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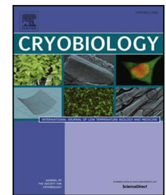

Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma

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ABSTRACT

Addition of seminal plasma (SP) prior to cryopreservation may influence stallion sperm cryosurvival. The objective of this study was to investigate the addition of pooled SP from "good" or "bad" freezer stallions to spermatozoa selected by single layer centrifugation (SLC) prior to cryopreservation on post-thaw sperm quality. Semen from 12 stallions was collected; 5 mL was frozen as control (C) and the remainder was processed by SLC to remove SP and was divided into three aliquots: i) SLC sample without SP (SLC); ii) SLC plus pooled SP from "good freezer" stallions (SLC-GF); iii) SLC plus pooled SP from "bad freezer" stallions (SLC-BF). After thawing, the following parameters were evaluated: chromatin integrity (DNA fragmentation index; %DFI), mitochondrial membrane potential (MMP), membrane integrity (MI), reactive oxygen species (ROS) and sperm kinematics. The %DFI was reduced ($P < 0.0001$) in SLC samples compared to controls. The SLC group showed a lower proportion of spermatozoa with low MMP and a higher proportion of spermatozoa with high MMP than other groups ($P < 0.0001$), and had lower hydrogen peroxide content than control. Sperm kinematics were not different. In conclusion, selection by SLC prior to cryopreservation improved post-thaw sperm quality; inclusion of SP from "good" and "bad" freezer stallions did not have an additional beneficial effect.

1. Introduction

Cryopreservation is an invaluable tool to preserve spermatozoa of any species for an indefinite period. Artificial insemination (AI) with frozen semen has become increasingly frequent for breeding most domestic animals, including the horse. Using frozen semen has two major advantages; first, by ensuring the availability of spermatozoa when needed for insemination [23,30]; second, the ability to transport frozen semen over long distances without deterioration. Specifically for equine breeding, the possibility of utilizing frozen semen provides greater flexibility and facilitates supply during periods of intensive demand for fresh semen or when the stallion is needed for competition as well as for stud purposes [23,30].

In horse breeding, stallions are selected mainly according to their performance, with little regard to semen quality or the ability of their spermatozoa to survive cryopreservation. As a result, some ejaculates are of poor quality and do not survive storage or freezing. This considerable variation among stallions causes difficulties in presenting frozen stallion semen as a realistic alternative to fresh semen for artificial insemination [12,24]. However, cryopreservation would offer several advantages: the availability of semen would not depend on the presence of the stallion or on his ability to produce an ejaculate on demand; there would be more flexibility when performing the insemination, and semen from particular individuals would be widely available rather than being restricted to studs within transport distance for fresh semen.

Although there are many reasons for wanting to use frozen stallion semen, it is still not possible to freeze all stallion ejaculates successfully. Not only does freezability vary among stallions, but there is also variation between ejaculates from a given stallion [24,40]. Thus there is a requirement to develop better cryopreservation techniques. Furthermore, maintaining membrane stability, acrosome integrity and avoiding the accumulation of ROS during storage are important for establishing a healthy pregnancy. The role of seminal plasma (SP) in maintaining sperm viability and acrosome integrity during cryopreservation has still not been determined. Some researchers claim

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beneficial effects of SP on cryosurvival [28] whilst others have seen detrimental effects [1,25]. Including small amounts of stallion SP (0.6%–20%) was found to have a beneficial effect on sperm parameters [16,31]. Inclusion of SP from stallions known as "good freezer" was reported to improve the post-thaw sperm quality of stallions known as "bad freezer". In contrast, adding SP from a stallion with low post-thaw motility to semen from stallion with high post-thaw motility reduced the progressive motility without affecting the viability [2]. However, variable quantities of the stallions' own seminal plasma may have been included in these various studies, making interpretation of the results difficult. "Washing" of spermatozoa by centrifugation in the presence of extender does not remove all the seminal plasma proteins coating the spermatozoa [22].

One way of overcoming the problem of the stallions' own SP being present is to prepare the sperm samples by colloid centrifugation, such as Single Layer Centrifugation (SLC) [22]. This is a valuable technique to select good quality spermatozoa i.e. those with good morphology, viability and chromatin integrity. Thus combining SLC with the addition of small quantities of SP from stallions of known freezability could help to improve cryosurvival of stallion spermatozoa [15]. The hypotheses were that the quality and origin of seminal plasma added would affect sperm cryotolerance, and that colloid centrifugation isolates a population of spermatozoa whose cryosurvival is better than the population as a whole. The aim of the current study, therefore, was to evaluate the efficiency of applying SLC and adding SP from good or bad freezer stallions prior to cryopreservation on sperm quality after thawing.

2. Materials and methods

2.1. Animals and husbandry

Twenty warmblood stallions, 4–18 years old (mean 7.6 ± 3.9 years), of proven fertility, were housed under standard husbandry condition at Brandenburg State Stud Neustadt/Dosse, Germany. The semen was subjected to a test freezing protocol to confirm the freezability as described below.

