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Spermatozoa recovery and post-thawing quality of brown bear ejaculates is affected for centrifugation regimes

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Abstract Sperm cryopreservation protocols for brown bear (*Ursus arctos*) require the centrifugation of semen samples to increase sperm concentration and to clean urine in contaminated samples. We evaluated the effect of centrifugation regimes (time and relative centrifugal force—RCF) on the quantity of sperm recovered and the quality of post-thawed sperm. Thirteen brown bears were electroejaculated. The ejaculates were diluted 1:1 in Tris–citric acid–glucose (TCG) extender and centrifuged with different RCF/time combinations: 600×*g*, 1,200×*g* and 2,400×*g*, for 3, 6 or 12 min. After centrifugation, spermatozoa were diluted in TES–Tris–fructose extender with egg yolk and glycerol (final glycerol concentration of 8%) and frozen in 0.25-mL straws. In the post-thawed semen, motility was assessed by CASA, and acrosomal status (PNA-FITC), viability (SYBR-14 with propidium iodide) and chromatin status (SCSA) were determined by flow cytometry. The longest centrifugation time (12 min) significantly decreased some motility parameters. Sperm recovery significantly decreased in brown bear at 600×*g*. Our results suggest that brown bear spermatozoa are more sensitive to long centrifugation times than to high RCF. Centrifugation regimes showed no effects on the post-thawing chromatin status. We recommend preparing the brown bear semen for freezing by centrifugation 1,200×*g* or 2,400×*g* for 6 min, after electroejaculation and dilution 1:1 in TCG extender, since these procedures increase the spermatozoa recovery without harmful effects on the post-thawed quality of brown bear spermatozoa.

Keywords Centrifugation · Cryopreservation · Brown bear · Spermatozoa

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Introduction

Damage caused by sperm-handling procedures is accumulative, and small successive injuries may cause an important decrease in fertilizing ability (Woelders 1997). Centrifugation is one of these procedures and found to be an essential step in the cryopreservation protocol of some species, either to concentrate diluted samples (bear, Ishikawa et al. 2002; Kojima et al. 2001), or to wash urine-contaminated semen (horse, Griggers et al. 2001; human, Makler et al. 1981; Kim and Kim 1998). Moreover, some authors propose the semen centrifugation to remove the effects deleterious of seminal plasma (dog, Rota et al. 1995; goat, Pellicer-Rubio et al. 1997; ram, Ritar and Salamon 1982; stallion, Jasko et al. 1991; Carver and Ball 2002; Sieme et al. 2004; bull, Way et al. 2000). The effects of centrifugation on spermatozoa may be influenced by many factors, such as centrifugation time, centrifugal forces, dilution rate, semen extender and individual variability. Several authors working with bear semen have used different centrifugation regimes: 500×*g* for 5 min (Okano et al. 2006), 200×*g* for 8 min (Spindler et al. 2004), and we have used 600×*g* for 6 min (Nicolas et al. 2011). A variety of centrifugation regimes for dog has been reported: 400×*g* for 5 min (Kawakami et al. 2002), 700×*g* for 5 min (Schafer-Somi et al. 2006), 700×*g* for 10 min (Okano et al. 2004a), and 700×*g* for 6 min (Rota et al. 1995). Physical forces may cause mechanical stress and deleterious effects during centrifugation of spermatozoa. It is known that there is a great variability among species regarding sensitivity to injury caused by centrifugation. Thus, spermatozoa from rat (Cardullo and Cone 1986), human (Ng et al. 1992; Alvarez et al. 1993; Aitken and Clarkson 1988) and mouse (Katkov and Mazur 1998) are sensitive to centrifugal forces, but spermatozoa from other species are not (dog, Okano et al. 2004b; stallion, Cochran et al. 1984; Sieme et al. 2004; Brinsko et al. 2000; Crockett et al. 2001; Waite et al. 2008; bull, Pickett et al. 1975; and boar Carvajal et al. 2004; Matas et al. 2007).

Several studies suggest that the damage induced in spermatozoa during centrifugation is mainly due to time factor. Thus, Aitken and Clarkson (1988) have reported that applying centrifugation for long periods of time will cause a production of reactive oxygen species on human sperm, resulting in lipid peroxidation. This damage caused by centrifugation can be prevented by the use of antioxidants (Parinaud et al. 1997).

