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Sperm quality improvement of cryopreserved boar semen through colloidal centrifugation gradient

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Abstract: Boar semen cryopreservation is limited due to the lower thermal shock resistance of sperm cells. A colloidal centrifugation gradient is an enhancement to cryopreservation that could improve the frozen boar semen quality. Cryopreserved boar semen quality was evaluated using a commercial colloidal centrifugation gradient. A total of 15 ejaculates from 5 boars were evaluated across two treatments: a control without colloidal centrifugation and a treatment with commercial colloidal centrifugation. A manual freezing method used two freezing curves in liquid nitrogen. Sperm motility was assessed by Computer-Assisted Semen Analysis, as well as sperm capacitation-like membrane destabilisation at 30 min and 150 min after thawing using Merocyanine 540 (M540) for samples incubated at 37 °C. Spectrophotometry measured lipid peroxidation indirectly by the amount of malondialdehyde; reactive oxygen species production was also determined for sperm samples incubated for 30 minutes. The gradient centrifugation treatment improved ejaculate sperm motility and membrane destabilisation. The proportion of morphologically normal sperm was higher in the gradient than in the control. A total of 74% of spermatozoa retained normal morphology. Lipid peroxidation was lower in the colloidal centrifugation treatment. MDA was lower with gradient (16.4 ± 2.5 vs 22.3 ± 2.5 μmol/30 × 10^6 sperm; $P < 0.05$). Intracellular reactive oxygen species (ROS) and M540 positivity did not differ significantly between the treatments at either time point. The centrifugation process improved the quality parameters of frozen boar semen after thawing.

Keywords: animal science; cryopreservation; frozen-thawed sperm; oxidative stress; reproduction

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Colloid centrifugation can eliminate sperm with motility problems, morphoanomalies, and compromised plasma and acrosomal membrane integrity in boar and other domestic species (Papas et al. 2020). The centrifugation separates anomalous cells based on cell density, that is, changes in the cell isopycnic point, which measure movement velocity through a colloid when subjected to centrifugation (Qiu and Mao 2011).

Sperm separation techniques are widely used in assisted human reproduction (Pinto et al. 2021). Analogous approaches have been adopted for livestock, including pigs, to eliminate inferior quality cells in an ejaculate or to select superior quality cells for assisted reproduction such as artificial insemination, *in vitro* fertilisation, or intracytoplasmic sperm transfer. The frozen semen industry in particular values separation techniques for animals of high genetic value but with inferior sperm quality.

Furthermore, since laboratory sperm handling can cause structural damage in bulls (Viquez et al. 2020, 2021), separating non-viable sperm in boar samples prior to cryopreservation produces high-quality sperm samples (Nagata et al. 2019; Zuidema et al. 2021). Considering that non-viable sperm suffer higher levels of reactive oxygen species (ROS), sperm separation could improve the quality of prepared doses (Hai et al. 2024).

In pigs and other livestock species, semen colloidal centrifugation can separate sperm cells from seminal plasma to isolate a sperm subpopulation (Sterbenc et al. 2019; Valverde et al. 2021). During centrifugation, cells move along a density gradient, instead of actively moving through the colloid (Mortimer 2000). After centrifugation, the sperm pellet is resuspended in an extender (washed medium). Percoll® was the first colloid used for sperm selection. However, its use was restricted due to possible toxicity to sperm (Malvezzi et al. 2014). Since, new colloids have been developed for different species (Sterbenc et al. 2019; Cojkic et al. 2024).

Until recently, colloid tube centrifugation was limited by the volume that could be processed and by the time to prepare the different layers of density gradient centrifugation. We hypothesised that single-layer colloidal gradient centrifugation prior to freezing enriches with robust boar spermatozoa and improves post-thaw motility and membrane status while reducing lipid peroxidation versus non-centrifuged controls. The objective then of this study was to evaluate seminal quality of cryopreserved boar sperm with a colloidal centrifugation gradient.