2.2. Collection and classification of stallion's freezability

Semen was collected using a lubricated Hannover artificial vagina pre-warmed to 42-44 °C. The semen was filtered through gauze into a measuring cylinder to remove the gel. Sperm concentration was evaluated using a SpermaCue photometer (Minitüb, Tiefenbach, Germany); subsequently a 500 μL aliquot from the gel-free fraction of the ejaculate was immediately extended 1:1 with Equiplus extender (Minitüb, Tiefenbach, Germany) that had been warmed to 38 °C, for evaluation of the fresh semen. Sperm motility was estimated subjectively in 5 μL of extended semen placed on a warm slide under an 18 × 18 mm coverslip using a phase contrast light microscopy, and also after 1:4 dilution with the same extender, using a computer-assisted sperm analyzer (AndroVision; Minitüb, Tiefenbach, Germany).

2.3. The test freezing protocols

Semen was obtained from stallions of known freezability according to the stud records; test freezing was conducted to confirm the freezability, before the start of the present experiment. Sperm freezing was done as previously described by Schober et al. [38] with slight modifications. Briefly, the diluted semen (1:2) was centrifuged at 750 × g for 10 min, after which the supernatant was removed using a water-driven vacuum pump. The resulting sperm pellet was resuspended with 1 mL of Gent freezing extender; according to the manufacturer's website the extender contains egg yolk, 5% glycerol and antibiotics (Minitüb, Tiefenbach, Germany). Thereafter, an equal volume of freezing extender was added to double the initial volume of the resuspended pellet. Straws were filled automatically using an automatic filling and sealing machine (MPP Uno; Minitüb, Tiefenbach, Germany). The straws were frozen using a computer controlled freezer (Ice Cube 14S; Minitüb, Tiefenbach, Germany). The freezing procedure included a slow cooling phase (0.3 °C/min) from 20 °C down to 5 °C. Subsequently a rapid cooling phase (10 °C/min), from 5 °C to −25 °C then after the second rapid phase (25 °C/min) to cool down the sample from −25 °C to −140 °C was used. After 1 h the straws were taken out of the Ice Cube and plunged directly into liquid nitrogen (−196 °C); they were stored in liquid nitrogen until required for analysis.

One week later one straw from each ejaculate was thawed by immersing in a 37 °C water bath for 30 s. Post-thaw total and progressive motility were evaluated using the AndroVision (Minitüb, Tiefenbach, Germany). According to the stud evaluation, the stallion was classified as a "good freezer" if the total motility was ≥60% and the progressive motility was ≥40, and as a "bad freezer" if the total motility was ≤50% and the progressive motility was ≤30.

Seven stallions were excluded from the study because the post-thaw motility values were midway between the thresholds for good and bad.

2.4. Preparation of seminal plasma

This part of the experiment was conducted separately as a preparatory step. Seminal plasma was harvested from fresh gel-free semen by centrifugation at 2000 × g for 10 min. The supernatant was aspirated into sterile tubes and checked for the presence of spermatozoa; if spermatozoa were seen, the samples were centrifuged again at 3500 × g until the SP became sperm-free. Thereafter the SP was filtered into labeled 4 mL tubes using a membrane syringe filter size 0.2 μm, and stored at −80 °C until used. Equal volumes of SP from "good freezer" (n = 7) or "bad freezer" (n = 6) stallions, were pooled.

2.5. Effect of seminal plasma

Twelve stallions were used for this experiment, according to availability during the study period. Six of them were confirmed as "good freezers" and the others as "bad freezers". Four ejaculates were obtained from each stallion, except for one stallion where only three were obtained (n = 47). Semen was collected twice a week during the period January–February 2015.

2.6. Preparation of spermatozoa

2.6.1. Sperm concentration

The concentration of spermatozoa was evaluated before freezing using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) according to the manufacturer's instructions: (50 μL) of each sample was mixed with 5 mL of reagent S100 in a sample cup. A cassette containing propidium iodide (PI) was loaded with the mixture and inserted into the fluorescence detector.

2.6.2. Semen processing and freezing

Gel-free semen was evaluated for volume, concentration and motility immediately after collection as described above. The concentration was evaluated using the Nucleocounter SP-100. Five mL of the ejaculate were extended to a concentration of 200×10^6 spermatozoa/mL using Gent Extender for fresh semen (Minitüb, Tiefenbach, Germany). According to the manufacturer's website, this extender contains egg yolk and antibiotics. The samples were prepared for freezing using the previously described protocol, (control (C) samples; Fig. 1). The concentration of the remaining sample was adjusted to 100×10^6 spermatozoa/mL, and used for SLC with the colloid Equicoll (formerly known as Androcoll-E) [33].

The method was described by Morrell et al. [32]. Briefly, 15 mL of extended semen at a concentration of 100×10^6 spermatozoa/mL was layered carefully over 15 mL of Equicoll, previously equilibrated to

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