An efficient centrifugation protocol must aim at achieving the least sperm damage while assuring high sperm recovery in the pellet. The total number of recovered spermatozoa afters centrifugation was significantly influenced by relative centrifugal force (RCF) and centrifugation time (boar, Carvajal et al. 2004; mouse, Katkov and Mazur 1998). To our knowledge, the effects of centrifugation on brown bear spermatozoa have not been fully elucidated. Thus, it is necessary to validate a specific protocol for centrifuging brown bear semen. We hypothesized that centrifugation may influence semen quality, and that high centrifugation forces and long centrifugation times would be detrimental to sperm, even though recovery rates increased. Thus, the objective of this study was to evaluate the effects of different centrifugation regimes on both the quantity of sperm recovered after supernatant removal and the sperm quality after freezing.

The present study must be situated in the context of the conservation of the endangered Cantabric brown bear (~150 individuals restricted to two isolated populations in the north of Spain) and in the development of artificial reproduction techniques to establish germplasm banks to aid in preservation of these populations (Anel et al. 2008).

Materials and methods

All chemicals were of at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise specified. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005 and with European Union Regulation 2010/63.

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) in 2007 and 2008. 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 teleanaesthesia with a combination of zolazepam HCl, tiletamine HCl (7 mg/kg of Zoletil 100; Virbac, Carros, France) and ketamine (2 mg/kg of Imalgene 1000; Rhone-Mérieux, Lyon, France). After immobilization, they were weighed and monitored during the time of anaesthesia (pulse, saturation of peripheral oxygen and respiration). The pubic region was cleaned, the prepuce and penis were washed with sterile physiological saline and the rectum emptied of faeces. Then, electroejaculation was carried out, using a PT Electronics electroejaculator (Boring, OR, USA). The transrectal probe was 320 mm long, with a diameter of 26 mm. Electric stimuli were given at intervals of 3 s of shock and three of rest, until ejaculation [10 V and 250 mA, in average, according to Garcia-Macias et al. (2006)]. The bladder was catheterized during semen collection to prevent urine contamination. The ejaculates were collected as isolated fractions to prevent urine contamination or low cellular concentration in graduated glass tubes (the volume is recorded). Fractions of reduced concentration (<200× 10^6 cells/mL), low motility (<50%) or urine contaminated (>80 mg urea/dL) were rejected as explained below. All valid fractions of the same electroejaculation were mixed (are named as an ejaculate). Forty ejaculates were obtained which were distributed as follows: 22 in experiment 1, 9 in experiment 2 and 9 in experiment 3.

Experimental design

Experiment 1

Our objective was to assess the overall effect of centrifugation on post-thawing quality of undiluted semen. Nine ejaculates of brown bear semen were used. Each ejaculate was divided into two aliquots, which were processed differently: (1) centrifugation (600×*g*, 6 min) and resuspension in the same supernatant (CENTR) and (2) no centrifugation keeping the semen with seminal plasma (NO CENTR).

Experiment 2

We evaluated the effects of several centrifugation regimes, applied previously to cryopreservation, on the post-centrifugation recovery and post-thawing quality of brown bear spermatozoa. The factors assayed were RCF (600×*g*, 1,200×*g* and 2,400×*g*) and centrifugation time (3, 6 and 12 min), resulting in a factorial design with nine centrifugation regimes: 600/3, 600/6, 600/12, 1,200/3, 1,200/6, 1,200/12, 2,400/3, 2,400/6 and 2,400/12. For this purpose, 22 ejaculates were used that were divided into nine aliquots, one for each of the tested regimes. All the aliquots were diluted 1:1 (*v*/*v*) with TCG extender [Tris (200 mM), citric acid (63 mM), glucose (70 mM), benzylpenicillin (1,000 IU/mL), dihydrostreptomycin (1 mg/mL)] and centrifuged using a microtube centrifuge (MiniSpin plus, Eppendorf®, Hamburg, Germany). After centrifugation, the supernatant was removed and the sample cryopreserved as indicated in the “Spermatozoa cryopreservation” section.