MATERIAL AND METHODS

Ethical approval

The study was conducted during the year 2024 in the Animal Reproduction Laboratory of the School of Agronomy, Costa Rica Institute of Technology. The project was approved by the University Committee of the Center for Research and Development in Sustainable Tropical Agriculture, under Section 08/2023 and Article 5.0 del DAGSC-075-2023. The study was conducted in accordance with the laws and regulations for live animal studies in Costa Rica. Furthermore, the study adhered to the ARRIVE guidelines (https://arriveguidelines.org/).

Animals and semen collection

Fifteen ejaculates were collected from five two-year-old boars of the Duroc (n = 3) and Pietrain (n = 2) breeds employed in artificial insemination programs. The animals were kept in individual cages (23 ± 2 °C) and fed twice daily a standard breeder diet that met their nutritional needs (2.5 kg/day) and provided with water *ad libitum*. The semen was collected by the double-glove technique. At least one ejaculate was collected per week. The sperm-rich fraction of each ejaculate was maintained in a water bath at 37 °C with a 1 : 1 (v : v) Best Thawing Solution (BTS) medium dilution (Minitüb, Tiefenbach, Germany). At least 50 ml of the dilution was prepared for each ejaculate. The diluted semen was deposited in Falcon tubes (50 ml) and covered to avoid direct light. The ejaculates were refrigerated and transported at 17 °C in sealed polystyrene boxes to the Animal Reproduction Laboratory.

Sperm processing and treatment descriptions

Each ejaculate was split into two paired arms: (*i*) Control (no colloid) and (*ii*) Gradient (single-

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layer colloid centrifugation; Androcoll-P-Large, Minitüb, Tiefenbach, Germ+any). All downstream steps were identical between arms.

After preparation, samples were examined for sperm morphology using a UB203 microscope (UOP/Proiser R+D) fitted with a 10× eyepiece and a 40× negative phase-contrast objective. Images were acquired with a video camera at 768 × 576 pixels without any retouching. For each sample, 200 cells were evaluated, and the abnormality percentage was calculated according to the ICAR Guidelines, assuming that the sum of all abnormal categories plus normal sperm equals 100%. The centrifugal separation treatment began with adding 15 ml of commercial colloidal centrifugation medium in a 50 ml Falcon tube, followed, at a 45-degree angle, by a 15 ml aliquot of the semen sample (concentration: 50 × 10^6/ml). To prepare the control treatment, a 30 ml aliquot of diluted semen was used without colloid. Then both treatments were continuously centrifuged at 500 × *g* for 20 min at 17 °C.

When completed, the colloidal supernatant was mostly removed, leaving 1–2 mm of the colloid on the sperm pellet. The pellet was aspirated using a sterile Pasteur pipette and placed in a new Falcon tube. For each treatment, sperm concentration was determined using a sperm photometer, and cryopreservation procedures followed for each treatment.

Freezing media and dilutions

Dose dilution occurred at two stages. An initial analysis of refrigerated semen doses was performed using a CASA-Mot system ISAS® v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain), followed by centrifugation at 2 400 × *g* (17 °C) for 3 min (Heraeus Sepatech Megafuge 1.0R; Hanau, Germany). Pelletised sperm and exposed supernatant were removed. TRIS-egg yolk (TRIS-Y containing ~20% egg yolk) diluent was used to achieve a concentration of 1.5 × 10^9 sperm/ml. The sample was then stored at 5 °C for 150 min (equilibration time). After equilibration, the second dilution step involved adding TRIS-egg yolk (20%) and glycerol (5%) (TRIS-Y-G) along with a detergent (Equex STM; Nova Chemical Sales Inc., Scituate, Mass, USA). This step resulted in a final concentration of 1 × 10^9 sperm/ml.

