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Effects of Centrifugation Before Freezing on Boar Sperm Cryosurvival

GEMA CARVAJAL, CRISTINA CUELLO, MARIA RUIZ, JUAN MARÍA VÁZQUEZ, EMILIO ARSENIO MARTÍNEZ, AND JORDI ROCA

From the Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, Campus de Espinardo, University of Murcia, Murcia, Spain.

ABSTRACT: Current protocols for boar sperm cryopreservation require the centrifugation of semen in order to separate sperm cells from the seminal plasma. This study evaluated the influence of different centrifugation regimes on both sperm recovery and yield (percentage of viable sperm with an intact acrosome relative to the initial sperm population) after centrifugation (experiment 1) as well as the influence of different centrifugation regimes on boar sperm cryosurvival (experiment 2). In both experiments, sperm-rich fractions from 3 boars were diluted, pooled, and cooled to 17°C before centrifugation. In experiment 1, the *g*-forces tested were 400, 800, 1600, and 2400 × *g* for 3 or 5 minutes, using the standard regime (800 × *g* for 10 minutes) as a reference. Sperm recovery (Bürker Chamber) and yield (triple fluorescent stain of PI/R123/FITC-PNA [DNA-specific fluorochrome propidium iodide/mitochondria-specific fluorochrome rhodamine-123/acrosome-specific fluorochrome fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin]) were calculated. The highest recovery and yield ($P < .05$) values were achieved using 2400 × *g* for 5 or 3 minutes and 1600 × *g* for 5 minutes, which showed no differences ($P > .05$) from the reference in terms of sperm yield. In experiment 2, cooled semen was centrifuged using 3 different regimes: C1 (2400 × *g* for 3 minutes), C2 (1600 × *g* for 5 minutes), and C3 (800 × *g* for 10 minutes). Pellets were diluted in lactose-egg yolk (LEY)-glycerol-Equex STM (1×10^9 cells/mL) and frozen in 0.5-mL straws. After thawing, sperm quality was assessed after 30 and 150 minutes of incubation (37°C). Centrifugation regimes C1 and C2 showed significantly ($P < .05$) higher postthaw sperm motility (assessed with a computer-assisted semen analysis system), viability (evaluated as for experiment 1), and percentage of uncapacitated sperm (assessed with a chlortetracycline assay) than did C3. In addition, C1 had the highest ($P < .05$) oocyte penetrating ability (assessed with the homologous in vitro penetration test performed with immature oocytes). Malondialdehyde production, assessed with the thiobarbituric acid reactive species test, was unaffected ($P > .05$) by the centrifugation regime used. We conclude that high *g*-force (2400 × *g*) and short centrifugation time (3 minutes) do not affect sperm recovery and yield and that, moreover, they have a positive effect on the cryosurvival of boar sperm. Therefore, we recommend the use of short-term centrifugation with a relatively high *g*-force (2400 × *g* for 3 minutes) in boar sperm cryopreservation protocol.

Key words: Cryopreservation, g-force, frozen-thawed sperm, semen quality.

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The low fertility of sows following artificial insemination (AI) with frozen-thawed boar spermatozoa is well documented (Johnson et al, 2000), and it is largely due to the loss of fertilizing ability of spermatozoa during the cryopreservation process. In fact, only 50% of the spermatozoa from an ejaculate survive the freezing and thawing process (Almlid and Hofmo, 1996).