Experiment 3

To evaluate the performance of centrifugation regimens, determining the most suitable combination to allow maximum amount of sperm with less cell damage, an additional experiment was designed. We have evaluated the effect of high RCF on nine ejaculates divided into five aliquots, assaying 600×*g*, 1,200×*g*, 2,400×*g*, 4,800×*g* and 9,600×*g* for 6 min (600/6, 1,200/6, 2,400/6 , 4,800/6, 9,600/6). The samples were processed as in experiment 2, changing only the centrifugation regimes applied.

Spermatozoa cryopreservation

Q5 Post-centrifugation sperm pellets were resuspended with the same volume of TTF extender at 20°C [TES solution (300 mOsm/kg) and Tris solution (300 mOsm/kg) mixed to pH 7.1, adding 4% final volume of D-fructose solution (300 mOsm/kg), 8% glycerol, 20% egg yolk, 2% EDTA and 1% Equex Paste (Minitüb, Tiefenbach, Germany)] (Anel et al. 2010). After 1:1 dilution, the sample has a rate of 4% glycerol. Tubes with diluted samples were put in glasses containing 100 mL of water at room temperature, and transferred to a refrigerator at 5°C, thus the temperature decreased slowly to 5°C (70–80 min). Next, the sample at 4% glycerol is diluted 1:1 (*v*/*v*) using the TTF extender at 12% glycerol, in order to reach a final glycerol concentration of 8%. A final concentration of 100×10^6 spz/mL was obtained by adding more TTF extender at 8% glycerol. 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 650-16 Planer^{plc}, Planer, Sunbury, UK) at −20°C/min down to −100°C, and then transferred to liquid nitrogen containers. The cryopreserved samples were stored in liquid nitrogen for a minimum of 1 week. Thawing was performed by plunging the straws in water at 65°C for 6 s.

Semen evaluation

Sperm concentration was assessed (Bürker hemocytometer, Marienfeld GmbH, Marienfeld, Germany) using CASA (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). The total number of spermatozoa was obtained from the sperm concentration determined before and after centrifugation and the volume of the ejaculate. The recovery rate (total spermatozoa in the pellet/total spermatozoa in pre-centrifugation sample, in percent) was calculated.

The motility and kinematics parameters were evaluated using a computer-assisted semen motility analysis system (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). 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 min. Then, a 5-µL sperm sample was placed in a Makler counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope (×10) with a warmed stage (38°C). The standard settings were: 25 frames/s; 5–80 μm^2 for head area and curvilinear velocity >10 µm/s to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analysed afterwards. Reported parameters were total motility (TM, in percent), Q6 progressive motility [PM, in percent; spermatozoa were considered progressive if curvilinear velocity (VCL) >25 and STR >80], average path velocity (in micrometers per second), VCL (in micrometers per second), straight-line velocity (VSL, in micrometers per second), linearity (LIN, in percent) and amplitude of lateral head displacement (ALH, in micrometers).

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 sperms/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 min in the dark. We detected three populations corresponding to viable spermatozoa (green), moribund

Q7 **Table 1** Effect of different treatments before cryopreservation (centrifugation and resuspension in the same supernatant—CENTR—and no centrifugation—NO CENTR) on brown bear sperm motility and quality parameters (mean±SEM)

Treatment	CENTR	NO CENTR
TM (%)	33.8±8.1	41.3±4.1
PM (%)	20.6±5.3	22.2±2.4
VCL (μm/s)	102.5±7.3	101.4±5.0
VSL (μm/s)	39.5±4.0	38.4±2.2
LIN (%)	38.3±2.1	37.7±1.8
ALH (μm)	4.4±0.3	4.4±0.3
ACRO+	6.7±1.0	5.5±1.3
VIAB	40.2±7.6	45.7±3.7

Q8 Different letters indicate significant differences among treatments ($P<0.05$)

TM total motility, *PM* progressive motility, *VCL* curvilinear velocity, *VSL* straight-line velocity, *LIN* linearity of the curvilinear trajectory, *ALH* amplitude of lateral head displacement, *ACRO+* percentage of acrosome-damaged spermatozoa, *VIAB* percentage of viable spermatozoa

spermatozoa (red+green) and dead spermatozoa (red). We recorded the percentage of viable spermatozoa (VIAB).

To evaluate the sperm acrosomes, we used the double stain PI/PNA-FITC. Sperm samples were diluted in PBS (5×10^6 sperms/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 (ACRO+) as those green-stained.

