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Effect of different thawing times of equine semen on the motility, vigor, morphology, and membrane integrity of the sperm cell

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Summary: The objective of this study was to examine the changes in motility, vigor, morphology, and cell membrane integrity of frozen equine spermatozoa subjected to three different thawing times. In total, 36 straws (0.5 mL of frozen semen) from 12 different stallions of four horse breeds (Appaloosa, Crioula, Mangalarga Marchador, and American Quarter Horse) were evaluated. Frozen semen straws were randomly distributed into three groups: (1) thawing for 30 s at 37 °C (G3037, n = 12), (2) thawing for 20 s at 46 °C (G2046, n = 12), and (3) thawing for 60 s at 37 °C (G6037, n = 12). To standardize sample evaluation, the semen from the straws was placed on a glass slide and sealed with a coverslip after thawing. Subsequently, the samples were filmed for 30 s with an optical microscope coupled to a high-resolution camera. Three experienced technicians evaluated the semen separately using the same film and parameters. No significant differences in motility, vigor, morphology, or cell membrane integrity were observed between groups. Therefore, we concluded that equine semen can be thawed at the temperatures and times applied in this study without having any negative effect on motility, vigor, morphology, or cell membrane integrity.

Keywords: stallion, frozen semen, membrane integrity, sperm cryopreservation, thaw semen

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Introduction

Equideoculture has had significant growth in the national and international markets in recent decades. Echinoculture has a relevant role in the Brazilian economy as it directly employs more than 640000 people, indirectly generates 3.2 million jobs, and brings approximately 4.2 billion US dollars per year into the economy (*Lima* et al. 2006). Brazil has the third largest horse population on the planet, with high performance animals mainly produced. Alternative methods to improve genetics and reproductive rates are sought after, and the use of reproductive biotechnologies such as artificial insemination (AI) with frozen semen is encouraged (*Papa* et al. 2005).

AI has been successful in reproductive practices, which has led to increasing interest in improving the programs that employ this technology. Semen freezing studies aim to improve the performance of stallions, find favorable conditions for transporting semen, reduce disease transmission, and allow the use of genetic material for an indeterminate amount of time without affecting reproduction (*Avanzi* et al. 2006, *Aman* and *Graham* 1992).

Despite the advantages that frozen semen provides, some limitations are inherent in the freezing or thawing processes. Sudden changes in temperature in both processes can damage sperm cells through the formation of intracellular ice crystals, oxidative stress, and osmotic stress due to changes in the plasma membrane or DNA, resulting in a cell mortality rate of 10 to 50% (*Graham* 1996, *Watson* 2000, *Ball* et al. 2001).

To minimize the above problems, freezing protocols with slow and fast speed curves (rates) are used. In rapid freezing methods, complete dehydration of the sperm does not occur, causing ice crystals to form inside the cells and cause lesions (*Graham*, 1996). In slow freezing methods, there is sufficient time for dehydration of the spermatozoa so that no ice crystals are formed; however, this method leads to higher concentrations of solutes and changes in pH and osmolarity of the medium, which can be damaging for the cells (*Watson* 1995).

In fast freezing methods, the semen is centrifuged for 10 min at 2200 rpm and the supernatant is discarded by dilution with a cryoprotectant. Subsequently, the semen is packed in straws (0.5 mL per straw) and stabilized at 5 °C for 20 min. Afterwards, the straws are cooled for 15 to 20 min at a distance of 3 to 6 cm above the liquid nitrogen in a special box. The straws are then immersed in the liquid nitrogen and stored in a cryo-cylinder (*Papa* et al. 2011). For slow freezing procedures, the temperature change occurs in steps. After the

initial steps of centrifugation, removal of the supernatant, dilution with a cryoprotectant, and packaging of the semen in straws, the samples are subjected to a temperature drop of 2 °C per minute until 22 °C. Thereafter, the temperature is lowered from 22 to 10 °C at a rate of 0.3 °C per minute. From 10 to 4 °C, the temperature is reduced at a rate of 0.2 °C per minute. Then, the temperature must be reduced at a speed of 60 °C per minute until reaching the temperature of –140 °C. Finally, the straws are immersed in liquid nitrogen and stored in a cryo-cylinder (*Brinsko* et al. 2011).

After freezing, care should be taken to minimize sperm lesions. Cryopreserved spermatozoa frozen by the rapid freezing method should be thawed rapidly to avoid recrystallization. On the other hand, cryopreserved spermatozoa frozen by the slow freezing method, should be thawed slowly so that there is sufficient time to rehydrate the sperm cells. Some factors such as the freezing technique, container type, straw thickness, heat conductivity, and temperature are determinants for defining the thawing technique (*Mazur*, 1984, *Amann* and Pickett 1987, *Pickett* and *Amann* 1992, *Graham* 1996).

