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Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa

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Abstract

Centrifugation is a crucial procedure in sperm cryopreservation protocols of brown bear (*Ursus arctos*), because the semen must be processed to increase sperm concentration and/or clean urine-contaminated samples. The efficacy of three media for centrifugation (Maxifreeze [IMV technologies, L'Aigle, France], Cushion Fluid Equine, and PureSperm [Nidacon, Gothenburg, Sweden]) on the quality of bear spermatozoa was evaluated. In experiment one, two cushioned media used for protecting against mechanical stress during centrifugation were analyzed. In experiment two, a density gradient based on PureSperm was assessed in relation to the maximum retrieval and the quality of fresh spermatozoa, and the freezability of the spermatozoa selected in this density gradient was studied in experiment three. Finally, the selection of frozen-thawed sperm using PureSperm was analyzed in experiment four. Our results indicate that the use of dense isotonic cushion solutions (Maxifreeze, Cushion Fluid Equine) in centrifugation did not improve the quality of recovered spermatozoa compared with standard centrifugation. However, a density gradient prepared with PureSperm improved the quality of spermatozoa in fresh semen and frozen-thawed semen, but the spermatozoa selected from the fresh sample with this density gradient did not show a better resistance to freezing with this density gradient in comparison with the control sample.
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Keywords: Centrifugation; Density gradient; Cryopreservation; Brown bear; Spermatozoa

1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is the last autochthonous bear population in the Iberian Peninsula (approximately 150 individuals restricted to 2 isolated populations in the North of Spain), and it is considered to be at risk of extinction (Real Decreto 439/1990, regulation of the National Catalogue of Endangered Species). Adapting artificial reproductive techniques to the brown bear and to establishing germ plasm banks should be a priority in the recovery plans for this population.

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Damage caused by semen processing procedures is accumulative, and small injuries may result in important deleterious changes at the end of the process. Centrifugation of spermatozoa is necessary before cryopreservation in many species to reach an adequate sperm concentration (bear [1]), remove seminal plasma (dog [2], stallion [3,4], goat [5]), and clean urine-contaminated samples (horse [6]). Indeed, our previous experience with the brown bear has shown that most samples do not achieve a concentration high enough for the cryopreservation procedure [7]. Although some studies have shown the beneficial effects of seminal plasma on semen processing and cryopreservation (deer [8], ram [9]), others have reported deleterious effects (dog [2], goat [10], ram [11], stallion [3,4], bull [12]).

Centrifugation techniques have represented a compromise between the need to recover as many spermatozoa as possible and the damage caused by pelleting the sperm. The outcome of centrifugation of brown bear spermatozoa depends on the extender and the dilution rate used for prefreezing preparation [13], although we found that brown bear sperm is highly resistant to various combinations of time and relative centrifugal force [14].

Cushioned centrifugation methods have been developed in species, such as boar [15] and horse [16] to separate spermatozoa from seminal plasma efficiently. In this method, a nonionic iodinated compound, iodixanol, is used as a cushion for centrifuging sperm to minimize possible damage caused by the centrifugation process.

Density gradient centrifugation has been suggested as a means of selecting animal spermatozoa for artificial breeding [17]. Density gradient centrifugation is a widely used technique for the separation of motile from nonmotile sperm, for the removal of contaminating agents, such as bacteria and viruses, obtaining a final sperm suspension free of seminal plasma, leukocytes, microbial contamination, and other debris [18]. The PureSperm (Nidacon, Gothenburg, Sweden) density gradient centrifugation technique is designed to select viable and morphologically intact human spermatozoa for assisted reproductive technologies (ART), such as artificial insemination (AI) and in vitro fertilization [19].

In brown bear, centrifugation is necessary to process seminal samples in cryopreservation protocols. Cushioned centrifugation and centrifugation in a density gradient have been developed to solve specific problems associated with centrifugation in certain domestic animal species. We have studied the application of these techniques in brown bear sperm.

Thus, the objectives of this study were: (1) to examine whether sperm centrifugation on a cushion solution improves the quality of recovered sperm; (2) to assess the suitability of PureSperm density gradient centrifugation of fresh semen for improving the sperm quality of brown bear spermatozoa; (3) to evaluate the freezability of the spermatozoa selected by the PureSperm density gradient; and (4) to assess the suitability of density gradient centrifugation of cryopreserved spermatozoa for improving post-thaw sperm quality.

2. Materials and methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise specified.

2.2. Animals and sample collection

Semen samples from 13 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July). The animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m), and fed with a diet based on chicken meat, bread, and fruits.