Sperm chromatin status was assessed by the SCSA test using the metachromatic staining acridine orange (AO; Polysciences Inc., Warrington, PA). 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 (DFIt) and high DNA stainability (HDS) were determined according to Garcia-Macias et al. (2006).

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-ssDNA (650 long pass filter), and the FL1 photodetector channel to read the green emission light of FITC and AO-dsDNA (530/30 band pass filter). In all cases we assessed 10,000 events per sample with a flow rate of 200 cells/s.

Statistical analysis

The results are shown as means and standard errors. Data were normalized using arc-sine transformation. Statistical analyses were performed with the SAS/STATTM package, Version 8 (SAS Institute Inc., Cary, NC, USA). The effects of different factors (centrifugation regime, seminal plasma manipulations) on parameters of post-thawing sperm quality were analysed using linear mixed-effects models (MIXED procedure) considering ejaculate as a random effect. For the analysis of sperm recovery rate (recovered spermatozoa in the pellet/spermatozoa in pre-centrifugation sample, in percent), data of experiments 2 and 3 were grouped. Differences among groups were considered significant when $P<0.05$.

Table 2 Motility parameters (mean±SEM) of post-thawed brown bear semen processed after centrifugation by nine different regimes: three times 3, 6 and 12 min combined with three RCF: 600×*g*, 1,200×*g* and 2,400×*g*

RCF	Min	TM (%)	PM (%)	VCL (μm/s)	VSL (μm/s)	LIN (%)	ALH (μm)
600×*g*	3	70.6±3.2	28.8±2.3	95.5±4.7	32.1±1.9	33.0±1.1	4.4±0.2
	6	69.4±3.5	30.0±2.6	99.7±5.1	34.7±2.2	34.0±1.0	4.5±0.2
	12	69.5±3.6	27.0±2.5	90.4±4.7*	30.4±2.0*	32.7±1.1	4.2±0.2
1,200×*g*	3	70.7±3.4	30.5±2.5	98.1±5.5	33.6±2.2	33.4±1.0	4.5±0.2
	6	71.8±3.5	30.3±2.2	96.2±4.8	33.2±1.7	33.7±0.8	4.4±0.2
	12	68.5±4.5	28.4±2.7	92.6±4.4*	31.5±1.9*	33.5±1.3	4.2±0.2
2,400×*g*	3	69.8±4.1	29.9±2.6	98.4±4.3	33.6±1.7	33.3±1.1	4.5±0.2
	6	70.1±3.5	29.3±2.2	94.0±4.4	32.2±1.6	33.7±1.0	4.3±0.2
	12	70.4±3.3	26.7±2.3	94.6±3.8*	30.9±1.6*	32.0±1.2*	4.4±0.2

TM total motility, *PM* progressive motility, *VCL* curvilinear velocity, *VSL* straight-line velocity, *LIN* linearity of the curvilinear trajectory, *ALH* amplitude of lateral head displacement

*$P<0.05$, significant differences from 600×*g* for 6 min

Q9

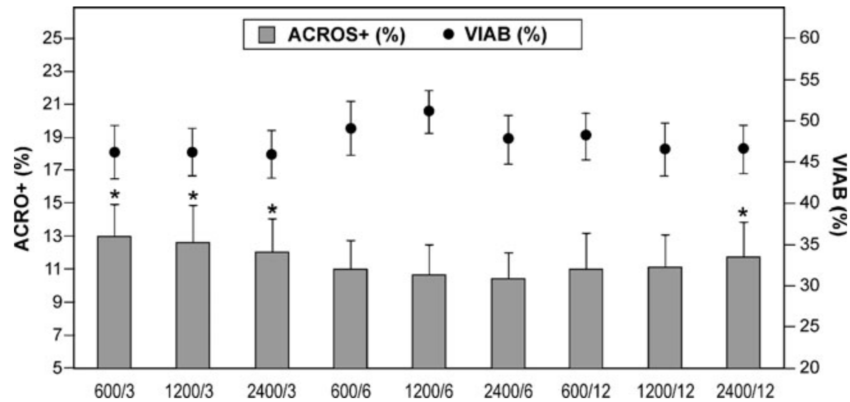

Fig. 1 Effect of different centrifugation regimes (RCF/time in minutes) on the percentage of acrosome-damaged spermatozoa (*ACROS+*) and percentage of viable spermatozoa (*VIAB*), in frozen-thawed brown bear semen (mean±SEM). *Asterisk* indicates significant differences with 600×*g* for 3 min

Results

The fresh semen samples of the brown bear selected for this experiment yielded a volume of 3.2±0.8 mL and a sperm concentration of 186.7±79.4×10^6 spermatozoa/mL. The average quality of fresh semen was TM 68.8%±4.5, PM 36.3%±4.2 and sperm viability 74.9%±3.0.