From the first protocols used (Pursel and Johnson, 1975; Westendorf et al, 1975), many papers have been published in which several steps of the cryopreservation process have been evaluated, such as cooling and warming rates, cold shock and holding time during cooling, cryoprotective agents and their concentration, and packaging systems (for reviews, see Bwanga, 1990; Holt, 2000; Johnson et al, 2000; Watson, 2000). However, very little attention has been paid to the centrifugation step, which the sperm are subjected to before being frozen. Centrifugation is a necessary step in boar sperm cryopreservation protocol that is used to concentrate the sperm population so that they can be rediluted with freezing extenders. Reviews of boar sperm cryopreservation research show that a variety of centrifugation regimes have been used: 300 × *g* for 10 minutes (Pursel and Johnson, 1975), 800 × *g* for 10 minutes (Westendorf et al, 1975), 800 × *g* for 15 minutes (Paquignon and Courot, 1976), and 1400 × *g* for 5 minutes (Larsson et al, 1977). Two of those freezing methods, the Beltsville (Pursel and Johnson, 1975) and the Hülsenberg (Westendorf et al, 1975), are the basis for boar sperm cryopreservation protocols currently in place for commercial use. In these protocols, the centrifugation step is performed with the centrifugation regime unchanged from the original.

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Correspondence to: Dr Jordi Roca, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, Campus de Espinardo, University of Murcia, E-30071 Murcia, Spain (e-mail: roca@um.es).

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On the other hand, it has been shown that for several species, such as the rat (Cardullo and Cone, 1986), human (Ng et al, 1990), and mouse (Katkov and Mazur, 1998), centrifugation is a potentially sperm-damaging step during semen processing. Specifically, it has been demonstrated (Aitken and Clarkson, 1988) that centrifugal pelleting of unselected human sperm populations caused the production of reactive oxygen species (ROS) within the pellet, which induced irreversible damage to spermatozoa and the impairment of their in vitro fertilizing ability. Studies on the effect of different centrifugation regimes on human sperm (Shekarriz et al, 1995) have concluded that the time of centrifugation is more critical than the *g*-force for inducing sperm damage; thus, the use of short-term centrifugation is recommended in the preparation of sperm for assisted reproductive techniques.

To our knowledge, studies on the effect of centrifugation on frozen-thawed boar spermatozoa have not been reported. Therefore, the purposes of this report were to evaluate 1) the effect of different centrifugation regimes on the quantity of sperm recovered after supernatant removal and their viability, and 2) the effect of different centrifugation regimes before freezing on the motility, viability, capacitation-like changes, oocyte penetrating ability, and lipid peroxidation of frozen-thawed spermatozoa.

Materials and Methods

Reagents

Equex STM was purchased from Nova Chemical Sales Inc (Scituate, Mass). All other reagents were obtained from the Sigma-Aldrich Chemical Co (St Louis, Mo).

Animals and Sperm Cryopreservation

Ejaculates were collected weekly by the gloved-hand method from 3 mature Pietrain boars (Agropor AI Center, Murcia, Spain) for 8 weeks. Sperm-rich fractions were extended (1:1 [vol/vol]) in Beltsville Thawing Solution (BTS) (Pursel and Johnson, 1975). After collection, sperm characteristics (sperm concentration, subjective sperm motility, acrosome integrity, and normal morphology) were microscopically evaluated by standard laboratory techniques (Martín Rillo et al, 1996), and only ejaculates with more than 75% motile spermatozoa and more than 80% normal acrosomal ridges were used. Immediately after evaluation, the diluted sperm-rich fractions were pooled and slowly cooled to 17°C for 240 minutes. At 17°C, diluted spermatozoa were centrifuged (Megafuge 1.0 R, Heraeus, Germany; the centrifugation regimes used are described in the experimental design), and all of the supernatants were discarded.

Spermatozoa were cryopreserved using a modification of the straw-freezing procedure described by Westendorf et al (1975) adapted to 0.5-mL straws by Thurston et al (1999). After centrifugation, the pellets were diluted to a concentration of 1500 $\times 10^6$ cells/mL with lactose-egg yolk (LEY) extender (pH 6.2, and 330 ± 5 mOsmol/kg), composed of 80% (v/v) β-Lactose solution (310 mM in distilled water), 20% (v/v) egg yolk and 100 μg/mL kanamycin sulfate. After further cooling to 5°C for 120 minutes, diluted spermatozoa were resuspended to a final concentration of 1000 $\times 10^6$ cells/mL with LEY-glycerol-Orvus-ES-Paste (LEYGO) extender (pH 6.2 and 1650 ± 15 mOsm/kg), consisting of 92.5% (v/v) LEY, 6% (v/v) glycerol and 1.5% (v/v) Equex STM (equivalent to Orvus ES Paste; Graham et al, 1971). French straws (0.5 mL; Minitüb, Landshut, Germany) were filled with diluted and cooled spermatozoa. Sperm packaged in this manner were frozen from 5°C to −5°C at a rate of 6°C/min, frozen from −5°C to −80°C at a rate of 40°C/min, held for 30 seconds at −80°C, and then cooled from 70°C/min to −150°C using a programmable cell freezer (IceCube 1810, Minitüb, Germany). After reaching −150°C, the straws were plunged into liquid nitrogen and stored in a liquid nitrogen container.

