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Seminal plasma interference in the kinetics and viability of cryopreserved canine sperm

Interferência do plasma seminal na cinética e viabilidade de espermatozoides caninos criopreservados

Interferencia del plasma seminal en la cinética y viabilidad de los espermatozoides caninos criopreservados

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Abstract

Most researchers have frozen dog semen using methodologies described for other species. Consequently, these studies have shown that the thawed semen of dogs is of low quality, with conception rates lower than that of other species. Therefore, in this study, we evaluated different freezing protocols for canine semen, using three adult Bulldog Campeiro males, aged 2 to 5 years, with proven fertility. Five semen samples were collected from each animal using the penile bulb digital manipulation method. The collected samples were divided into: group 1, the samples were diluted directly in Botudog® commercial freezing medium (Botupharma Biotecnologia Animal) and group 2, the samples were centrifuged at 600 g for 10 min and the pellet was resuspended in Botudog®, totaling a concentration end of 200 x 10^6 sperm per ml. The samples were packaged in 0.5 mL straws at a concentration of 100 x 10^6 viable sperm. The samples remained for 1 h in stabilization at 4 °C and transferred to nitrogen vapor for 20 min, immersed in nitrogen, stored in a cryogenic cylinder, and thawed at 46 °C for 20 s. It was found that the total motility (MT, %), path speed (VAP, µm/s), progressive motility (PM, %), progressive linear speed (VSL; µm/s), curvilinear speed (VCL; µm/s), linearity (LIN, %), percentage of fast sperm (RAP, %), and plasma and acrosomal membrane integrity were higher in group 1, with samples not being centrifuged. These data demonstrate that the canine semen freezing protocol, using the Botudog® diluent, does not recommend the centrifugation of the ejaculate, prior to freezing.

Keywords: Canine semen; Freezing; Sperm analysis.

Resumo

A maioria dos pesquisadores tem congelado o sêmen de cães, utilizando metodologia descrita para outras espécies. Assim, estes estudos têm demonstrado que o sêmen descongelado de cães é de baixa qualidade, com taxas de concepção inferiores a de outras espécies. Assim, este

trabalho avaliou diferentes protocolos de congelação para sêmen canino, utilizando 3 machos, adultos, da raça Buldogue Campeiro, com idades entre 2 a 5 anos e fertilidade comprovada. Realizou-se 5 colheitas de sêmen de cada animal, pelo método de manipulação digital do bulbo peniano. As amostras colhidas foram divididas em: grupo 1, as amostras foram diluídas diretamente em meio de congelação comercial Botudog® (Botupharma Biotecnologia Animal) e grupo 2, as amostras foram centrifugadas a 600g/ 10min e o *pellet* ressuspendido em Botudog®, totalizando uma concentração final de $200x10^6$ espermatozoides por mL. As amostras foram envasadas em palhetas de 0,5 mL com concentração de $100x10^6$ espermatozoides viáveis. As amostras permaneceram por 1 hora em estabilização a 4ºC e transferidas para o vapor de nitrogênio durante 20 minutos, por fim, mergulhadas em nitrogênio e armazenadas em botijão criogênico e descongeladas a 46ºC/20s. Verificou-se que as motilidade total (%), motilidade progressiva (%), velocidade de trajeto (VAP, µm/s), velocidade linear progressiva (VSL; µm/s), velocidade curvilínea (VCL; µm/s), linearidade (%), percentagem de espermatozoides rápidos (%) e integridade de membrana plasmática e acrossomal foram superiores no grupo 1, onde as amostras não foram centrifugadas. Estes dados demonstram que o protocolo para congelação de sêmen canino, utilizando o diluente Botudog®, não preconiza a centrifugação do ejaculado, previamente a congelação.
Palavras-chave: Análises espermáticas; Congelação; Sêmen canino.

