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Comparison of single layer centrifugation and magnetic activated cell sorting for selecting viable boar spermatozoa after thawing

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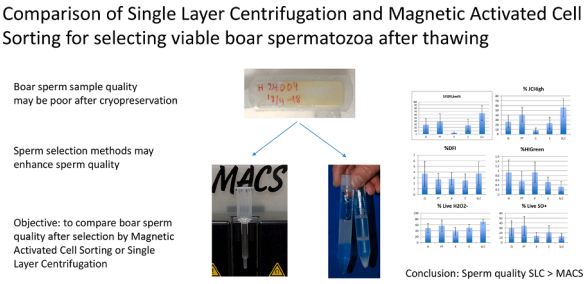
HIGHLIGHTS

- Thawed boar semen samples were split between MACS and SLC treatment groups.
- Membrane integrity and mitochondrial potential were higher in SLC samples than controls.
- MACS selected samples had fewer spermatozoa with immature chromatin than controls.
- In general, sperm quality was better in SLC samples than in MACS selected samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Sperm selection techniques, such as magnetic activated cell sorting (MACS) and colloid centrifugation, are reported to select good quality spermatozoa from semen samples of various species. Although the sperm quality of fresh boar semen is usually good, cryopreservation has a negative effect on parameters such as plasma membrane integrity and mitochondrial activity. Therefore, the objective of the present study was to determine whether MACS or centrifugation through a single layer of colloid (Single Layer Centrifugation, SLC) would be beneficial in enriching thawed boar sperm samples for viable spermatozoa with active mitochondria and good chromatin integrity. Frozen samples from three boars, three ejaculates per boar, were thawed and split. One part was selected by MACS, one was prepared by SLC, and the remainder served as the control. Controls and the selected sperm samples were evaluated for sperm quality (plasma membrane integrity, chromatin integrity, mitochondrial membrane potential and production of reactive oxygen species). Although several aspects of sperm quality were improved in the SLC-selected sperm samples compared to control, the flow-through MACS samples were only improved in having a lower proportion of spermatozoa with immature chromatin (Hi green fluorescence) compared to the labeled control. Sperm quality in the SLC samples was better than in the flow-through samples from MACS. Therefore, despite promising reports of the use of MACS for selecting good quality spermatozoa from semen in other species, the method was not useful for improving sperm quality in the thawed boar sperm samples in this experiment.

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1. Introduction

During natural service good quality sperm cells are physiologically selected in the female reproductive tract to achieve successful conception (Suarez, 2007). Selection of good quality sperm cells is imperative for fertilization, embryonic development and birth of healthy offspring. During assisted reproductive techniques (ART) only a part of the ejaculate is used, and therefore selection of good quality spermatozoa is essential to ensure that sufficient spermatozoa reach the oocyte. Cryopreservation causes irreparable damage to sperm cells, resulting in decreased motility, viability, fertilizing capacity, deterioration of acrosomal and plasma membrane integrity, and deterioration of deoxyribonucleic acid (Bucak et al., 2010). Moreover, boar spermatozoa are unique in many aspects from other species as they are highly sensitive to cold shock and sperm viability decreases severely when the temperature is reduced below 15 °C (de Leeuw et al., 1991). Therefore, researchers worldwide are engaged in improving the quality of thawed boar semen to achieve optimum conception rate and litter size. Selection of good sperm cells following cryopreservation could be one of the options for improving sperm quality in thawed boar semen samples.

A number of techniques have been developed to separate viable sperm from the ejaculate for use in assisted reproduction technologies (ART): these selection techniques include swim-down, swim-up, migration-sedimentation, density gradient centrifugation, magnetic activated cell sorting, and glass wool filtration. Single Layer Centrifugation (SLC) is a modification of colloid centrifugation that has been used to select robust spermatozoa from sperm samples in a variety of species (Morrell and Rodriguez-Martinez, 2009), employing only one layer of colloid instead of the multiple layers needed to create a density gradient. This technique permits selection of the best boar spermatozoa in terms of motility, morphology, viability (Morrell et al., 2009; Van Wienen et al., 2011) and survival during storage (Morrell et al., 2009).