The animals were immobilized by teleanesthesia with zolazepam HCl, tiletamine HCl (zoletil 1001; Virbac, Carros, France) 7 mg/kg and ketamine (Imalgene 10001; Rhône-Mérieux, Lyon, France) 2 mg/kg. After immobilization, they were weighed and monitored during anesthesia (pulse, saturation of peripheral oxygen, and breathing). Before electroejaculation, the pubic region was cleaned, the penis was washed with sterile physiological saline, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination of the ejaculate. Electroejaculation was carried out with a PT Electronics electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average). Urine-contaminated ejaculates (> 80 mg urea/dL) were rejected.

2.3. Experimental design

2.3.1. Experiment one: cushioned centrifugation

We evaluate the protective effects of two cushioned media during centrifugation of brown bear sperm. Nine

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brown bear ejaculates were used in this experiment. Immediately after collection, fresh semen was divided into three aliquots. All aliquots were diluted with the same volume of the TCG self-made extender [13], made up of by 200 mM Tris, 70 mM glucose, 63 mM citric acid, 1000 UI/mL benzylpenicillin, and 1 mg/mL dihydrostreptomycin. One aliquot was centrifuged without cushioned medium (control), another aliquot was centrifuged with Cushion fluid (Minitübe, Tiefenbach, Germany) and the other aliquot was centrifuged using Maxifreeze (IMV technologies, L'Aigle, France). Both media were used as follows: semen extended 1:1 in TCG was placed in a centrifuge tube, cushion media was introduced under the semen (10% of the volume of semen). All three aliquots were centrifuged at 600*g* for 6 minutes and supernatant was removed after centrifugation. Also the cushion media remaining in the bottom layer was removed with a syringe and long injection needle.

2.3.2. Experiment two: PureSperm gradient applied to fresh semen

Thirteen brown bear ejaculates were used in this experiment. Immediately after collection, fresh semen was divided into two aliquots: one aliquot (centrifugation control; C-Control) was diluted with the same volume of TCG and centrifuged at 600*g* for 6 min, the other aliquot was treated with PureSperm 100.

The bottom fraction of the PureSperm gradient (90%) was prepared by mixing 9 mL of commercially supplied PureSperm solution with 1 mL of TCG medium. The upper fraction (45%) was obtained by dilution of the 90% solution with an equal volume of TCG medium and stored at 5 °C until used. To prepare the gradient for sperm purification, 3.5 mL of 90% PureSperm was pipetted into the bottom of the centrifuge tube and 2.2 mL of 45% PureSperm was carefully layered over the bottom fraction.

The semen sample was placed on top of the upper layer of the PureSperm gradient and centrifuged at 600*g* for 20 min. The resulting pellet, placed in the bottom of the tube, was aspirated and diluted 1:4 in TCG and centrifuged at 600*g* for 6 min (PureSperm pellet; PS-Pellet). Semen placed in the interface, between the bottom and the upper fraction, was analyzed in the same way as the previous sample (PureSperm interface; PS-Interface).

2.3.3. Experiment three: freezability of selected spermatozoa in PureSperm gradient

The three types of samples obtained in the previous experiment, C-Control, PS-Pellet, and PS-Interface, were cryopreserved (13 straws per sample type) and the samples kept in liquid nitrogen for at least 6 wk. The quality of the frozen-thawed semen was analyzed.

2.3.4. PureSperm gradient applied to freeze-thawed semen

Eight brown bear ejaculates, diluted with the same volume of TTF extender (Section 2.4.), were cryopreserved. Immediately after thawing, the semen was divided into three aliquots. One control aliquot was centrifuged at 600*g* for 6 min, the supernatant was discarded, and the pellet was diluted with the same volume of TCG and analyzed (frozen-thawed centrifugation control, FT_C-Control). The second aliquot was centrifuged with PureSperm: the pellet (FT_PS-Pellet) and the sample in interface between fractions (FT_PS-Interface) was processed in the same way as described in Section 2.3.2. The last aliquot was maintained in TTF extender without centrifugation (FT_No-Centr) for 45 min (handling time for the other aliquots) and then was analyzed. In this experiment there were not enough samples for SCSA analysis.