Resumen

La mayoría de los investigadores han congelado el semen de perro, utilizando la metodología descrita para otras especies. Así, estos estudios han demostrado que el semen descongelado de los perros es de baja calidad, con tasas de concepción inferiores a las de otras especies. Así, este trabajo evaluó diferentes protocolos de congelación de semen canino, utilizando 3 machos adultos Bulldog Campeiro, de 2 a 5 años y con fertilidad comprobada. Se tomaron cinco muestras de semen de cada animal, utilizando el método de manipulación digital del bulbo del pene. Las muestras recolectadas se dividieron en: grupo 1, las muestras se diluyeron directamente en medio congelador comercial Botudog® (Botupharma Biotecnologia Animal) y grupo 2, las muestras se centrifugaron a 600g / 10min y el pellet se resuspendió en Botudog®, totalizando una concentración final de 200x106 espermatozoides por ml. Las muestras se empaquetaron en pajuelas de 0.5 mL con una concentración de 100x106 espermatozoides viables. Las muestras permanecieron 1 hora en estabilización a 4ºC y se transfirieron a vapor de nitrógeno durante 20 minutos, finalmente, se sumergieron en nitrógeno y se almacenaron en cilindro criogénico y se descongelaron a 46ºC / 20 s. Se

encontró que la motilidad total (%), la velocidade progressiva (%), la velocidad de trajeto (VAP, µm/s), la velocidad lineal progresiva (VSL; µm / s), la velocidad curvilínea (VCL; µm / s), la linealidad (%), el porcentaje de espermatozoides rápidos (%) y la integridad de la membrana plasmática y acrosomal fueron mayores en el grupo 1, y las muestras no se centrifugaron. Estos datos demuestran que el protocolo para la congelación de semen canino, utilizando el diluyente Botudog®, no recomienda la centrifugación del eyaculado antes de la congelación.

Palabras clave: Análisis de esperma; Congelación; Semen canino.

1. Introduction

The first successful freezing of canine semen was described by Rowson (1954), while Platz & Seager (1977) reported the first pregnancy resulting from artificial insemination (AI) with the use of frozen canine semen. Since then, studies have been carried out to determine the best conditions for the cryopreservation of canine sperm. However, many factors interfere with the semen refrigeration and freezing process of dogs, and no methodology seems to be ideal for all dogs and all ejaculates (Souza et al., 2007).

In this context, some dogs have semen with greater resistance to the cryopreservation process. This can occur due to genetic differences in the composition of the sperm cell membrane, resulting in better or worse cryoprotectant permeability, changes in water movement, intracellular ice formation, and cell death (Jarosz, et al., 2016). This confirms that individual variability in this species is of great importance (Mascarenhas, et al., 2014).

Most researchers have frozen dog semen using the methodology described for other species. Thus, they demonstrated that the thawed semen of dogs is of low quality, and has conception rates lower than those of other species. Therefore, there is a limitation in the use of frozen semen. Another factor affecting fertility rates of cryopreserved canine semen is the difficulty in identifying the ideal time for artificial insemination in females and the location of semen deposition in the female genital tract (England, 1993).

In addition, genetic mapping studies in dogs have shown similarity between genetic changes in this species and in humans, favoring the use of the dog as an experimental model. Thus, it is necessary to standardize the cryopreservation protocols of canine sperm cells to adapt mainly to the those of the human sperm (Thirumala, et al., 2003; Souza, et al., 2007).

In addition to the importance of the dog as an experimental model, the reasons for using frozen semen are numerous, such as the possibility of transporting semen over long

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distances, preserving it for an indefinite period, and permitting the reduction of expenses, which would occur with the need to move the animal. It also reduces the need of sending the female to the place where the male is, which is an exhausting procedure that can reflect on the fertility rate (Puja, et al., 2019). Moreover, a study on the cryopreservation of canine semen can serve as a basis for research on endangered canid species, such as the Brazilian maned wolf (*Chrysocyon brachyurus*), field fox (*Pseudalopex sp.)* (Silva, et al., 2000) and the dog do mato (*Cerdocyon thous*) (Soares, et al., 2002).

Therefore, this study aimed to evaluate the kinetics and sperm viability of canine semen subjected to two freezing protocols with and without centrifugation of the ejaculates and subsequent dilution in the commercial Botudog® medium.

2. Material and Methods

This experimental study was approved by the Research Ethics Committee Involving Institutional Experimental Animal, protocol number 34604/2018, approved on March 21, 2018.

The harvesting and evaluation of fresh semen were carried out at the Animal Reproduction Laboratory of UNIPAR, municipality of Umuarama, state of Paraná, Brazil.

Sperm kinetic and viability evaluations were performed using the CASA system at the Faculty of Veterinary Medicine and Animal Science, São Paulo State University, in the Department of Animal Reproduction and Veterinary Radiology, located in the city of Botucatu, state of São Paulo, Brazil.

