



Effect of plasma separation techniques and sperm selection on sperm recovery and viability of cooled pony stallion semen for 48h at 5°C

[Efeito das técnicas de separação de plasma e de seleção de esperma na recuperação e viabilidade do sêmen de garanhão pônei resfriado por 48h a 5°C]

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ABSTRACT

Separation techniques of seminal plasma [centrifugation (SC) and Sperm Filter® (SF)] and sperm selection [Androcoll-E (SCA) and filtration glass wool (GW)] were used in 24 ejaculates from 6 stallions. In experiment 1, the ejaculates were allocated into control (no spin), centrifugation at 600 g x 10min, SF and GW. In experiment 2, semen was submitted to SC, SGA and filtered through GW. Following the treatments in both experiments, samples were kept chilled at 5°C to 50 x 10⁶ sperm/ml for 48h. The variables measured on fresh and cooling semen were pH, motility, membrane viability function by 6-carboxyfluorescein diacetate and propidium iodide (CFDA / PI), viability or vitality (eosin / nigrosine) and mitochondrial activity. In experiment 1, centrifugation to remove seminal plasma resulted in greater damage to sperm than separation by sperm filter, and selection by glass wool was more efficient in separating viable cells and maintaining viability during cooling. In experiment 2 Androcoll-E and glass wool treatments resulted in higher (P <0.0001) motility, membrane function, mitochondrial activity, and viability than centrifuged semen. Both selection by Androcoll- E and glass wool improved the quality of semen pony stallions for preservation for up to 48h to 5°C.

Keywords: semen, equine, cooling

RESUMO

As técnicas de separação do plasma seminal (centrifugação, SpermFilter) e de seleção espermática (Androcoll-E e filtração por lã de vidro) foram aplicadas em 24 ejaculados de seis garanhões da raça Pônei Brasileiro. Após coleta e separação da fração gel, os ejaculados foram diluídos 1:1 com diluente à base de leite em pó. No experimento 1, os ejaculados foram distribuídos em controle (sem centrifugação), centrifugação a 600g x 10min, SpermFilter e filtração por lã de vidro. No experimento 2, o sêmen foi submetido aos procedimentos: centrifugado (SC), centrifugado com Androcoll-E e filtrado por lã de vidro. Após os procedimentos de ambos os experimentos, as amostras foram mantidas refrigeradas a 5°C, com 50 x 10⁶ espermatozoides/mL, por 48h. As variáveis mensuradas a fresco, 24h e 48h foram: pH, motilidade, funcionalidade de membrana, viabilidade por diacetato de carboxifluoresceína e iodeto de propídio (CFDA/PI, vitalidade (eosina/nigrosina) e atividade mitocondrial. Já osmolaridade e morfologia espermática foram avaliadas somente imediatamente após a coleta. No experimento 1, a centrifugação para retirada do plasma seminal resultou em maiores danos aos espermatozoides do que a separação por SpermFilter. A filtração por lã de vidro mostrou-se mais eficiente em separar células viáveis e manter a viabilidade durante o resfriamento. No experimento 2, os tratamentos com Androcoll-E e filtrado por lã de vidro foram superiores (P<0,0001) ao sêmen centrifugado quanto à motilidade, à funcionalidade de membrana, à atividade mitocondrial e à viabilidade, tanto nas amostras de sêmen fresco como de sêmen refrigerado. O Androcoll-E e a lã de vidro permitiram manter por 48h, a 5°C, o sêmen de garanhões pôneis utilizando-se diluente à base de leite.

Palavras-chave: equino, sêmen, separação espermática

Recebido em 7 de abril de 2020

Aceito em 7 de julho de 2020

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INTRODUCTION

Cooled or frozen semen in horses has become an important biotechnology applied to the reproduction management of this species (Ball *et al.*, 2001). Expansion in the use of ponies in recreational, therapeutic, and recreational activities with children has increased the demand for ponies in the last few years. Studies about reproduction of some breeds demonstrate reduced daily sperm production due to their size (Neves *et al.*, 2006).