Magnetic activated cell sorting (MACS) is a procedure to eliminate apoptotic cells from an ejaculate. This occurs by identification of phosphatidylserine residues on apoptotic sperm cells by annexin V-conjugated superparamagnetic microbeads (Grunewald and Paasch, 2013) and separation of bound spermatozoa in a magnetic field. The MACS method is effective in selecting motile, viable, morphologically normal human spermatozoa that display notable cryopreservation tolerance and higher fertilization potential (Said et al., 2006; Aziz et al., 2007) It can also be used to eliminate human sperm with high DNA fragmentation, and therefore may help to improve reproductive outcomes in couples undergoing ART (Pacheco et al., 2020). However, a recent report indicated that although enrichment for non-apoptotic boar spermatozoa with normal morphology could be achieved with MACS, sperm motility was impaired by this procedure (Mrkun et al., 2014). The latter authors concluded that the MACS selection technique would not be useful for boar spermatozoa. In contrast, the results of a different study indicated that sperm motility of fresh boar spermatozoa could be improved by selection with MACS (Chung and Son, 2016).

Separation of spermatozoa from seminal plasma and cryomedium is required if thawed spermatozoa are to be used for *in vitro* fertilization (IVF). Sperm quality is crucial for ART since good gametes are required to produce good quality embryos (Vandael and van Soom, 2011); thus, the sperm preparation method used should preferably select good quality spermatozoa as well as separating them from the cryomedium and seminal plasma. Therefore, the present study was designed to compare the quality of thawed boar spermatozoa following selection by SLC or MACS.

2. Materials and methods

2.1. Animals and sample preparation

Three mature boars (Hampshire) between 2 and 5 years old were selected for the study based on normal semen quality and proven fertility. All the boars were housed and fed according to Swedish husbandry standards at a commercial boar semen collection unit (Köttföretagen; Hållsta, Sweden). Three ejaculates from each boar were collected into a plastic bag inside an insulated thermos flask at weekly intervals by the gloved-hand technique. Only ejaculates with at least 70% motile spermatozoa and 75% morphologically normal spermatozoa were used. Semen was extended (1:1, v/v) in Beltsville thawing solution (BTS) (Pursel and Johnson, 1975) and then frozen according to Saravia et al. (2005). The BTS - diluted semen was cooled to +16 °C for 3 h. After that semen was centrifuged twice at 800 x g for 10 min and the supernatant was discarded. Sperm concentration was determined and the sperm sample was again extended at a ratio 2:1 with a second extender (Extender II: 80 mL of 11% β-lactose and 20 mL egg yolk). The semen was further cooled to +5 °C for 2 h and finally mixed at ratio 2:1 with a third extender (extender III: 89.5 mL Extender II; 9 mL glycerol and 1.5 mL of Equex STM). The spermatozoa were packed at +5 °C in a cold handling cabinet in multiple FlatPacks (MFPs). The MFPs were transferred to a programmable freezer set at +5 °C. The cooling/freezing rate was as follows: 3 °C/min from +5 °C to -5 °C, 1 min of holding time for crystallization, and thereafter 50 °C/min from -5 to -140 °C. The samples were then immediately plunged into liquid nitrogen for storage. Before analysis, the flatpacks were thawed for 20 s at 35 °C; the sperm concentration was determined using a Nucleocounter (Chemometec, Allerød, Denmark) (Hansen et al., 2006). Briefly, an aliquot (50 μL) of each sperm sample was mixed with 5 mL Reagent S100 and loaded into a cassette containing propidium iodide (PI; reagents supplied by Chemometec). After inserting the cassette into a fluorescence detector, the sperm concentration was displayed on the instrument.

2.2. Sperm selection

2.2.1. Single layer centrifugation (SLC)

The sperm concentration of each sperm sample was adjusted with BTS to provide $100*10^6$/mL. Four mL Porcicoll Small (available from JM Morrell, Swedish University of Agricultural Sciences, Uppsala, Sweden) were poured into a 12 mL conical centrifuge tube and 4.0 mL diluted thawed semen was pipetted carefully on top. The remainder of the sample served as a control. The tube was centrifuged at 300 g for 20 min, the supernatant was removed and the sperm pellet was aspirated from beneath the remaining colloid for resuspension in BTS (Morrell et al., 2009).

