Effect of Removing Seminal Plasma Using a Sperm Filter on the Viability of Refrigerated Stallion Semen

Carlos Ramires Neto, Gabriel Augusto Monteiro MSc, Rafaela Fatima Soares, Cesar Pedrazzi DVM, Jose Antonio Dell’aqua Jr. PhD, Frederico Ozanan Papa PhD, Marco Antonio Alvarenga PhD

A School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, São Paulo, Brazil
b Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, São Paulo, Brazil
c CEAPE Tecnologia Veterinária Ind., Sorocaba, São Paulo, Brazil

Abstract

Cooling of equine semen obtained from some stallions results in lower seminal quality and viability when the seminal plasma (SP) is present. The objective of this study was to evaluate the effect of the removal of SP using a Sperm Filter on the viability of cooled stallion semen. For this purpose, 31 stallions were used. Their ejaculates were divided into three groups: CN, semen was diluted with an extender; FLT, SP was removed by filtration; and CT, SP was removed by centrifugation and cooled to 15°C for 24 hours. Sperm kinetics and plasma membrane integrity were evaluated immediately after collection (T0) and after 24 hours of refrigeration (T1). No difference (P > 0.05) was noted at T1 for total sperm motility (TM), progressive sperm motility, or plasma membrane integrity when semen samples from all the stallions were analyzed. However, when samples from stallions termed “bad coolers” (TM < 30% at T1) were analyzed, a difference was observed in TM and progressive sperm motility for CN compared with FLT and CT at T1. Sperm recovery was greater when SP was removed using the filter (FLT) to that when the SP was removed by centrifugation (CN) (89% vs. 81%). Thus, we concluded that filtering with a Sperm Filter is an efficient and practical method for removal of SP from stallion ejaculates, with lower sperm loss than centrifugation. We also found that the presence of SP reduces the quality and viability of cooled semen from stallions whose semen is sensitive to the process of refrigeration.

1. Introduction

Cooling of stallion semen reduces the metabolic activity of the sperm and thereby increases the time that the sperm remains fertile [1]. Cooled semen can be stored and transported [1], and the rates of conception obtained using cooled semen are similar to those obtained with fresh semen [2]. This semen processing approach, together with artificial insemination, minimizes the spread of diseases and allows for the breeding of geographically distant animals [3,4]. Although it provides benefits in terms of reducing postbreeding endometritis [5], seminal plasma (SP) has been reported as being deleterious to the in vitro preservation of refrigerated semen [6-9].

SP is a fluid produced by the rete testis, epididymis, and accessory glands, and is expelled in fractions during ejaculation. Its function involves the transport and supply of metabolic substrates to the sperm and also participates in...
the process of sperm maturation [10]. It also contains substances that protect and stimulate the cells [1]; however, owing to its variable composition among individuals, SP can be beneficial or deleterious to sperm quality [2]. Thus, it is sometimes necessary to remove SP from seminal samples to increase the time that the cooled semen is viable [7,11].

Centrifugation is the technique normally used to remove SP and to concentrate sperm from the ejaculate; however, the intensity and time of centrifugation can interfere negatively with the motility, plasma membrane integrity (PMI), and quantity of sperm recovered. Siene et al. [12] observed that in some stallions, the process of centrifugation damages the cells. Dell’aqua et al. [13] showed that centrifugation at 600 g for 10 minutes provided the best quality and the least sperm loss during the centrifugation process.

Recently, a new method for removal of SP from stallion ejaculate using a filter made of a synthetic hydrophilic membrane (Sperm Filter, CEAFEPE Tecnologia Veterinária Ind., Sorocaba, São Paulo, Brazil) was proposed [14]. This filter was shown to be efficient for the retention and preservation of the viability of stallion sperm, allowing only the passage of SP. Afterward, the retained spermatozoa are resuspended with an extender until the desired sperm concentration per milliliter of semen is reached.

The aim of this study was to evaluate the effect of removing SP using the Sperm Filter on cooled stallion semen.

2. Material and Methods

2.1. Collection of Semen and Division into Groups

Thirty-one stallions, ranging in age from 5 to 20 years, of the Quarter Horse, Mangalarga Marchador, and Thoroughbred breeds were used.

