**Application**

Apart from thoroughbred breeding, where natural mating is the method of choice, most sporthorse breeding involves the use of artificial insemination (AI) with cooled semen. The availability of frozen semen would facilitate the use of AI in horses if post-thaw sperm quality can be improved. Cryopreservation of stallion semen is possible for some ejaculates but post-thaw sperm quality varies considerably. Using a sperm preparation protocol that is less harsh when removing some of the seminal plasma, such as reducing centrifugation force, could benefit sperm cryosurvival. This study evaluates whether centrifugation through high or low-density colloids yields better post-thaw sperm quality compared to sperm washing, which is the conventional method of preparing stallion semen for freezing.

**Introduction**

Cryopreserving stallion ejaculates can be challenging as not all sperm samples yield good sperm quality on thawing. One possibility is that centrifuging the semen to increase the sperm concentration prior to freezing damages the spermatozoa. Colloid centrifugation provides an alternative method for separating spermatozoa from seminal plasma which does not cause damage. Whereas centrifugation through a high density colloid selects the most robust sperm which survive cryopreservation better than the unselected samples (Hoogewijs et al., 2011), some spermatozoa are lost in the process. Centrifugation through a low density colloid was used to separate spermatozoa from seminal plasma without selecting for good quality spermatozoa (Malaluang et al, 2024) and produced sperm samples of comparable quality to sperm washing for cooled storage. Objective: to compare the effect of centrifugation through high or low density Equicoll and sperm washing prior to freezing on post-thaw sperm quality

**Materials and Methods**

Three ejaculates from each of 10 stallions at a commercial stud in Sweden was collected using an artificial vagina. An aliquot of each ejaculate was extended 1:1 in Kenny´s extender without antibiotics and was transported to the laboratory at the Swedish University of Agricultural Sciences in an insulated box. On arrival, the concentration was adjusted to 100 x106/mL with Kenny´s extender and each sample was split into three portions. One portion (10 mL) was centrifuged at 800x g for 10 minutes as the control (sperm washing; Malaluang et al., 2024). The other two portions (10 mL) were each layered over 15 mL high or low density Equicoll in a 50 mL tube and centrifuged at 300 x g for 20 minutes (Malaluang et al., 2024). After centrifugation the resulting sperm pellets were resuspended in EquiPlus Freeze (Minitube International, Tiefenbach, Germany) to a sperm concentration of 200 x106/mL and were used to fill 0.5 mL plastic straws. These were frozen in a programmable freezer (Cryochamber, Cryologic, Australia) and plunged into liquid nitrogen for storage. After at least 24h in liquid nitrogen, the straws were thawed in a water bath at 37 °C for 30 sec. Sperm quality was analysed using computer assisted sperm analysis and flow cytometry for membrane integrity after staining aliquots with SYBR14/propidium iodide. Chromatin integrity was assessed using the Sperm Chromatin Structure Assay. Sperm quality means were compared using a mixed model (PROC MIXED) with the SAS software (version 9.4; SAS Institute Inc., Cary, NC), followed by Scheffé adjustment for multiple comparisons.

**Results**

Sperm yields after centrifugation were similar for sperm washing and centrifugation through low density Equicoll but was less for centrifugation through high density Equicoll (P<0.001). Mean total motility and membrane integrity were similar in all treatments. The DNA fragmentation index was lower for samples prepared by Single Layer Centrifugation with high density Equicoll than for the other two treatments, indicating selection for spermatozoa with intact chromatin.

|  |  |  |  |
| --- | --- | --- | --- |
| Variable | Sperm washing |  SLC with high density colloid | SLC with low density colloid |
| Yield (%) | 68.4 b | 41.8 ab | 80.2 a |
| Total Motility (%) | 35±2.3 | 31±2.3 | 33±2.3 |
| Membrane intact (%) | 51±2.3 | 50±2.3 | 49±2.3 |
| %DFI | 16±1.8b | 7±1.8ab | 13±1.8a |

Notes: SLC = Single Layer Centrifugation; %DFI = DNA fragmentation index. Different superscript letters indicate statistical significance within the row.

**Conclusions**

Centrifugation through low density Equicoll offers an effective alternative to sperm washing for preparing stallion semen for freezing, as it maintains sperm quality while significantly increasing yield. This approach could be particularly advantageous for equine breeding programs aiming to maximize sperm recovery without compromising post-thaw motility or membrane integrity. Although SLC through high density Equioll improved DNA integrity, the reduced yield suggests this method may be better suited for specific applications where chromatin integrity is critical, such as in cases of subfertile stallions or for intracytoplasmic sperm injection (ICSI). Further research could evaluate the long-term fertility outcomes of these methods and assess their cost-effectiveness in commercial breeding programs.

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