The specific reasons for declining viability of the sperm cell during cooling and freezing processes involve several factors, among them osmotic and oxidative stress, pH changes and osmolarity that may be a consequence of toxic products eliminated by dead cells pre-processing (Ball, 2008). The production of oxygen-reactive species is high in the presence of cells damaged by the cryopreservation process, non-viable spermatozoa, or with morphological defects, among which we highlight intermediate part changes (Ball & Vo, 2001). Damage to the sperm membrane and mitochondria is greater when equine semen is subjected to cooling or cryopreservation.

The decrease in mitochondrial sperm activity was verified in humans as a potential cause to increase the production of oxygen reactive species (ROS) (Wang *et al.*, 2003). These events that cause changes in all cellular components can lead to cell death via apoptosis or necrosis. Thus, the increase of dead cells causes an increase in the cascade effect of free radical production, leading to cytoplasmic damage and DNA fragmentation (Sharma & Agarwal, 1996; Agarwal *et al.*, 2003).

The use of several techniques such as the seminal plasma (centrifugation and SpermFilter®), spermatric separation by concentration gradients (Percoll®, Androcoll-E®), or spermatric selection in columns (glass wool and Sephadex) sought in the first circumstance the reduction of the deleterious effects of seminal plasma during cooling and, in the other cases, a greater number of viable cells after the cryopreservation process. These are some of the alternatives available to improve the quality of equine semen (Sieme *et al.*, 2003; Alvarenga *et al.*, 2012; Morrell, 2012; Morrell *et al.*, 2016).

When comparing methods of sperm concentration, Alvarenga *et al.* (2012), found that centrifugation can induce lesions in the sperm cell. The use of synthetic hydrophilic membrane with 2 µm pores (SpermFilter) is as efficient as conventional centrifugation, as well as centrifugation in colloidal solution to remove seminal plasma, besides being more suitable. It is known that glass wool filtration and the centrifugation usually used to remove seminal plasma from stallion semen methods are effective in separating spermatozoa with good motility and with increased capacity after freezing. However, the data are still conflicting when compared to the technique of colloidal centrifugation and with large volumes of semen (Morrell & Rodriguez-Martinez, 2010).

The hypothesis in experiment 1 is that centrifugation causes irreversible sperm damage and negative impact on parameters during cooling. The hypothesis in experiment 2 is that glass wool has a similar capacity to Androcoll-E for sperm selection in the semen of pony stallions. The objective was to evaluate different techniques for seminal plasma separation and sperm selection before cooling at 5°C on the viability of the semen of pony stallions during 24 and 48h of cooling.

MATERIAL AND METHODS

The study was conducted during the breeding season in the southern hemisphere. Four Brazilian pony stallions aged 9 to 13 years, that were fed once a day with oats and alfalfa, pasture, and water ad libitum all day, served as semen donors. All stallions were allocated to paddocks and kept under the same management conditions for exhaustion of the extragonadal reserves, as well as for the twice weekly regular ejaculate collection. An estrus female served as a dummy, and the ejaculate was collected with an artificial vagina (Hannover model, minitube, Tiefenbach, Germany). The free ejaculate of the gel fraction was measured for volume, total motility, concentration, pH and osmolarity and then diluted 1: 1 in diluent with skimmed milk powder (Equidil – UFSM, Santa Maria, Brazil).

Extender: The extender, produced by the Embryolab Laboratory of the Federal University of Santa Maria, is a modification of the Kenney diluent (Kenney, 1975), composed of 4.9 g glucose (Merck 1.08337.1000), skimmed milk powder, 4g) and distilled water qsp, without the

use of antibiotics. Semen evaluation: In duplicate samples were taken for the thiazolyl blue reduction test ((3- (4,5-dimethylthiazolyl-2) -2,5-diphenyltetrazolium bromide)) (MTT) (Aziz *et al.*, 2005), hyposmotic test, osmolarity, pH, morphology and vitality.

Sperm motility was evaluated subjectively (Varner *et al.*, 1989). Aliquots of 5.0µL of the semen were deposited on slide on cover slip, both heated at 38°C. The test was performed by warm phase contrast (400X) optical microscopy (38°C). The spermatozoa were evaluated in different fields of the slide. The total proportion of mobile spermatozoa was estimated immediately after preparation of the sample at room temperature of 20-22°C, as well as after maintenance at 5°C for 24h and 48h of cooling. A 50-sec video was delivered to three experienced reviewers who did not know the treatments. Evaluators estimated motility and an arithmetic mean was used.