2.2.2. Magnetic activated cell sorting (MACS)

For the separation, $25*10^6$ spermatozoa were mixed with 1 mL Dead cell removal particles (Miltenyi Biotec, Bergisch Gladbach, Germany). From this suspension, 1 mL was applied to a Large Selection (LS) column in a SuperMACS II instrument (Miltenyi) that had previously been equilibrated with 7 mL Annexin binding buffer (Miltenyi). Then 7.5 mL Annexin binding buffer was applied to the column, and the flow-through fraction was collected. After that, the column was removed from the SuperMACS II, another 5 mL was applied, and the eluate fraction was collected. The concentration in the collected fractions was determined using the Nucleocounter. The sperm concentration was adjusted to not more than $2*10^6$ cells/mL using Annexin binding buffer, Also, the remainder of the original sample (labeled control), mixed with dead cell removal particles, was diluted to $2*10^6$ cells /mL using Annexin binding buffer.

2.3. Sperm evaluation

2.3.1. Membrane integrity

Aliquots of all samples at a sperm concentration of approximately 2×10^6 mL^{-1} in Beltsville Thawing Solution (BTS) were stained with 0.07 μM SYBR14 and 24 μM PI (Live-Dead® Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA). After incubating in the dark at 38 °C for 10 min, the samples were evaluated using a FACSVerse flow cytometer

(BDBiosciences; Franklin Lakes, NJ, USA). Excitation was induced with a blue laser (488 nm). Green fluorescence from SYBR14 (FL1) was detected with a band-pass filter (527/32 nm) and red fluorescence (FL3) from PI was measured using a band-pass filter (700/54 nm). A total of 30,000 events was evaluated for each sample. After gating to identify spermatozoa, the cells were classified as membrane intact (SYBR14+/PI-), or membrane damaged (SYBR14-/PI+ or SYBR14+/PI+). For the purposes of this study, only proportions of membrane intact spermatozoa are reported.

2.3.2. Mitochondrial membrane potential

Aliquots of all samples (2×10^6 spermatozoa in 300 μl PBS) were stained with 12 μM of 3 mM of the lipophilic cationic probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probes). After incubating the mixture at 37 °C for 30 min in the dark, analysis was carried out using a FACSVerse flow cytometer (BDBiosciences). Excitation of stained cells was obtained with a blue laser (488 nm); emitted fluorescence was detected using both FL1 (527/32 nm) and FL2 (586/42 nm) filters with compensation applied between channels. Evaluation of 30,000 cells was followed by gating to identify spermatozoa and classifying them into two groups: spermatozoa with high MMP (orange fluorescence) and those with low MMP (green fluorescence). Only high MMP results are reported here.

2.3.3. Sperm chromatin structure assay

The protocol described by Evenson and Jost (2000) was used with slight modifications. Briefly, spermatozoa stored frozen in TNE buffer were thawed on ice; 100 μl sperm suspension was mixed with 200 μl of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) detergent solution (0.08 N HCL, 0.1% Triton X-100; pH 1.2). After incubation at room temperature for 30 s, 600 μl of Acridine Orange (AO, Sigma-Aldrich) staining solution (200 $mmolL^{-1}$ Na_2HPO_4, 0.1 $molL^{-1}$ citric acid buffer, pH 6.0, 1 $mmolL^{-1}$ EDTA, 150 $mmolL^{-1}$ NaCl and 6 μgmL^{-1} AO) were added. Within 3–5 min, the samples were analyzed using a flow cytometer (FACSVerse, BDBiosciences). For each sample, a minimum of 10,000 events were analyzed at a speed of 200 cells s^{-1} after excitation with a blue laser (488 nm). The FSC (Forward scatter), SSC (Side scatter), FL1 (green fluorescence) and FL3 (red fluorescence) were collected. The DNA Fragmentation Index (%DFI, the ratio of cells with denatured, single-stranded DNA to total cells acquired) as well as the proportion of cells with high green fluorescence, representing the high DNA stainability (HDS) population, were calculated for each sample using FCS Express version 5 (De Novo Software, Pasadena, CA, USA).

