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ARTICLE

Single layer centrifugation of stallion spermatozoa improves sperm quality compared with sperm washing

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Abstract This study compared the effect on semen quality of different handling methods used in the preparation of stallion semen doses for artificial insemination. The three methods were (i) extending the ejaculate to $30–50 \times 10^6$/ml, (ii) single layer centrifugation (SLC) and (iii) sperm washing (centrifugation without a colloid). An additional treatment was to add seminal plasma (SP) in various proportions to some SLC preparations. The resulting samples were evaluated for sperm motility by computer assisted sperm analysis, membrane integrity using the Nucleocounter SP-100 and chromatin integrity by the sperm chromatin structure assay. SLC samples consistently had better sperm quality than the extended samples. Sperm washing did not confer any beneficial effect compared with the extended samples and these samples had significantly worse sperm quality than the SLC samples (motility, $P < 0.01$; viability, $P < 0.001$). There was no evidence to suggest that adding SP to the SLC samples could enhance sperm motility for more than a few hours. Longer term cold storage of spermatozoa in the presence of small concentrations of SP resulted in a reduction in total motility and progressive motility compared with SLC alone. High concentrations of SP were detrimental to sperm survival.

KEYWORDS: chromatin integrity, seminal plasma, SLC, sperm motility, sperm washing, stallion

Introduction

In some countries, e.g. The Netherlands (Colenbrander et al., 2003), Germany and Austria (Pagl et al., 2006), it is standard practice to centrifuge stallion semen and resuspend the sperm pellet in extender prior to preparing sperm doses for artificial insemination (AI). It is claimed that the removal of seminal plasma (SP) enhances the survival of the spermatozoa (Aurich, 2005; Parlevliet and Colenbrander, 1999). However, in other countries, for example, UK (Allen, 2005), Sweden, France (Batellier et al., 1998) and Italy (Rota et al., 2004), it is not standard practice to centrifuge the semen unless the sperm concentration is very low, in which case it may be necessary to remove some seminal plasma to provide the required sperm dose for AI in a suitable volume (up to 20 ml of extended semen). There is some debate about whether centrifugation of extended semen actually

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damages the spermatozoa, for example by creating reactive oxygen species that may cause lipid peroxidation (Parinaud et al., 1997).

Recently, a new technique has become available for selecting the most robust spermatozoa from stallion ejaculates as well as separating the spermatozoa from seminal plasma (Morrell et al., 2008). The new technique, single layer centrifugation (SLC) through a species-specific formulation of glycidoxypropyltrimethoxysilane-coated silica, Androcoll-E, has been shown to select spermatozoa with good motility, normal morphology, intact membranes and good chromatin integrity (reviewed by Morrell and Rodriguez-Martinez, 2009). Moreover, the selected spermatozoa retain their motility, viability and chromatin integrity longer than unselected spermatozoa (Johannisson et al., 2009).

Critics of the SLC technique claim that, while it is beneficial for sperm longevity to remove some of the SP, it is not necessary to remove it all, nor is it necessary to select the best spermatozoa prior to AI since morphologically abnormal and/or damaged spermatozoa should be filtered out during passage through the reproductive tract of the mare, as has been shown for the sow (Ardon et al., 2008). Others state that removal of all the seminal plasma from an AI dose could be detrimental to fertility, since the presence of seminal plasma in the uterus of the mare may be required to ensure adequate sperm transport to the site of fertilization in the oviduct (Troedsson et al., 2005), for preparation of the uterine endometrium for the eventual implantation of the conceptus (Robertson, 2005) or for the up- or down-regulation of breeding-induced endometritis (Troedsson et al., 2001). Clearly these hypotheses require careful investigation to aid improvements in the sperm quality in AI doses.

The present study was designed to compare the effect of: (i) no centrifugation; (ii) centrifugation though Androcoll-E (SLC); and (iii) sperm washing (centrifugation without a colloid), on the kinematics, viability and chromatin integrity of stallion spermatozoa. In an additional treatment group, different proportions of SP were added to aliquots of SLC-selected spermatozoa, to investigate the effect of its presence on the aforementioned parameters of sperm quality.

Materials and methods

Animals and semen collection

Warmblood stallions and one riding pony of breeding age and documented fertility were housed under standard husbandry conditions at a commercial stud, the Swedish National Stud, Flyinge, Sweden, (17 stallions, 5—25 years, mean age 9.7 ± 5.7 years). Semen was collected up to three times a week during the normal breeding season, as part of the usual semen collection routine of the stud. The stallions mounted a phantom and ejaculated into a warmed artificial vagina (Colorado or Missouri type, depending on which worked best for each individual), the semen being collected into a warm glass bottle fitted with a filter to capture gel. In all cases, the ejaculate was first extended 1:1 with warm semen extender (INRA96; IMV, L'Aigle, France) at 37°C before removal of an aliquot for the experiment. The remainder of each ejaculate was processed and supplied to customers for AI according to their usual procedure.

Sperm concentration and sperm viability

Sperm concentration and viability were measured with a Nucleocounter SP-100 (Chemometec, Denmark), according to the manufacturer's instructions, as described previously (Morrell et al., 2010). For sperm concentration, an aliquot (50 μl) of each sample was diluted with 5 ml reagent S100 and, after mixing, was loaded into a cassette containing propidium iodide. The cassette was inserted into the fluorescence detector and the computer reported the total number of cells in the sample (T, million). For sperm viability, a further 50-μl aliquot of the sample was diluted with phosphate buffered saline (5 ml, pH 7.1; Chemometec), before loading into another cassette and inserting into the fluorescence detector. This time the instrument reported the number of non-viable cells. The viable count was determined by subtracting the non-viable cells from the total number of cells and expressing the result as a percentage of the total number of cells.

Single layer centrifugation

The method was as described previously (Morrell et al., 2010). Briefly, 4 ml Androcoll-E (patent applied for; SLU, Sweden) were pipetted into a centrifuge tube and an aliquot of extended semen (3.0 or 4.5 ml of extended semen containing approximately 100×10^6 spermatozoa/ml) was layered on top. After centrifugation at 300*g* for 20 min, the supernatant and most of the colloid were discarded and the sperm pellet was transferred to a clean centrifuge tube containing 3 ml extender. No SP was transferred with the sperm pellet for normal SLC. For treatments requiring the addition of SP, the top 1.5 ml of the supernatant after SLC was reserved. Once the remaining supernatant had been removed, aliquots of the reserved SP (diluted with INRA96 where required) was used to resuspend the sperm pellet, either with or without fresh extender. Thus the sperm pellet was resuspended in a portion of its own seminal plasma.

Sperm washing

Aliquots (3.0 ml) of the stock sperm suspension were centrifuged at 300*g* for 20 min. After removal of the supernatant (seminal plasma and extender), the sperm pellet was resuspended in 3 ml fresh extender.

Subjective estimation of motility

Aliquots (5.0 μl) of all sperm samples were examined by phase contrast light microscopy (400×) using a heated microscope stage (38°C), immediately after preparation and again after 24 h. Approximately 200 spermatozoa in several fields were examined on each occasion. One observer, who was unaware of the identity of the samples, performed all motility assessments. The samples were stored overnight