Different thawing protocols have been proposed, including defrosting at 37 °C for 30 s or at even higher temperatures for shorter periods of time, such as 46 °C for 20 s or 75 °C for 7 s (*Sieme* 2011). The professionals who apply these thawing protocols are generally in the field, therefore, there is little time or capacity for preparing or performing techniques in the laboratory, which can be an additional difficult factor. The aim of the present study was to find a longer thaw time for equine semen, than previously used, which does not cause damage to the spermatozoa.

The objective of this study was to subject frozen equine semen to three different thaw times and to examine the effects of these thaw times on motility, vigor, sperm morphology, and cell membrane integrity.

Materials and Methods

Location and stallions

A total of 36 straws (0.5 mL) of frozen semen from 12 stallions from four horse breeds (Appaloosa, Crioulo, Mangalarga Marchador, and American Quarter Horse) were used, all of which were fertile and had good freezing rates. The study was conducted at the Animal Reproduction Laboratory of the Experimental Farm Gralha Azul, PUCPR, Brazil (latitude: 25° 39' 27" S, longitude: 49° 18' 29" W).

The stallions were kept in a semi-extensive grazing system in individual pastures during the summer, or on cultivated Lolium multiflorum during the day and in a pen at night during the winter. Semen from the 12 stallions was collected during the breeding season (September to December in the Southern Hemisphere) using an artificial vagina model Botucatu (Botupharma®, Botucatu, Brazil) and a water temperature of 50 °C, with either a mare in estrus or a mannequin. After collection, the semen was evaluated (*CBRA Manual*, 2013) and diluted using a commercial diluent based on powdered milk (BotuSêmen®, Botupharma®, Botucatu, Brazil) at a ratio of 1:1 (semen:diluent).

For removal of seminal plasma, the semen was centrifuged at 2200 rpm for 10 min. After concentration calculations and removal of the supernatant, the commercial BotuCrio® cryoprotectant diluent (Botupharma®, Botucatu, Brazil) was added to the semen at a concentration of 100 million spermatozoa per straw.

To freeze the samples, the straws were maintained for 20 min at 5 °C in a refrigerator, after which the straws were placed in a liquid nitrogen vapor for 20 min (6 cm above the liquid nitrogen) and were then submerged in liquid nitrogen and stored in a cryo-cylinder at –196 °C.

Thawing techniques and semen evaluation

Thirty-six frozen sperm straws were randomly distributed into three groups: one thawed for 30 s at 37 °C (G3037, n = 12), the second thawed for 20 s at 46 °C (G2046, n = 12), and the third thawed for 60 s at 37 °C (G6037, n = 12). After thawing, the semen from each straw was placed on a slide and sealed with a coverslip. To standardize the sample evaluation process, an optical microscope (magnification 400×) coupled to a high-resolution camera was used to film each sample for 30 s. Each sample was separately evaluated by three experienced technicians, each using the same filmed material. The following characteristics were evaluated: vigor (intensity of the mass movement of sperm cells with values from 1 to 5; 1 = spermatozoa only exhibiting oscillatory movement, 5 = spermatozoa with rectilinear movement and high speeds), sperm motility (0–100 %), and sperm morphology. Semen was placed on slides and stained using the Cerovski method. Two hundred sperm cells were counted and classified into normal cells or cells with major or minor defects. The sperm morphology test follows the criteria proposed by the *Colégio Brasileiro de Reprodução Animal* (*CBRA)* (2013) and samples were subjected to the hypoosmotic test. Here, the semen samples were diluted in double distilled water at 37 °C in a ratio of 1:20 (semen:double-distilled water) and analyzed in a Neubauer chamber where 200 spermatozoa were counted and classified according to their membrane integrity. Cell membrane integrity was evaluated according to the Manual of andrology and manipulation of equine semen (Manual para exame andrológico e avaliação de sêmen animal) (Botupharma, 2014).

Statistical analysis

For the statistical analysis of the data, the D'Agostino and Pearson normality test was applied. The results were tabulated and evaluated according to the ANOVA method and are presented as the mean ± standard deviation. The level of significance was 5 % ($\alpha = 0.05$). The calculations were performed using GraphPad Prism version 6.01 for Windows statistical software (San Diego, California, USA).

Results and Discussion

There was insignificant variation in motility ($P = 0.5796$) and sperm vigor ($P = 0.2204$) between groups after thawing the

semen (Table 1), as well as in characteristic sperm morphologies (normal cells, minor defects, and major defects with P = 0.6907, 0.8763, and 0.7302, respectively) (Table 2). Table 3 shows the percentage of cell membrane damaged spermatozoa and normal cells after thawing, for each of the three groups. Differences in the percentages of normal and abnormal cells (P < 0.0001) were observed within groups but no differences were observed between the groups for the hypoosmotic test (P = 0.8200). The thawing method used can cause irreversible damage to sperm, such as accelerated loss of motility and vigor (*Graham*, 1996). In the present study, sperm samples thawed via three different methods were evaluated for changes in motility, vigor, morphology, and cell membrane damage. Our results did not show any significant differences between the three methods used, indicating that thawing at 37 °C for 60 s (less the conventional method) can also be employed (Table 1). The emphasis on this time and temperature given as 60 s allows sufficient time for the spermatozoa to be revived, thereby eliminating the use of only the traditional thawing methods, such as 37 °C for 30 s and 46 °C for 20 s (*Sieme* 2011). *Cochran* et al. (1984) tested two different techniques for thawing semen straws (0.5 mL at 37 °C for 30 s and 75 °C for 7 s). Thawing at 75 °C for 7 s resulted in better motility and vigor parameters than thawing at 37 °C for 30 s. However, the authors emphasized the importance of time precision (7 s) for thawing, since 2 additional seconds at 75 °C would be enough to cause irreversible damage to the sperm cells.