2.3.4. Reactive oxygen species

Aliquots (300 μL), extended to a sperm concentration of $2 \times 10^6 mL^{-1}$ using BTS, were stained with Hoechst 33,258 at 0.4 μM (HO; Sigma, Stockholm) for classification into live and dead, 0.4 μM hydroethidine (HE; Invitrogen Molecular Probes, Eugene, OR, USA) for detection of superoxide ($SO_2\bullet$) and 20 μM dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen Molecular Probes) for detection of hydrogen peroxide (H_2O_2). The samples were incubated at 37 °C for 30 min before analyzing by FACSVerse (BDBiosciences) flow cytometer (FC). Excitation was with a blue laser (488 nm) and a Violet laser (405 nm). Detection of green fluorescence (FL1) was via a band-pass filter (527/32 nm), red fluorescence (FL3) was measured using a band-pass filter (700/54 nm), and blue fluorescence (FL5) was detected via a band-pass filter (528/45 nm). In total, 30,000 sperm specific-events were evaluated. After gating for spermatozoa in the FSC-SCC dotplot, they were classified as living superoxide ($SO_2\bullet$) negative or hydrogen peroxide (H_2O_2) negative, living $SO_2\bullet$ or H_2O_2 positive, or dead using dot-plots of HO vs HE for $SO_2\bullet$ and HO vs DCFDA for H_2O_2. Only the ROS content of living spermatozoa is reported here.

2.4. Statistical analysis

All statistical analyses were performed with SAS® software version 9.4 (SAS 158 Institute, V.9.4, Cary, North Carolina, USA), using the MIXED procedure for linear mixed models. A repeated effect of ejaculate, nested within treatment was tested. The correlations accounted for by specifying a correlation structure AR(1) among residuals to consider that time intervals between samplings were not exactly the same. Boar was used as a SUBJECT= optional statement parameter to define which observations belong to the same subject. The residuals from the observations generated from the mixed models were tested for normal distribution using PROC UNIVARIATE in SAS 9.4. Data on, % LR DCFDA, % UL HE, % LR HE, and % UR DCFDA deviated from a normal distribution and were log-transformed. However, to improve clarity and avoid redundancy, the respective log-transformed values are presented as non-log values throughout the remainder of this paper.

The model used, included the fixed effects of treatment (5 classes), ejaculate (3 classes) and the interaction between treatment and ejaculate. Boar was set as a random effect.

All results are expressed as Least-square means value ± standard error and p-values <0.05 were considered statistically significant. Scheffe adjustment for multiple-post ANOVA comparisons was used.

3. Results

The samples arising from MACS (eluate and flow-through) showed differences from labeled control (Tables 1 and 2), as follows: spermatozoa in the flow-through sample had higher MI and high MMP and lower Hi green fluorescence than the eluate. The proportions of live ROS-producing spermatozoa were greater in flow-through than in the eluate. Flow-through was different to the labeled control only in having lower Hi Green fluorescence ($p < 0.0024$).

For the SLC versus control (Tables 1 and 2), SLC samples showed higher MI, high MMP and proportion of live hydrogen peroxide negative spermatozoa and a lower proportion of live superoxide positive spermatozoa than controls.

Comparing the effect of treatment (flow-through versus SLC) on sperm quality, SLC samples had higher MI, a higher proportion of live superoxide negative spermatozoa and a lower proportion of live superoxide positive spermatozoa than flow-through samples. The %DFI and Hi green were not different between treatments.

There was a significant effect of boar on MI ($p = 0.02$), %DFI ($p < 0.035$) and ROS production ($p < 0.001$), and significant boar x treatment

Table 1
Effect of post-thaw selection method on sperm quality of boar spermatozoa (LS Means ± SE).

Sperm quality	Labeled control	Flow-through	Eluate	Control	SLC
MI (%)	28.53 ± 9.21 ^{a}	38.89 ± 9.21 ^{bc}	3.60 ± 9.21 ^{ab}	27.21 ± 9.21 ^{d}	64.98 ± 9.2 ^{cd}
%DFI	3.68 ± 6.08	2.68 ± 6.08	2.84 ± 6.08	2.47 ± 6.08	3.72 ± 6.08
Hi green (%)	0.92 ± 0.11 ^{a}	0.56 ± 0.11 ^{ab}	1.09 ± 0.12 ^{b}	0.53 ± 0.11	0.51 ± 0.11
High MMP (%)	25.07 ± 6.08	41.27 ± 6.08 ^{a}	8.22 ± 6.08 ^{a}	22.98 ± 6.08 ^{b}	55.84 ± 6.08 ^{b}

Note: MI = membrane integrity, %DFI = DNA fragmentation index, Hi Green = high green fluorescence (immature chromatin), MMP = mitochondrial membrane potential.
Statistical significance between treatments (within a row) denoted by similar superscript letters, as follows:
For MI: a labeled control vs eluate $p < 0.02$, b flow-through vs eluate, $p < 0.0001$, c flow-through vs SLC $p < 0.014$. d control vs SLC $p < 0.0001$,
High green fluorescence: a labeled control vs flow-through, $p < 0.048$; eluate vs flow-through, $p < 0.0024$; High MMP: a flow-through vs eluate $p < 0.0005$; b Control vs SLC $p < 0.0005$

Table 2
Effect of post-thaw selection method on production of reactive oxygen species in boar spermatozoa (LS Means ± SE).