Five semen collections were obtained from three sexually mature animals of Bulldog Campeiro breed aged between 2 and 5 years, using the method of digital manipulation of the penile bulb, with weekly intervals, totaling 15 harvests.

After semen collection, the samples were subjectively evaluated under an optical microscope for total motility (TM, %) and sperm vigor (1-5). Subsequently, the sperm concentration was evaluated in a Neubauer chamber at a dilution of 10 μL of the ejaculate in 190 μL of 10% formaldehyde saline.

Dogs considered fit for the experiment had total motility ≥ 90%, vigor 3 (rating 1-5), and sperm concentration ≥ 200 × 106 sperm per ejaculate.

After the initial screening, each ejaculate was divided into two rates, each of them: 1) Group 1: samples diluted directly in the commercial freezing medium Botudog® (Botupharma Animal Biotechnology).

2) Group 2: The samples were centrifuged at 600 g for 10 min, and then the pellet was resuspended in the commercial Botudog® freezing medium.

The sperm concentration in the diluted samples was adjusted to 200 million total spermatozoa per mL of diluter and filled in 0.5 mL French straws (IMV® TecnologiesL'Aigle Cedex, France), previously identified with the animal's name and harvest number. Thus, each reed contained a total of 100 × 106 sperm. The samples were destined for refrigeration, arranged in a Styrofoam box (Botuflex®, Botupharma Biotecnologia Animal), previously refrigerated at 5 ºC and maintained for 1 h at a temperature of 5 ºC. Next, a freezing curve was generated on the liquid nitrogen vapor (N2L) by packing the straws in a conventional 40 L polystyrene box at a fixed distance of 6 cm from the N2L level, with the N2L column being 4 cm, remaining for a period of 20 min. After this period, the straws were directly immersed in liquid nitrogen, frozen, and then racked for storage in cryobiological cylinders. For the standardization of post-thaw analyses, the analyses were started after a minimum period of three days of storage (Crespilho, et al. 2014).

Post-thaw sperm analysis

For all analyses, the straws were thawed in a water bath at 46 °C for 15 s. Post-thaw analyses of sperm kinetics were then performed using the CASA system (HTM – IVOS 12, Hamilton ThorneResearch, Beverly, MA, USA) and evaluation of the plasma and acrosomal membrane integrity by flow cytometry using the BD LSR Fortessa (Becton Dickinson, Mountain View, CA, USA). For the evaluation of sperm kinetics, five fields were analyzed for each sample, with the aid of the CASA system (HTM – IVOS 12, Hamilton Thorne Research, Beverly, MA, USA).

The parameters evaluated were: total sperm motility (MT, %), progressive sperm motility (MP, %), path speed (VAP, μm/s), linearity (LIN, %), progressive linear speed (VSL, μm/s), curvilinear speed (VCL, μm/s), and percentage of sperm with rapid movement (RAP, %).

Evaluation of sperm characteristics by Flow Cytometry

For flow cytometry analyses, the BD LSR Fortessa equipment (Becton Dickinson, Mountain View, CA, USA) was used, equipped with lasers: blue 488 nm, 100 mW, red 640 nm, 40 mW and violet 405 nm, 100 mW. The data were evaluated using BD FACSDiva ™

software v6.1 and WinList 6.0 (Verify software house). The samples were analyzed at an acquisition rate of 800 events/s, with 10 000 cells per sample. Cellular debris and particles were excluded from the acquisition and analysis by adjusting the threshold and by marking with Hoescht 33342 (100µg / mL) excited by the violet laser.

Simultaneous assessment of plasma and acrosomal membranes

The combination of the propidium iodide (P4170 - Sigma Aldrich), FITC-PSA (L0770 - Sigma Aldrich), and Hoechst 33342 (14533 - Sigma Aldrich) probes was used. For each 200 µL sample of semen diluted in TALP-PVA at a concentration of 5 × 106 sperm/mL, 5 µL of H3342 (100 µg/mL), 5 µL of IP (50 µg/mL), and 1 µL of FITC-PSA were added (100µg / mL), and then homogenized and incubated for 15 min in the dark at 37 °C.

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System program (SAS, Institute Inc., 1999; Cary, USA). First, the Shapiro-Wilk test (Proc-Univariate) was used for normal data analysis and the Chi-Square test (Proc-GLM) for the analysis of homogeneity of variations. Means and standard deviations of the experimental groups and their relations with the study groups were determined using analysis of variance (Proc-GLM) with a significance level of 5% ($P < 0.05$).

