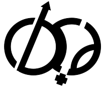

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Androcoll™-P-Large selects boar spermatozoa with good membrane integrity from the sperm-rich fraction of the ejaculate

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Abstract

The study investigated sperm membrane integrity (as a measure of sperm viability) and sperm motility in spermatozoa taken from different portions of the ejaculate, namely the first 10 ml (P1) of sperm-rich fraction (SRF) and from the rest of the ejaculate up to the appearance of gel (P2), both before and after centrifugation on a single layer of Androcoll-P-Large (SLC). Thus there were 4 treatment groups: P1, P2, SLC P1 and SLC P2. Sperm motilities were not different between the various treatment groups, except that the SLC samples had higher linear + non-linear motility than the non-SLC-selected samples. Sperm membrane integrity, in contrast, was significantly higher in P2 than in P1 ($P < 0.001$), and was also higher in both SLC-selected groups than in the uncentrifuged groups ($P < 0.001$). There was a significant correlation between membrane integrity and linear + non-linear motility ($P < 0.001$). These results indicate that the spermatozoa found in P2 have better membrane integrity than those in P1 when used as fresh spermatozoa, and furthermore, that SLC selects the most robust spermatozoa regardless of their origin in the ejaculate. Thus, in situations where P1 is collected separately for sperm cryopreservation purposes, the remainder of the SRF could be used for fresh AI doses, particularly where SLC can be used to select the most robust spermatozoa. These findings have practical importance for the swine insemination industry.

Keywords: Androcoll™-P, boar sperm viability, SLC.

Introduction

The boar ejaculate consists of several distinct fractions, composed of different proportions of the secretions from various accessory glands (Einarsson, 1970) plus spermatozoa and secretions from the epididymides (Lavon and Boursnell, 1975). Most spermatozoa (80-90%) are found in the middle portion, known as the sperm-rich fraction (SRF; Lavon and Boursnell, 1975). There appears to be considerable variation between different countries and even between semen stations regarding which portions of the ejaculate are collected for the production of liquid semen AI doses, varying in the proportion of seminal vesicle secretion that is included (Wallgren, unpublished data). There have been reports that the spermatozoa contained in the first 10 ml of SRF, (the so-called Portion 1 or P1), are those that colonize the sperm reservoirs in the female and ultimately fertilize the oocytes, rather than those from the rest of the ejaculate (P2; Rodriguez-Martinez *et al*., 2005). Other studies indicate that the spermatozoa in P1 are better able to survive manipulations such as cryopreservation than P2 (e.g. Pena *et al*., 2003). Thus, to achieve the best results in cryopreservation, some authors suggest that only P1 spermatozoa should be used (Saravia *et al*., 2010). However, their studies have compared spermatozoa in P1 with those in the remainder of the same ejaculate (P2) that have been exposed to large quantities of accessory gland secretions (Rodriguez-Martinez *et al*., 2005). By definition, P1 spermatozoa cannot be compared to spermatozoa in the SRF of the same ejaculate, since P1 is a subset of SRF. Major differences in the seminal plasma of P1 and P2 have been identified that could account for these differences in sperm resilience, for example differences in the protein levels and types, bicarbonate levels and pH (Rodriguez-Martinez *et al*., 2009). The amount of accessory gland secretion included with the SRF appears to vary with the individual collector, i.e. the collector decides when to stop collecting the SRF into the collection vessel. Therefore, there is an interest in comparing the sperm characteristics in specifically-defined portions of the ejaculate.

A new method for selecting the most robust spermatozoa from the rest of the ejaculate and separating spermatozoa from seminal plasma has been developed recently at the Swedish University of Agricultural Sciences (SLU; Morrell and Rodriguez-Martinez, 2009). This method, Single Layer Centrifugation (SLC) through species-specific formulations of silane-coated silica colloids (Androcoll™), has been shown to be effective for a number of species (reviewed by Morrell and Rodriguez-Martinez, 2009), particularly for stallion ejaculates of medium quality (Morrell *et al*., 2009a, b). The SLC method has been scaled-up to allow larger volumes of semen to be processed (Morrell *et al*., 2009c). A preliminary study investigating the effects of SLC on fresh boar spermatozoa found that spermatozoa from P1

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had lower mean subjective motility than spermatozoa from the SRF of different ejaculates from the same boars (Morrell *et al*., 2009c). It would be interesting to establish whether spermatozoa in the remainder of the ejaculate (P2) have the same viability and survival as those in P1 when used as fresh spermatozoa, despite their differential exposure to seminal plasma. Furthermore, if it is possible to select the spermatozoa with better membrane integrity from P2 by SLC, it would be possible to use both portions of the ejaculate effectively, i.e., to use P1 for cryopreservation and P2 from the same ejaculate for liquid AI doses. Therefore, the current study was conducted to compare sperm membrane integrity (as a measure of sperm viability) and motility of spermatozoa from P1 and in the remainder of the ejaculate up to the appearance of gel (P2), before and after SLC, using split ejaculates. Both P1 and P2 were stored as fresh liquid samples.