ROS population	Labeled control	Flow-through	Eluate	Control	SLC
Live superoxide negative (%)	20.58 ± 6.94	24.40 ± 6.94 [a]	24.64 ± 6.94	30.04 ± 6.94 [b]	59.93 ± 6.94 [ab]
Live superoxide positive (%)	29.78 ± 3.16 [a]	33.97 ± 3.16 [b]	14.09 ± 3.16 [ab]	21.29 ± 3.16 [c]	12.81 ± 3.16 [c]
Live hydrogen peroxide negative (%)	49.69 ± 6.72	57.64 ± 6.72 [ab]	38.64 ± 6.72 [a]	50.41 ± 6.72 [c]	71.20 ± 6.72 [bc]
Live hydrogen peroxide positive (%)	0.62 ± 0.29	0.72 ± 0.29	0.11 ± 0.29	1.01 ± 0.29	1.59 ± 0.29

Note: ROS = reactive oxygen species; SLC = Single layer Centrifugation.
Statistical significance between treatments (within a row) denoted by similar superscript letters, as follows:
For live superoxide negative, a Flow-through vs SLC $p < 0.0001$; b control vs. SLC, $p < 0.0001$.
For live superoxide positive, a Labeled control vs elute $p < 0.0004$; b flow-through vs eluate $p < 0.0001$, c flow-through vs SLC $p < 0.0001$.
Live hydrogen peroxide negative, a flow-through vs eluate $p < 0.0011$, b flow-through vs SLC $p < 0.0234$; c control vs SLC $p < 0.0003$.

interactions for live superoxide positive spermatozoa ($P = 0.0012$) and for live hydrogen peroxide negative spermatozoa ($P = 0.04$).

4. Discussion

The purpose of the study was to compare the two selection methods SLC and MACS for their ability to select good quality thawed boar spermatozoa. The results showed that whereas sperm quality was improved in the SLC-selected sperm samples compared to controls in several aspects, it was only marginally improved in the flow-through MACS samples compared to the labeled control in having a lower proportion of spermatozoa with immature chromatin (Hi green fluorescence). Sperm quality in the SLC samples was better than in the flow-through samples.

This is the first study, to our knowledge, comparing MACS and SLC for selecting boar spermatozoa. Our results are in agreement with previous studies on SLC, in which selected sperm samples had higher membrane integrity than controls and improved ROS production (Morrell et al., 2009; Martinez-Alborcia et al., 2012, 2013). However, our results are in contrast to the study of Mrkun et al. (2014) in which enrichment for non-apoptotic boar spermatozoa with normal morphology could be achieved with MACS. The reason for the difference between our study and the study by Mrkun et al. (2014) is not known but could be due to the latter using fresh semen compared to the frozen sperm samples used in our study, or on differences between individual boars or ejaculates. Boar had a significant effect in our study.

There is a striking difference between the few studies on boar spermatozoa selected by MACS and the studies on human spermatozoa. Reports on selecting human spermatozoa by MACS indicated that membrane integrity, motility, normal morphology and chromatin integrity were improved in samples selected by MACS. The reason for the differences between the two species is not known. Levels of %DFI were very low in our study, in agreement with other studies on boar spermatozoa (Evenson and Wixon, 2006), far lower than the values reported for human spermatozoa. Therefore, it might have been difficult to detect slight changes in %DFI in selected samples occurring at the threshold of detection.

There are some reports of the use of MACS to prepare human spermatozoa for intracytoplasmic sperm injection (ICSI); no differences in fertilization, pregnancy, embryo quality, implantation and live birth rates were found between controls and selected sperm samples (Nadalini et al., 2014; Romany et al., 2010). These results are surprising since the improved quality of the selected samples, particularly DNA, should have been associated with fewer early embryo losses. However, sperm quality is only one of the factors involved in human fertility problems (Oseguera-López et al., 2019) and the sperm preparation technique alone cannot solve all of them.