Experiment 1

The centrifugation of undiluted sample not showed significant differences in motility parameters and sperm quality with respect to non-centrifuged sample (Table 1).

Experiment 2

Post-thawing sperm motility was influenced by the centrifugation regime employed (Table 2). VCL, VSL and ALH significantly decreased when using 600/12, 1,200/12 and 2,400/12 centrifugation regimes in comparison with 600/6. LIN decreased with the 2,400/12 regime (P=0.043) with regard to 600/6. In relation to flow cytometry analyses (Fig. 1), ACRO+ was lower when using 600/6 regime than when using 600/3, 1,200/3, 2,400/3 or 2,400/12. The different centrifugation regimes had no significant effect on viable spermatozoa percentage of post-thawed bear semen.

Experiment 3

The parameters of post-thawing spermatozoa quality (motility, Table 3; SCSA, Table 4; ACRO+ and VIAB, Fig. 2) did not show significant differences with regard to centrifugation RCF at 6 min. It is noteworthy that the parameter DFIt showed a progressive increase with increasing RCF (600×*g*, 6.3; 9,600×*g*, 8.2).

The regression model applied to evaluate the percentage of recovered spermatozoa in the pellet in relation to RCF for 6 min had a poor fit due to the high between-sample variability (R^2=0.1, P=0.016; Fig. 3). To evaluate sperm recovery, data of experiment 1 and 2 were used in regression model. The total number of recovered spermatozoa increased linearly with RCF (Fig. 3), although it does not change significantly.

Discussion

Sperm-handling procedures may cause sperm damage. Preventing such damage is especially important when

Table 3 Effect of centrifugation regimes 600×*g*, 1,200×*g*, 2,400×*g*, 4,800×*g* and 9,600×*g* for 6 min on post-thawed motility of brown bear semen (mean±SEM)

RCF	TM (%)	PM (%)	VCL (μm/s)	VSL (μm/s)	LIN (%)	ALH (μm)
600×*g*	72.5±5.1	29.5±3.8	90.8±6.6	32.2±2.8	34.6±1.5	4.1±0.3
1,200×*g*	77.2±4.9	30.7±3.2	89.5±5.6	31.6±1.8	34.3±1.6	4.0±0.3
2,400×*g*	76.8±4.1	31.4±3.1	88.3±5.7	31.2±1.8	34.5±1.7	4.0±0.3
4,800×*g*	76.2±3.9	30.9±2.5	85.0±4.6	31.1±1.9	35.6±1.6	3.8±0.2
9,600×*g*	74.3±4.7	29.7±3.2	91.8±6.5	32.7±2.1	35.3±1.5	4.1±0.4

No significant differences were found among RCF (P<0.05)

TM total motility, *PM* progressive motility, *VCL* curvilinear velocity, *VSL* straight-line velocity, *LIN* linearity of the curvilinear trajectory, *ALH* amplitude of lateral head displacement

Table 4 Effect of centrifugation regimes 600×*g*, 1,200×*g*, 2,400×*g*, 4,800×*g* and 9,600×*g* for 6 min on post-thawed sperm chromatin status of brown bear semen (SCSA)

RCF	DFIt	HDS
600×*g*	6.3±0.7	4.4±0.7
1,200×*g*	6.8±0.6	5.5±1.1
2,400×*g*	7.1±1.0	5.0±1.0
4,800×*g*	6.9±0.7	4.9±0.5
9,600×*g*	8.2±2.2	5.7±1.4

No significant differences were found among treatments ($P<0.05$)

