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Prolongation of stallion sperm survival by centrifugation through coated silica colloids: a preliminary study

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Abstract

Difficulties to be overcome in the widespread use of artificial insemination (AI) in mares are low sperm survival and poor sperm quality, which are encountered frequently among breeding stallions. Therefore, a method is needed to prolong the useable life of stallion spermatozoa destined for AI. In a preliminary study using 8 ejaculates from one stallion, density gradient centrifugation or centrifugation through a single layer of silica colloid appeared to prolong sperm motility compared to uncentrifuged spermatozoa, thereby potentially extending the useable life of treated stallion spermatozoa for AI. Furthermore, there was an improvement in sperm morphology, with the number of morphologically normal spermatozoa increasing from 42 to 60.5% and with the removal of approximately 60% spermatozoa with head or tail defects from the original population. No difference between the two centrifugation methods, in terms of yield or duration of spontaneous motility, could be detected in this study. Either of these methods of colloidal centrifugation could be a useful aid to preparing stallion spermatozoa for artificial breeding techniques, including AI.

Keywords: colloidal centrifugation, sperm survival, stallion.

Introduction

Artificial insemination of mares is less widespread than in other domestic animals due to problems of sperm survival during transport and considerable variation in ejaculate quality between stallions. Most insemination doses are transported cooled in a styrofoam container, usually at approximately 7°C (Malmgren, 1998), for up to 24 hours, with insemination taking place up to 36 hours after semen collection. Viable sperm losses are considerable by the time AI is performed, a problem which needs to be addressed (Colenbrander *et al*., 2003). Extending the useable live of the spermatozoa beyond 36 hours would be a considerable advantage to horse breeders.

Efforts have been made to prolong stallion sperm survival by using different semen extenders. However, no single semen extender appears to be universally suitable, and modifications of Professor Kenney´s skimmed milk formulation are still the most widely used extenders for stallion semen (Kenney *et al.*, 1975). Similar problems are not seen with production animals (dairy cattle and pigs) because males for semen production in these species can be selected for semen quality and sperm viability, rather than for the animal´s performance in competition, as in the case of equines (Colenbrander *et al*., 2003; Morrell, 2006). In contrast, the problem of using unselected donors is observed frequently in human assisted reproduction (Morrell, 2006).

Over the last 15 years, the technique of density gradient centrifugation has been used to select sub-populations of highly motile human spermatozoa for immediate use in *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) or, occasionally, for intrauterine deposition. Originally, silica particles coated with polyvinylpyrrolidone (PVP) were used, but owing to problems of reported toxicity (Avery and Greve, 1995) more recent formulations have used silane-coated silica particles in the density gradient (Mortimer, 2000). A recent study reported that senescence of human spermatozoa could be delayed by centrifugation on a density gradient (Morrell *et al.*, 2004). Density gradients have also been used to prepare bovine spermatozoa for *in vitro* fertilization (Rodriguez-Martinez *et al.*, 1997; Samardzijia *et al.*, 2006) and to enhance stallion semen quality in a preliminary study (Macpherson *et al.*, 2002). A modification of the density gradient technique, centrifugation through a single layer of colloid, has also been reported for human spermatozoa (Sharma *et al.*, 1997; Zhang *et al*., 2004).

The objective of the preliminary study reported here was to compare two methods of stallion sperm preparation: (i) a density gradient centrifugation, or (ii) a new technique - centrifugation on a “single layer” (i.e. with only one layer of colloid), for their effect on the survival of stallion spermatozoa. In both cases, the formulation of the density gradient or single layer was optimized especially for stallion spermatozoa, and contained silane-coated silica.

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Materials and Methods

Animals and husbandry

A standardbred stallion, 12 yr old, was housed under standard husbandry conditions at the Division of Reproduction, SLU, Uppsala, Sweden. Semen was collected up to three times a week over a 6-week period during the normal breeding season (April to August in Sweden) using an artificial vagina (Missouri) according to standard methods. The ejaculate was filtered and the gel fraction discarded. Aliquots of 10 ejaculates, collected for educational purposes, were made available for this study.