For morphological evaluation, a smear was performed with 5µL of semen sprayed on a slide. After drying, the sample was fixed and stained with Spermac dye solutions (Minitub do Brasil, Porto Alegre, Brazil). To determine the percentage of live and dead spermatozoa, 5µL samples of semen were used to prepare smears that were stained with eosin-nigrosin (Dott & Foster 1972) (Minitub, Porto Alegre, Brazil). The fluorescence technique to determine sperm viability used 6-carboxyfluorescein diacetate (C504, Sigma-Aldrich) and propidium iodide (P4170, Sigma-Aldrich) (CFDA / PI) (Harrison & Vickers, 1990; Arruda *et al.*, 2002).

The mitochondrial activity (MTT test) of the semen samples was evaluated with (3 [4,5-dimethylthiazol-2-il] -2,5-diphenyltetrazolium) according to the predefined protocol (Aziz *et al.*, 2005). The reading was performed on a microplate reader (MR 96A) with the use of a 540 nm filter.

The 50mL volume tubes containing the semen samples were cooled in a refrigerator at 5°C. The tubes were placed in a tilted position on a tube rack maintaining the cooling curve of 0.5°C per minute. A digital thermohydrometer (ITHT 2210, São Paulo, Brazil) was used to calibrate the surface temperature of the tubes with a fine cable kept inside the refrigeration chamber. The samples were homogenized, and a sample was withdrawn to determine the motility, pH,

osmolarity, MTT, hyposmotic test, sperm viability and vitality test (eosin / nigrosin) at each 24h of cooling. The sperm membrane functionality was evaluated (Lagares *et al.*, 2000) under increasing phase contrast of 400X. The classification of sperm cells was performed according to known protocol described by Neild *et al.* (2000).

Experiment 1: For this study, 24 ejaculates (6 ejaculates / stallion) from four (4) stallions of Brazilian breed ponies during the breeding season served to compose the experimental design. The ejaculates were distributed in four equal fractions and named according to the groups established for cooling: Control: semen diluted and not centrifuged at the concentration of 50 x 10⁶ cells /mL; diluted semen centrifuged: at 600 x g /min for 10min, diluted semen separated with SpermFilter (Alvarenga *et al.*, 2012) and diluted semen separated by glass wool. The preparation of the glass wool column (Sigma-Aldrich, 20411) followed the methodology described by Engel *et al.* (2001) with modifications. The glass wool (250mg) was placed and compressed in 3mL syringe without a rubber plunger and in the washed and steam sterilized sequence.

Prior to use, the assembled sterile glass wool column was washed with 10mL of the same diluent used in the remainder of the semen procedures. The syringe was then placed in the upright position and the 1: 1 diluted semen was poured into the upper portion of the syringe. Due to gravity, the spermatozoa with viability flowed through the column and were deposited in tubes of 15mL located immediately below the syringe. After the filtration process, the material retained in the glass wool was washed with 10mL of Petri dish diluent to evaluate the concentration of retained cells, motility, pH, osmolarity, hyposmotic test and supra vital coloration (eosin / nigrosin).

Experiment 2: The experiment was performed with 24 ejaculates obtained from four stallion ponies of the Brazilian Breed (6 ejaculates / stallion) during the breeding season. The collection, evaluation and dilution of the ejaculate was performed as described in experiment 1. In this experiment, the semen was distributed in the following treatments.

Androcoll-E centrifuged semen (SCA), a 15mL aliquot of the 1: 1 diluted ejaculate was deposited

on 15mL of an Androcoll-E column (Morrell *et al.*, 2009) in a 50mL centrifuge tube. This sample was centrifuged at 300 g x 20min, the supernatant (plasma, diluent and colloid) discarded and the pellet resuspended in diluent to obtain the concentration of 50×10^6 sperm /mL. After adjusting the concentration, the sample was refrigerated at 5°C for 48h. *Filtered semen by glass wool (SFL)*: A 15ml aliquot was subjected to gravity filtration in a 10mL syringe containing glass wool pressed to the 1cc mark. The filtered semen was subjected to a new determination of the concentration, and the diluent was added to the sample to adjust the concentration to 50×10^6 spermatozoa /mL and refrigerated at 5°C for 48h. *Centrifuged semen (SC)*: 15mL of the diluted 1: 1 semen was centrifuged at 600 g x 10min and soon after the concentration was adjusted to 50×10^6 sperm /mL. Samples were refrigerated at 5°C for 48h.