Pugliesi et al. (2014) retested these thawing methods. They confirmed that semen thawed at 75 °C for 7 s responded better. However, the authors recommended thawing at 37 °C for 30 s because higher temperatures require more precise timings to avoid the risk of damage to sperm.

Acipreste et al. (2014) compared two protocols for thawing dog semen. The first one at 37 °C for 60 s (slow) and the second at 75 °C for 7 s (rapid). They reported better performance when the spermatozoa were rapidly thawed, emphasizing that thawing at 75 °C for 7 s reduces the formation of intracellular ice crystals, whereas when slowly thawed, larger temperature variations occur, increasing the lipid and protein deposition in the spermatic cell, thereby reducing their viability (*Soderquist* et al. 1997, *Peña* and *Linde-Forsberg* 2000).

Our hypoosmotic test results showed no differences between the three thawing methods (Table 3), with changes from 33.92 % to 37.42 % observed. Any of the tests can be used without significant loss of the temperature and time of thawing on the spermatozoa. Plasma membrane integrity is an important spermatozoa characteristic for the female reproductive tract (*Pickett* and *Amann* 1992) as changes in this membrane lead to a loss of cellular homeostasis, impairing the survival and fertilizing capacity of the spermatozoa (*Parks* and *Graham* 1992).

Semen freezing and thawing procedures can damage cells due to sudden changes in temperature, resulting in the formation of ice crystals, cellular oxidation, DNA damage, and osmotic stress and cell toxicity from some cryoprotectants. These cellular changes can dramatically increase the number of spermatozoa with pathologies, not only from freezing, but also thawing (*Graham* 1996, *Watson* 2000, *Ball* et al. 2001).

In the present study, three different thawing methods were evaluated; no differences in sperm morphology were observed between the three, confirming that any of the methods described here can be used in equine AI (Table 2). Our results show that a thawing time of 60 s is now an option within the field. The professional can opt for thawing at 37 °C for 30 s knowing that if this time was exceeded for another 30 s, there

Table 3 Hypoosmotic test for cell membrane integrity in stallions semen after thawing straws for 30 seconds at 37 °C (Group thawed straws, G3037), for 20 seconds at 46 °C (G2046) and for 60 seconds at 37 °C (G6037).

Groups	Sperm membrane integrity	(%)
G3037	Normal	66,08 ± 13,65a
	Altered	33,92 ± 13,65b
G2046	Normal	62,58 ± 14,96a
	Altered	37,42 ± 14,96b
G6037	Normal	64,67 ± 12,21a
	Altered	35,33 ± 12,21b

Different letters indicate significance at the level of P < 0.05.

Table 1 Motility and vigor of stallion spermatozoa after thawing (0.5 mL) straws for 30 seconds at 37 °C (Group thawed straws, G3037), for 20 seconds at 46 °C (G2046) and for 60 seconds at 37 °C (G6037)

Groups	G3037 (%)	G2046 (%)	G6037 (%)	P value
Motility (0 to 100 %)	44.17 ± 13.79[a]	48.33 ± 10.30[a]	43.33 ± 13.03[a]	0.5796
Vigor (1 to 5)	2.54 ± 0.62[a]	2.91 ± 0.51[a]	2.58 ± 0.55[a]	0.2204

Table 2 Sperm morphology of stallion semen after thawing straws for 30 seconds at 37 °C (Group thawed straws, G3037), for 20 seconds at 46 °C (G2046) and for 60 seconds at 37 °C (G6037).

Sperm morphology	(G3037) (%)	G2046 (%)	G6037 (%)	P value
Normal cells	75,25 ± 8,45[a]	73,17 ± 11,33[a]	71,50 ± 11,82[a]	0,6907
Minor Defects*	15,08 ± 8,42[b]	17,17 ± 9,85[b]	16,17 ± 11,26[b]	0,8763
Major defects*	9,66 ± 4,33[c]	10,67 ± 5,36[c]	11,33 ± 5,67[c]	0,7302

* Colégio Brasileiro de Reprodução Animal Manual (2013) [7]/Different letters indicate significance at the level of P < 0.05

would still be no damage to the sperm cells. The objective for the present study has been achieved, since the results obtained are consistent with the norms proposed by the *CBRA* (2013).

Conclusion

It was concluded that equine semen can be thawed at the temperatures and times used in the present study (37 °C for 60 s) without affecting the motility, vigor, cell membrane, or morphology of the sperm cells.

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