Media

Kenney´s extender: to 100 ml water were added glucose (4.9 g), skimmed milk powder (2.4 g), dihydrostreptomycin (0.15 g), and penicillin (0.15 g).

Colloid: silane-coated silica colloid in a buffered salt solution (Androcoll-E™, patent applied for) of two different densities was used for the density gradient. The higher density mixture was also used for the single layer.

Sperm concentration

The concentration of spermatozoa in the original ejaculate was measured using a Spermacue photometer (Minitube, München, Germany). The semen was extended approximately 1:1 with Kenney´s extender at 37°C. The sperm concentration of both the extended ejaculate and the centrifuged sperm preparations was counted using a Bürker counting chamber.

Density gradient centrifugation

Aliquots of stallion ejaculates were prepared on a density gradient as described previously (Morrell and Geraghty, 2006). Briefly, a density gradient was prepared by pipetting 2 ml of the higher density layer into a centrifuge tube and carefully layering 2 ml of the lower density layer on top; an aliquot (1.5 ml) of semen, extended to a sperm concentration of not more than 100 million spermatozoa per ml, was pipetted on top of the upper layer. The gradient was centrifuged at 300 x *g* for 20 min, after which the supernatant and most of the gradient material was discarded. The sperm pellet was placed in a clean centrifuge tube containing Kenney´s extender (5 ml) and was washed by centrifuging for 10 min at 500 x *g*. Following washing, the sperm pellet was resuspended in Kenney´s extender (1 ml).

Single layer centrifugation

The method was similar to that for density gradient centrifugation with the exception that 4 ml of the higher density material were placed in the centrifuge tube instead of two layers of different densities (2 ml of each density).

Subjective estimation of motility

Aliquots (0.5 µl) of the extended ejaculate and sperm preparations were examined by phase contrast light microscopy (x400) for subjective motility assessment, using a heated stage at 38°C, immediately after preparation and once daily over the subsequent 4 days. When assessing sperm motility in samples which were stored at 4°C, the sperm preparations were allowed to equilibrate at room temperature (22ºC) for 15 min before removing aliquots for motility assessment. The motility determinations were performed by one operator to achieve as much standardization as possible with this subjective method of assessment.

Sperm morphology

Smears of extended ejaculates and the sperm preparations after centrifugation were made on clean glass slides. In addition, a few drops of each sperm suspension were fixed with buffered formaldehyde solution (Hancock, 1952). The slides and fixed samples were stored at room temperature (approximately 22°C) until required for staining and analysis. Wet smears and Williams-stained slides were examined by phase contrast microscopy and light microscopy by skilled technicians for the following morphological defects in a standard number of spermatozoa: (i) formol saline (200 spermatozoa); proximal cytoplasmic droplets, detached heads, acrosome defects, nuclear pouches, midpiece defects, and tail defects (bent, coiled or double bend). The proportions were used to calculate the total pathological heads, and the number of morphologically normal spermatozoa. (ii) Williams stained preparations (500 spermatozoa): focusing on sperm head abnormalities such as pear-shaped heads, heads narrow at the base, heads with abnormal contour, undeveloped heads, detached heads, narrow (tapering) heads, heads of variable size, as well as the total number of pathological heads. The method used was the modification of William´s method (Williams and Utica, 1920) described by Lagerlöf (1934) without methylene blue. Spermatozoa which were otherwise normal but exhibited distal cytoplasmic droplets were counted as having normal morphology.

Experimental design

Aliquots of extended ejaculates (n = 8 ejaculates) were prepared by density gradient and single layer preparation as described above. Samples were taken for motility assessment or morphology analysis both before and after centrifugation. The sperm preparations were stored either at 4°C in the wells of a specially designed styrofoam tray (seven ejaculates) or

at room temperature (22°C; one ejaculate). In addition, on two occasions, 10 ml of extended (non-centrifuged) ejaculate was stored at 4°C, together with the 1 ml aliquots. Subjective motility evaluations were made daily until the motility had dropped to approximately 20%. This level was chosen arbitrarily as the cut-off point.

Statistical analysis

The sperm yields obtained from each density gradient and single layer were calculated by expressing the number of spermatozoa obtained as a percentage of the initial load. A one-way analysis of variance (Campbell, 1974) was performed on the yields obtained for the two methods from each ejaculate.