After collection with an artificial vagina, semen from each ejaculate was diluted 1:1 with a skim milk–based extender (Botu-Sêmen; Botupharma, SP, Brazil), and the ejaculates were divided into three groups: CN, FLT, and CT. For the CN samples, the semen was further diluted with Botu-Sêmen until reaching a concentration of 50 x 10^6 sperms/mL. For the FLT samples, the semen was filtered using the Sperm Filter (Pat req. US2010/0099075), and the sperms retained in the filter were resuspended with Botu-Sêmen to a final concentration of 50 x 10^6 sperms/mL. For the CT samples, the semen was centrifuged to 600 x g for 10 minutes, and the pellet was resuspended in Botu-Sêmen at a final concentration of 50 x 10^6 sperms/mL.

2.2. Cooling Semen

Immediately after collection and processing, the semen samples from the three groups were conditioned in the same commercial Botu-Flex (Botupharma, Brazil) refrigeration system for 24 hours at 15°C.

2.3. Analysis of the Semen

The semen samples were analyzed immediately after collection and processing (T0) and 24 hours after refrigeration (T1) at 15°C (Botu-Flex).

The following motility parameters were analyzed: total sperm motility (TM; %), progressive sperm motility (PM; %), rapid sperm (%), velocity of trajectory (μm/s), linear progressive velocity (μm/s), and curvilinear velocity (μm/s), using Computer-Assisted Semen Analysis (Hamilton-Thorne, Beverly, MA). In addition, the PMI (%) was analyzed using epifluorescence microscopy (Leica Microsystems, DMLB, Germany) with the fluorescent probes 6-carboxy-fluorescein diacetate and iodine propidium [15].

The semen, after filtration and centrifugation, was diluted to the same initial volume to calculate sperm loss, and a Neubauer chamber was used to determine concentration of the rediluted aliquots.

2.4. Classification of the Stallions

First, all stallions (n = 31) were analyzed in the same group (experiment 1). Then, these same animals were subdivided into two categories, based on observed sperm motilities after cooling (experiment 2) (24 hr/15°C). The stallions were classified as sensitive to the seminal refrigeration process (“Bad coolers”) if they showed less than 30% TM in CN after cooling (T1) and were classified as “Good coolers” if they showed more than 30% TM in CN at T1.

2.5. Statistical Analysis

All the sperm parameters were compared with analysis of variance using the program GraphPad InStat 3 (GraphPad Software, La Jolla, CA). Differences were considered significant at a probability level of P < .05 [16].

3. Results

In experiment I, when values from all stallions were included in the analysis, there were no differences (P > .05) for any parameter measured (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>TM (%)</th>
<th>PM (%)</th>
<th>VAP (μm/s)</th>
<th>VSL (μm/s)</th>
<th>VCL (μm/s)</th>
<th>RAP (%)</th>
<th>PMI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN T0 (n = 31)</td>
<td>71.7 ± 12.4</td>
<td>33.8 ± 11.4</td>
<td>114.1 ± 15.8</td>
<td>88.1 ± 9.6</td>
<td>61.0 ± 14.4</td>
<td>54.7 ± 14.7</td>
<td>71.7 ± 12.4</td>
</tr>
<tr>
<td>FLT T0 (n = 31)</td>
<td>72.3 ± 13.1</td>
<td>34.6 ± 12.5</td>
<td>113.1 ± 17.3</td>
<td>87.0 ± 11.6</td>
<td>61.2 ± 16.8</td>
<td>57.6 ± 12.1</td>
<td>72.3 ± 13.1</td>
</tr>
<tr>
<td>CT T0 (n = 31)</td>
<td>68.2 ± 13.8</td>
<td>31.2 ± 11.2</td>
<td>113.9 ± 16.8</td>
<td>90.2 ± 16.8</td>
<td>58.9 ± 15.8</td>
<td>35.6 ± 13.3</td>
<td>68.2 ± 13.8</td>
</tr>
<tr>
<td>CN T1 (n = 31)</td>
<td>37.1 ± 23.3</td>
<td>14.2 ± 13.2</td>
<td>93.8 ± 20.1</td>
<td>70.6 ± 16.8</td>
<td>28.8 ± 24.6</td>
<td>35.3 ± 15.3</td>
<td>37.1 ± 23.3</td>
</tr>
<tr>
<td>FLT T1 (n = 31)</td>
<td>51.0 ± 19.3</td>
<td>21.8 ± 12.4</td>
<td>96.8 ± 19.6</td>
<td>73.3 ± 14.7</td>
<td>41.0 ± 21.6</td>
<td>45.5 ± 13.4</td>
<td>51.0 ± 19.3</td>
</tr>
<tr>
<td>CT T1 (n = 31)</td>
<td>47.9 ± 20.3</td>
<td>17.1 ± 10.7</td>
<td>87.9 ± 17.9</td>
<td>66.7 ± 13.3</td>
<td>35.1 ± 18.9</td>
<td>47.2 ± 13.8</td>
<td>47.9 ± 20.3</td>
</tr>
</tbody>
</table>