Statistical analysis was performed using SAS® 9.3 statistical analysis software (SAS Institute Inc., Cary, NC, USA) Means, GLM and GLIMMIX procedure. The analysis compared the effect of treatments [Study 1: Control, centrifuged, sperm filter and glass wool; Study 2: *Androcoll-E centrifuged semen (SCA)*, *Filtered semen by glass wool (SFL)*, *Centrifuged semen (SC)*], time (0h, 24h and 48h) and their interaction (time*treatment). [and stallions were included in the model as a block. All ejaculates were distributed equally in treatments (Subsampling) repeat measures.

Percentage of intact cells and defects were observed to have normal distribution and analyzed using the procedure GLIMMIX fitted to the normal distribution. Least square means \pm standard error described the response variable. Results with *p* value <0.05 were considered statistically significant. Graphs were achieved

using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The committee of ethics in animal experimentation approved all procedures performed in these experiments according to protocol number CEUA/UFSM 2017130318 according to the original project, "Use of non-enzymatic antioxidant in equine semen cooled after sperm selection."

RESULTS

The mean volume of the ejaculates was 22 ± 4 mL and the mean concentration in the ejaculate was $205 \pm 11.6 \times 10^6$ sperm /mL. The total mean motility of the ejaculates was $73.9 \pm 1.6\%$. The spermatic morphology of the ejaculates was $73.7 \pm 4.2\%$ of spermatozoa with intact morphology. Osmolarity was similar between ejaculates and between treatments. The pH was also similar between the fresh groups for the control (7.52), centrifuged (7.55), glass wool (7.54) and SpermFilter (7.53) ($P = 0.96$) groups. After 24h cooling, the pH did not differ between the control groups (7.25), centrifuged (7.26), glass wool (7.22) and SpermFilter (7.14) ($P = 0.08$). At 48h cooling, pH was higher in the control group (7.87) compared to centrifuged (7.61), glass wool (7.40) and SpermFilter (7.53) ($P < 0.0001$).

Experiment 1: The ejaculates, whose semen was separated with glass wool had a higher percentage of intact cells after the filtering procedure ($P = 0.04$) for multiple comparison. In Table 1, it is possible to verify that the ejaculates submitted by glass wool treatment exhibited an above low percentage of defects ($P < 0.001$). Ejaculates whose spermatozoa were centrifuged presented a higher percentage of cells with tail defects after processing compared to the ejaculates of the control group (non-centrifuged), SpermFilter or glass wool ($P < 0.05$).

Table 1. Mean percentage (\pm SE) of defects and normal spermatozoa in smears stained with Spermac dye in 24 ejaculates of Brazilian stallion ponies pre-cooling (0h), submitted to four different treatments

Treatments	Defects				
	Acrossoma	Head	Middle Piece	Tail	Normal
Control	1.43 ± 0.31^a	2.2 ± 0.28^{bc}	2.31 ± 0.37^{ab}	$14.13 \pm 0.76b$	$78.39 \pm 0.97b$
Centrifuged	2.16 ± 0.39^a	3.07 ± 0.39^a	2.92 ± 0.39^a	$19.43 \pm 1.17a$	$72.4 \pm 1.51c$
Glass wool	0.80 ± 0.17^b	1.62 ± 0.32^{ab}	1.14 ± 0.21^c	$7.78 \pm 0.99b$	$88.63 \pm 1.22a$
Sperm Filter	1.52 ± 0.28^{ab}	2.55 ± 0.24^c	1.99 ± 0.3^b	$14.05 \pm 0.92c$	$79.87 \pm 0.99b$
<i>P Value</i>	0.0296	0.0055	0.0004	<0.0001	<0.0001

^{a,b,c} Different letters in the same column indicate difference ($P < 0.05$) between treatments.

Effect of plasma...

Motility was significantly different among treatments at 0, 24 and 48h ($P = 0.0015$, $P = 0.0001$ and $P < 0.0001$, respectively). Motility parameters and comparisons between treatments at 0, 24 and 48h post-treatment are shown in Table 2. The hyposmotic test revealed a higher

percentage of spermatozoa with intact plasma membrane after cooling for 48h in the ejaculates submitted to the separation in glass wool (Table 3). The membrane functionality gradually reduced with the cooling time in all treatments.

