Pferdeheilkunde – Equine Medicine 35 (2019) 5 (September/October) 437–441 DOI 10.21836/PEM20190506

Equine semen cryopreservation: comparison between centrifugation and filtration

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Summary: The study aimed to compare two techniques for plasma semen removal from 11 stallions using semen centrifugation and semen filtration. Two hundred and thirteen semen samples were frozen and evaluated. The samples were distributed into two groups: centrifuged semen group (GSC) and filtered semen group (GSF). The semen was evaluated fresh and after freezing based on sperm movement (vigor) and sperm motility. After the analysis of the fresh ejaculate, the samples were submitted to centrifugation (600 g/10 minutes) or filtration through synthetic hydrophilic membrane SpermFilter and were frozen. Significant differences were found for the characteristics of motility ($P = 0.0002$) and vigor ($P = 0.0221$) between GSC and GSF groups in the pre-freezing time. Sperm motility was observed in 74.6% (GSC) and 79.4% (GSF) in pre-freezing time. After freezing phase, sperm motility resulted in 63.4% (GSC) and 65.8% (GSF) ($P = 0.2303$); and vigor showed a reduction in the number of samples classified for score 4, and a predominance of the samples classified in scores 2 and 3 in both groups with no difference between groups. It was concluded that the different methods of seminal plasma removal used (centrifugation or filtration) did not influence sperm quality after freezing; the semen filtration was better in the pre-freezing moment showing the best technique for the use of fresh semen.

Keywords: centrifugation, filtration, semen, freezing, horse

Citation: Felicio L. C. S., Felicio G. B., Kozicki L. E., Scheffer Pereira J. F., Zielinski B. L., Scheffer Pereira da Silva M. T., Gassenferth G., Gaievski F. R., Ollhoff R. D. (2019) Equine semen cryopreservation: Comparison between centrifugation an filtration. Pferdeheilkunde 35, 437–441; DOI 10.21836/PEM20190506

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Received: April 11, 2019 | **Accepted after revision:** July 3, 2019

Introduction

The aim of using cryopreserved equine semen is to preserve the genetic material of the stallion (indefinitely), to eliminate geographical barriers and to reduce the transmission of diseases. Seminal processing techniques that maximize fertility and longevity can be applied with the goal of increasing sperm concentration, preventing cell damage, and benefiting sperm survival through seminal plasma separation (*Alvarenga* et al. 2016)

The quality of frozen semen is determined by several factors, such as intrinsic characteristics of each stallion, type of diluent used, freezing curve and applied technique for seminal plasma removal (*Ramires Neto* et al. 2013b). After harvest, dilution and analysis of the semen, the freezing process includes the removal of seminal plasma to reduce its adverse effect on long-term fertility and to concentrate spermatozoa (*Brinsko* et al. 2000, *Akcay* et al. 2006, *Loomis* 2006, *Hoogewijs* et al. 2010).

Sperm cell concentration and seminal plasma separation can be performed by centrifugation at 2200 rpm for 10 minutes, or filtration through a synthetic hydrophilic membrane (eg. SpermFilter®, Botupharma, Botucatu, Brazil) (*Ramires Neto* et al. 2013b, *Brinsko* et al. 2000, *Akcay* et al. 2006, *Loomis* 2006, *Hoogewijs* et al. 2010, *Weiss* et al. 2004). Centrifugation is the most common method of sperm concentration; however, the strength and duration of the rotations may negatively interfere on the motility and integrity of the sperm membrane (*Hoogewijs* et al. 2010). With the synthetic hydrophilic membrane for seminal plasma filtration, the diluted semen is placed in the filter and by soft movements on a petri dish, the plasma is removed by capillarity and the spermatozoids are retained in the membrane. After separation, the filtered cells are homogenized with the calculated volume of the cryoprotective diluent, resuspending the spermatozoa (*Alvarenga* et al. 2016). This way, the adverse effects of centrifugation are avoided, reducing the damage against spermatic membrane (*Alvarenga* et al. 2010).

The hypothesis of the present study is that the removal of seminal plasma by the filtration method leads to improvements on the quality of cryopreserved equine semen in comparison to the centrifugation technique, once its adverse effect on the plasma membrane is lower.

The aim of this study was to compare the centrifugation and filtration techniques of equine semen through the evaluation of motility and vigor, before and after freezing, in order to determine the influence of each method of seminal plasma separation on the semen quality after thawing.

Materials and Methods

The present study was conducted strictly at the field level, having as a reference the activities of a great number of professionals, who work daily in the conditions of commercial horse breeding farms worldwide. For this, 213 ejaculates of 11 Quarter Horse Mile stallions, 8 to 15 years old were used. The animals were kept in a semi-extensive grazing system + supplementation of commercial ration, mineral salt and alfalfa hay, located in Presidente Prudente (São Paulo State, Brazil, latitude 22° 07' 32" S; longitude 51° 23' 20" W). The stallions' semen were collected at 48-hours intervals between February and June 2017, totaling 213 samples of frozen semen evaluated.