TM, total motility; PM, progressive motility; VAP, velocity of trajectory; VSL, linear progressive velocity; VCL, curvilinear velocity; RAP, rapid sperm; PMI, plasma membrane integrity; CN, semen containing seminal plasma; FLT, semen filtered to remove seminal plasma; CT, semen centrifuged to remove seminal plasma; T0, before refrigeration; T1, after refrigeration.
In stallions classified as “Bad coolers” (experiment 2), no difference was observed (P > 0.05) for any of the parameters between the three groups (CN, FLT, and CT) at T0. However, 24 hours after refrigeration (T1) at 15°C (Table 2), values for TM, PM, and RAP were higher (P < 0.05) in groups in which the SP had been removed (FLT and CT) as compared with the group containing SP (CN). In addition after 24 hours of storage, the PMI was higher in the centrifuged sample (CT) than in the group with SP (CN; Table 2).

In experiment 2, no differences (P < 0.05) were observed in any semen parameter evaluated at T0 for stallions classified as “Good Coolers” (Table 3). However, velocity of trajectory was higher (P < 0.05) in CN than in CT, and the PMI was superior (P < 0.05) in the filtered group (FLT) than in CN, after 24 hours of seminal refrigeration (Table 3).

Higher percentage of sperm cells were recovered (P < 0.05) when filtering was used (89.4 ± 7.4%) compared with centrifugation (80.9 ± 5.5%). The filtration process was accomplished in approximately 5 minutes.

4. Discussion

The presence of SP was believed to be essential for the process of fecundation. However, recent publications have reported that when sperms from the epididymis are processed in an extender, they will have quality and fertility equal to or superior to sperms that have mixed with SP at ejaculation [17,18].

There is great disagreement among authors regarding the influence of SP on the viability of cooled stallion semen. Love et al. [19] reported an increase in the fragmentation of sperm DNA when SP was present in cooled stallion semen, which could lead to a reduction in fertility. Troedsson et al. [10] reported that SP functions to reduce chemotaxis of polymorphonuclear cells in the uterine endometrium and consequently reduces postbreeding endometritis. Some authors report that the total removal of SP from the ejaculate was deleterious to sperm quality: showing that 5%–20% of SP increases fertility and sperm viability time [2,20,21].

Moore et al. [20] have shown that the presence of SP was not relevant to the quality and viability of cooled semen when it was processed immediately after collection. However, when the semen was exposed to room temperature before the cooling process, the presence of SP had deleterious effects. Brinsko et al. [7] concluded that the removal of SP did not influence, or, in some situations, could negatively influence, sperm kinetics of refrigerated stallion semen. A similar result was observed in the present study, when analyzing only the animals with good performance, no difference was observed in most of the evaluated sperm parameters. However, the removal of SP was beneficial in preserving the PMI for sperms from this group of stallions.

In stallions with semen sensitive to the process of refrigeration (“Bad coolers”), we noted a beneficial effect of the removal of SP on sperm quality and viability, corroborating the results of Brinsko et al. [7]. In the present study, after 24 hours of refrigeration, a larger decrease in TM, PM, and RAP was observed for the samples with SP compared with those without. In addition, a higher level of PMI was observed for the centrifuged group compared with those with SP remaining. Love et al. [22] observed a significant increase in sperm DNA fragmentation rates 20 hours after refrigeration in animals classified as “Bad coolers” compared with animals resistant to the semen refrigeration process.