Table 2. Mean percentage of total (\pm SE) subjective motility of 24 ejaculates of Brazilian pony stallions submitted to immediate evaluation after processing (0h), cooled for 24h and 48h at 5°C

Time	Control	Centrifuged	Sperm Filter	Glass wool	P value
0h	70.0 \pm 2.8 ^b	71.1 \pm 2.78 ^b	73.8 \pm 3.14 ^b	82.2 \pm 1.9 ^a	0.0015
24h	35.1 \pm 2.81 ^c	39.2 \pm 2.4b ^c	43.3 \pm 2.4 ^b	51.6 \pm 2.1 ^a	0.0001
48h	16.38 \pm 2.8 ^b	18.33 \pm 2.94 ^b	25.0 \pm 2.02 ^a	27.2 \pm 1.9 ^a	0.001

^{a,b,c} Different letters on the same line indicate difference between groups ($P < 0.05$).

Table 3. Mean values (\pm SE) of the membrane function determined by the hyposmotic swelling test, in 24 ejaculates of Brazilian stallion ponies chilled for 24h or 48h at 5°C in diluent with 50×10^6 skimmed milk powder spermatozoa /mL.

Time	Control	Centrifuged	Sperm Filter	Glass wool	P value
0h	66.6 \pm 3.0 ^b	69.1 \pm 3.1 ^b	70.7 \pm 2.2 ^b	76.2 \pm 2.7 ^a	0.001
24h	41.7 \pm 2.7 ^b	44.2 \pm 1.9 ^b	44.9 \pm 1.9 ^{ab}	48.2 \pm 2.7 ^a	0.02
48h	21.2 \pm 3.2 ^b	24.6 \pm 1.3 ^b	25.6 \pm 2.4 ^b	31.8 \pm 2.2 ^a	0.008

^{a,b} Different letters on the same line indicate difference between groups ($P < 0.05$).

The mitochondrial activity test (MTT) demonstrates a reduction in sperm viability during the cooling period at 5°C in all treatments. The ejaculates submitted to separation through glass wool presented higher number of cells with mitochondrial activity during the cooling (Figure 1). The absorbance of the glass wool group (0.921 \pm 0.04) differed ($P = 0.008$) from the ejaculates treated with SpermFilter (0.850 \pm 0.05), centrifuged (0.833 \pm 0.05) and control (0.777 \pm 0.04).

In the MTT evaluation after 24h of cooling, the ejaculates treated with glass wool (0.695 \pm 0.03) were similar to the SpermFilter (0.630 \pm 0.04), which did not differ from the centrifuged group (0.579 \pm 0.04) and control (0.556 \pm 0.05) ($P = 0.0295$). After 48h of cooling, the mitochondrial activity remained higher ($P = 0.004$) for the group filtered by glass wool (0.513 \pm 0.03). This same parameter evaluated in the ejaculates treated with SpermFilter (0.407 \pm 0.02) and centrifuged (0.364 \pm 0.02) presented similarity of results and the same occurred in the ejaculates centrifuged and control (0.299 \pm 0.03).

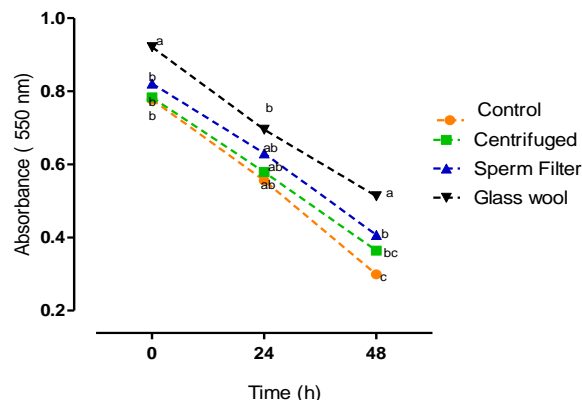


Figure 1. Mitochondrial activity assessed by the reduction of MTT in semen samples of Brazilian stallion ponies refrigerated at 5°C for 48h ($P = 0.001$).