3. Results

The data of sperm kinetics after the freeze/thaw process in a commercial Botudog® thinner, using the following parameters: MT, MP, VAP, LIN, VSL, VCL, and RAP, are described in Table 2.

According to the data presented in Table 1, significant differences were observed in all parameters between the groups studied ($P<0.05$), showing that the non-centrifugation of the canine ejaculate provided better parameters for sperm kinetics in samples subjected to freezing in the commercial Botudog® diluting medium. Sperm viability assessment was used for the evaluation of cell viability of the evaluated groups and the plasma and acrosomal membrane integrity parameters. In group 1 (not centrifuged), we found a higher percentage of intact cells than in group 2 (centrifuged) ($P <0.05$) (Table 2).

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Table 1. Mean values and standard deviation of total sperm motility (TM, %), progressive sperm motility (PM, %), path speed (VAP, µm/s), linearity (LIN, %), progressive linear speed (VSL, µm/s), curvilinear speed (VCL, µm/s), and percentage of sperm with rapid movement (RAP, %) frozen / thawed dog.

	Group 1 (NC)	Group 2 (C)
TM (%)	53,23 ± 8,0 [a]	47,08 ± 10,82 [b]
PM (%)	33,0 ± 6,63 [a]	32,15 ± 8,91 [b]
LIN (%)	61,15 ± 7,61 [a]	56,0 ± 11,28 [b]
VAP µm/s	72,72 ± 4,22 [a]	70,46 ± 6,10 [b]
VCL µm/s	78,57 ± 16,6 [a]	76,27 ± 16,04 [b]
VSL µm/s	68,78 ± 5,97 [a]	66,80 ± 7,63 [b]
RAP (%)	46,69 ± 5,71 [a]	37,69 ± 6,22 [b]

Group 1 (NC) = not centrifuged e 2 (C) = centrifuged. Different lowercase letters on the same line indicate significant difference ($P < 0.05$). Source: Authors.

Table 2. Mean and standard deviation of percentage of intact plasma and acrosomal membranes (IPAM, %), in the Group 1 and Group 2.

	1 (NC)	2 (C)
IPAM (%)	52,28 ± 8,41 [a]	41,93 ± 14,48 [b]

Group 1 (NC) = not centrifuged e 2 (C) = centrifuged. Different lowercase letters on the same line indicate significant difference ($P < 0.05$). Source: Authors.

4. Discussion

The use of frozen canine semen has the advantage of maintaining the fertilizing capacity in animals of high genetic interest, for an indeterminate period, in addition to protecting them from the stress caused by transport for mating purposes. Moreover, it permits access to cryopreserved genetic material from dogs around the world (Silva, et al., 2003).

Consequently, cryopreservation of canine semen has been widely used despite the difficulties encountered during this process, with protocols that maintain sperm kinetics and viability, making this biotechnology commercially applicable (Silva, et al., 1995; Lima, et al., 2019).

Thus, several methodologies have been described for the freezing of dog semen, with various tested diluting media, protocols, and different freezing speeds. Regarding the diluting media, the most described ones use citrate, skimmed milk, TRIS, Lactose, Clone, Laiciphos 478®, Biociphos W482®, and a diluent based on natural coconut water (DBAC) and powder (ACP-106 and ACP106c). However, the results are controversial (Santos, et al., 2016). In our experimental study, the commercial Botudog® dilution medium, composed of egg yolk and glycerol, was used, and showed encouraging results, given the post-thaw sperm quality, compared to the traditional TRIS-yolk medium, as described by Lima, et al. (2019).

Another important observation for the cryopreservation of canine semen is at the time of ejaculate collection. Previous studies report that the canine ejaculate must be collected in fractions for the control of the prostatic fluid (Mascarenhas, et al., 2014), since its separation during the harvest seems to be a relatively easy process (Rijsselaere, et al., 2002, Verstegen, et al., 2005). However, in practice, there may be a mixture of fractions and centrifugation would be the solution to such a problem, in addition to promoting the concentration of sperm. Associated with this, the total concentration of sperm in the ejaculate is influenced by age, frequency of ejaculation, libido, and the size of the testis and its daily sperm production (Amann, 1981; Olar, et al., 1983; Kutzer, 2005).