Sperm freezing process

The previously prepared samples were manually vacuum-packed into 0.5 ml straws (Minitüb; Tiefenbach, Germany) and sealed in a thermal display case (5 °C) with an automatic sealer (Ultraseal 21™; Minitüb, Tiefenbach, Germany). Two freezing phases were used. During the first, straws were placed in liquid nitrogen vapour for 20 min using a perforated aluminium tray 4 cm above the liquid nitrogen to promote controlled precooling and minimise intracellular ice. During the second phase, straws were placed in liquid nitrogen (N_2) at –196 °C for 5 minutes. Finally, the straws were placed in N_2 tanks for 15 days of storage. Both treatments underwent identical freezing/thawing.

Flow-cytometry assays (M540/YO-PRO-1) and ROS (CM-H_2DCFDA)

The protocol was used to assess capacitation-like membrane lipid destabilisation. The Merocyanine 540 (M540, M24571; Invitrogen, Molecular Probes, Willow Creek Road, Eugene, Oregon, USA) and the Yo-Pro-1 (YP1, 4-[(3-Methyl-2(3H)-benzoxazolylidene) methyl]-1-[3-(trimethylammonio) propyl]-quinolinium diiodide) (Y3603; Invitrogen, Molecular Probes, Willow Creek Road, Eugene, Oregon, USA) staining kit was used.

To prepare the M540, a stock solution was created by diluting 100 mg of the vial in 1.755 µl of dimethyl sulfoxide (DMSO). A 5 µl portion of this solution was stored at –20 °C. Then 500 µl of phosphate buffered saline, PBS (Kitazato, Tokyo, Japan), was added to an aliquot, and a concentration of 1 mM M540 was determined. YP1 was used for a 1 : 40 (v : v) dilution of the DMSO stock solution (5 µl YP1 + 200 µl of PBS) for a concentration of 25 µM. The final solution was stored for seven days at 4 °C. Figure S1 in the Electronic Supplementary Material (ESM) shows the representative flow cytometry gating for capacitation-like membrane destabilisation (Merocyanine 540; x-axis) and membrane-compromised sperm (YO-PRO-1; y-axis).

The Guthrie and Welch (2006) protocol uses fluorochrome CM-H_2DCFDA[5-(y-6)-chloromethyl-2',7'-diacetate dichlorodihydrofluorescein, acetyl ester] to determine intracellular ROS. Esterases cleave the fluorochrome molecule CM-H_2DCFDA

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(C6827; Invitrogen, Molecular Probes, Eugene, Oregon, USA) releasing 2',7'-dichlorodihydrofluorescein (H_2DCF), which is oxidised by the ROS producing DCF (dichlorofluorescein). It further emits the calculated fluorescence of 530 nm in response to 488 nm. The 1 mM CM-H_2DCFDA stock solution (50 mg) was prepared using DMSO (86.53 µl) and stored at –20 °C until use. Butylhydroperoxide (TBHP, 70% H_2O, $C_4H_{10}O_2$, B2633; Sigma Aldrich, St. Louis, MO, USA) was used to calculate ROS produced by spermatozoa. Also, TBHP (7.3 M) was used as a stock solution to prepare a new 1M mixture by adding double-distilled water. The obtained solution was 1 mM (1 µl of TBHP-1 M in 1 000 µl of diluted sample). ROS were quantified with CM-H_2DCFDA (1 mM stock; excitation 488 nm/emission 530 nm). Positive control: tert-butyl hydroperoxide (TBHP; $C_4H_{10}O_2$, 1 mM final); negative control: vehicle.

Preparation of MDA-586® kit solutions

To measure malondialdehyde (MDA), the MDA working solution (20 µM) was prepared by diluting 3 µl of the MDA standard in 1.5 ml of ddH_2O (1/500 dilution) and refrigerated at –4 °C. For the working solution R1, a 1 : 3 (v : v) mixture of methanol: R1 was mixed and refrigerated at 4 °C. The control sample solution was prepared by mixing methanol with acetonitrile 1 : 3 (v : v). For the Fe^{2+}/ascorbate solution, 11 mg of $FeSO_4 \cdot 7\ H_2O$ and 40 mg of sodium ascorbate were dissolved in 10 ml of ddH_2O (4 mM Fe^{2+} and 20 mM ascorbate). All solutions were sealed with Parafilm immediately after preparation and stored at 5 °C until use. MDA was measured as an index of lipid peroxidation with the MDA-586® kit (BIOXYTECH® MDA-586). For each straw, duplicate test wells and duplicate control wells were run; $FeSO_4 \cdot 7\ H_2O$ and sodium ascorbate were used for the Fe^{2+}/ascorbate reagent. Results are expressed as µmol per 30×10^6 sperm.