2.4. Sperm cryopreservation

The semen samples from experiments one (Section 2.3.1.), three (Section 2.3.3.), and four (Section 2.3.4) were resuspended with the same volume of TTF extender (Tes-Tris-fructose 300 mOsm/kg, pH 7.1, 8% glycerol, 20% egg yolk, 2% EDTA, and 1% Equex Paste) at 20 °C [20]. Tubes with diluted samples (at 4% glycerol) were placed in glasses containing 100 mL of water at room temperature, and transferred to a refrigerator at 5 °C, the temperature thus decreasing slowly to 5 °C (70 to 80 minutes). A second 1:1 dilution was then performed at 5 °C, using the TTF extender at 12% glycerol to reach a glycerol concentration of 8%. More TTF extender at 8% glycerol was added to obtain a final concentration of 100 $\times$ 10^6 spermatozoa/mL. After 1 h of equilibration at 5 °C, the semen was packaged into 0.25-mL plastic straws, and the samples were frozen in a programmable biofreezer (Kryo 560-16 II Planer, Planer Plc, Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples (experiments one [9 straws per sample type], three [13 straws per sample type], and four [8 straws per sample type]) were stored in liquid nitrogen for a minimum of 1 mo. Thawing was performed by plunging the straws in water at 65 °C for 6 sec.

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2.5. Semen evaluation

Sperm concentration was assessed using a Bürker hemocytometer (Marienfeld, GmbH, Marienfeld, Germany) and CASA (ISAS, Integrated Semen Analyzer System; Proiser, Valencia, Spain). Concentration was determined before and after centrifugation and the recovery rate (recovered spermatozoa in the pellet/spermatozoa in precentrifugation sample, %) was calculated.

The quality of the samples was evaluated before and after freezing/thawing. The kinematics parameters were assessed using a computer assisted semen motility analysis system (Integrated Semen Analyzer System; Proiser). The samples were diluted ($10–20 \times 10^6$ cells/mL) in a buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300 mOsm/kg) with 1% egg yolk, and warmed on a 37.5 °C plate for 5 minutes. Then, a 5-μL sperm sample was placed in a Makler cell counting chamber (10-μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase-contrast microscope (magnification $\times$ 10) with a warmed (38 °C) stage. The standard settings of the CASA used correspond to the values of dog spermatozoa defined by ISAS following the manufacturer's recommendations. The settings used to define progressive motility are specific to bears and are defined in our previous experience [20]. The settings were as follows: 25 frames/sec; 5 to 80 μm^2 for head area; curvilinear velocity >10 μm/sec to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analyzed afterward. Reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if curvilinear velocity [VCL] $>$ 25 and STR $>$ 80), average path velocity (VAP, μm/sec), VCL (μm/sec), and linearity (LIN, %).

Sperm viability and acrosomal status were analyzed according to García-Macías et al. [21], by flow cytometry. Sperm viability was evaluated using the double stain SYBR-14 with propidium iodide (PI) (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain). Sperm samples were diluted with PBS down to 5×10^6 sperm/mL, and 300 μL was transferred to a polypropylene tube to which we added 3 μL PI (3 mg/mL in water) and 1.5 μL SYBR-14 (1 mM in DMSO). The tubes were kept at 37 °C for 20 minutes in the dark. We detected three populations corresponding to viable spermatozoa (green), moribund spermatozoa (red + green), and dead spermatozoa (red). We recorded the percentage of viable spermatozoa (VIAB).

To evaluate the sperm acrosomes, we used the PI/PNA-FITC double stain. Sperm samples were diluted in PBS (5×10^6 sperm/mL), and 300 μL were transferred to a polypropylene tube, adding 2.5 μL PI (1 mg/mL in water) and 2.5 μL PNA-FITC (0.2 mg/mL in water). We obtained the percentage of spermatozoa with damaged acrosomes (dACRO) as those green-stained.

Sperm chromatin status was assessed, in PureSperm experiments (see 2.3.3.), by the SCSA test using the metachromatic acridine orange staining procedure (AO; Polysciences, Inc., Warrington, PA, USA). This dye fluoresces green when combined with intact double-strand DNA (dsDNA), and red when combined with single-strand DNA (ssDNA). The total DNA fragmentation index (DFI) and high DNA stainability (HDS) were determined according to Alvarez et al. [22].

For flow cytometry evaluation (viability, acrosomal status, and chromatin status) we used a FACSCalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with an argon ion laser (488 nm). Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). We used the FL3 photodetector channel to read the red emission light of PI and AO-single-strand DNA (650 long pass filter), and the FL1 photodetector channel to read the green emission light of SYBR-14, FITC and AO-double-strand DNA (530/30 band pass filter). In all cases we assessed 10 000 events per sample with a flow rate of 200 cells/sec.

2.6. Statistical analysis

The results are shown as means and standard errors. Statistical analyses were performed with the SAS/STAT package, Version 9.1 (SAS Institute, Inc., Cary, NC, USA). The effects of different factors on parameters of sperm quality were analyzed using linear mixed-effects models (MIXED procedure) considering ejaculate as a random effect. Differences among groups were considered significant when $P < 0.05$.