Table 4 shows the spermatic viability determined by the supra-vital coloration (eosin-nigrosin). Ejaculates that had seminal plasma separated for cooling by the SpermFilter technique or that underwent spermatic separation with glass wool presented greater sperm viability. This difference was verified both fresh and after 24 and 48h of cooling. Experiment 2: The osmolarity after the semen processing was similar between treatments (SC = 303.6; SCA = 300.4 and SFL = 302.5). The pH (mean \pm SE) was also similar between SC treatments (7.52 \pm 0.03), SFL (7.54 \pm 0.02) and SCA (7.53 \pm 0.02) in fresh semen (P = 0.83), in the 24h of SC cooling (7.16 \pm 0.03), SFL (7.15 \pm 0.04) and SCA (7.12 \pm 0.05). However, in the 48h of cooling, the pH of the SC (7.84 \pm 0.09) was higher (P = 0.001) than the SFL (7.49 \pm 0.05) and SCA (7.5 \pm 0.04). Centrifugation with Androcoll-E and separation with glass wool resulted in a higher percentage of normal cells, as well as reduction in the percentage of cells with tail changes (P < 0.0001) (Table 5).

The results of motility, membrane functionality (HOST), sperm viability (CFDA / PI), mitochondrial activity (MTT reduction) and sperm vitality (eosin / nigrosin) are expressed as means \pm SE in Table 6. Sperm selection by Androcoll-E and glass wool were able to maintain a higher number of viable cells during cooling. Although they differed from each other, treatment with Androcoll-E resulted during the cooling

period in higher mitochondrial activity and sperm viability.

DISCUSSION

Seminal plasma affects sperm longevity, and particularly in stallions that produce semen with limited tolerance of storage, semen quality can be improved through modifications of semen handling procedures (Kareskoski & Katila, 2008). This study is a pioneer in bringing together different techniques of concentration and sperm separation in semen of stallions of the Brazilian Pony breed. The use of centrifugation for concentration and removal of seminal plasma for equine semen cooling has been widely discussed in many studies (Brinsko *et al.*, 2000; Raphael *et al.*, 2008).

In the literature, it is also possible to identify studies that attribute deleterious effects to the sperm membrane, or loss in the supernatant (Martin *et al.*, 1979; Jasko *et al.*, 1991b; Dell'aqua Jr. *et al.*, 2001; Keller *et al.*, 2001), as well as interactions with extender (Ferrer *et al.*, 2012). The analysis of the results obtained shows that the centrifugation of the semen without the use of colloids can cause damage to the sperm cell, corroborating other studies (Sieme *et al.*, 2003). However, these factors should always be considered taking into account the extender used in the dilution of the ejaculate to perform the centrifugation (Len *et al.*, 2010).

Table 4. Mean values (\pm SE) of the sperm viability determined by the eosin / nigrosin test in 24 ejaculates of Brazilian stallion ponies chilled at 5°C in diluent with skimmed milk powder (Equidil / UFSM, Santa Maria/RS, Brazil) at a dose of 50x10⁶ sperm /mL

Time	Control	Centrifuged	Sperm Filter	Glass wool	P Value
0h	56.1 \pm 2.5 ^b	55.4 \pm 2.4 ^b	57.4 \pm 2.6 ^b	64.4 \pm 1.9 ^a	0.007
24h	35.0 \pm 2.4 ^c	34.7 \pm 3.5 ^c	36.8 \pm 2.7 ^{ab}	41.8 \pm 2.5 ^a	0.03
48h	19.55 \pm 2.3 ^b	18.2 \pm 2.8 ^b	24.0 \pm 2.2 ^{ab}	27.3 \pm 2.3 ^a	0.04

^{a,b,c} Different letters on the same line indicate difference between groups (P < 0.05).

Table 5. Mean percentage (\pm SE) of defects and normal spermatozoa visualized on smears stained with Spermac dye in 24 ejaculates of Brazilian stallion ponies after processing at the initial (0h) pre-cooling time subjected to selection by centrifugation, Androcoll-E or by glass wool

Groups	Defect				Normal
	Acrossoma	Head	Middle piece	Tail	
Centrifuged	1.24 \pm 0.19 ^a	2.73 \pm 0.36 ^a	1.6 \pm 0.25 ^a	19.87 \pm 1.13 ^a	78.39 \pm 0.97 ^b
Androcoll-E	0.65 \pm 0.12 ^b	1.41 \pm 0.29 ^b	0.91 \pm 0.13 ^a	7.52 \pm 0.89 ^b	89.5 \pm 1.1 ^a
Glass wool	0.71 \pm 0.14 ^b	1.6 \pm 0.38 ^b	1.4 \pm 0.34 ^a	9.1 \pm 0.81 ^b	86.8 \pm 1.28 ^a
P Value	0.02	0.03	0.22	0.001	0.001

^{a,b} Different letters in the same column indicate statistical difference (P < 0.05).