DFIt total DNA fragmentation index, *HDS* high DNA stainability

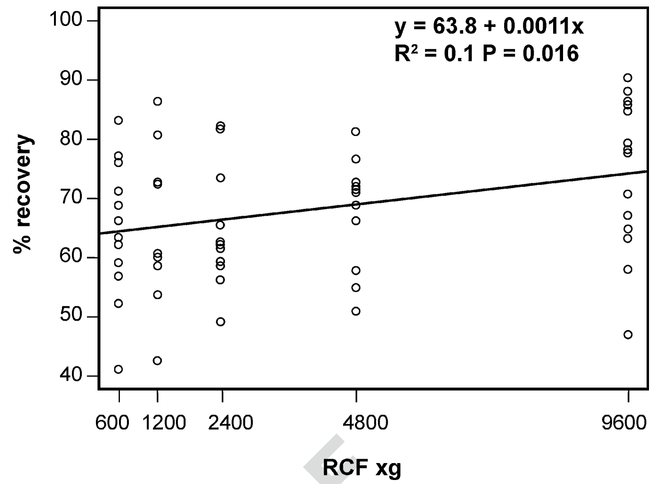

Fig. 3 Regression model of RCF (600×*g*, 1,200×*g*, 2,400×*g*, 4,800×*g* and 9,600×*g* for 6 min) on brown bear sperm recovery rate (recovered spermatozoa in the pellet/spermatozoa in pre-centrifugation sample, in percent). The linear model is shown within the figure

working with valuable samples, such as the Cantabric brown bear, an endangered species.

Although there are no studies regarding how centrifugation affects bear spermatozoa, studies in other species have indicated that time might be more critical than RCF (boar, Carvajal et al. 2004; rat, Varisli et al. 2009; mouse, Katkov and Mazur 1998). Agreeing with these authors, we have shown that long-time centrifugation (12 min) decreased some motility parameters in post-thawed brown bear spermatozoa, whereas RCF did not seem to influence post-thaw sperm characteristics. These facts suggest a time-dependent detrimental effect on sperm quality rather than a centrifugal force effect. Previous studies concluded that moderate RCF (720×*g* for 5 min) was the best strategy to centrifuge dog semen, and that high RCF was potentially harmful to its motility and viability (Rijsselaere et al. 2002). Katkov and Mazur (1998) also recommended centrifuging mouse semen using RCF below 800×*g*, since it rendered the maximum number of motile spermatozoa. Nevertheless, brown bear spermatozoa showed high resilience to high RCF, and our results indicated that relatively high RCF (1,200×*g* and 2,400×*g*) did not reduce either post-thaw motility or viability in brown bear samples. Our results are more similar to those obtained by Carvajal et al. (2004) in boar, indicating that 2,400×*g* enabled high sperm recovery to be achieved whilst maintaining sperm viability.

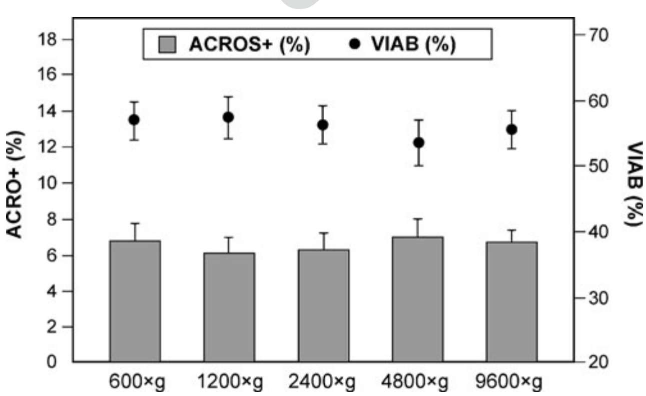

Fig. 2 Effect of different centrifugation RCF during 6 min on the percentage of acrosome-damaged spermatozoa (*ACROS+*) and percentage of viable spermatozoa (*VIAB*), in frozen-thawed brown bear semen (mean±SEM). No significant differences were found among treatments ($P<0.05$)

The percentage of post-thaw acrosome-damaged spermatozoa in brown bear was higher, in general, when centrifuged during short times (3 min) in comparison with values observed at centrifugation times of 6 or 12 min. These contradictory results might be caused by the fact that shorter times for any RCF leave more spermatozoa in the supernatant, and centrifugation may cause a differential pelletization of low-quality spermatozoa while a higher proportion of spermatozoa with intact acrosomes remain in the supernatant.