3. Results

The fresh brown bear semen samples showed the following values for volume and cell concentration (mean $\pm$ SD): 2.9 $\pm$ 0.5 mL; $152.2 \pm 28.4 \times 10^6$ spermatozoa/mL.

3.1. Cushioned centrifugation

With regard to the fresh samples, no significant differences were found in the proportion of spermatozoa recovered from the Maxifreeze (43.5 $\pm$ 10.3%),

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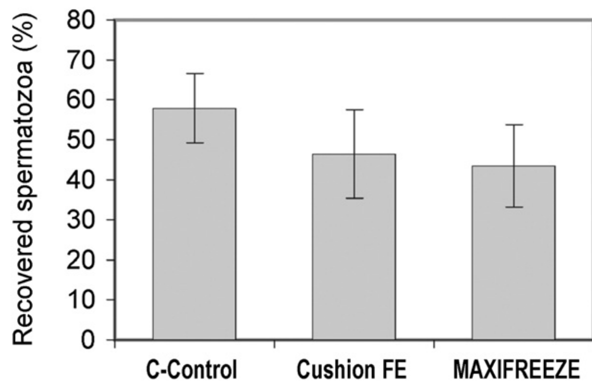

Fig. 1. Proportion of spermatozoa recovered in the pellet after centrifugation of sample (spermatozoa in the pellet/spermatozoa in precentrifugation sample, %) diluted with TCG (C-Control) or using cushioned media (Cushion Fluid Equine or Maxifreeze [IMV technologies, L'Aigle, France]). Data are shown as mean ± SEM. No significant differences were found ($P > 0.05$).

Cushion FE (46.4 ± 11.0%) or C-Control methods (57.8 ± 8.7%) (Fig. 1).

After centrifugation, the quality of spermatozoa recovered from the Maxifreeze, Cushion FE, and C-Control methods showed no significant differences but they were different from the fresh sample (Table 1). VAP observed in the centrifuged samples was lower than in the fresh sperm samples ($P < 0.05$) and VCL of C-Control was decreased with regard to fresh sample ($P = 0.025$). Both cushioned centrifugation samples had lower viability and a higher percentage of acrosome-damaged cells than the fresh semen ($P < 0.05$). The post-thawing results (Table 2) showed that C-Control motility was significantly higher than the Maxifreeze (TM $P < 0.001$; PM $P < 0.001$) and Cushion FE (TM $P < 0.002$; PM $P < 0.042$) samples. These cushioned centrifugation methods did not differ with regard to viability or acrosomal status ($P > 0.05$).

Table 2
Quality of frozen-thawed brown bear spermatozoa after centrifugation of fresh semen diluted with TCG (C-Control) or using cushion media (Cushion Fluid equine or Maxifreeze).

Parameter	C-control	Cushion FE	Maxifreeze
TM (%)	69.3 ± 6.3[a]	53.9 ± 6.4[b]	48.8 ± 6.2[b]
PM (%)	30.5 ± 3.2[a]	25.6 ± 3.6[b]	21.9 ± 3.2[b]
VAP (μm/sec)	45.8 ± 2.3	45.8 ± 2.1	44 ± 2
VCL (μm/sec)	83.9 ± 4.6	82 ± 4	82.1 ± 4.2
LIN (%)	34.7 ± 1.5[ab]	36 ± 1.2[a]	33.9 ± 1.2[b]
VIAB (%)	56.8 ± 3.4	51.9 ± 3.4	52.7 ± 3.7
dACRO (%)	6.3 ± 0.8	5.7 ± 0.5	6.9 ± 0.8

Manufacturer information: Cushion Fluid Equine, ; Maxifreeze, IMV technologies, L'Aigle, France. Data are shown as mean ± SEM. Different letters in each row indicate significant differences ($P < 0.05$).
dACRO, percentage of cells with damaged acrosome; FE, ; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TCG, ; TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

3.2. *PureSperm gradient applied to fresh samples*

The proportion of spermatozoa recovered from the bottom of the PureSperm gradient after centrifugation (24.4 ± 3.6%) and spermatozoa recovered from the interface (13.9 ± 3.9%) was significantly lower than the total number of sperm recovered by centrifuging with TCG (control: 66.5 ± 4.2%) (Fig. 2). PureSperm significantly improved the quality of sperm samples. Total motility and progressive motility were significantly higher for PS-Pellet samples than the fresh sample (TM $P = 0.004$; PM $P = 0.047$) (Table 3). The percentage of viable spermatozoa in PS-Pellet sample

Table 1
Quality of brown bear spermatozoa after centrifugation of fresh semen diluted with TCG (C-Control) or using cushion media (Cushion Fluid Equine or Maxifreeze).