Frozen sperm samples were thawed in a circulating water bath at 37°C for 20 seconds. After thawing, sperm suspensions were diluted at 37°C in BTS (1:1 [vol/vol]) and held in the water bath for 150 minutes.

Sperm Quality Parameters

Objective Sperm Motility—A 4-μL sperm sample was placed on a prewarmed (37°C) Makler chamber (Sefi Medical Instruments, Haifa, Israel) and analyzed for kinematics using a computer-assisted semen motility analysis system (Sperm Class Analyzer; Microptic, Barcelona, Spain). Observations were made at a magnification of 100× on a positive phase-contrast microscope (Labophot; Nikon, Tokyo, Japan) with a warmed (39°C) stage. Three fields per sample were analyzed, and the total number of spermatozoa analyzed in each sperm sample was a minimum of 100 spermatozoa. The computer-assisted sperm analysis (CASA)-derived motility characteristics studied were curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, μm), mean dance (DNM, mean value of VCL × ALH, μm2/s), and beat cross-frequency (BCF, Hz). The definition of these descriptors can be found in previous publications (eg, Davis and Siemers, 1995). Total sperm motility (TSM, %) was defined as the percentage of spermatozoa that showed an average path velocity greater than 10 μm/s, and rapid motile spermatozoa (RMS, %) was defined as the percentage of spermatozoa that showed an average path velocity greater than 50 μm/s.

Plasma Membrane Integrity, Mitochondrial Function, and Acrosomal Integrity—Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity. These characteristics were analyzed simultaneously using a modification of a triple fluorescent procedure described by Graham et al (1990), which includes the DNA-specific fluorochrome propidium iodide (PI), the mitochondria-specific fluorochrome rhodamine-123 (R123), and the acrosome-specific fluorochrome fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin assay (FITC-PNA). Three hundred spermatozoa were counted at 1000× magnification (Eclipse E800, Nikon) using a BV-2A filter (a 400- to 440-nm excitation filter, an emission wavelength of 455 nm, and a 470-nm barrier filter). Four staining patterns could be discerned:

1) viable sperm with an intact acrosome (VS, with no staining of the head and the midpiece of the sperm tail stained green), 2) viable sperm with an acrosome reaction (VS-AR, with both the midpiece of sperm tail and the acrosome region stained green), 3) nonviable sperm with an intact acrosome (DS, with the sperm head stained red), and 4) nonviable sperm with an acrosome reaction (DS-AR, with the sperm head stained red and the acrosome region stained green. Values were expressed as percentages.

Capacitation-Like Changes—Capacitation-like changes were evaluated by fluorescent microscopy with chlortetracycline (CTC) staining (Maxwell and Johnson, 1997) that included the antibiotic CTC to visualize the course of capacitation and the acrosome reaction. Two hundred spermatozoa were counted at 1000× magnification (Eclipse E800, Nikon), using a BV-2A filter (a 400- to 440-nm excitation filter, an emission wavelength of 455 nm, and a 470-nm barrier filter). Sperm were classified according to CTC staining patterns using the nomenclature described by Fraser et al (1995): F (uniform fluorescent head—uncapacitated and acrosome intact), B (fluorescence-free band on the postacrosomal region—capacitated), and AR (nonfluorescent head or a thin fluorescent band on the equatorial segment—acrosome reacted). Values were expressed as percentages.