The semen harvest was carried out in an artificial vagina model Botupharma (Botupharma®, Botucatu, Brazil), using the closed collection methodology, with the temperature of the artificial vagina around 40 °C. For the harvest semen, mares in estrus or manikins were used. After evaluations the semen was diluted in Botu-Semen® commercial diluent (Botupharma®, Botucatu, Brazil), based on skimmed milk, in the ratio of one part semen to one part diluent (1:1). The samples were randomly distributed and two groups were formed: centrifuged semen group (GSC) and filtered semen group (GSF) before sperm evaluation.

The evaluation of semen quality followed criteria of subjective analysis of sperm motility and vigor. The semen samples were evaluated by two experienced veterinarians, before and after freezing, to determine the intensity and speed of sperm cell movement through vigor (vigor 1 = spermatozoa with exclusively oscillatory movements; 5 = spermatozoa with rectilinear and very fast progressive movement) (*Colégio Brasileiro de Reprodução Animal*, 2013) and sperm motility (0–100 %), placing the sample between the slide and coverslip using the 200 × magnification under an optical microscope. The sperm concentration was performed through the Neubauer camera to establish the number of inseminating doses for each ejaculate. After collection and analysis, the semen was subjected to the seminal plasma separation procedure to perform the concentration of the sperm cells by two methods: 1) Centrifugation (600 g for 10 minutes) by Baby Fanne® centrifuge (São Paulo, Brazil) (n = 151). 2) Filtration through synthetic hydrophilic membrane (SpermFilter® Botupharma, Botucatu, São Paulo, Brazil) (n = 62). After centrifugation or filtration of the seminal plasma the sperm cells were resuspended in Botucrio commercial cryoprotective diluent (Botupharma, Botucatu, Brazil) and conditioned in 0.5 ml straws with the spermatic concentration of 100×10^6 spermatozoa with progressive movements.

For the cooling curve, the straws were placed at 5 °C for 20 minutes. After stabilization, they were placed in liquid nitrogen vapor at 6 cm from the nitrogen level for 20 minutes. After this period, they were immersed in liquid nitrogen and stored in a cryobiological cylinder. Thawing was done in a water bath at 46 °C for 20 seconds.

Statistical Analysis

The sperm motility data were analyzed by ANOVA and for median comparison test by Bonferroni. The spermatic vigor between the groups was evaluated using the Chi-square test. In both tests, the significance level of 5 % ($P < 0.05$) was adopted. The analysis was performed using the Statgraphics Centurion XVI statistical software (Version 16.2.04, Virginia, USA).

Results and Discussion

The objective of the present study was to compare two methods of seminal plasma removal and check its influence on sperm motility and vigor. Differences ($P = 0.0002$) in sperm motility were observed between the GSC and GSF groups in the pre-freezing phase (Table 1), but no differences ($P = 0.2303$) were found in sperm motility after freezing. (Table 1)

The difference between the groups in the pre-freezing of semen could be associated with intrinsic factors of the GSF stallions, by presenting higher sperm motility values in relation to the GSC group. Such divergence may be related to the seminal quality and fertility of each stallion, including characteristics such as sperm morphology and age of animals (*Fernandes* and *Pimentel* 2002). Similar values of sperm motility were found in both groups on freshly diluted semen before and after seminal plasma removal by centrifugation or hydrophilic membrane (*Alvarenga* et al. 2010, *Ramires Neto* et al. 2013a).

Between the pre and post freezing times statistical differences in both GSC ($P < 0.0001$) and GSF ($P = 0.0001$) groups were observed. Lower values of sperm motility were observed in studies comparing semen before and after freezing. However, this reduction may be related to the freezing process, and not to the seminal plasma removal method used (*Ramires Neto* et al. 2013a).

The cryopreservation process has adverse effects on the spermatozoa due to thermal stress causing reduction of metabolism, loss of integrity and acrosome membrane, cell dehydration, ice crystal formation, irreversible and possibly lethal

Table 1 Sperm motility before and after freezing of the centrifuged equine semen (GSC) and filtered semen groups (GSF). | *Beweglichkeit des Spermas vor und nach Einfrieren in der GSC- und der GSF-Gruppe.*

Sperm motility (%)	Before freezing x ± s (%)	After freezing x ± s (%)
GSC (n=151)	74.67±0.710 ^{aA}	63.48±1.168 ^{aB}
GSF (n=62)	79.44±0.976 ^{bA}	65.89±1.293 ^{aB}
Value P	0.0002	0.2303

Different uppercase letters on the same line indicate statistical difference ($P < 0.005$) between before and after freezing times.

damage (*Brinsko* et al. 2000, *Oliveira* et al. 2013). Some authors differ from these results indicating that damage is experienced by the sperm cell during the centrifugation process, such as plasma membrane lesions, circular movements and cells agglutination (*Brinsko* et al. 2000, *Hoogewijs* et al. 2010, *Sieme* et al. 2003). However, these damages were minimized using the centrifugal force of 600 g for 10 minutes (*Dell'aqua* et al. 2001). In agreement with this study, some studies have shown that the removal method of seminal plasma by filtration or centrifugation did not negatively influence the motility or plasma membrane integrity in fresh (*Ramires Neto* et al. 2013b, *Alvarenga* et al. 2010), refrigerated (*Alvarenga* et al. 2010, *Neto* et al. 2013a) or frozen semen (*Ramires Neto* et al. 2013b).