Results

The mean motility of the ejaculates was 71.7 ± 7.5%, and the mean sperm concentration was 137.3 ± 40.9 x 10^6 spermatozoa per ml. The mean proportion of motile spermatozoa increased from approximately 72% before centrifugation to 90% after centrifugation (subjective assessment). The mean number (± SD) of spermatozoa obtained from the single layers and density gradients were 33.6 ± 9.8 and 31.5 ± 11.2 million spermatozoa respectively (not significant). The yield (number of spermatozoa in the pellet as a proportion of the original load) varied from 19 to 42%, and was not different between the two preparation methods.

The proportion of morphologically normal spermatozoa was increased by colloidal centrifugation, from 42% before centrifugation to 60.5% after centrifugation. The incidence of morphologically abnormal spermatozoa in the sample was reduced by single layer centrifugation, with the proportions of head and tail defects being reduced by approximately 60% (from 8.5 to 5% for head defects, from 13.0 to 5% for tail defects). The complete results of one analysis, before and after single layer centrifugation, are shown in Table 1.

Table 1. Proportion of spermatozoa with defects (%) before and after centrifugation through a single layer of colloid (n = 1 ejaculate).

Defect	Before centrifugation	After centrifugation
Proximal droplets	12	4.5
Detached heads	0.5	1
Abnormal acrosome	0.5	2.5
Nuclear vacuole	1	3
Midpiece defect	3	1
Bent tail (hairpin)	1	0.5
Coiled tail	-	-
Double bend tail	-	-
Pear shaped head	5	2.6
Narrow-base head	6.4	1.4
Abnormal outline	0.4	0.2
Undeveloped head	0.4	0.2
Detached head	0.2	0.2
Narrow (tapered) head	0.4	0.6
Variable size	1.8	4

The sperm motility results from the seven occasions when the prepared spermatozoa and aliquots of the extended semen were stored at 4°C immediately after preparation are shown in Fig. 1. Preparing the spermatozoa by centrifugation, either on a density gradient or on a single layer, prolonged sperm survival (as judged by the proportion of motile spermatozoa) by approximately three days compared to the non-centrifuged spermatozoa in the extended ejaculate. Small differences in sperm survival between the two methods were observed from day to day, although these were not statistically significant. On the two occasions when 10 ml aliquots of uncentrifuged semen were stored at 4°C, it was observed that the proportion of motile spermatozoa was considerably greater in the larger volume than in the 1 ml aliquots (35% compared to 10%).

For the ejaculate stored at room temperature, sperm motility in the single layer preparation was prolonged compared to the extended ejaculate but had fallen to 5% at 72 h after collection (Fig. 2).