In Vitro Penetrating Ability—In vitro penetrating ability was assessed using the homologous in vitro penetration (hIVP) test with immature oocytes (Martínez et al, 1996). Two hundred forty oocytes per replicate (80 oocytes per centrifugation regime) were processed. The fixed oocytes were stained with 1% lacmoid and examined for evidence of sperm penetration under phase-contrast microscopy at 400× magnification. Immature oocytes with a broken oolemma or abnormal-looking cytoplasm were classified as degenerated (unhealthy oocytes) and were not evaluated. Healthy immature oocytes at the germinal vesicle stage were considered penetrated when spermatozoa with swollen or unswollen heads and their corresponding tails were found in the vitellus. The oocyte penetrating ability parameters evaluated were as follows: oocyte penetration rate (percentage of the number of oocytes penetrated/the total number of viable oocytes, %) and the number of spermatozoa per penetrated oocyte (the mean number of spermatozoa in penetrated oocytes).

Lipid Peroxidation Determination

Lipid peroxidation levels were quantified using the endpoint generation of malondialdehyde (MDA) with the thiobarbituric acid reactive substance (TBARS) test (Comaschi et al, 1989) and were expressed as the simple concentration of MDA (pmol MDA/10^8cells). The MDA concentration was measured by a spectrophotometer (UNICAM PU 8610 Kinetics Spectrophotometer; PHILIPS, Cambridge, United Kingdom) at 534 nm.

Experimental Design

Experiment 1: Effect of the Centrifugation Regimes on Boar Sperm Recovery and Yield—The factors incorporated in this experiment (4 × 2 factorial) were as follows: *g*-force (400 × *g,* 800 × *g,* 1600 × *g,* and 2400 × *g*) and centrifugation time (3 and 5 minutes). The standard centrifugation regime, 800 × *g* for 10 minutes (Westendorf et al, 1975) was used as a reference. The diluted and pooled sperm-rich fractions were divided into 9–50 mL aliquots and transferred to 50 mL-Corning centrifugation tubes. After centrifugation at 17°C, the supernatants were discarded, and pelleted spermatozoa were gently rediluted with BTS to the initial volume (50 mL). Before (BC) and after centrifugation (AC), the sperm concentration (SC; Bürker Chamber) and the percentage of viable sperm with intact acrosome (VS; using the triple fluorescent stain) were assessed. This experiment was performed 3 times. Sperm recovery (SR) and sperm yield (SY) were expressed in percentages and were calculated as follows:

$$SR = \frac{SC\ AC}{SC\ BC} \times 100$$

$$SY = \frac{SC\ AC \times VS\ AC}{SC\ BC \times VS\ BC} \times 100$$

Experiment 2: Effect of Centrifugation Regimes Before Freezing on Boar Sperm Cryosurvival and Postthaw Lipid Peroxidation—To perform this experiment, 2 centrifugation regimes of experiment 1 were chosen: 2400 × *g* for 3 minutes (C1) and 1600 × *g* for 5 minutes (C2). The standard centrifugation regime, 800 × *g* for 10 minutes (Westendorf et al, 1975), was used as a reference (C3). The diluted and pooled sperm-rich fractions were divided into 3 aliquots and transferred to 50-mL Corning centrifugation tubes. After centrifugation at 17°C, the samples were processed following the cryopreservation protocol previously described. The postthaw evaluation was performed as follows: motility, viability, and capacitation-like change parameters were assessed at 30 and 150 minutes. Oocyte penetrating ability parameters were evaluated just after thawing. MDA production was measured after 0, 30, and 150 minutes of postthawing. This experiment was performed 5 times.