We have shown that the centrifugation regime affects brown bear sperm recovery. This was expected, since higher RCF and longer times would allow more spermatozoa to pellet resulting in higher percentages of sperm recovery. Conversely, when the semen sample was centrifuged for short times combined with low RCF, a higher sperm loss was caused. These results coincide with studies in other species (dog, Rijsselaere et al. 2002; mouse, Katkov and Mazur 1998; boar, Carvajal et al. 2004).

Our data suggested high male-to-male variability among bears regarding sperm recovery that has perhaps influenced the results. This fact could not be studied in depth because of the difficulty in replicating each male, due to the characteristics of the population. Nevertheless, it seems that whereas some bears rendered low yields for all the centrifugation regimes, some of them showed high recoveries, and others had an irregular response for different centrifugation regimes. This might be due to individual changes in seminal plasma or sperm membrane composition, resulting in variable pelleting times and a tendency to form either loose or compact pellets. In fact, Rijsselaere et al. (2002) studied the effect of centrifugation on dog semen, reporting high variability among ejaculates regarding the sperm concentration in the supernatant. No explanation could be given for these findings. In this way, other studies must be developed to identify factors of variation that could influence the process of centrifugation in brown bear sperm.

In conclusion, for brown bear sperm, centrifuging for a long time (12 min) was detrimental to post-thaw motility parameters, whereas shorter times and lower RCF decreased sperm recovery. Therefore, considering that brown bear semen freezability seems to be resilient to high RCF, we suggest applying 6 min of centrifugation and medium-high RCF (1,200×*g* or 2,400×*g*) for centrifuging brown bear semen after electroejaculation and dilution 1:1 in TCG extender, since these regimes increases the spermatozoa recovery without apparently harmful effects on the post-thawed quality of brown bear spermatozoa.

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References

Aitken RJ, Clarkson JS (1988) Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 9:367–376

Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero PP, Storey BT (1993) Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. Hum Reprod 8:1087–1092

Anel L, Alvarez M, Martinez-Pastor F, Gomes S, Nicolas M, Mata M, Martinez AF, Borragan S, Anel E, de Paz P (2008) Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. Reprod Domest Anim 43(Suppl 4):9–17

Anel L, Gomes-Alves S, Alvarez M, Borragan S, Anel E, Nicolas M, Martinez-Pastor F, de Paz P (2010) Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. Theriogenology 74:643–651

Brinsko SP, Crockett EC, Squires EL (2000) Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 54:129–136

Cardullo RA, Cone RA (1986) Mechanical immobilization of rat sperm does not change their oxygen consumption rate. Biol Reprod 34:820–830

Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J (2004) Effects of centrifugation before freezing on boar sperm cryosurvival. J Androl 25:389–396

Carver DA, Ball BA (2002) Lipase activity in stallion seminal plasma and the effect of lipase on stallion spermatozoa during storage at 5 degrees C. Theriogenology 58:1587–1595

Cochran JD, Amann RP, Froman DP, Pickett BW (1984) Effects of centrifugation, glycerol level, cooling to 5 degrees C, freezing rate and thawing rate on the post-thaw motility of equine sperm. Theriogenology 22:25–38

Crockett EC, Graham JK, Bruemmer JE, Squires EL (2001) Effect of cooling of equine spermatozoa before freezing on post-thaw motility: preliminary results. Theriogenology 55:793–803

Garcia-Macias V, Martinez-Pastor F, Alvarez M, Borragan S, Chamorro CA, Soler AJ, Anel L, de Paz P (2006) Seasonal changes in sperm chromatin condensation in ram (*Ovis aries*), Iberian red deer (*Cervus elaphus hispanicus*), and brown bear (*Ursus arctos*). J Androl 27:837–846

Griggers S, Paccamonti DL, Thompson RA, Eilts BE (2001) The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology 56:613–622

Ishikawa A, Matsu M, Sakamoto H, Katagiri S, Takahashi Y (2002) Cryopreservation of the semen collected by electroejaculation from the Hokkaido brown bear (*Ursus arctos yesoensis*). J Vet Med Sci 64:373–376

Jasko DJ, Lein DH, Foote RH (1991) The repeatability and effect of season on seminal characteristics and computer-aided sperm analysis in the stallion. Theriogenology 35:317–327

Katkov II, Mazur P (1998) Influence of centrifugation regimes on motility, yield, and cell associations of mouse spermatozoa. J Androl 19:232–241