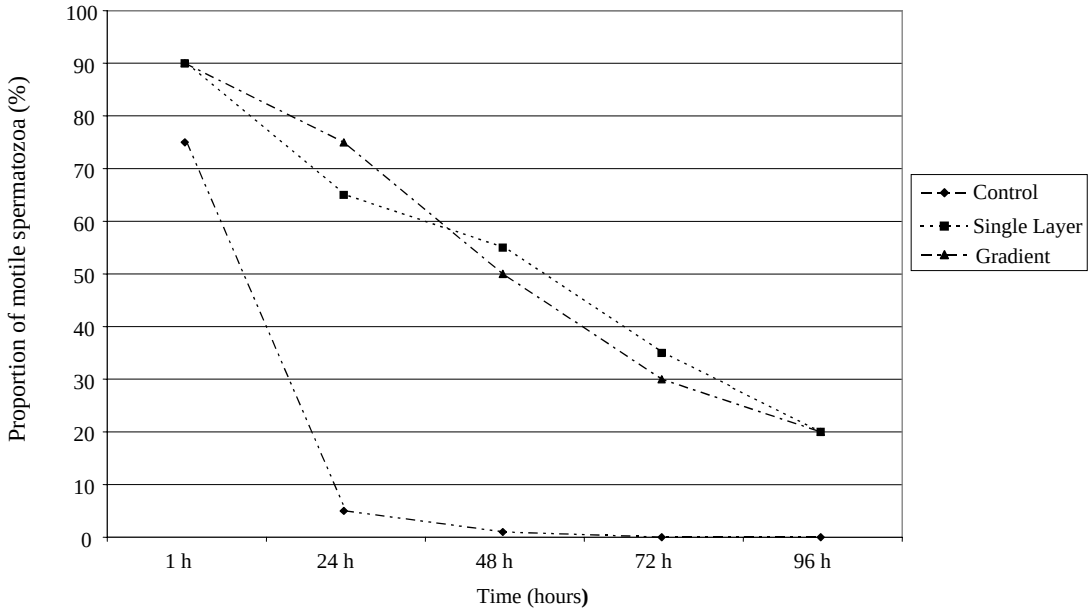

Figure 1. Survival of stallion spermatozoa after centrifugation on a colloid stored at 4°C (n = 7).

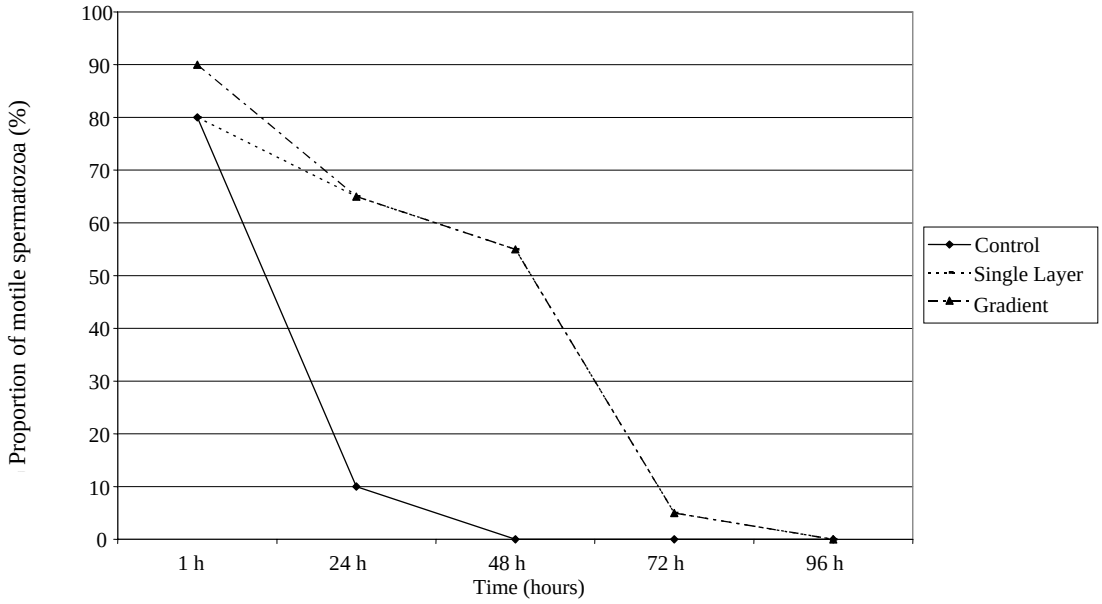

Figure 2. Survival of stallion spermatozoa after centrifugation on a colloid stored at room temperature (22°C; n = 1).

Discussion

The ejaculate characteristics were found to lie mostly within the normal ranges for stallion semen (Watson, 1990). The results reported in the present study indicate that there was little difference between the two methods of sperm centrifugation in terms of number and proportion of motile spermatozoa in the pellets, in contrast to human spermatozoa where the two-layer gradient preparation was superior to the single layer (Sharma *et al.*, 1997; Zhang *et al.*, 2004). In addition, both density gradient centrifugation and single layer preparation of stallion spermatozoa prolonged the length of time over which the spermatozoa remained viable, when stored either at 4°C or at room temperature (22°C). The number of morphologically abnormal spermatozoa was reduced by single layer preparation. Small differences in sperm survival between the two methods were observed from day to day, although these were not statistically significant.

