela citometria de fluxo caracterizadas pelas diversas funções exercidas pelo espermatozoide.
2. As populações espermáticas são alteradas pelo processo de criopreservação – refrigeração e congelação/descongelação, levando a diminuição da população das células íntegras frequentemente por modificações na integridade da membrana plasmática.
3. Ocorreu uma diminuição da população das células íntegras gerada pelo processo de criopreservação em todos os animais avaliados.
4. A qualidade seminal pós congelação, avaliada pelas técnicas descritas no presente estudo, não esta relacionada as taxas de fertilidade. O cão 1 não foi o de melhor fertilidade como o esperado, nem mesmo com o aumento da dose inseminante.
5. O número de espermatozoides viáveis utilizados é fundamental para o sucesso da IATC com sêmen congelado em cães.

ANEXOS

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Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

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- Either cite a P value (recommended for Abstract and for Results) or use the term 'significant' (recommended for Discussion), but generally avoid doing both.
- Terms with a specific statistical meaning (i.e. significant, tended and correlated), should only be used in a strict statistical context.
- Numbers less than 10 are written as a word, unless followed by an abbreviation for unit of measure, e.g. five embryos, 5 min

### ***Use the following expressions***

- transrectal palpation, not rectal palpation
- nucleus transfer, not nuclear transplant
- estrus (noun) synchronization, but, estrous (adjective) behavior
- sperm can be used as both noun and adjective
- 120 to 125, not 120-125
- treatment by period, not treatment X period
- gravity: 100 X g (in lieu of speed for centrifugation)
- magnification: X 100
- identification number of an animal: No. 10, but 30 animals: n = 30
- 3 d, Day 3 (define Day 0)

### ***Standard definitions***

- oogonium: female gamete before meiosis
- oocyte, primary: female gamete from onset of the first maturation division (meiosis) to extrusion of the first polar body
- oocyte secondary: female gamete from onset of second meiosis to extrusion of the second polar body
- ovum: female gamete from the end of both meiotic divisions until the union of the male and female pronuclei (differs from the common use of ovum as a general term for any female gamete)
- germinal vesicle: nucleus of the ovum
- zygote: a fertilized ovum, from fusion of the male and female gamete to completion of first cleavage
- embryo: a conceptus from the 2-cell stage to the stage when cell migration and differentiation are largely complete
- fetus: a conceptus after organogenesis is mostly complete (primarily increasing in size)
- conceptus: an embryo or fetus with all its membranes and accessory structures
- abortion: expulsion of a conceptus incapable of independent life
- premature parturition: expulsion (before full term) of a conceptus capable of

independent life

- stillbirth: avoid this term (use fetal death or abortion)

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The following abbreviations may be used in the text without definition (note that abbreviations exclude periods):

AI	ANOVA	ADP	ATP	BSA
cAMP	CL	DEAE-cellulose	DMSO	DNA
eCG	EDTA	EGF	ELISA	FSH
GH	GnRH	hCG	HEPE	ShMG
IVC	IVF	IVM	LH	MOET
MSH	mRNA	NAD	NADH	PBS
PGF2 $\alpha$	PGFM	PIPES	PRID	PRL
RIA	RNA	SDS-PAGE	SCNT	TRH
TRIS	tRNA	TSH		

### *Units of Measure*

cpm - counts per min  
 dpm - disintegrations per min  
 g - gram  
 ga - gauge of hypodermic needle  
 h - hour  
 kg - kilogram  
 L - liter  
 mL - milliliter  
 $\mu$ L - microliter  
 m - meter  
 min - minute  
 mo - month  
 s - second  
 v:v - volume ratio  
 wk - week  
 wt/vol - weight per volume  
 y - year

### *Routes of treatment*

id - intradermal  
im - intramuscular  
iu - intrauterine  
iv - intravenous  
sc - subcutaneous  
po - oral

*Statistical expressions*

ANOVA - analysis of variance  
CV - coefficient of variation  
df - degrees of freedom  
F - variance ratio  
NS - not significant  
P - probability  
SD - standard deviation  
SEM - standard error of the mean  
r - correlation coefficient  
r<sup>2</sup> - coefficient of regression

# ANIMAL REPRODUCTION SCIENCE

An International Journal

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- **Audience p.1**
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## DESCRIPTION

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ISSN: 0378-4320

*Animal Reproduction Science* publishes original research and timely reviews on topics relating to **reproduction** and **fertility in animals**. This includes both fundamental research and applied studies, including management practices that increase our understanding of the **biology** and **manipulation** of reproduction. The focus is on animals that are useful to humans including food- and fibre-producing; companion/recreational; captive; and endangered species including zoo animals, but excluding laboratory animals unless the study reveals new information that impacts our basic understanding of the biology or manipulation of reproduction applicable to the animals listed above.

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## IMPACT FACTOR

2015: 1.377 © Thomson Reuters Journal Citation Reports 2016

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