Post-thaw analysis

The thawing protocol used a 37 °C water bath for 20 seconds. For each analysis, one straw from each treatment was thawed. Each sample was incubated in an oven at 37 °C for up to 150 minutes. Semen quality variables were analysed using a CASA system at 30 and 150 min after thawing. The amount of MDA was determined from the thawed samples (1×10^9 spermatozoa) and diluted 1 : 2 (v : v) with BTS for a final concentration of 500×10^6 spermatozoa. Exactly 60 µl (30×10^6 spermatozoa) were transferred to an Eppendorf tube containing 500 µl of BTS at 37 °C. Subsequently, the volume was brought to 1 000 µl with BTS. For each straw, 4 samples were prepared to measure MDA concentration (2) and for use as controls (2).

Then 100 µl of semen was added to each sample respectively, and finally the Fe^{2+}/ascorbate solution was added at a ratio of 1 : 100 [solution (1 µl)/sample (100 µl)]. The samples were incubated on a black rack for 30 min at 37 °C in darkness. The percentage of sperm recovery was calculated as follows: (% sperm quality before colloidal gradient centrifugation × 100)/(% sperm quality after colloidal gradient centrifugation) or (% sperm quality before cryopreservation × 100)/(% sperm quality after thawing).

Statistical analysis

Homoscedasticity was assessed using Levene's test, and normal distribution was assessed using the normal probability distribution test for the sperm variables analysed.

Differences in the semen quality between fresh and frozen-thawed spermatozoa subjected to colloid centrifugation and the control group without colloid centrifugation were evaluated using a mixed analysis of variance (ANOVA), in which the use or absence of gradient centrifugation, the individual boar, and the interaction between the two were considered fixed factors. ANOVA determined differences between ROS generation and plasma membrane destabilisation. ANOVA also evaluated the effect of individual boars and of the gradient centrifugation on MDA concentration in frozen-thawed samples. The mixed model considered the boar and the colloid centrifugation procedure as fixed effects.

Pairwise comparisons between treatments were performed using the Tukey-Kramer test with $P < 0.05$. Results are presented as the mean ± standard error of the mean. IBM SPSS, v29.0.0, for Windows (SPSS Inc., Chicago, IL, USA) was used for all analyses.

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RESULTS

There was an effect ($P < 0.05$) of the commercial colloidal gradient centrifugation on sperm parameters with respect to the control, without the colloidal layer. Differences ($P < 0.05$) were found in sperm concentration and quality between the commercial colloid centrifugation group and its respective control group. There was no significant difference in total normal spermatozoa and in the plasma membrane and acrosome integrity (Table 1). A decrease in the number of total sperm cells recovered (% sperm quality before colloidal gradient centrifugation × 100)/(% sperm quality after colloidal gradient centrifugation) was observed after application of the colloidal gradient centrifugation (56.6 ± 5.10%); however, the average value for the total variable of normal sperm recovered was 73.8 ± 4.30%.

There was an effect of commercial colloidal centrifugation treatment on total motility and post-thaw progressive motility ($P < 0.05$). These differences were observed at both 30 and 150 min after thawing. There was also an effect ($P < 0.05$) on the integrity of the plasma membrane and the acrosome. Differences ($P < 0.05$) were observed between the control and commercial colloidal centrifugation treatments at both post-thaw times (Table 2).

Differences ($P < 0.05$) were found between the treatments in the recovery percentages of both total motile sperm and progressively motile sperm, and sperm showing the plasma membrane integrity. In all cases, there was a higher recovery percentage in the samples processed with the commercial centrifugation gradient treatment, and these differences were observed at both 30 and 150 min after thawing (Table 3).