Table 6. Mean (\pm SE) of Total motility, hyposmotic swelling test, MTT absorbance, viability, and sperm vitality in ejaculates of Brazilian stallion ponies at zero (initial), 24 and 48h after cooling at 5°C submitted to selection by centrifugation, Androcoll-E or glass wool

Time	Treatment ^a			P value
	Centrifuged	Androcoll-E	Glass wool	
		<i>Total motility (%)</i>		
0h	63.3 \pm 2.1 ^b	85.0 \pm 2.9 ^a	78.5 \pm 2.2 ^a	0.001
24h	36.6 \pm 2.5 ^b	48.0 \pm 2.9 ^a	46.2 \pm 2.4 ^a	0.002
48h	16.2 \pm 2.6 ^b	35.3 \pm 2.4 ^a	27.0 \pm 2.1 ^a	0.001
		<i>Hyposmotic swelling test (%)</i>		
0h	63.9 \pm 3.2 ^b	72.6 \pm 1.7 ^a	74.2 \pm 2.2 ^a	0.001
24h	38.6 \pm 2.6 ^b	48.4 \pm 2.1 ^a	42.1 \pm 2.3 ^{ab}	0.02
48h	20.3 \pm 1.7 ^b	32.4 \pm 2.1 ^a	28.9 \pm 1.9 ^a	0.03
		<i>MTT absorbance (nm)</i>		
0h	0.692 \pm 0.03 ^b	0.885 \pm 0.04 ^a	0.873 \pm 0.04 ^a	0.005
24h	0.569 \pm 0.04 ^b	0.683 \pm 0.04 ^a	0.656 \pm 0.03 ^a	0.04
48h	0.377 \pm 0.03 ^b	0.564 \pm 0.03 ^a	0.452 \pm 0.02 ^{ab}	0.02
		<i>CFDA/PI (%)</i>		
0h	51.8 \pm 2.0 ^b	66.85 \pm 1.9 ^a	65.4 \pm 2.6 ^a	0.001
24h	30.9 \pm 2.3 ^b	45.35 \pm 2.6 ^a	43.0 \pm 3.1 ^a	0.001
48h	17.4 \pm 3.6 ^b	34.9 \pm 1.6 ^a	24.9 \pm 3.2 ^b	0.01
		<i>Eosin-nigrosin (%)</i>		
0h	48.6 \pm 1.65 ^b	62.7 \pm 2.1 ^a	62.8 \pm 1.8 ^a	0.001
24h	31.5 \pm 1.5 ^b	43.0 \pm 2.2 ^a	38.5 \pm 2.5 ^a	0.002
48h	16.1 \pm 2.1 ^c	37.6 \pm 2.1 ^a	29.5 \pm 2.2 ^a	0.03

^{a,b} Different letters on the same line indicate statistical difference ($P < 0.05$).

SpermFilter is characterized as being an efficient technique in sperm concentration and for removing seminal plasma without causing damage to cells (Alvarenga *et al.*, 2012). Conducting the evaluation of the equine semen using SpermFilter was more effective in maintaining the viability of the refrigerated semen in a similar way to conventional centrifugation, with the advantage of increasing the number of retrieved spermatozoa (Ramires Neto *et al.*, 2013). Increased changes in equine semen were associated with decreased fertility (Jasko *et al.*, 1991a). The higher number of viable and intact spermatozoa after filtration by glass wool of frozen bovine semen resulted in better in vitro fertilization capacity (Lee *et al.*, 2009).