Parameter	Fresh	C-control	Cushion FE	Maxifreeze
TM (%)	69.4 ± 6.4	74.8 ± 6.4	70.8 ± 4.3	65.4 ± 5.1
PM (%)	26.1 ± 4	33.9 ± 5.5	35.8 ± 3.4	30.5 ± 3.3
VAP (μm/sec)	88.5 ± 7.5[a]	64.4 ± 2.2[b]	66.5 ± 2.8[b]	64.9 ± 2.3[b]
VCL (μm/sec)	122.7 ± 10[a]	101.4 ± 8.3[b]	104.1 ± 8.7[ab]	107.9 ± 7.2[ab]
LIN (%)	46.3 ± 2.9	41.2 ± 3.1	42.7 ± 2	39 ± 1.6
VIAB (%)	79.9 ± 2.3[a]	71.5 ± 3.4[ab]	69.9 ± 3.8[b]	68.8 ± 4.2[b]
dACRO (%)	1.7 ± 0.5[a]	4.4 ± 0.5[ab]	5 ± 0.6[b]	6.5 ± 1.4[b]

Manufacturer information: Cushion Fluid Equine, ; Maxifreeze, IMV technologies, L'Aigle, France. The fresh semen is the control sample. Data are shown as mean ± SEM. Different letters in each row indicate significant differences ($P < 0.05$).
dACRO, percentage of cells with damaged acrosome; FE, ; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TCG, ; TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

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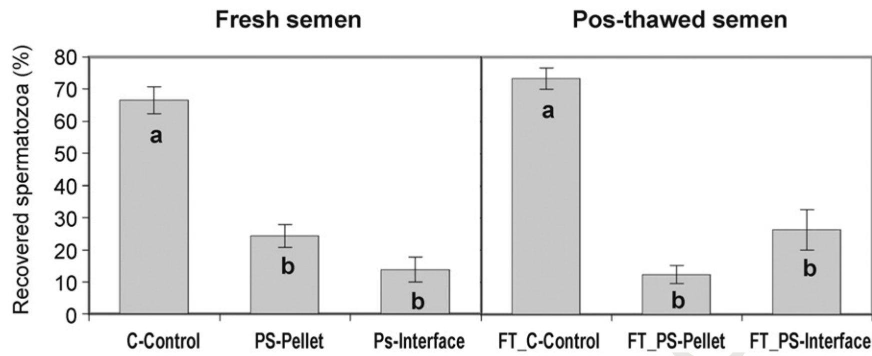

Fig. 2. Proportion of spermatozoa recovered in the pellet after centrifugation of fresh or post-thawed semen (spermatozoa in the pellet/ spermatozoa in the precentrifugation sample, %) diluted with TCG (C-Control) or using PureSperm [Nidacon, Gothenburg, Sweden] (2 samples were acquired: PS-Pellet and PS-Interface). Data are shown as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

was higher than in the fresh sample ($P < 0.001$) or C-Control ($P = 0.0164$) (Table 3). The percentage of acrosome damaged spermatozoa was lower in PS-Pellet and C-Control samples than in the fresh sample ($P < 0.05$) (Table 3). Moreover, the spermatozoa of PS-Interface had worse values for TM, PM, VAP, VCL, and viability than PS-Pellet, C-Control, and fresh samples ($P < 0.05$). Also, the percentage of spermatozoa with damaged acrosome was significantly higher in the PS-Interface sample than for C-Control and PS-Pellet ($P < 0.05$) (Table 3).

3.3. Freezability of PureSperm gradient treated samples

Motility, viability, and acrosome status of PS-Pellet and C-Control sperm samples did not differ after thawing (Table 4). Spermatozoa recovered from the PS-Interface showed significantly lower values of TM, PM, and viability ($P < 0.05$) than the other analyzed samples (Table 4). Post-thawing chromatin assessment showed higher values for the DFI parameter in PS-Interface spermatozoa in comparison with spermatozoa recovered from PS-Pellet ($P = 0.006$) (Table 5). In the analysis of recovery rates for sperm quality parameters, we observed that the acrosome of sperm selected by PureSperm are particularly susceptible to freeze damage, when compared with that observed in the sperm of the interface (Fig. 3).

3.4. PureSperm gradient applied to freeze-thawed samples

The percentage of spermatozoa recovered from FT_PS-Pellet (12.5%; Fig. 2) was significantly lower than that recovered from FT_C-Control and FT_PS-Interface (73.3 and 26.4%, respectively). FT_PS-Pellet spermatozoa showed significantly higher values for motility parameters (TM, PM, VAP, VCL, and VCL) than FT_C-Control and FT_PS-Interface spermatozoa ($P < 0.05$) (Table 6). The viability of the FT_PS-Pellet sperm sample was better than the other samples ($P <$

Table 3
Quality of brown bear spermatozoa after centrifugation of fresh semen diluted with TCG (C-Control) or using PureSperm (two samples were acquired: PS-Pellet and PS-Interface).