The proxy of lipid peroxidation, MDA concentration, was significantly different ($P < 0.001$) between the treatments in this study. Control samples without commercial colloidal centrifugation had the mean MDA concentration of 22.3 ± 2.5 µmol/30 × 10^6 sperm, while the group treated with commercial colloidal centrifugation had the mean concentration of 16.4 ± 2.5 µmol/30 × 10^6 sperm (Figure 1).

There were no differences ($P > 0.05$) between the two sperm separation groups in the induced fluo-

Table 1. Sperm parameters (mean ± standard error of the mean) of frozen-thawed boar semen with centrifugation with a commercial colloidal gradient and control treatment (without gradient)

Parameter	Control	Gradient
Total sperm (×10^9)	5.35 ± 1.10^a	3.03 ± 0.91^b
Normal sperm (%)	60.4 ± 8.4^b	78.3 ± 9.1^a
Normal total sperm count (×10^9)	3.40 ± 1.13	2.51 ± 0.92
Total motility (%)	74.8 ± 2.2^b	77.5 ± 1.7^a
Progressive motility (%)	34.1 ± 2.2^b	41.4 ± 3.0^a
Plasma membrane integrity (%)	85.9 ± 3.9^a	83.1 ± 3.9^b

^{a,b}Different subscript letters indicate significant differences between treatments ($P < 0.05$)
Control: the same ejaculate processed identically but without the colloidal layer (no-gradient)

Table 2. Seminal quality of frozen-thawed boar semen (mean ± standard error of the mean) with commercial colloidal gradient and control treatment (without gradient) at different post-thaw times

Parameter (%)	Min	Control	Gradient
Total motility	30	37.7 ± 9.6^b	56.5 ± 10.9^a
	150	20.2 ± 4.6^b	35.0 ± 6.4^a
Progressive motility	30	28.3 ± 8.1^b	47.3 ± 9.7^a
	150	16.0 ± 4.3^b	29.0 ± 5.3^a
Plasma membrane integrity	30	35.0 ± 4.9^a	51.2 ± 8.1^a
	150	33.9 ± 5.0^a	29.0 ± 6.9^b

^{a,b}Different superscript letters in the same row indicate differences between centrifugation treatments ($P < 0.05$)

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Table 3. Percentages of sperm recovery (mean ± standard error of the mean) according to the control and commercial colloidal gradient treatment

Parameter (% recovery)	Min	Control	Gradient
Total motility	30	47.8 ± 10.1[b]	59.4 ± 13.1[a]
	150	25.6 ± 5.4[b]	40.3 ± 7.4[a]
Progressive motility	30	82.9 ± 22.7[b]	95.6 ± 25.5[a]
	150	45.5 ± 13.1[b]	67.2 ± 13.9[a]
Plasma membrane integrity	30	39.7 ± 5.8[b]	59.19 ± 6.4[a]
	150	38.1 ± 3.9[b]	50.7 ± 6.5[a]

[a,b]Different superscript letters in the same row indicate differences between centrifugation gradients ($P < 0.05$)
Recovery was calculated using the formula: (% sperm quality before cryopreservation × 100)/(% sperm quality after thawing)

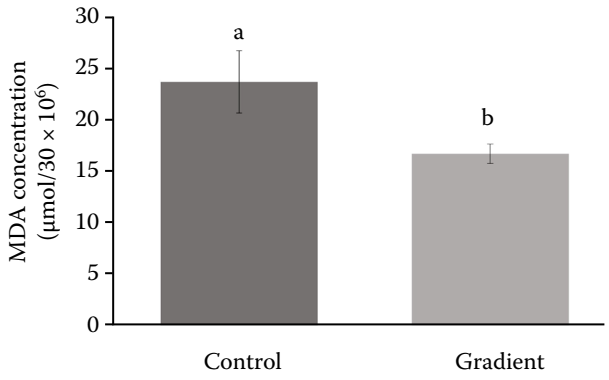

Figure 1. Malondialdehyde (MDA) concentration ± standard error of the mean in thawed samples from previous centrifugation, commercial colloidal centrifugation gradient and control treatments
[a,b]Different superscript letters indicate differences between the treatments ($P < 0.05$)