In contrast, a meta-analysis (Gil et al., 2013) of the use of MACS to prepare human spermatozoa for ART reported 5 studies in which MACS samples had been compared to either density gradient (3 studies) or swim-up prepared samples (2 studies). However, two of these studies involved semen from male factor infertility patients, two involved normal semen samples and one was with frozen semen. The results indicated that there was a beneficial effect in pregnancy rate of preparing the samples by MACS compared to the other methods, although there was no decrease in the miscarriage rate, which might have been expected from an improvement in chromatin integrity. Unfortunately it is not possible to determine whether other unreported studies have been carried out where no beneficial effect was found from preparing the sperm samples by MACS.

In studies with bull spermatozoa, magnetic nanoparticles coated with antibody to ubiquitin or with lectin that binds to glycan exposed on the sperm surface were used to remove damaged spermatozoa. Similar conception rates were obtained for the nanoparticle-purified sperm samples and the controls, despite using only half the sperm number of the selected samples for IVF (Odhiambo et al., 2014). Similarly, cat or bull spermatozoa selected using nanoparticles had similar fertilization rates in IVF to controls (Durfey et al., 2019). These results are not surprising since the spermatozoa that are being removed are dead or damaged and would not be those that fertilize the oocyte in IVF; therefore, no impact on fertilization rates would be expected. Usually the sperm dose added in IVF is in excess of the minimum number required, in order to maximize production of blastocysts (Ward et al., 2003). A more relevant aspect to investigate for animal semen would be whether sperm quality deteriorated more slowly in stored sperm samples selected by MACS than in controls after the removal of damaged spermatozoa that are potential sources of reactive oxygen species, or whether pregnancy rates following embryo transfer were different between treatments. Such studies have not been reported yet, to our knowledge. In contrast, SLC-selected stallion sperm samples remain motile longer than controls (Morrell et al., 2010) and retain their fertilizing capacity (Lindahl et al., 2012) for prolonged periods.

It would have been interesting to calculate the sperm yield from the two preparation methods. Although the sperm concentration in the resulting samples was measured, the volume of the samples obtained was not recorded; therefore, it was not possible to calculate sperm yields or recovery rates. However, in previous experiments, sufficient thawed spermatozoa were recovered for IVF e.g. boar spermatozoa processed by SLC with Androcoll-P (Martinez-Alborcia et al., 2013); bull sperm processed by MACS (Odhiambo et al., 2014). Sperm motility evaluation could provide additional information. Note that Androcoll-P has now been re-named as Porcicoll.

Previously, Mrkun et al. (2014) stated that MACS was inappropriate for boar spermatozoa, and Nagata et al. (2018) considered that the MACS technique required further refinement before it could be useful for processing boar spermatozoa. The results presented here are in agreement with these conclusions since they did not indicate a substantial improvement in boar sperm quality except for a reduction in spermatozoa with immature chromatin. Furthermore, any sperm selection method for boar spermatozoa has to be capable of processing large volumes of semen if it is to be of interest outside the research laboratory. As yet, there are no published reports of MACS being used to prepare boar semen for artificial insemination (AI). On the other hand, a scaled-up SLC was used to process boar semen for AI on a commercial farm, with good results in terms of pregnancy rate and litter size (Morrell et al., 2021). Use of MACS requires specialist equipment that is not normally available on semen collection stations whereas SLC requires only a centrifuge with a swing-out rotor, which could already be

available on the semen collection station.

5. Conclusion

Sperm quality in thawed boar sperm samples selected by SLC was clearly improved, in contrast to selection by MACS, where there was only a decrease in the proportion of spermatozoa with immature chromatin. Despite promising results with MACS for human sperm samples, this method was not useful for improving sperm quality in the thawed boar sperm samples used in this experiment.

CRediT authorship contribution statement

Sourabh Deori: Conceptualization, Formal analysis, Investigation, Writing – review & editing, Visualization, Funding acquisition, Data curation. **Theodoros Ntallaris:** Data curation, Writing – review & editing, Methodology. **Margareta Wallgren:** Investigation, Writing – review & editing. **Jane M. Morrell:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition. **Anders Johannisson:** Conceptualization, Formal analysis, Investigation, Writing – review & editing, Visualization, Data curation, Funding acquisition.

Declaration of Competing Interest

JMM is the inventor and one of the patent holders for the colloid formulation used in this study.

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