Parameter	Fresh	C-control	PS-pellet	PS-interface
TM (%)	58.8 ± 5.5^{c}	65.3 ± 6.4^{bc}	79.9 ± 3.6^{b}	29.5 ± 6.4^{a}
PM (%)	34.8 ± 4^{c}	36.3 ± 3.4^{bc}	44.5 ± 2.6^{b}	17.1 ± 4^{a}
VAP (μm/sec)	78.3 ± 6.9^{b}	67 ± 5.3^{b}	69.6 ± 3.7^{b}	47.7 ± 6.2^{a}
VCL (μm/sec)	110.4 ± 9.5^{b}	107.5 ± 9^{b}	116 ± 6.7^{b}	82.9 ± 10.8^{a}
LIN (%)	45.8 ± 2.3^{b}	43.3 ± 2.1^{ab}	41 ± 1.6^{ab}	38 ± 3.9^{a}
VIAB (%)	76.3 ± 2.9^{bc}	68.7 ± 6.4^{b}	82.4 ± 2.5^{c}	50.3 ± 5.7^{a}
dACRO (%)	5.3 ± 1.1^{ac}	3.2 ± 0.5^{b}	2.4 ± 0.4^{b}	7 ± 0.7^{a}

Manufacturer information: PureSperm, Nidacon, Gothenburg, Sweden. The fresh semen is the control sample. Data are shown as mean ± SEM. Different letters in each row indicate significant differences ($P < 0.05$).
dACRO, percentage of cells with damaged acrosome; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TCG, ; TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

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Table 4
Quality of frozen-thawed brown bear spermatozoa after centrifugation of fresh semen diluted with TCG (C-Control) or using PureSperm (two samples were acquired: PS-Pellet and PS-Interface).

Parameter	C-control	PS-pellet	PS-interface
TM (%)	49.2 ± 5.9[a]	62.9 ± 5.4[a]	22.5 ± 5.0[b]
PM (%)	26.5 ± 3.4[a]	31.3 ± 2.6[a]	11.8 ± 2.8[b]
VAP (μm/sec)	58.5 ± 6.2[ab]	60.1 ± 3.3[a]	45.4 ± 5.8[b]
VCL (μm/sec)	103.2 ± 11.0[ab]	109.4 ± 5.3[a]	81.6 ± 10.7[b]
LIN (%)	34.9 ± 3.0	36.0 ± 1.0	33.4 ± 4.0
VIAB (%)	46.1 ± 3.0[a]	51.3 ± 2.9[a]	37.0 ± 5.3[b]
dACRO (%)	7.6 ± 1.1	7.3 ± 1.5	7.9 ± 1.6

Manufacturer information: PureSperm, Nidacon, Gothenburg, Sweden. Data are shown as mean ± SEM. Different letters in each row indicate significant differences ($P < 0.05$).
dACRO, percentage of cells with damaged acrosome; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TCG, ; TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

0.05). The FT_PS-Pellet sample showed a lower percentage of acrosome damaged cells ($P < 0.05$).

4. Discussion

The aim of these studies was to assess the suitability of two cushioned centrifugation solutions and a density gradient centrifugation technique in preparation methods of brown bear semen for freezing.

Several authors have reported the beneficial effects of cushioned centrifugation on semen quality (boar [15], horse [23,24]). However, when we applied these systems in the centrifugation of bear semen, we observed that the samples with standard centrifugation showed better motility than the cushioned ones. In this sense, Matás et al. [15], analyzing thawed boar semen, found that total and progressive motility and VCL, VSL, and VAP were higher using the standard centrifugation than after using the cushioned centrifugation techniques, although they observed a significant effect of the centrifugation regime on the number of viable spermatozoa, with a lower proportion of dead sperm for the cushioned method. In our study, the highest recovery of spermatozoa was also achieved using the standard centrifugation method. However, other authors describe that the percentage of recovered spermatozoa with cushioned methods improve with regard to standard centrifugation (horse [23], boar [15]); which may be due to the fact that these authors used different centrifugation forces and higher times or the specificity of the medium for the species analyzed.