The post-thaw sperm motility data found in this study are in agreement with the acceptable parameters for total motility (50%) and progressive (30%) in the qualitative analysis of frozen equine semen (*Alvarenga* et al. 2016), where the motility of more than 60% in both experimental groups was observed.

Regarding the sperm vigor, the number of samples classified in each score (1–5) (Colégio Brasileiro de Reprodução Animal, 2013) is represented in Figure 1. In the pre-freezing evaluation, samples classified in scores 3 and 4 of spermatic vigor prevailed in both groups; however, 11 samples were classified in score 2 in the pre-freezing GSC group. It was found that no sample scored 5 for sperm vigor throughout the experiment. Differences in spermatic vigor were observed between the GSC and GSF groups before freezing ($P = 0.0221$) (Figure 1).

In the post-freezing time, there was a reduction in the number of samples classified in score 4, and a predominance of the samples classified in scores 2 and 3 in both groups, but with no difference between the post-freezing groups ($P = 0.9953$).

The freezing process influenced the sperm vigor scores in each group. There were differences between pre and post freezing times. Lower sperm vigor scores were verified at the time after freezing ($P = 0.0001$). Similarly to sperm motility, vigor was also affected by temperature difference, reducing the metabolism and sperm motility (*Oliveira* et al. 2013). The sperm vigor data are directly related to percentages of sperm motility in both groups. The GSC group in the pre-freezing phase presented some samples of sperm vigor with score 2, as well as lower percentage of sperm motility; whereas GSF showed the highest vigor scores and higher percentages of sperm motility. In the present study, the filtration technique satisfactorily removed seminal plasma prior to freezing. Data showed that sperm recovery was close to 90% using the hydrophilic membrane, and lower in the methodology of centrifugation (80%) (*Ramires Neto* et al. 2013a, *Ramires Neto* et al. 2013b, *Alvarenga* et al. 2010).

The hypothesis of the present study could be partially confirmed, once the filtration of the semen provided a greater recovery of spermatozoa after thawing, besides requiring less time for execution. It is suggested that for semen under low quality, semen filtration should be used, aiming to maintain the minimum quality for freezing (*Alvarenga* et al. 2010, *Ramires Neto* et al. 2013a).

Conclusion

Under horse breeding farms conditions as this study was carried out, it was concluded that the used plasma seminal removal techniques did not influence sperm quality after freezing; the semen filtration method was more efficient for use of fresh semen; the performance of spermatozoa after thawing proved to be better in the methodology of seminal plasma removal by filtration.

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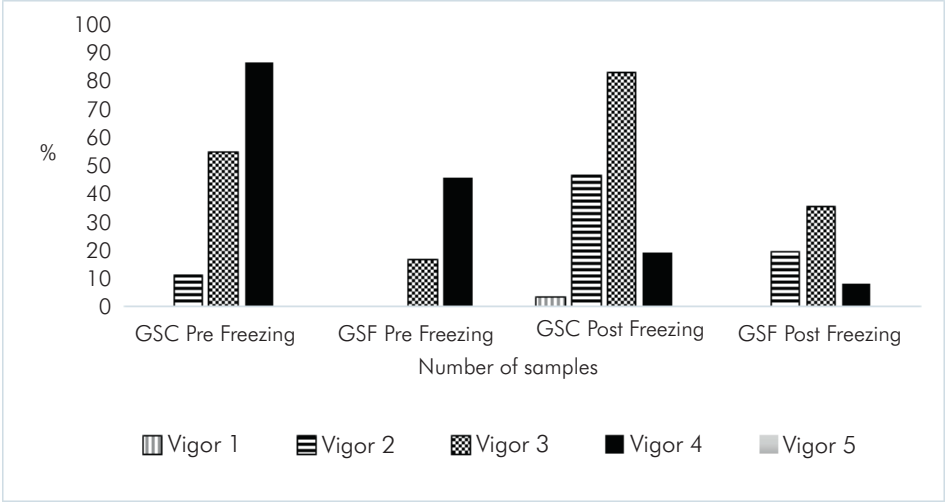

Fig. 1 Number of samples regarding sperm vigor score of equine semen for the GSC and GSF groups in the pre and post freezing phase. | *Anzahl der Proben (%) in den verschiedenen Sperma-Vitalitätsstufen (Vigor) der GSC- und GSF-Gruppe, jeweils vor und nach der Gefrierphase.*

*Colégio Brasileiro de Reprodução Animal (*2013). Manual para exame andrológico e avaliação de sêmen animal. 3. ed. Belo Horizonte: CBRA

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