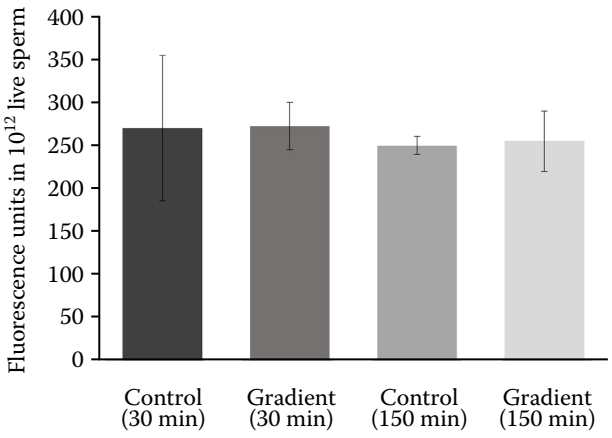

Figure 2. Induced fluorescence intensity ± standard error of the mean expressed in fluorescence units (10^{12} live spermatozoa) in cryopreserved samples with centrifugation gradient at different times (30 and 150 min after thawing)

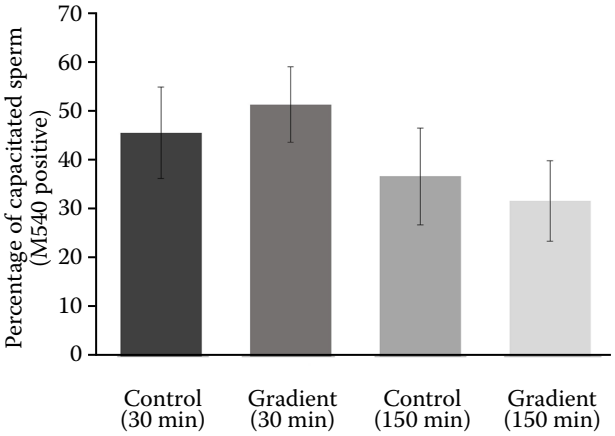

Figure 3. Percentages of sperm capacitation ± standard error of the mean in cryopreserved samples with centrifugation gradient at different times (30 and 150 min after thawing)

rescence intensities, which indicate intracellular ROS production (Figure 2). No interaction effect of the time treatment was observed.

No differences ($P > 0.05$) were determined between the treatments with regard to plasma membrane destabilisation (%) after thawing in doses previously subjected to a capacitation-like lipid peroxidation procedure of sperm membranes (Figure 3).

DISCUSSION

In the present study a 1 : 1 dilution (semen volume to commercial colloidal centrifugation volume, using 15 ml for each volume) was used, which is relatively high compared to volumes of other centrifugation colloids such as Puresperm® or Porcipure®. Colloidal centrifugation is particu-

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larly useful for genetically high-quality boars with low-quality of sperm because it can improve the sperm quality (Morrell 2019).

Preservation inflicts not only lethal damage to sperm but also sublethal damage to most sperm cells that survive, because membrane integrity and cellular metabolism are compromised (Hai et al. 2024). Such damage reduces post-thaw cryopreserved sperm functionality (Nagata et al. 2019). In equine spermatozoa, Androcoll-E conserved most sperm quality indicators compared to freshly ejaculated spermatozoa (Gutierrez-Cepeda et al. 2023). For porcine and equine species, the percentages of normal spermatozoa in Androcoll-P (Large-Porcicoll) and Androcoll-E gradients were higher compared to controls without gradients.

The use of commercial colloidal centrifugation with Androcoll-P-Large medium did not impair the ejaculate quality. This finding suggests that this colloid does not alter motility or plasma membrane integrity. These sperm quality results are consistent with previously reported observations for equine sperm (Johanisson et al. 2009; Morrell et al. 2009). Significantly lower motility, however, was also reported in this species in samples treated with the Androcoll-E gradient before freezing; this could be due to the physical process of cryopreservation (Johannisson et al. 2009). This result could be caused by an individual effect, where motility would improve in some sperm but not in others.