Given the need to centrifuge the samples, we look for other ways to improve quality and prepare sperm to assisted reproduction and cryopreservation. Gradient centrifugation techniques have been widely used in several species to improve the quality of seminal samples (human [25,26], bull [27], ram [28], marmoset [29], stallion [17]). Immediately after the centrifugation of brown bear semen with PureSperm gradient, we noticed that spermatozoa recovered from the PureSperm pellet has higher motility than the fresh sample. Our data do not agree with the results of Centola et al. [30], when compared sperm separation methods (Percoll and PureSperm) of human spermatozoa and they obtained better values for progressive velocities (fresh vs. PureSperm) but no differences were found in the percentage of motile sperm. Mousset-Siméon et al. [31] also observed that PureSperm led to a high progressive motility and concluded that it could be considered for the selection of fresh and frozen-thawed human spermatozoa in assisted reproduction techniques. In this regard, we note that it is difficult to compare assays developed with different methods (centrifugation force and times) or species because the physical dynamics may affect sperm recovery differently.

It is reasonable to speculate that selecting highly motile spermatozoa before freezing may reduce the loss of motility, viability, and acrosome integrity during cryopreservation [26]. Brown bear semen selected for prefreezing with PureSperm showed an improvement in progressive motility, viability, and acrosome status but did not show a reduction in the deleterious effects of cryopreservation. Esteves et al. [26] observed that selecting a highly motile human sperm population before freezing enhances overall post-thaw spermatozoa quality. Our post-thawing results demonstrated that the preparation of the brown bear sperm by the PureSperm method did not significantly improve the ability to cryopreserve, which is inconsistent with the improvement in quality observed in prefreezing spermatozoa. We ob-

Table 5
Sperm chromatin status of frozen-thawed brown bear spermatozoa after centrifugation of fresh semen diluted with TCG (C-Control) or using PureSperm (2 samples were acquired: PS-Pellet and PS-Interface).

Sample	tDFI	HDS
C-control	4.6 ± 0.7[ab]	3.2 ± 1
PS-pellet	3.2 ± 0.7[b]	1.1 ± 0.2
PS-interface	7.6 ± 1.6[a]	2.3 ± 0.5

Manufacturer information: PureSperm, Nidacon, Gothenburg, Sweden. Different letters in each column indicate significant differences between samples ($P < 0.05$). Data are shown as mean ± SEM.
HDS, high DNA stainability; TCG, ; tDFI, total DNA fragmentation index.

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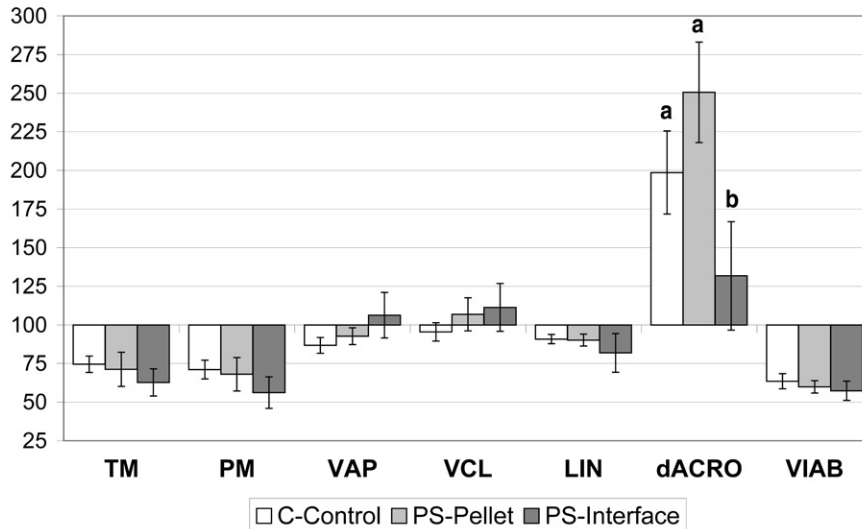

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Fig. 3. Changes in semen quality between fresh and thawed samples (mean ± SEM) diluted with TCG (C-Control) or using PS. The value is calculated as the ratio post-thawed data/prefreezing data and is represented as a percentage deviation from a reference value of 100. Different letters in each row indicate significant differences ($P < 0.05$). dACRO, percentage of cells with damaged acrosome.; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

served that the acrosomes of PureSperm selected sperm are particularly susceptible to freezing damage, compared with interface sperm. This fact could be explained by possible subtle damage caused by PureSperm in brown bear spermatozoa, which could become less resistant to cryopreservation. However, PureSperm has been shown to have no toxic effects on somatic human cells [32] although we note that the spermatozoa is a special cell, with a high heterogeneity in cell membranes.