Statistical Analysis

Data were analyzed using the SPSS 10.0 PC statistics package (SPSS Inc, Chicago, Ill). The sperm parameters evaluated both in experiment 1 (*g*-forces and centrifugation time) and in experiment 2 (centrifugation regime and postthawing evaluation time) were analyzed using a 2-way analysis of variance. In the second experiment, when no significant effect of postthawing evaluation time was observed, the results were combined and analyzed as a complete data set. When the analysis of variance showed a significant effect, the values were compared by the Tukey test. In the second experiment, the percentage of oocytes penetrated and the number of spermatozoa per penetrated oocyte were analyzed using the chi-square test and an analysis of variance, respectively. Values are presented as the mean ± the standard error of the mean, and the level of statistical significance was considered $P < .05$.

Results

Experiment 1: Effect of Different Centrifugation Regimes on Boar Sperm Recovery and Yield

The *g*-force and centrifugation time had a significant ($P < .05$) influence on both sperm recovery and yield (Fig-

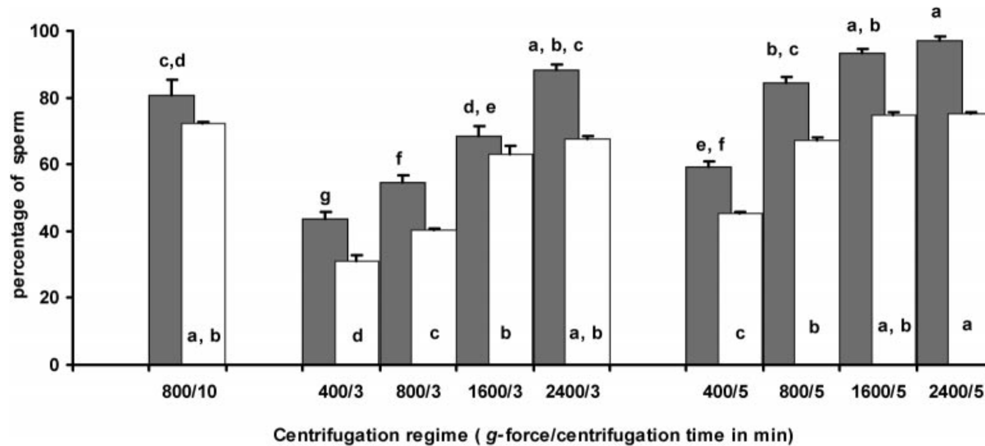

Figure 1. Effect of different centrifugation regimes (*g*-force/time in minutes) on sperm recovery (▤) and yield (□) of diluted (1:1 [vol/vol] with Beltsville Thawing Solution [BTS]) pooled sperm-rich fractions of boar semen cooled to 17°C; **(a–g)** indicate significant differences among groups ($P < .05$).

ure 1). The *g*-force and time interaction was also significant ($P < .001$).

Three regimes (2400 × *g* for 5 minutes, 1600 × *g* for 5 minutes, and 2400 × *g* for 3 minutes) provided the highest ($P < .05$) postcentrifugation sperm recovery. Two of them, 2400 × *g* for 5 minutes (97.26% ± 0.94%) and 1600 × *g* for 5 minutes (93.40% ± 0.93%) reached significantly ($P < .05$) higher sperm recoveries than the reference, 800 × *g* for 10 minutes (80.58% ± 4.6%).

The highest ($P < .05$) value for postcentrifugation sperm yield was achieved ($P < .05$) both when using these 3 centrifugation regimes, 2400 × *g* for 5 minutes (75.03% ± 0.50%), 1600 × *g* for 5 minutes (74.70% ± 0.79%), and 2400 × *g* for 3 minutes (69.57% ± 0.77%), and when using the reference regime (72.28% ± 0.29%).

The low speed/short time of centrifugation (400 × *g* for 5 minutes, 800 × *g* for 3 minutes, and 400 × *g* for 3 minutes) pelleted about half of the sperm; consequently, the sperm recovery and yield achieved with them were significantly ($P < .05$) lower than with the reference.