Kawakami E, Morita Y, Hori T, Tsutsui T (2002) Lectin-binding characteristics and capacitation of canine epididymal spermatozoa. J Vet Med Sci 64:543–549

Kim SC, Kim HW (1998) Effects of nitrogenous components of urine on sperm motility: an in vitro study. Int J Androl 21:29–33

Kojima E, Tsuruga H, Komatsu T, Murase T, Tsubota T, Kita I (2001) Characterization of semen collected from beagles and captive Japanese black bears (*Ursus thibetanus japonicus*). Theriogenology 55:717–731

Makler A, David R, Blumenfeld Z, Better OS (1981) Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. Fertil Steril 36:507–511

Matas C, Decuadro G, Martinez-Miro S, Gadea J (2007) Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 67:1087–1091

Ng FL, Liu DY, Baker HW (1992) Comparison of Percoll, mini-Percoll and swim-up methods for sperm preparation from abnormal semen samples. Hum Reprod 7:261–266

Nicolas N, Alvarez M, Gomes-Alves S, Mata-Campuzano M, Borragán S, Martinez-Pastor F, de Paz P, Anel L (2011) Effects on brown bear (*Ursus arctos*) spermatozoa freezability of different extender and dilution ratios used for pre-freezing centrifugation. Eur J Wildl Res 57:259–266

Okano T, Murase T, Tsubota T (2004a) Electroejaculation and semen cryopreservation of free-ranging Japanese black bears (*Ursus thibetanus japonicus*). J Vet Med Sci 66:1371–1376

Okano T, Murase T, Asano M, Tsubota T (2004b) Effects of final dilution rate, sperm concentration and times for cooling and glycerol equilibration on post-thaw characteristics of canine spermatozoa. J Vet Med Sci 66:1359–1364

Okano T, Murase T, Yayota C, Komatsu T, Miyazawa K, Asano M, Tsubota T (2006) Characteristics of captive Japanese black bears (*Ursus thibetanus japonicus*) semen collected by electroejaculation with different voltages for stimulation and frozen-thawed under different conditions. Anim Reprod Sci 95:134–143

Parinaud J, Le LD, Vieitez G, Griveau JF, Milhet P, Richoilley G (1997) Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit) following ejaculation. Hum Reprod 12:2434–2436

Pellicer-Rubio MT, Magallon T, Combarnous Y (1997) Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kilodalton glycoprotein with triglyceride lipase activity. Biol Reprod 57:1023–1031

Pickett BW, Sullivan JJ, Byers WW, Pace MM, Remmenga EE (1975) Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil Steril 26:167–174

Rijsselaere T, Van SA, Maes D, de Kruif A (2002) Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 57:1669–1681

Ritar AJ, Salamon S (1982) Effects of seminal plasma and of its removal and of egg yolk in the diluent on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. Aust J Biol Sci 35:305–312

Rota A, Strom B, Linde-Forsberg C (1995) Effects of seminal plasma and three extenders on canine semen stored at 4 degrees C. Theriogenology 44:885–900

Schafer-Somi S, Kluger S, Knapp E, Klein D, Aurich C (2006) Effects of semen extender and semen processing on motility and viability of frozen-thawed dog spermatozoa. Theriogenology 66:173–182

Sieme H, Katila T, Klug E (2004) Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenology 61:769–784

Spindler RE, Huang Y, Howard JG, Wang P, Zhang H, Zhang G, Wildt DE (2004) Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda. Reproduction 127:547–556

Varisli O, Uguz C, Agca C, Agca Y (2009) Various physical stress factors on rat sperm motility, integrity of acrosome, and plasma membrane. J Androl 30:75–86

Waite JA, Love CC, Brinsko SP, Teague SR, Salazar JL Jr, Mancill SS, Varner DD (2008) Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 70:704–714

Way AL, Griel LC Jr, Killian GJ (2000) Effects of accessory sex gland fluid on viability, capacitation, and the acrosome reaction of cauda epididymal bull spermatozoa. J Androl 21:213–219

Weiss S, Janett F, Burger D, Hassig M, Thun R (2004) The influence of centrifugation on quality and freezability of stallion semen. Schweiz Arch Tierheilkd 146:285–293 Q10

Woelders H (1997) Fundamentals and recent development in cryopreservation of bull and boar semen. Vet Q 19:135–138