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Colloid centrifugation selects normal spermatozoa from polymorphic bull ejaculates: a case study

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Summary

Semen from a Western Finncattle bull exhibiting a highly polymorphic spermiogram was processed by colloid centrifugation using Androcoll-B, a species-specific silane-coated silica colloid. In the first experiment Single Layer Centrifugation (SLC) was used to identify which density colloids were needed to separate different cell populations. Colloids of the two chosen densities were then used in a density gradient resulting in two sperm sub-populations, one containing nearly all normally sized spermatozoa, and the other enriched for the macrocephalic spermatozoa. Microcephalic spermatozoa did not appear in either of the selected sub-populations. Using a combination of SLC and DGC with this species-specific colloid, it was possible to separate the spermatozoa into different sub-populations, that is, a sub-population containing nearly all normally sized spermatozoa, and another one enriched for the macrocephalic spermatozoa. Thus, colloid centrifugation could be used to select sufficient normal spermatozoa from a highly polymorphic ejaculate for AI, if desired.

Introduction

Currently bull sires for the artificial insemination (AI) industry are chosen principally on the basis of their genetics, e.g. for growth and conformation in the case of beef bulls, rather than on semen quality. Thus it is only when the bulls arrive at the semen collection station that problems with semen quality, including sperm morphology, are identified. Some morphological defects are “compensable” ie the presence of morphologically abnormal spermatozoa in the ejaculate can be compensated for by increasing the sperm number in the insemination dose (Chenoweth, 2005). However, other defects are non-compensable, raising the question of what to do to try to improve sperm quality.

Colloid centrifugation, in the form of density gradient centrifugation (DGC), has been used for approximately two decades to improve sperm quality for human patients receiving fertility treatment. However, the low volume of semen that can be processed using DGC and the lack of species-specific colloid formulations were barriers to its use in animal breeding. In 2006 a new technique using only one layer of colloid - Single Layer Centrifugation (SLC) using species-specific colloid (Androcoll, with a suffix to denote the species) was developed. This method has been used to improve sperm quality in stallion (Morrell et al., 2008, 2009a and b) and boar (Morrell et al., 2009c; Morrell & Wallgren 2010) semen samples. SLC-selected stallion spermatozoa from problem ejaculates have generated pregnancies when used for AI (Morrell et al., 2011). Androcoll-B was found to be equally effective at preparing bull spermatozoa for in vitro fertilization (IVF) when used as SLC as for DGC (Thys et al., 2009).

The SLC method has been scaled up to process larger volumes of stallion and boar semen than are possible with DGC (Morrell et al., 2009d; Morrell et al., 2011). Thus, SLC with Androcoll-B could offer a practical method for improving bull semen quality at the semen collection station.

A Finncattle bull at an AI center was found to have a highly polymorphic spermiogram, with macrocephalic and microcephalic spermatozoa present (Figure1), together with a range of spermatozoa of approximately normal size (Revay et al., 2010). Progressive motility was estimated to be 25%. Both diploid and haploid spermatozoa were confirmed by flow cytometry (Revay et al., 2010). The purpose of the present study was to determine whether apparently normal spermatozoa could be selected from these semen samples by SLC or DGC using Androcoll-B.

Materials and Methods

Spermatozoa

Ejaculates from the bull were collected and cryopreserved at the Artificial insemination (AI) station in Hollola, Finland. Straws (250 μL) were sent to SLU for subsequent colloid centrifugation and analysis. The straws were thawed in water at 37°C for 12 seconds, after which the semen was extended 1:1 with Buffer B (patent applied for).

Media

Colloid: a stock solution of silane-coated silica in a buffered salt solution (Androcoll-B; patent pending) was used to prepare single layers or density gradients, with layers of different densities made by diluting the stock colloid with Buffer B to varying extents from 40-90%.

Density gradient centrifugation

The method has been described previously (Thys et al., 2009). Briefly, density gradients were 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 (0.5 mL) of thawed semen was pipetted on top of the upper layer. Occasionally three or four layers were used, depending on the experimental design (see later), initially the volume of each layer was 1.3 or 1 mL respectively but eventually four layers of 0.5 mL were used. The gradients were centrifuged at 300 x ***g*** for 20 minutes, after which the seminal plasma and most of the gradient material was discarded. The sperm pellet was transferred to a clean centrifuge tube containing 250 μL Buffer B (see above).

Single layer centrifugation

The method was similar to that for density gradient centrifugation with the exception that 4 mL of the colloid was pipetted into the centrifuge tube instead of two layers of different densities (2 mL of each density) (Thys et al., 2009).

Sperm morphology

Pre-stained slides (Testsimplets; Online Diagnostics, Germany) were used to stain the spermatozoa for microscopic examination. The proportion of macrocephalic spermatozoa in 200 spermatozoa was recorded.

Experimental Design

The semen was cryopreserved at the AI station in Hollola, Finland in the usual manner and the straws were sent to SLU for further examination. Prior to centrifugation, the straws of extended semen were thawed in water at 37°C for 12 seconds and the semen was extended with Buffer B. Single layers of colloid of different densities ranging from 1.052 to 1.117 g/mL (40-90% of the stock colloid), were used to identify suitable densities for subsequent use in a density gradient. The different densities were tested once each but not all densities were tested on the same day. Colloid centrifugation was carried out as described previously, with the resulting sperm pellet being resuspended in Buffer B. Pre-stained slides (Testsimplets; Online Diagnostics, Germany) were used for morphological examination.