Effect of different centrifugation regimes before freezing namely C1 (2400 × g*/for 3 minutes), C2 (1600 ×* g*/for 5 minutes), and C3 (800 ×* g*/for 10 minutes; reference) on sperm motility indexes and kinetics parameters of frozen-thawed boar spermatozoa (values are the average of 30 and 150 minutes of postthawing incubation time)*[*†]

Motility Indexes and Kinematics Parameters	C1	C2	C3
TSM (%)	45.6 ± 3.4 A[‡]	35.7 ± 3.4 A	34.9 ± 3.4 B
RMS (%)	25.2 ± 3.4 A	19.4 ± 3.4 B	17.0 ± 3.4 B
VCL (μm/s)	76.3 ± 4.6	73.7 ± 4.6	70.1 ± 4.6
VSL (μm/s)	52.9 ± 3.7	50.6 ± 3.7	49.4 ± 3.7
VAP (μm/s)	59.6 ± 4.1	57.1 ± 4.1	54.7 ± 4.1
LIN (%)	68.7 ± 1.5	68.4 ± 1.5	68.3 ± 1.5
STR (%)	86.4 ± 1.7	86.2 ± 1.7	87.4 ± 1.7
ALH (μm)	2.8 ± 0.1 A	2.8 ± 0.1 A	2.5 ± 0.1 B
DNM (μm2/s)	0.062 ± 0.006	0.074 ± 0.006	0.053 ± 0.006
BCF (Hz)	12.3 ± 0.2	12.4 ± 0.2	12.7 ± 0.2

[*] Results are given as least square mean ± SEM.

[†] TSM indicates total sperm motility; RMS, rapid motile spermatozoa; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory (ratio of VSL/VCL); STR, straightness; ALH, amplitude of lateral head displacement; DNM, mean dance (mean value of VCL × ALH); and BCF, beat cross-frequency.

[‡] Values within columns with different letters differ significantly ($P < .05$).

Experiment 2: Effect of Centrifugation Regimes Before Freezing on Boar Sperm Cryosurvival and Postthaw Lipid Peroxidation

The centrifugation regime used had a significant influence on all postthawing sperm quality parameters. Sperm motility indexes (TSM and RMS) were significantly ($P < .05$) higher for frozen-thawed spermatozoa centrifuged with C1 before freezing than for the rest of the regimes (Table). In relation to kinetics parameters, the only difference noted among centrifugation regimes was for ALH, which was higher ($P < .05$) when using C1 and C2 than when using C3.

The centrifugation regimes C1 (55.10% ± 1.17%) and C2 (57.50% ± 1.48%) showed significantly ($P < .05$) higher percentages (Figure 2) of frozen-thawed viable spermatozoa with an intact acrosome (VS) than did C3 (49.80% ± 1.94%). Likewise, lower percentages ($P < .05$) of nonviable spermatozoa with an intact acrosome (DS) were observed for C1 (17.10% ± 0.38%) and C2 (17.80% ± 0.48%) than for C3 (23.60% ± 1.22%).

Capacitation-like change results are shown in Figure 3. The centrifugation regimes C1 (47.15% ± 2.09%) and C2 (48.15% ± 1.45%) showed significantly ($P < .05$) higher percentages of frozen-thawed uncapacitated spermatozoa (F) than did C3 (40.70% ± 2.14%). Besides, C3 (33.65% ± 2.19%) had a significantly ($P < .05$) higher percentage