Our results are consistent with those previously observed in horses with Androcoll-E (Morrell et al. 2009) and Bovicoll in cattle (Cojkic et al. 2024). The results suggest that using gradient centrifugation can be an effective method for improving sperm quality parameters in frozen-thawed samples containing low-quality ejaculates, based on sperm selection. Separating most of the seminal plasma using simple (i.e. without a colloid) centrifugation has improved some sperm quality parameters such as motility, reflected in increased sperm survival during artificial insemination (Tanga et al. 2021). This procedure, however, does not discriminate between viable and non-viable sperm and thus it does select for the best sperm (Meitei et al. 2021). In addition, simple centrifugation favours sperm lipid peroxidation (Gualtieri et al. 2021). The comparative advantage of colloidal centrifugation, on the other hand, is that it can remove non-viable sperm prior to cryopreservation (Morrell 2019). For AI centres, gradient selection can be deployed selectively, e.g. for high-value boars or ejaculates with borderline baseline quality to boost post-thaw function. Although total sperm recovery decreases, the higher proportion of functionally competent spermatozoa may offset yield losses in dose formulation. Facilities should balance per dose cell numbers against enhanced post-thaw performance and consider costs/logistics for routine integration.

Androcoll-P produces less MDA after thawing than in samples not centrifuged with this gradient. This phenomenon could be related to the lower presence of non-viable sperm (whose membrane integrity has diminished or that suffered greater morphological abnormalities) in samples, which consequently results in fewer extracellular ROS substances (Alyethodi et al. 2021), since non-viable sperm generate and release ROS substances into the environment (Silvestre et al. 2021; Qamar et al. 2023). Lipid peroxidation is a harmful process that reduces motility and decreases sperm fertilisation capacity in many species, including humans (Hussain et al. 2023). Lipid peroxidation occurs spontaneously in mammalian spermatozoa, in humans it is much higher in ejaculates with a higher proportion of non-viable spermatozoa (Aitken and Krausz 2001).

The analysis of plasma membrane integrity indicated that the partial removal of non-viable and morphologically abnormal sperm prior to freezing with the commercial colloidal centrifugation improved sperm survival and quality after thawing. In addition, they could affect the fluidity of the sperm membrane and chromatin integrity (Johannisson et al. 2009). Intracellular ROS production (measured in fluorescence units) increased with incubation time as the number of viable sperm decreased. Sperm centrifuged with the commercial colloidal gradient did not generate more intracellular ROS than non-centrifuged sperm. This phenomenon indicates that the effect of the longer centrifugation time required by this gradient does not modify the intracellular ROS generation. It can be concluded, therefore, that centrifugation with the gradient colloid does not generate more oxidizing agents or as a consequence of greater mitochondrial activity (Guthrie and Welch 2006).

Furthermore, the present study demonstrated that samples centrifuged with commercial colloidal centrifugation did not show any greater sensitivity to sperm capacitation measured with M540. In boars, Carvajal et al. (2004) showed that

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a brief, high-*g* centrifugation step prior to cryopreservation improves post-thaw motility, viability, and oocyte penetration capacity without increasing the lipid peroxidation, supporting the premise that optimised pre-freeze handling enhances cryosurvival. Using density-gradient methods, Noguchi et al. (2015) reported that a two-step Percoll DGC applied to frozen-thawed semen increased motility, preserved plasma and acrosomal membrane integrity, and improved IVF outcomes relative to simple centrifugation. More recently, Lian et al. (2021) demonstrated that DGC purification with BoviPure alters porcine sperm quality and composition, providing mechanistic insight into downstream functional differences; however, their analyses were conducted on fresh AI-dose semen rather than on explicitly cryopreserved samples.

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Conflict of interest

The authors declare no conflict of interest.

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