Results

The sperm samples contained both haploid and diploid polymorphic spermatozoa (Figure 1). With SLC using various densities, almost all of the spermatozoa were able to pass through the single layers of colloid of density 1.052 and 1.065 g/mL (Table 1) whereas almost no spermatozoa passed through a single layer of density 1.117 g/mL (data not shown). The highest proportion of macrocephalic spermatozoa was found in the pellet after single layer of colloid of density 1.078 g/mL (49% macrocephalic), decreasing again to 27% macrocephalic when the colloid density was increased to 1.091 g/mLUsing colloid densities of 1.091 g/mL and 1.0715 g/mL Androcoll-B for DGC, it was possible to obtain two sperm sub-populations, one containing nearly all normally sized spermatozoa, and the other enriched for the macrocephalic spermatozoa (Table 2). Microcephalic spermatozoa did not appear in either of the selected sub-populations.

Discussion

SLC using Androcoll-B of different densities was used to identify which colloid densities the different sub-populations of spermatozoa could pass through. In this way it was possible to select suitable colloid densities for subsequent use in DGC to separate the different sperm sub-populations.

At first, a four-layer density gradient (1.052 g/mL, 1.065 g/mL, 1.078 g/mL 1.104 g/mL Androcoll-B)) was used (1 mL in each layer), but the sperm concentration was too low to allow identification of the small numbers of spermatozoa at each interface. When a four layer gradient was prepared, with 0.5 mL colloid in each layer, the layers were too narrow to allow the different sub-populations to be harvested without contaminating each other. Therefore, for the sperm concentration available here, it was only feasible to use two-layer density gradients. However, using colloid densities of 1.0715 g/mL and 1.091 g/mL, it was possible to obtain two sperm sub-populations, one containing nearly all normally sized spermatozoa, and the other enriched for the macrocephalic spermatozoa. The microcephalic spermatozoa were selected out by the lowest density colloid and therefore did not appear in either of the selected sperm populations, although there were small numbers of spermatozoa that had a smaller than usual head size. Thus, using DGC, it might be possible to select sufficient normal spermatozoa for AI from a highly polymorphic ejaculate, if desired. It should be noted that the "rolled head, nuclear crest, giant head syndrome" is suspected to be genetic in origin (Chenoweth, 2005); therefore, breeding from an affected bull might not be desirable unless the offspring are not themselves intended for breeding. However, in the case reported here, the bull was shown to be fertile with unprocessed semen, since six out of 12 inseminated cows were pregnant two months after AI. This level of fertility suggests that the abnormal spermatozoa were not reaching the site of fertilization, possibly because of selection mechanisms in the female reproductive tract enabling mainly normal spermatozoa to reach the site of fertilization (Suarez, 2007). These factors are interesting *per se,* indicating that more research is needed to investigate the ability of apparently "normal" spermatozoa from such males to fertilize oocytes and initiate normal development. The use of colloid centrifugation could be useful in this context.

Previous studies using DGC with frozen bull spermatozoa of normal morphology have achieved a yield of more than 50% (e.g. Thys et al., 2009). The yield of normal spermatozoa was not calculated in the present study but since 64.5% of the spermatozoa were not categorised as either macrocephalic or unusually small, it can be assumed that they were normal for the purposes of estimating yield. Thus one could expect to obtain 7.5 to $10x10^6$ spermatozoa from one straw, assuming that the original sperm number per straw was

approximately 15-20 $x10^6$. Since small numbers of bull spermatozoa (< $2x10^6$) are routinely inseminated, for example when using sexed spermatozoa, it would be feasible to use colloid centrifugation to select sufficient normal spermatozoa from a highly polymorphic ejaculate for AI if desired, although the possible genetic nature of the syndrome might suggest that it is better not to use the bull for producing future breeding stock. However, the principle that normal spermatozoa can be separated from polymorphic ejaculates remains of interest for reproductive researchers.

In conclusion, using a combination of SLC and DGC, it was possible to separate the spermatozoa into different sub-populations, that is, a sub-population containing nearly all normally sized spermatozoa, and another one enriched for the macrocephalic spermatozoa. Thus, colloid centrifugation could be used to select sufficient normal spermatozoa from a highly polymorphic ejaculate for AI if desired, although the possible genetic nature of the syndrome might suggest that it is better not to use the bull for producing future breeding stock.

Conflict of Interest

JMM and HR-M have a patent for Androcoll-B and SLC.

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Table 1: Proportions (%) of spermatozoa of different sizes before and after single layer centrifugation of bull spermatozoa.

Sample	Description	Macrocephalic	Smaller than usual head size	Remainder
Uncentrifuged		25	6	69
SLC 1	Pellet	35.5	1	63.5
	Colloid (small numbers only)	23	4	73
SLC 2	Pellet	49	2	49
	Colloid	14	3	82
SLC 3	Pellet	27	3.5	69.5
	Colloid	15	3,5	80

Note: the following densities of Androcoll-B were used for Single Layer Centrifugation (SLC): SLC 1 1.052g/mL, SLC 2 1.078 g/mL, SLC 3 1.091 g/mL.

Table 2: Proportions (%) of spermatozoa of different sizes in uncentrifuged and DGC-selected samples of bull spermatozoa.

Sample	Description	Macrocephalic	Smaller than usual head size	Remainder
Uncentrifuged		32	3	65
DGC	Sperm pellet	34	1.5	64.5
	In bottom layer of colloid	17	1.5	81.5
	Interface between colloid layers	8	0	92
	Above top layer of colloid (small numbers)	10	6.5	83.5

DGC = density gradient centrifugation with Androcoll-B of the following densities: bottom layer 1.091 g/mL; top layer 1.0715 g/mL.

Figure 1: Range of head-size exhibited by spermatozoa from the Western Finncattle bull