Biomimetics in Action: Practical Applications of Single Layer Centrifugation for equine breeding

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Abstract

Single Layer Centrifugation (SLC) of spermatozoa through a species-specific colloid has been shown to be effective in selecting the best spermatozoa from stallion semen. The method is easier to use and less time-consuming than density gradient centrifugation (DGC), and has been scaled-up to allow whole ejaculates to be processed in a practical manner. The major applications for SLC-sperm selection are as follows: to improve sperm quality in artificial insemination (AI) doses, particularly for "problem" stallions; to increase the "shelf-life" of normal sperm samples, either by processing the fresh semen before preparing AI doses, or by processing the stored semen dose to extract the best spermatozoa; to circumvent the problem of spermatozoa that do not tolerate cooling to 4-6°C; to improve cryosurvival by removing dead and dying spermatozoa prior to cryopreservation, or selecting the live spermatozoa post-thawing; to select morphologically normal spermatozoa with intact chromatin from sub-fertile stallion semen for ICSI, thus increasing the number of blastocysts; to remove pathogens (viruses, bacteria); to accelerate the process of flow cytometric sex selection by removing the dead and dying sperm before passage through the laser beam; to conserve rare breeds. These applications are discussed and practical examples provided.

Although there have been numerous reports on the use of SLC to improve sperm quality, as shown by laboratory assays [8-13] there are few reports on fertility in the field. The purpose of this review, therefore, is to examine potential practical applications of SLC in the equine breeding industry and report preliminary fertility data for some of these applications.

Advantages of SLC for preparing stallion sperm doses for AI

The following benefits of using SLC to prepare animal spermatozoa have been reported previously:

i) SLC-selected stallion spermatozoa show better progressive motility, normal morphology, viability and chromatin integrity than their unselected counterparts [8,9,14];

ii) Sperm quality (motility, viability and chromatin integrity) declines more slowly during cold storage than in unselected semen [10,11,15];

iii) SLC-selected spermatozoa retain progressive motility when stored at room temperature (approximately 22°C) for 48-72h [8];

iv) SLC can be scaled-up to prepare whole stallion ejaculates without loss of sperm quality [6];

v) SLC-selected samples have better sperm quality than samples produced by "sperm washing" and retain this improved quality for longer [16];

Keywords: SLC; Androcoll-E; equine breeding; stallion semen

Introduction

Biomimetics, the use of technologies and/or processes that mimic a naturally occurring event, can be used to advantage in animal breeding [1]. Evidence for selection mechanisms within the female reproductive tract that permit only morphologically normal spermatozoa with intact membranes and good chromatin integrity to pass up to the site of fertilization ("good" spermatozoa) has been discussed frequently over the last two decades [2]. Some semen preparation techniques mimic this selection in vitro and thus fit the description of biomimetics. The purpose of this review is to introduce Single Layer Centrifugation (SLC) as one such biomimetic technique and to describe its potential applications in the equine artificial insemination (AI) industry.

SLC as a method of processing semen first made its debut when [3] reported their attempts to process human ejaculates using one layer of Percoll, rather than two or more layers of colloid of different densities, as in the conventional density gradient centrifugation (DGC). These techniques of DGC and SLC have been described in detail previously [1]. The results of Sharma et al. [3] showed that although it was possible to improve sperm quality in human ejaculates of low initial quality, no improvement in sperm quality was seen when "normal" human ejaculates were processed in this manner. Sieme et al. [4] obtained similar results using one layer of Percoll™ for stallion semen.

The first indication that sperm quality in "normal" stallion ejaculates could be improved by SLC was given in a preliminary study using Androcoll-E, a new species-specific colloid formulation for stallion spermatozoa [5]. This formulation incorporated silane-coated silica in an optimised salt solution instead of Percoll™ (polyvinylpyrrolidone-coated silica) to which various salt solutions had been added. The original SLC methodology was later modified to enable larger volumes of ejaculate to be processed without compromising sperm quality in the resulting preparations [6], a feature that was not possible with DGC without a drop in sperm yield [7]. The initial stage of preparing a scaled-up SLC with Androcoll-E and stallion semen is shown in (Figure 1). It was possible to prepare an average gel-free stallion ejaculate using six to eight 50-mL centrifuge tubes using the SLC-Large method [6].

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vi) SLC does not adversely affect sperm capacitation status [13];

vii) SLC can be used post-thawing to select the motile, viable spermatozoa from cryopreserved semen doses [12,17];

viii) Spermatozoa can be separated from viruses in the semen (Blomqvist et al., unpublished data),

ix) Bacterial contamination can be removed from the ejaculate, thus potentially allowing a reduction in the use of antibiotics in semen extenders [17].