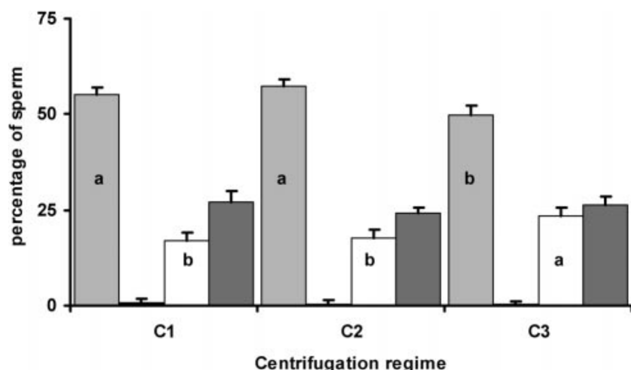

Figure 2. Effect of different centrifugation regimes used before freezing (C1: 2400 × *g* for 3 minutes, C2: 1600 × *g* for 5 minutes, and C3: 800 × *g* for 10 minutes as a reference) on sperm viability (plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity) of frozen-thawed boar spermatozoa. Four sperm populations were observed: VS (▤; viable sperm with an intact acrosome), VS-AR (■; viable sperm with acrosome reaction), DS (□; nonviable sperm with an intact acrosome), and DS-AR (▥; nonviable sperm with acrosome reacted). Each bar (±SEM) represents the average of 30 and 150 minutes of postthawing incubation time; **(a, b)** indicate significant differences among groups ($P < .05$).

of frozen-thawed capacitated spermatozoa than did C1 (27.05% ± 2.16%) and C2 (26.75% ± 1.86%).

The oocyte penetrating ability parameters, in terms of the oocyte penetration rate and the number of spermatozoa per penetrated oocyte (Figure 4), were significantly ($P < .05$) higher for frozen-thawed spermatozoa centrifuged with C1 before freezing than for those centrifuged using the C2 and C3 regimes.

The different centrifugation regimes had no effect on MDA production ($P > .05$), a measure of lipid peroxidation. All of the centrifugation regimes showed the same behavior: relatively low levels of MDA 0 and 30 minutes after postthawing incubation time and a significant increase ($P < .05$) after 150 minutes (Figure 5).

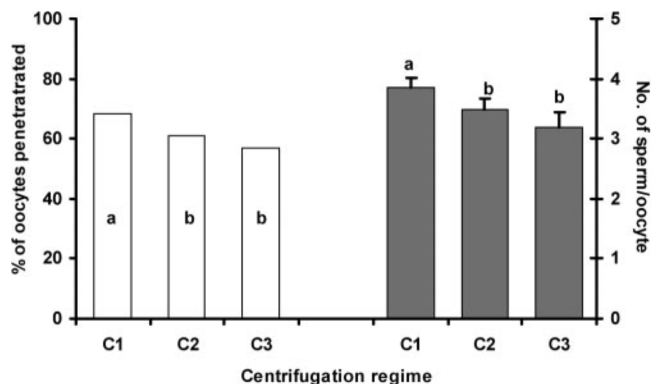

Figure 4. Effect of different centrifugation regimes used before freezing (C1: 2400 × *g* for 3 minutes, C2: 1600 × *g* for 5 minutes, and C3: 800 × *g* for 10 minutes as a reference) on the oocyte penetration rate (□) and the number of spermatozoa per oocyte penetrated (▥) of frozen-thawed boar spermatozoa. The homologous in vitro penetration (hIVP) test was performed with immature oocytes; **(a–b)** indicate significant differences among groups ($P < .05$).

Discussion

Centrifugation is one of the common sperm preparation techniques for both experimental and practical programs. In the boar, centrifugation is a necessary step to take before freezing. Centrifugation facilitates the removal of seminal plasma and concentrates the spermatozoa to prepare the sperm for redilution with cryopreservation extenders. Cryopreservation of boar sperm with current methods brings about losses of viable sperm, usually more than 50%, which do not survive the freeze-thaw procedure (Almlid and Hofmo, 1996). This decrease is