Morrell et al. [33] found that preparing spermatozoa by PureSperm density gradient centrifugation is advantageous in prolonging human sperm survival and maintaining DNA integrity, presumably by removing sources of reactive oxygen species. The PureSperm technique can enrich the human sperm population by separating out those with nicked DNA and with poorly condensed chromatin [34]. According with it, we have shown that the selected spermatozoa in PureSperm pellet post-thawing had less chromatin damage than sperm that remained in the interface between gradients.

Another option to improve the quality of the semen sample is to make the selection after the freezing and thawing process. The aim of sperm preparation should be to minimize the risk of abnormal spermatozoa being used for fertilization and thus optimize the results of different assisted reproduction techniques [30,35]. Our

Table 6
Quality of brown bear spermatozoa after centrifugation of frozen-thawed semen diluted with TCG (FT_C-Control) or using PureSperm (two samples were acquired: FT_PS-Pellet and FT_PS-Interface).

Parameter	FT_No-Centr	FT_C-control	FT_PS-pellet	FT_PS-interface
TM (%)	39.9 ± 5.1[b]	38.5 ± 7.7[b]	72.3 ± 7.2[a]	33.8 ± 5.3[b]
PM (%)	17.6 ± 2.9[b]	16.1 ± 4.8[b]	37.2 ± 9.6[a]	12.3 ± 3.4[b]
VAP (μm/sec)	54.6 ± 3.7[b]	59.4 ± 3.9[b]	76.2 ± 3.3[a]	49 ± 4[b]
VCL (μm/sec)	104 ± 7[b]	107.5 ± 6.1[b]	141.1 ± 7.2[a]	91.8 ± 7[b]
LIN (%)	32.5 ± 1.9	32.1 ± 2.6	33.9 ± 4.2	30.9 ± 2.1
VIAB (%)	55.6 ± 3.2[b]	63.8 ± 3.7[b]	83.8 ± 3.7[a]	54.3 ± 3.6[b]
dACRO (%)	8.5 ± 1.5[b]	6 ± 1.2 [bc]	1.2 ± 0.4[a]	4.6 ± 0.6[c]

Manufacturer information: PureSperm, Nidacon, Gothenburg, Sweden. The frozen-thawed semen without centrifugation is the control sample (FT_No-Centr). Data are shown as mean ± SEM. Different letters in each row indicate significant differences ($P < 0.05$).
dACRO, percentage of cells with damaged acrosome; LIN, linearity of the curvilinear trajectory (%); PM, progressive motility (%); TCG, ; TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, percentage of viable spermatozoa.

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results show that the population selected by the PureSperm method in the seminal sample, after thawing, showed better motility, viability, and acrosomal status than the control sample. The maintenance of motility and acrosome integrity of bull spermatozoa after double freezing and thawing interjecting the two cryopreservation steps with sperm selection using PureSperm density gradient centrifugation have been reported [36].

Samardzija et al. [37] have examined the effect of Bovipure density gradient preparations (similar to PureSperm according to these authors) on the separation of frozen/thawed semen from bulls and they have observed significant differences between the evaluated parameters before and after sperm processing. Their in vitro fertilization (IVF) and culture (IVC) results suggest that BoviPure is a good alternative for sperm separation in bovine IVF.

Most filtration and gradient systems result in loss of spermatozoa and therefore, it may be that the proportion of selected functional cells is a critical factor of these techniques.

In the present study, a high proportion of the spermatozoa were lost on the gradient and, on average, only 24.4 ± 3.6% of fresh sperm and 12.5 ± 2.8% of post-thawed sperm were recovered in the pellet after PureSperm density gradient centrifugation. Maxwell et al. [36] obtained a higher percentage (35.8%) of freeze-thawed bull sperm. In special situations, such as Cantabric population of brown bear in Spain, where semen is difficult to obtain and each spermatozoon is very valuable, a low recovery percentage is not acceptable. In postthawing application of PureSperm centrifugation, we observed that a percentage of cells were located between both gradients of PureSperm (26.4%). We recovered these spermatozoa despite the fact that their quality was lower than the pellet, but similar to control sample, to improve the recovery rate. This sample should be independently stored, labeled as interface spermatozoa and could be applied with assisted reproductive technologies to optimize these genetic resource.

In conclusion, the use of dense isotonic cushion solutions (Maxifreeze and Cushion Fluid Equine) did not improve the quality of brown bear semen compared with standard centrifugation. PureSperm density gradient centrifugation is suitable for improving qualitative spermatozoa characteristics in fresh semen and post-thawed semen, but the selected spermatozoa of fresh samples with this density gradient do not show a better resistance to freezing compared with the control sample. Sperm located between PureSperm gradients, despite their lower quality, should be stored to optimize the quantitative performance of the technique.

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