Since pregnancy rates in artificially inseminated mares have been linked to both normal morphology and good chromatin integrity of the spermatozoa in the original ejaculates [19], SLC-selected sperm doses should, theoretically, produce good pregnancy rates when used for AI. However, since factors other than sperm quality are involved in the fertility, it is often difficult to show an improvement in pregnancy rates under “standard” conditions, i.e. sperm doses from a normal stallion, processed and stored under optimal conditions, used for AI in normal mares by experienced personnel. A large sample size is needed to compensate for extraneous variables [20]. Such a fertility trial requires considerable time and resources and is beyond the scope of the present review. However, some initial observations have been made on the fertility of SLC-selected stallion spermatozoa, with particular reference to areas that are currently considered “challenging” in commercial horse breeding.

From the experimental observations listed above, several applications of SLC can be suggested, which are discussed below.

**Improvement of sperm quality from subfertile stallions**

Sperm doses from some “problem” stallions showed an improvement in fertility when SLC-selection was used, for example, pregnancy rates of <20% without SLC can rise to >50% after SLC, depending on the nature of the problem [21,22], although the number of mares inseminated was small. The problems in four of the five stallions reported in the latter study were large volume with low sperm concentration with or without poor motility (two stallions), poor morphology (1 stallion), and one case of presumed ampullary stasis. Although sperm quality in the ejaculates from the first three stallions was demonstrably better, sperm morphology was still sub-optimal in the SLC-samples from the stallion with ampullary stasis since the detached heads pelleted along with the normal spermatozoa. The chromatin damage was also increased in the SLC-samples compared to the uncentrifuged samples, which was in contrast to previous findings in a large number of stallions [14]. The presence of the detached heads may have been associated with the increased %DFI in this sample.

**Spermatozoa that show low tolerance to cooling**

In some stallion ejaculates, progressive motility rapidly declines to less than 20% when the semen is extended and cooled. SLC-selected sperm samples from such ejaculates showed a considerable improvement in progressive motility compared to the control samples (Figure 1). Thus two options are possible: SLC followed either by cooling to 4-6°C or storage at ambient temperature (20°C).

Samples from four such stallions have been processed by SLC and used for AI: one was used for AI immediately after processing [22]; the other three were cooled and transported to another stud for AI after 24h. All four mares conceived, indicating that stallion spermatozoa with a low tolerance to cooling in the presence of some seminal plasma benefit from its complete removal.

When the pregnancy data from these four stallions were combined with those for the four “problem” stallions mentioned in the preceding section, an overall pregnancy rate of 57% (12 out of 21 inseminated mares) is obtained, which is in agreement with the previous observations.

![Figure 1: Effect on sperm motility of Single Layer Centrifugation of spermatozoa that do not normally tolerate cooling.](image)

![Figure 2: Retention of progressive motility in SLC-selected stallion sperm samples compared to uncentrifuged controls. Time 0 is the day of semen collection.](image)
extension of the shelf life of normal semen doses

The common practice of collecting semen from stallions three times a week (Monday, Wednesday and Friday) causes some problems for both stallion and mare owners. Stallion owners may not be able to supply semen on every "standard" collection day, particularly from Sport horse stallions that may be competing. Furthermore, on any collection day, demand may exceed supply, particularly for popular stallions. Mare owners also face the difficulty of ordering semen at the stud where the mares are kept. Typically such insemination is retained along with the progressive motility, SLC-selected sperm samples were inseminated into eight oestrous mares after 48-72 h storage at 6°C and ambient temperature. In a different experiment, progressive motility was retained for longer and chromatin damage was reduced when INRA96 was used as a semen extender instead of Kenney’s extender [15].

To test the hypothesis that the fertility of SLC-selected sperm doses is retained along with the progressive motility, SLC-selected sperm samples were inseminated into eight oestrous mares after 48-72 h storage at 6°C. Conceptuses were identified by ultrasound examination at 16-18 days after ovulation in 3 out of 4 mares inseminated with 48 h storage at 6°C and ambient temperature. In a preliminary study with eight batches of frozen semen from stallions was used as a semen extender instead of Kenney’s extender [15].

The second option suggested by the motility results shown in Figure 2, to store the SLC-selected spermatozoa at ambient temperature. This possibility is currently under investigation, particularly for stallions whose spermatozoa do not tolerate cooling.

The third option provided by SLC for extending the shelf-life of semen doses is to perform the SLC on cooled semen after transport to the stud where the mares are kept. Typically such insemination doses arrive after overnight transport, approximately 15-24 h after semen collection. Initial laboratory experiments using Androcoll-E-Small with semen samples extended in Kenney’s extender showed that had been rejected on the basis of a subjective assessment of >35% morphology and chromatin integrity [26]. The SLC-sperm preparations from stallions classified as poor freezers were of better quality than controls prepared by cushion-centrifugation. However, for stallions classified as "good freezers", the yield of sperm doses was reduced in the SLC-group. This reduction would be a considerable disadvantage unless a concomitant reduction in sperm dose could be achieved because of the improved sperm quality.