In the present study, it was observed that the centrifugation process increased the percentage of sperm with damage to the plasma and acrosomal membrane integrity as well as decreased sperm kinetic parameters such as TM, PM, VAP, LIN, VSL, VCL, and RAP.

In this light, the centrifugation for 10 min at 600 × g, used in the present study, impaired the kinetics and viability of canine sperm. Associated with this, the diluting medium used in this study, Botudog®, had no effect on the damage caused by centrifugation. This shows that seminal plasma has beneficial effects on sperm kinetic and viability parameters. Other authors reported that seminal plasma prevents the formation of lipid peroxides that are harmful for the sperm membrane (Ochsendorf, 1999). However, controversial results have been reported in the literature; (Mascarenhas, 2014) and (Aurich, 2005) diverged from our studies when they reported that prolonged contact of sperm with seminal plasma is associated with decreased sperm motility and viability.

Other authors also reported that proteins and other components of canine seminal plasma have an affinity for heparin, which is indispensable for sperm formation and resistance of spermatozoa to cryopreservation, in addition to being related to individual differences in resistance to cryoinjuries (Threlfall, 2003; Kutzler, 2005; Souza, et al., 2007).

Seminal plasma is a complex biological fluid composed of ions (sodium, calcium, potassium, magnesium, and chlorine), energetic substrates (fructose, sorbitol, glycerylphosphorylcholine), and organic compounds (citric acid, amino acids, peptides, proteins, lipids, hormones, and cytokines) (Nguyen, et al., 2019). In addition, seminal plasma is essential for the survival of sperm in the female genital tract. Although studies have shown that seminal plasma is considered beneficial for the integrity of the plasma, acrosomal and mitochondrial membranes, there are reports that its presence in the ejaculate leads to a decrease in sperm motility (Rota, et al. 1995; Treulen, et al. 2012; Hori, et al. 2017), different from that observed in the present study, where the removal of seminal plasma by centrifugation at 600 × g for 10 min impaired sperm kinetics and viability.

Some authors explained that the reduction in sperm motility may be due to a decrease in the concentration of adenosine triphosphate in seminal plasma due to the activity of acid and alkaline phosphatase (Gunzel-Apel & Ekrod, 1991). Moreover, centrifugation and removal of the seminal plasma before dilution with cryoprotectants has no detrimental effect on function in refrigerated canine sperm (Goericke-Pesch, et al. 2012). The use of suitable cryoprotectants for the equine species was reported by Lima et al. (2019) when comparing the traditional TRIS-gemma and Botudog® media. These authors found that the Botudog diluting medium was more effective in maintaining the kinetics and viability of thawed canine semen.

Much has been discussed about the centrifugation of the ejaculate and resuspension of the pellet in the canine species, which is inherent to most semen cryopreservation protocols, associated with mechanical and oxidative injuries that are reflected in the loss of motility, as observed in the present study and semen fertility (Rijsselaere, et al., 2002; Aurich, 2005). If, on the one hand, the damage associated with centrifugation is proportional to its speed and duration, on the other hand, very gentle centrifugation will result in the loss of sperm, which remains suspended and is eliminated in the supernatant (Rijsselaere, et al., 2002).

Cunha and Lopes (1999) described that centrifugation at 800 × g for 15 min did not affect the quality of fresh canine semen, unlike that observed in the present study, in which centrifugation for 10 min at 600 × g was used (Tables 1 and 2). Rijsselaere, et al. (2002) evaluated the effect of four centrifugation speeds on semen quality and the loss of cells in the supernatant and concluded that centrifugation at 720 × g for 5 min resulted in minimal loss of cells without altering the seminal quality. Davis, et al. (2007) measured the concentration of reactive oxygen species (ROS) before and after centrifuging dog semen at 700 × g for 10 min and found a 200% increase in ROS concentration after centrifugation.

5. Conclusion

In conclusion, the canine semen freezing protocol, using the Botudog® diluent, does not recommend the centrifugation of the ejaculate prior to freezing.

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Carlos Renato de Freitas Guaitolini – 20%

Rosiara Rosaria Dias Maziero – 20%

André Maciel Crespilho – 10%

Camila de Paula Freitas Dell´Aqua – 10%

Maria Isabel Mello Martins – 10%

Gabriela Passamani da Cruz – 5%

Marcio Luiz Denck Tramontin – 5%