In the study of Alvarenga *et al.* (2012), the use of SpermFilter® and the colloid centrifugation technique were equally efficient, as well as centrifugation for the removal of seminal plasma from the stallions' semen. SpermFilter has advantages because it promotes less damage to the plasma membrane when compared to centrifugation. In addition, it reduces bacterial growth in semen and is more practical in the field since it does not require the use of a centrifuge. Our results obtained in experiment 1 demonstrated that the centrifugation was not able

to increase sperm motility when compared to the control group. Additionally, it was inferior to the semen submitted to plasma removal through the SpermFilter and the separation of cells by glass wool. The separation with SpermFilter promotes minor changes in cells, while centrifugation increased the percentage of cells with tail damage and these findings corroborate other studies (Alvarenga *et al.*, 2012; Ramires Neto *et al.*, 2013).

Mitochondrial activity was reduced in all treatments during the cooling period. However, after 48h of cooling, the mitochondrial activity remained higher for the group filtered by glass wool. The centrifuged and non-centrifuged ejaculates (control) showed inferior performance in the evaluated parameters when compared to the SpermFilter and glass wool groups, which also presented greater membrane functionality (HOST) and mitochondrial activity. Thus, it was evident that the glass wool method allows selection of spermatozoa with greater viability and the SpermFilter is an alternative to concentrate semen of pony stallions with less damage when compared to centrifugation. It was observed in this study that centrifugation could cause damage to sperm structure and reduce mitochondrial activity.

In experiment 2, centrifugation using Androcoll-E or glass wool separation proved to be more efficient than negative control (conventional centrifugation). This statement is based on the findings of higher number of normal cells, greater mitochondrial activity, and greater viability. The use of colloid centrifugation as Androcoll-E has been widely researched and excellent results have already been obtained for sperm selection both before and after preservation of equine semen (Johannisson *et al.*, 2009; Morrell *et al.*, 2009, 2011a, 2011b; Costa *et al.*, 2012; Macias-Garcia *et al.*, 2012). Obtaining similar parameters between centrifugation with glass wool and Androcoll-E suggests that both techniques can be used prior to cooling the semen of pony stallions. The highest number of normal spermatozoa selected through glass wool separation and Androcoll-E can be used as a justification to increase mitochondrial activity during the cooling period for both 24h and 48h.

Similar finding was reported by (Samper & Crabo, 1993) when affirming that the spermatic filtration can increase the number of intact spermatozoa and present a good correlation with fertility in Arabian and one Swedish Warmblood stallion. However, there are studies using Androcoll-E for selected sperm and did not differ from the non-treated semen in terms of viability, morphology, response to hypo-osmotic swelling test (HOS test) and mitochondrial membrane potential (Gamboa *et al.*, 2017).

Studies indicate that the mitochondria of the equine spermatozoon are more sensitive to osmotic alterations than the plasma membrane (Garcia *et al.*, 2011). Therefore, selecting spermatozoa with higher mitochondrial activity may be related to higher cold support. It is known that good stallions and bad freezers differ in levels of oxygen reactive species (ROS) after cryopreservation and have no relation to damage caused to the DNA of the nucleus. In turn, the best resistance to cryopreservation is seen in spermatozoa with greater mitochondrial potential (Yeste *et al.*, 2015). The MTT test described by Aziz *et al.* (2005) and used in this research is a reliable colorimetric assay to evaluate sperm viability and mitochondrial activity in horses.

The selection of spermatozoa by filtration in glass fibers has already been attributed as susceptible to contamination by glass wool debris and to

promote changes in pH and osmolarity of the medium (Morrell, 2012). In this study, these changes were not verified. Additionally, the results obtained with glass wool are also promising, since they have already been reported in other species (Johnson and Garvin, 1959; Paulson and Polakoski, 1977; Arzondo *et al.*, 2012).

Selection in glass wool was used by Arzondo *et al.* (2012) in frozen bovine semen. The authors state that the technique is fast, simple, inexpensive, highly effective in isolating spermatozoa and enriching the population of available and viable gametes, making it a useful tool for assisted reproduction technologies. It remains to be clarified if selected spermatozoa with glass wool submitted to refrigeration at 5°C and cryopreserved at -196°C show high viability after cryopreservation.

CONCLUSIONS

The separation technique of seminal plasma using the SpermFilter allows concentration of spermatozoa without damage. There was no difference between Androcoll-E and glass wool for semen cooled for 24h. However, selection by Androcoll-E resulted in higher percentage of viable spermatozoa and higher mitochondrial potential at 48h of cooling.

ACKNOWLEDGMENTS

CAPES/FAPERGS by the author scholarship.

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