Selecting viable spermatozoa after cryopreservation: SLC can be used to select spermatozoa with good motility, viability and chromatin integrity post-thawing [12,16]. Furthermore, SLC-Large can be used to improve sperm quality in sub-standard batches of frozen stallion semen. In a preliminary study with eight batches of frozen semen that had been rejected on the basis of a subjective assessment of >35% progressive motility, the motility (measured by the Qualisperm™ Motility Analyzer) was increased by a mean of 13±10% and viability (measured with the Nucleocounter SP-100) by a mean of 9±7% (Stuhmann & Morrell, unpublished data). Seven of these eight samples would have been acceptable for AI after SLC. The fertility of these “rescued” batches will be tested in an AI trial.

Frozen-thawed SLC-selected spermatozoa functioned normally when injected into in vitro matured equine oocytes using intracytoplasmic sperm injection [27]. SLC showed some advantages over DGC when preparing sperm samples from two sub-fertile stallions for ICSI [28] in that the number of blastocysts was increased by almost 50% in the oocytes injected with SLC-selected spermatozoa.

Table 1: Effect of SLC on sperm quality in stored semen doses (n=45).

| Sample | Total Motility (%) | Progressive motility (%) | Normal morphology (%) | %DFI |
|--------|-------------------|-------------------------|----------------------|------|
| Control | 76.4              | 59                      | 52.1                 | 65.5 | 15.4 |
| SLC    | 82.6              | 88                      | 59.6                 | 70   | 13.0 |
|        | P<0.001           | P<0.001                 | P<0.001              | P<0.001 | P<0.001 |

Note: %DFI is the DNA fragmentation index from the Sperm Chromatin Structure Assay.
versus DGC-selected spermatozoa (21 versus 15, respectively). A larger sample size would be required, however, to establish a statistical significance. In the absence of a reliable and repeatable method of performing conventional equine in vitro fertilization (IVF), ICSI is the only way to obtain offspring from some sub-fertile stallions and a way of making this labour-intensive procedure more efficient is potentially useful.

Removal of pathogens

Although all breeding stallions are tested for the presence of antibodies to EVA before they can be used for breeding, it is possible for stallions to shed the virus in semen and yet be sero-negative when tested for antibodies to the virus. Thus naïve mares are at risk of infection via semen doses from shedding stallions. It has been shown previously that spermatozoa could be separated from equine arteritis virus in stallion semen using DGC followed by swim-up [29]. However, the volumes of semen that can be processed by such a method are limited (approximately 1.5 mL), which would be impractical for processing equine semen doses. Recently, virus-free sperm samples were obtained from a shedding stallion by SLC with Androcoll-E, i.e. the SLC step alone appeared to sufficient to separate spermatozoa from virus in this case (JM Morrell, C Baule, M Wallgren & G Blomquist, unpublished data). However, complete removal may depend on the initial level of virus in the ejaculate; further work is in progress to identify levels of virus that can be removed by SLC.

Sexing spermatozoa

A major problem with flow cytometric separation of X- and Y-chromosome bearing spermatozoa, first achieved by Morrell et al. [30] and subsequently by Johnson et al. [31], is the slow speed of the process and the limited yield of sexed spermatozoa that can be processed within a reasonable time. Thus the likelihood of achieving adequate numbers of sexed spermatozoa for conventional equine AI with flow cytometry alone is low. Furthermore, the flow cytometric technique is not applicable to all males and there appears to be a difference in “sortability” between individuals [32,34]. The latter authors attributed this variation in “sortability” to the number of dead spermatozoa present in the sample. Thus SLC could be used in conjunction with sperm sexing, to remove the dead and dying spermatozoa first, thus speeding up the process sufficiently to make it commercially viable [32,33].

Conservation of rare breeds

Working with rare breeds, where the numbers of individuals available for breeding is reduced, poses several challenges. The small number of individuals may be closely related, in which case the changes of success are low, or they may be elderly or have low gamete quality. Since SLC has been shown to improve sperm quality in some problem stallions, it should be included as one of the potential tools to be used in trying to obtain offspring from these individuals.

Conclusion

SLC-selected spermatozoa have been shown to be capable of fertilization when used for AI in mares. The method has been used to improve the quality of some “problem” ejaculates, to extend the “shelf-life” of sperm doses for AI, and to improve sperm survival during cryopreservation. Since spermatozoa can be separated from viruses and bacteria in semen by SLC, it may be possible to enhance the biocompatibility of semen using this technique. SLC could also be potentially useful in conservation breeding and in sperm sexing by flow cytometry.

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