New seminal plasma removal method for freezing stallion semen

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1. Introduction

Semen freezing is a very important technique, because it can prevent the spread of diseases, eliminate geographical barriers and, above all, preserve genetic material indefinitely. However, damage to sperm during the semen freezing process can affect its subsequent fertility. This damage to sperm, when combined with the low resistance of the sperm of some stallions to cryopreservation [1], is one of the greatest barriers to widespread use of this biotechnology [2].

Post-thaw semen quality and fertility are influenced by several factors, namely the individual characteristics of each animal, technique used for seminal plasma removal, type of extender used, medium used for sperm dilution, freezing curve for the semen straws, type of straw storage used, semen thawing time and temperature, semen dose, and insemination technique used [3,4]. Because so many factors influence the success of sperm cryopreservation, no ideal protocol for all cases exists [5], although numerous studies have been conducted with the aim of improving the quality of equine frozen semen [6].

Of the steps involved in freezing equine semen, seminal plasma removal from the ejaculate before freezing, has been a major focus of research [7,8]. This step is essential for sperm cryopreservation [9,10]. The technique typically used in the removal of seminal plasma from stallion ejaculate is centrifugation; however, this procedure might cause mechanical damage to sperm [11]. After analyzing centrifugal force and duration, Dell'aqua et al. [12] reported that the sperm loss was minimized and sperm quality and integrity were maximized when semen was centrifuged at 600 × g for 10 minutes.

To concentrate semen without centrifugation and with less damage to sperm, Alvarenga et al. [13] proposed
a new method of removing seminal plasma from stallion ejaculate by filtering the semen through a filter composed of a synthetic hydrophilic membrane (Sperm Filter, BotuPharma, Botucatu, Sao Paulo, Brazil). The objective of the present study was to test the efficacy of the Sperm Filter in removing seminal plasma from stallion semen before freezing.

2. Materials and methods

2.1. Seminal plasma removal using the Sperm Filter

The Sperm Filter is a filter composed of a synthetic hydrophilic membrane with 2-μm pores; the filter is attached to a plastic ring that holds 50 mL of semen for filtration. The semen, diluted 1:1 with extender, was deposited on the membrane, and the filter was gently tapped over a 15-cm Petri dish. Because of the size of the pores and capillary action, 90 to 95% of the seminal plasma passed through the filter, and the sperm were retained. Then, an appropriate volume of extender was added to the filter, and the filter was moved to resuspend the sperm, as shown (Supplementary Fig. 1 and Supplementary File 1).

Filtration of 20 mL of diluted semen and subsequent resuspension required an average interval of 5 minutes to complete.

2.2. Experiment

Semen was collected (using an artificial vagina) from 31 healthy Quarter Horse, Mangalarga Marchador, and Thoroughbred stallions (5–20 years old). Collections were done on alternate days for 3 weeks before the study.

After semen collection, the ejaculate was first filtered through a nylon filter to remove gel and dirt and then diluted 1:1 with Botu-Semen (BotuPharma), a skim-milk-based extender. Sperm concentration was determined using a Neubauer chamber. The counts were performed in duplicate by microscopy (DMLB; Leica Microsystems, Wetzlar, Germany) and 10 μL of propidium iodide (0.5 mg/mL) and 10 μL of propidium iodide (0.5 mg/mL) [15]. In addition, a sperm count was performed on a sample before and after filtration and centrifugation using a Neubauer chamber. The counts were performed in duplicate by the same person, before and after semen processing.

2.3. Statistical analysis

Parameters before freezing were compared using analysis of variance (ANOVA), and after freezing, a paired Student t test was performed using GraphPad InStat Version 3 (GraphPad Software, La Jolla, CA, USA). The significance level was set at 5% [16].

3. Results

Before freezing, there were no differences (P > 0.05) among fresh semen in skim milk-based extender (G0), filtered semen (G1), and centrifuged semen (G2) for kinetic parameters and sperm plasma membrane integrity (Table 1). Additionally, for these parameters, there were no differences (P > 0.05) between G1 and G2 after thawing (Table 1). However, there were more sperm recovered in G1 versus G2 (89.4 ± 7.4% vs. 80.9 ± 5.5%).

### Table 1

Mean ± SD for sperm parameters of stallion G0, G1, and G2 before (M1) and after freezing (M2).