These preliminary findings are similar to those seen with human spermatozoa where density gradient preparation resulted in prolonged sperm survival (Morrell *et al*., 2004). Centrifugation of stallion semen without a colloid has been reported to extend sperm survival (Pagl *et al.*, 2006) by approximately 24 h, although there was no selection for motile spermatozoa in their experiment. In contrast, in the present study there was no selection for motile spermatozoa and they retained their motility for several days compared to the uncentrifuged controls.

The morphology results for stallion spermatozoa are in general agreement with the preliminary results reported for stallion spermatozoa by Macpherson *et al*. (2002). In their study using density gradients, Macpherson *et al*. (2002) found an increased proportion of morphologically normal spermatozoa, although the difference was not significant (P = 0.06). Our observations are in agreement with those of Macpherson *et al*. (2002) that density gradient preparation decreases the proportion of spermatozoa with midpiece defects or distal cytoplasmic droplets. However, in contrast to their study, our findings showed an increase in the proportion of morphologically normal spermatozoa.

The improved sperm survival where larger volumes (10 ml) of uncentrifuged extended semen were stored compared to 1 ml, was presumably due to slower cooling of the spermatozoa to 4°C in the larger volume. Although it was not possible to store larger volumes of centrifuged sperm preparations in this experiment, extrapolation of the results from the uncentrifuged extended semen would suggest that survival would also be improved for the larger volumes of centrifuged sperm suspensions, perhaps prolonging survival by a further 24 h. It might be possible to avoid this problem in the future by placing the receptacle containing the small volume of sperm preparation inside a container of water to resemble the volume of liquid being cooled in a standard semen dose.

An interesting result was that survival of the centrifuged spermatozoa was also prolonged at room temperature, although they did not survive for as long as at 4°C (one day less). This finding is in accordance with results observed by Battelier *et al*. (1998) for uncentrifuged semen stored at 15ºC, albeit using a different semen extender (INRA 96). This methodology could have important implications for transporting spermatozoa from ejaculates which apparently do not survive cooling, or in circumstances where freezing is not available to chill the cool packs used in the insulated boxes for semen transport. Further research is needed on storage temperatures for spermatozoa which have been prepared by colloidal centrifugation.

The possibility of using the single layer method, which avoids the necessity of layering a density gradient, is attractive for stud personnel because of the time-saving element and simplification of the protocol (Morrell, 2006). This preliminary study did not suggest any noticeable decrease in sperm survival or quality through using the single layer compared to a density gradient, in contrast to results obtained by Sharma *et al*. (1997) using human spermatozoa. On the contrary, a slightly larger yield of stallion spermatozoa was obtained from the single layers than from the density gradients in the study reported here. It is thought that the interfaces between layers of different densities play an important role in selecting which cells pass through, which could explain why more spermatozoa appeared in the pellet after centrifugation through a single layer than through a two layer gradient. However, the sample size of the present study is very small (n = 8 ejaculates from one stallion). A much wider study, encompassing a larger sample size and including additional tests for sperm quality, such as chromatin integrity, is to follow.

Initially, polyvinylpyrrolidone (PVP)-coated silica bead suspensions were used for the selection of spermatozoa. However, concern was raised that the PVP might have toxic effects on spermatozoa (Avery and Greve, 1995), and in 1996, Percoll™ was restricted to non-clinical use by its manufacturers (Mortimer, 2000). PVP-coated silica was subsequently superseded by non-toxic silane-coated silica preparations, which were incorporated into the formulations used in the study reported here. In addition to non-toxicity, use of a silane-coated silica colloid and optimized species-specific formulations confer several advantages: silane-coated silica is stable in salt solutions, thus permitting large batches of ready-made colloids of different densities and standardized formulations to be mass-produced in advance of their use, and it can be autoclaved, thus reducing endotoxin levels. Species-specific formulations permit better sperm selection and survival (Morrell *et al*.; unpublished observations).

In conclusion, these preliminary results suggest that sperm preparation by density gradient centrifugation or by the simplified single layer procedure, could offer an effective method for prolonging the survival of stallion spermatozoa for insemination. It is intended to expand the study to examine the effect of storage temperature on the survival of prepared spermatozoa and also to investigate the chromatin integrity of the prepared spermatozoa.

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