Materials and Methods

Animals and husbandry

Four mature boars (Swedish Yorkshire and Swedish Landrace), 18-24 months old, were used. They had been previously chosen according to normal semen quality (e.g. >50 x 10^9 total sperm number per ejaculate, initial motility >70%, morphologically normal spermatozoa >85%) and proven fertility after natural mating or AI with liquid-preserved semen. All boars were kept on straw bedding in individual pens at the Division of Reproduction, Department of Clinical Sciences, SLU, Uppsala. They were fed according to Swedish husbandry standards (Simonsson, 1994) and provided with water *ad libitum*. The experimental protocol had previously been reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden. Semen (3 ejaculates per boar) was manually collected by the gloved-hand technique, using a filter to remove the bulbo-urethral gland gel secretion. The first 10 ml of the sperm-rich fraction (P1) were collected separately, followed by the portion of the ejaculate up to the appearance of gel (P2). Aliquots of both sperm suspensions were extended immediately in modified Beltsville Thawing Solution (BTS) at 35°C.

Media

The extender was a modified Beltsville Thawing Solution (BTS; Pursel and Johnson, 1975), consisting of glucose (3.7 g), tri-sodium citrate (0.6 g), sodium hydrogen carbonate (0.13 g), sodium EDTA (0.13 g) and potassium chloride (0.08 g) added to 100 ml distilled water, i.e. the modification was that no antibiotics were added to the mixture. In addition, following SLC, the sperm pellets were resuspended in BTS with bovine serum albumin (BSA; 1.25 mg/ml) added, to prevent spermatozoa from aggregating or sticking to surfaces.

The colloid consisted of glycidoxypropyltrimethoxysilane-coated silica in a species-specific buffered salt solution (Androcoll™-P-Large; SLU, Sweden) at room temperature. This is a ready-to-use product; no preparation is required.

Sperm concentration

The concentration of spermatozoa in the extended semen was measured using a Nucleocounter SP-100 (Hansen *et al*., 2006). Briefly, 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 total number of cells in the sample was reported (T, million). The sperm concentrations in the sperm suspensions obtained after centrifugation were also determined in the same manner. The yield of motile spermatozoa in the suspensions was calculated as a proportion of the number of motile spermatozoa in the extended ejaculate.

Sperm membrane integrity

An aliquot (50 µl) of the sample was diluted with phosphate buffered saline (5 ml), pH 7.1 (Chemometec, Denmark), before loading into another cassette containing propidium iodide and inserting into the fluorescence detector. The instrument reported the number of non-viable cells (N, million). The viable count was determined by subtracting the non-viable cells from the total number of cells (T-N) and expressing the result as a percentage of the total number of cells.

Computer-assisted sperm motility analysis (CASA)

The motility of at least 200 spermatozoa was examined by computer-assisted sperm analysis (CASA) using a Mika Cell Motion Analyzer (MTM Medical Technologies Montreux, Switzerland) and a microscope equipped with a warm stage and phase contrast optics (20x objective, Optiphot-2, Nikon Nordic AB, Solna, Sweden). Aliquots (5 µl) of sperm samples were placed in a warmed Makler chamber with a depth of 10 µm (Sefi Medical Instruments, Haifa, Israel). The instrument settings had previously been established as appropriate for the species (Erikson *et al*., 2001). Although many kinematic parameters are reported, the ones recorded for this study were total motility (Motility), linear motility, and linear + non-linear motility; the latter two parameters being expressed as a proportion of the total motile population.

Single layer centrifugation (SLC)

The technique for SLC has been described

previously (Morrell *et al*., 2009d). Briefly, 15 ml Androcoll-P-Large was pipetted into a 50-ml Falcon tube and 15 ml of extended ejaculate were layered carefully on top. Following centrifugation at 300 g for 20 min, the supernatant (extender mixed with seminal plasma, interface and most of the colloid) was removed using a vacuum pump, and the sperm pellet was transferred to a clean centrifuge tube containing modified BTS with added BSA. The sperm concentration was adjusted to approximately 50 x 10^6/ml for CASA.

Experimental design

The following portions of each ejaculate (n = 12) were collected separately: (i) P1 (the first 10 ml of SRF), and (ii) P2. The samples were extended with warm modified BTS (35°C) either 1:5 (v/v; P1) or 1:1 (v/v) P2, and sperm concentration was measured with the Nucleocounter-SP100. After adjusting the sperm concentration to 100 x 10^6/ml with BTS, aliquots (15 ml) of the sperm suspensions were used for SLC as previously described. Following centrifugation, the sperm pellets were resuspended in BTS plus BSA, and both sperm membrane integrity and sperm motility were analyzed as previously described.