Centrifugation is the technique most often used to remove SP from stallion ejaculates. However, it can induce mechanical damage to the sperms, reducing their viability [23]. Dell’Acqua et al. [13] also demonstrated that a high

<table>
<thead>
<tr>
<th>Group</th>
<th>TM (%)</th>
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<th>VAP (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VCL (µm/s)</th>
<th>RAP (%)</th>
<th>PMI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN T0 (n = 12)</td>
<td>64.2 ± 13.2</td>
<td>30.1 ± 12.4</td>
<td>111.9 ± 12.4</td>
<td>85.6 ± 8.9</td>
<td>206.2 ± 21.3</td>
<td>54.2 ± 15.9</td>
<td>51.4 ± 17.6</td>
</tr>
<tr>
<td>FLT T0 (n = 12)</td>
<td>66.4 ± 15.8</td>
<td>33.0 ± 12.8</td>
<td>114.5 ± 12.4</td>
<td>88.0 ± 12.1</td>
<td>211.4 ± 19.7</td>
<td>59.3 ± 17.6</td>
<td>51.5 ± 13.1</td>
</tr>
<tr>
<td>CT T0 (n = 12)</td>
<td>61.8 ± 15.5</td>
<td>29.9 ± 12.3</td>
<td>113.8 ± 15.5</td>
<td>85.2 ± 16.2</td>
<td>206.8 ± 20.3</td>
<td>53.9 ± 17.7</td>
<td>51.8 ± 14.5</td>
</tr>
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</table>

Table 2

Mean values and standard deviations determined by Computer-Assisted Semen Analysis (Hamilton-Thorne, HTM-IVOS) for TM, PM, VAP, VSL, VCL, RAP, and PMI for “Bad coolers” (experiment 2) by groups CN, FLT, and CT

<table>
<thead>
<tr>
<th>Group</th>
<th>TM (%)</th>
<th>PM (%)</th>
<th>VAP (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VCL (µm/s)</th>
<th>RAP (%)</th>
<th>PMI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN T1 (n = 12)</td>
<td>36.1 ± 16.5A</td>
<td>13.4 ± 8.7A</td>
<td>89.6 ± 14.4</td>
<td>70.2 ± 17.3</td>
<td>174.5 ± 26.6</td>
<td>26.2 ± 18.2AB</td>
<td>37.5 ± 13.3AB</td>
</tr>
<tr>
<td>FLT T1 (n = 12)</td>
<td>33.2 ± 16.9A</td>
<td>12.4 ± 10.0B</td>
<td>85.3 ± 16.4</td>
<td>65.6 ± 16.5</td>
<td>171.0 ± 28.6</td>
<td>26.7 ± 19.7AB</td>
<td>44.3 ± 15.0A</td>
</tr>
</tbody>
</table>

Table 3

Mean values and standard deviations determined by Computer-Assisted Semen Analysis (Hamilton-Thorne, HTM-IVOS) for TM, PM, VAP, VSL, VCL, RAP, PMI for stallions with semen samples that were resistant to the seminal refrigeration process (experiment 2) by groups CN, FLT, and CT

TM, total motility; PM, progressive motility; VAP, velocity of trajectory; VSL, linear progressive velocity; VCL, curvilinear velocity; RAP, rapid sperm; PMI, plasma membrane integrity; CN, semen containing seminal plasma; FLT, semen filtered to remove seminal plasma; CT, semen centrifuged to remove seminal plasma; T0, before refrigeration; T1, after refrigeration. Values within different superscripts are significantly different (P < 0.05).
centrifuging speed can be deleterious to sperm integrity and that low centrifuging decreases sperm recovery. We concluded that centrifugation at 600× g for 10 minutes represented the best combination of sperm quality and quantity of sperm recovered.

Using the Sperm Filter, Alvarenga et al. [14] observed less damage than when centrifugation was used to remove the SP from previously cooled semen. This result contrasted with ours, probably because the cooling process caused the sperm to become more sensitive to mechanical damage. A higher sperm cell recovery was observed with the use of the Sperm Filter (89.4%) compared with centrifugation (80.9%). Loomis [21] noted a sperm loss similar to ours, but less damage than when centrifugation was used to remove the SP through centrifugation. The Sperm Filter method also allows stallion sperm to be concentrated without the necessity of expensive apparatus, such as centrifuges.

5. Conclusion

Based on the results of the present study, the Sperm Filter method used for removal of SP was effective in maintaining the sperm viability of refrigerated semen, similar to the system of centrifugation, with the advantage of increasing the number of recovered gametes. In this study, the presence of SP had deleterious effects on the quality and viability of sperm from stallions whose semen is sensitive to the process of refrigeration.

References