<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>PF (%)</th>
<th>PMI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0 (M1)</td>
<td>71.4 ± 17.7</td>
<td>32.8 ± 12.9</td>
<td>118.7 ± 19.5</td>
<td>90.4 ± 12.0</td>
<td>211.0 ± 37.1</td>
<td>58.8 ± 22.6</td>
<td>55.3 ± 15.6</td>
</tr>
<tr>
<td>G1 (M1)</td>
<td>76.0 ± 13.5</td>
<td>34.8 ± 14.2</td>
<td>106.3 ± 11.8</td>
<td>80.5 ± 9.1</td>
<td>186.1 ± 24.8</td>
<td>66.1 ± 18.0</td>
<td>58.3 ± 12.9</td>
</tr>
<tr>
<td>G2 (M1)</td>
<td>72.6 ± 19.7</td>
<td>33.4 ± 14.6</td>
<td>105.0 ± 14.3</td>
<td>80.7 ± 9.5</td>
<td>185.1 ± 20.9</td>
<td>64.7 ± 22.9</td>
<td>57.5 ± 16.0</td>
</tr>
<tr>
<td>G1 (M2)</td>
<td>50.8 ± 24.6</td>
<td>24.3 ± 14.9</td>
<td>84.9 ± 13.8</td>
<td>68.6 ± 10.0</td>
<td>148.5 ± 20.9</td>
<td>36.1 ± 22.9</td>
<td>30.2 ± 7.8</td>
</tr>
<tr>
<td>G2 (M2)</td>
<td>47.5 ± 25.4</td>
<td>22.5 ± 14.1</td>
<td>84.3 ± 13.1</td>
<td>68.7 ± 9.5</td>
<td>148.8 ± 21.8</td>
<td>35.1 ± 22.3</td>
<td>29.8 ± 11.2</td>
</tr>
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Abbreviations: G0, fresh semen in a skim milk-based extender; G1, sperm previously filtered for seminal plasma removal; G2, sperm previously centrifuged for seminal plasma removal; PF, progressive fast sperm; PM, progressive motility; PMI, plasma membrane integrity; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight linear velocity.
4. Discussion

In the present study, the filtering technique (G1) effectively removed seminal plasma from stallion semen before freezing. This step in equine semen cryopreservation is essential, because seminal plasma can be detrimental to sperm [17]; therefore, the sperm is concentrated, thereby facilitating storage of large numbers of sperm in small volumes [18]. Although centrifugation is commonly used to remove equine seminal plasma, this might damage sperm and thus reduce motility and fertility [2,11].

Many studies have sought to determine the best combination of centrifugal force and time for removing seminal plasma from semen [10,17,19–24]. In this study, we used a force/time relationship previously proposed [12]. Because there was no significant difference between G0 and G2, we concluded that the centrifugation protocol (600 × g for 10 minutes) did not damage the sperm and did not affect sperm kinetics. This relationship between the centrifugal force and duration of the process is directly linked to the damage caused to sperm and to the amount of sperm recovered [18]. High centrifugal forces cause sperm to adhere strongly to the pellet, which is harmful to sperm cells [23,25,26], and low centrifugal forces result in low sperm recovery [18].

The filter used in this study was a synthetic hydrophilic membrane filter (Sperm Filter), which was proposed by Alvarenga et al. [13] to remove seminal plasma from the ejaculate and cause less damage to sperm. The use of this filter did not damage the sperm, considering that there were no differences in sperm kinetics and plasma membrane integrity compared with fresh semen in a skim milk-based extender.

Sperm recovery from the centrifuged group (G2) confirmed a statement by Loomis [27], who reported a loss of approximately 25% when seminal plasma was removed by centrifugation. The higher sperm recovery rate obtained using the Sperm Filter (89%) compared with centrifugation (81%) facilitated the use of the ejaculate. These data are of great importance, because the total number of sperm present in the ejaculate and the percentage of sperm recovered after seminal plasma removal influence the number of doses of frozen-thawed semen that can be produced.

When comparing use of the Sperm Filter versus centrifugation, there were no significant differences in plasma membrane integrity. In contrast, Alvarenga et al. [13] reported a higher percentage of damage to the plasma membrane of sperm when centrifugation was used to remove the seminal plasma from equine semen. In that study, semen was previously cooled, thus causing the sperm to be more susceptible to mechanical damage.

In addition, no difference was observed between the filtration technique (G1) and centrifugation (G2) with regard to the kinetic parameters evaluated. However, the Sperm Filter had the advantages of a higher rate of sperm recovery and practicality, because it eliminates the use of costly equipment and is faster than centrifugation.

Recently, the cushioned centrifugation technique has been proposed to maximize sperm recovery from centrifuged equine semen [18,27–29]. This technique, and the Sperm Filter, avoids sperm packing, which might cause damage to sperm cells [22,26]. Future studies should compare the results obtained with the Sperm Filter and centrifugation techniques with cushioned centrifugation.

4.1. Conclusions

The Sperm Filter was as efficient as centrifugation in removing seminal plasma from stallion ejaculate and it had the advantages of a lower rate of sperm loss and greater practicality. Future studies should be performed to compare the filtering technique with other seminal plasma removal techniques, e.g., cushioned centrifugation. Fertility tests should also be performed on semen subjected to seminal plasma removal with the Sperm Filter.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2013.01.014.

References


Supplementary Fig. 1. Stallion semen, diluted with extender in a 1:1 ratio, was deposited on the membrane (A), and the filter was gently tapped over a 15-cm Petri dish (B). Because of the size of the pores and capillary action, seminal plasma passed through the filter, and sperm were retained (C).