Statistical analyses

Analysis of variance (PROC MIXED) and Spearman correlation were performed using Statistical Analysis Software (SAS version 9; SAS Institute Inc, Cary, NC, USA). The statistical model included the fixed effects of boar and treatment, and also the random effect of ejaculate nested within boar. The interaction between boar and treatment was not significant, and was thus omitted from the statistical model. A test for normality of residuals was performed. In all cases, significance was set at P < 0.05.

Results

The CASA motility results are shown in Fig. 1. There was no difference in total sperm motility between treatments for all groups before incubation, or for most of the treatment groups after incubation with the exception of SLC1. In contrast, linear + non-linear motility was significantly higher in the SLC samples than in the non-selected samples before incubation (P < 0.001). The proportions of motile spermatozoa showing linear motility were similar in the two uncentrifuged samples, and were higher after SLC (P < 0.001).

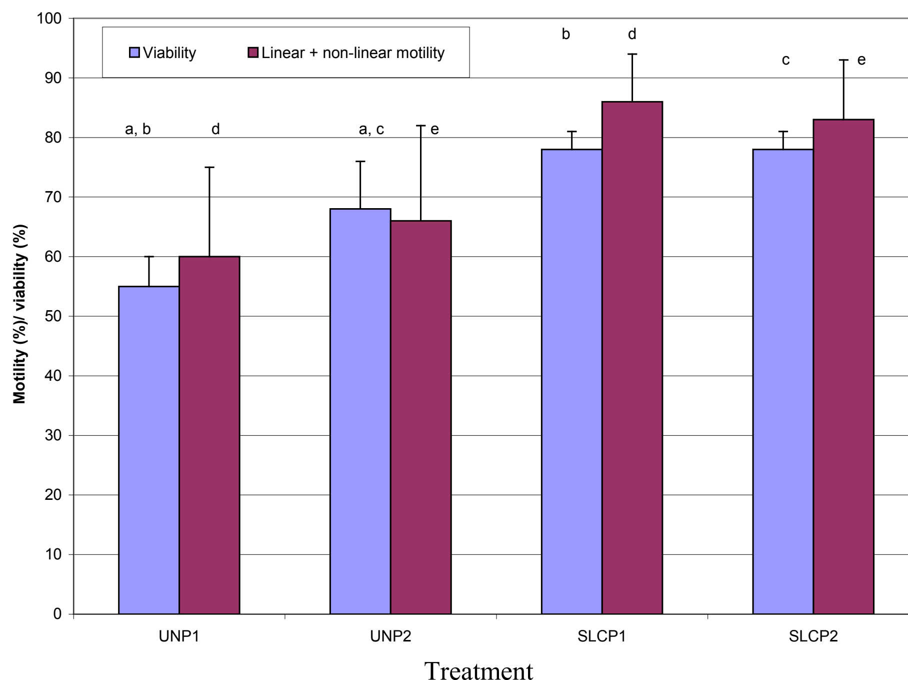

Figure 1. Mean (±SD) viability (%) and linear + non-linear motility (%) for different portions of the boar ejaculate, before and after Single Layer Centrifugation (n = 12).
UNP1 = Portion 1, no centrifugation; UNP2 = rest of the ejaculate (after removal of P1) up to the appearance of gel (Portion 2), no centrifugation; SLCP1 = Single Layer Centrifugation of Portion 1; SLCP2 = Single Layer Centrifugation of Portion 2. [a]Significant difference between UNP1 and UNP2 uncentrifuged (P < 0.001). [b]Significant difference between UNP1 and SLC1 (P < 0.001). [c]Significant difference between UNP2 and SLC2 (P < 0.001). [d]Significant difference between UNP1 and SLCP1 (P < 0.001). [e]Significant difference between UNP2 and SLCP2 (P < 0.001).

Membrane integrity (Fig. 1) was higher in UNP2 than in UNP1 ($P < 0.001$), and also higher in both SLCP1 and SLCP2 compared to UNP1 and UNP2 respectively ($P < 0.001$). These results showed the same pattern as the linear + non-linear motility, with a significant correlation between the two data sets ($r = 0.54$; $P < 0.001$) overall. There were too few data to see statistical differences within treatments. There were no significant differences between boars. The residuals from the ANOVA deviated only slightly from a normal distribution ($P < 0.02$-0.14), and thus no transformation of the variable was performed.

Discussion

The current study was conducted to compare sperm motility and viability (membrane integrity) in P1 and in P2 taken from the same ejaculates, both before and after SLC, using the scaled-up version of SLC reported previously. The results showed that the scaled-up SLC through Androcoll™-P-Large was able to select boar spermatozoa with intact membranes. This result is in keeping with previous results with stallion spermatozoa, where SLC through Androcoll™-E selected the most viable spermatozoa from the rest of the ejaculate (Johannisson *et al*., 2009). However, in a previous study on the viability of boar spermatozoa, in which the spermatozoa taken from the SRF were stained with SYBR-14/PI and their fluorescence analyzed by flow cytometry, no difference was detected in sperm membrane integrity after SLC-selection (unselected 92.6 ± 3.6% vs. SLC-selected 92.4 ± 3.6%; M. van Wienen, unpublished data). However, the mean viability obtained by the flow cytometry method for the unselected spermatozoa in the latter study was higher than previously reported values for this species (Garner and Johnson, 1995; Maxwell *et al*., 2000) and it would have been difficult to obtain a statistically significant increase in the SLC-selected sperm samples. According to the instructions accompanying the Nucleocounter SP-100, the dilution medium may affect the non-viable count, such that it may be slightly higher in phosphate buffered saline (as used in the experiment reported here) than in many semen extenders. However, it might be a more discriminating method than the SYBR14/PI method for boar spermatozoa.

In the present study, spermatozoa from P1 had similar total motility and progressive motility to those in P2, although membrane integrity was higher in P2 than in P1. However, sperm motility was not enhanced by SLC of P1 or P2, in contrast to our previous results (subjective motility) with boar spermatozoa (Morrell *et al*., 2009b), studies with stallion and bull ejaculated spermatozoa (reviewed by Morrell and Rodriguez-Martinez, 2009) and cat epididymal spermatozoa (Chatdarong *et al*., 2010). One explanation for this effect may be that the protein content of the extender (1.25 mg/ml BSA) was insufficient to protect the SLC-selected spermatozoa during storage (16-18°C for boar spermatozoa). The protein content of boar seminal plasma SP has been reported to lie within the range 5-59 g/l (Rodriguez-Martinez *et al*., 2009), which is far higher than the concentration of BSA used in this study. However, increasing the concentration of BSA caused problems for CASA analysis; the spermatozoa tend to aggregate and were thus not recognized as spermatozoa by the computer, and the velocity was reduced compared to unselected spermatozoa not exposed to BSA.

An explanation for the results presented here is that brief exposure to the SP of P2 may be beneficial to sperm membrane integrity, possibly by supplying proteins that help to stabilize sperm membranes. It has been observed previously that the various fractions of the boar ejaculate differ significantly in total protein content (Wallgren *et al*., unpublished data) and also in the type of protein (Calvete *et al*., 2005; Rodriguez-Martinez *et al*., 2009). Thus, there may be a crucial ratio between the number of spermatozoa and the amount of seminal plasma, or the amount of particular components of seminal plasma, which is important for membrane stabilization immediately after ejaculation and subsequent sperm survival. For example, Saravia et *al*. (2007, 2010) found that the concentration of bicarbonate in the different portions had more effect on destabilization of the sperm membranes than the amount of proteins or their types. Other authors have made similar hypotheses about a crucial ratio between sperm concentration and amount of seminal plasma (Johnson *et al*., 2000). Johnson *et al*. (2000) considered that the normal procedure of extending boar semen by approximately 1:10 (v/v semen: extender) immediately after ejaculation was sufficient to dilute out the factors in seminal plasma that adversely affect sperm survival, or alternatively that the initial contact between seminal plasma and spermatozoa at ejaculation was sufficient to initiate changes leading to sperm fertilizing capability. In support of our theory, it should be noted that spermatozoa from P1 are exposed to very little SP, being bathed mainly in epididymal fluid (Rodriguez-Martinez *et al*., 2005). The spermatozoa in P2 used in the current study would probably have had contact with secretions from the seminal vesicles, and also to some of the prostatic and bulbo-urethral gland secretions. SLC-selected sperm samples showed the best viability in the present study, suggesting that any membrane-stabilizing effect of SP components occurs immediately on exposure and is retained during subsequent centrifugation through Androcoll™-P-Large.

The results reported here indicate that the spermatozoa found in P2 have similar motility to those appearing fortuitously in P1, particularly after processing by SLC, and they may have better viability than those in P1 when stored at 16-18°C. Thus, if P1 is to be collected separately for sperm cryopreservation, the remainder of the P2 could be used for liquid AI doses, with or without processing by SLC to select the most robust spermatozoa. In this way, the ejaculate could be used most effectively. Furthermore, Androcoll™-P-Large was able

to select spermatozoa with intact plasma membranes, even from good quality ejaculates, regardless of their location in the SRF. These results are of practical importance for the swine insemination industry.

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