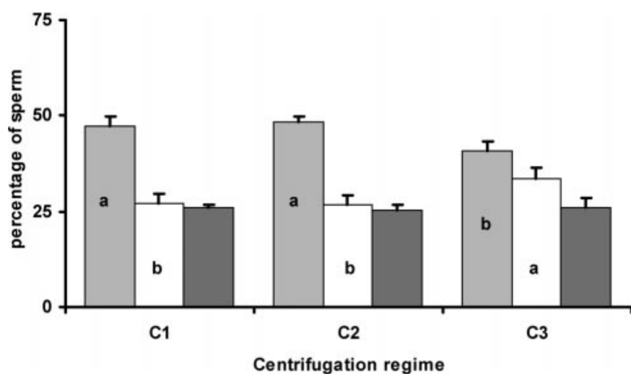

Figure 3. Effect of different centrifugation regimes used before freezing (C1: 2400 × *g* for 3 minutes, C2: 1600 × *g* for 5 minutes, and C3: 800 × *g* for 10 minutes as a reference) on capacitation-like changes of frozen-thawed spermatozoa. Sperm were classified into 3 groups: F (▤; uncapacitated and acrosome intact), B (□; capacitated), and AR (▥; acrosome reacted). Each bar (±SEM) represents the average of 30 and 150 minutes of postthawing incubation time; **(a, b)** indicate significant differences among groups ($P < .05$).

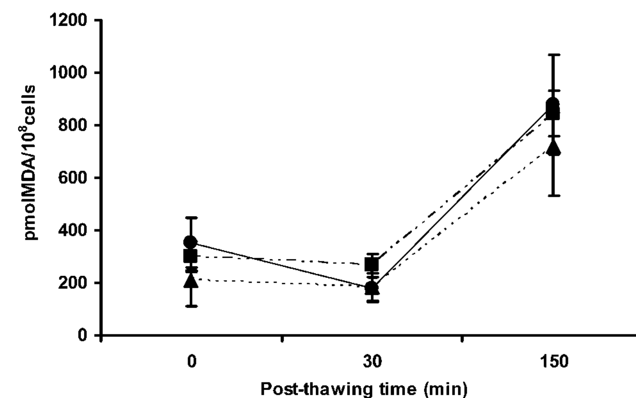

Figure 5. Effect of different centrifugation regimes used before freezing, namely C1 (2400 × *g* for 3 minutes; —■—), C2 (1600 × *g* for 5 minutes; - -▲- -), and C3 (800 × *g* for 10 minutes; reference; —●—), on malondialdehyde (MDA; indicator of lipid peroxidation) concentration (pmol MDA/10^8cells) produced by frozen-thawed boar spermatozoa 0, 30, and 150 minutes after postthawing incubation.

due to both temperature changes and the manipulation of sperm that occurs during centrifugation. Key to this issue is that centrifugation causes 2 problems: 1) the sperm loss because of removal of the supernatant (Katkov and Mazur, 1998), and 2) the physical damage caused to the sperm population (Alvarez et al, 1993).

In the first experiment, centrifugation with the standard centrifugation regime used in cryopreservation protocols, $800 \times g$ for 10 minutes (Westendorf et al, 1975), brought about a 19% loss of sperm caused by their being discarded with the supernatant, which was similar to the losses reported by Almlid et al (1987), 24%, and Eriksson et al (2002), 20%–25%. Our study shows that the centrifugation regime influences this sperm loss and that it therefore influences sperm recovery. Using the lowest *g*-forces and the shortest times of centrifugation (800 and $400 \times g$ for 3 minutes) led to the lowest sperm recovery, because of a lack of complete pelleting and, consequently, the loss of unpelleted cells upon supernatant removal. By contrast, using the highest *g*-forces and the longest times of centrifugation (2400 and $1600 \times g$ for 5 minutes) led to greater spermatozoa sedimentation. Moreover, the percentage of sperm recovered achieved with these regimes remained almost unchanged when using the highest *g*-force and reduced centrifugation time ($2400 \times g$ for 3 minutes). This suggests that it is possible to achieve high sperm recovery using a relatively high *g*-force and a short time of centrifugation.

On the other hand, the primary reason for centrifuging cell suspensions is to recover the maximum number of sperm from the initial population with minimum adverse effects on their viability. Centrifugation has been shown to be a potentially sperm-damaging step during human semen processing, inducing sublethal damage (Mortimer, 1991; Alvarez et al, 1993). In some mammals, such as the rat (Cardullo and Cone, 1986), human (Makler and Jakobi, 1981; Ng et al, 1990; Agarwal et al, 1994), and mouse (Schreuders et al, 1996), spermatozoa have been shown to be very sensitive to mechanical and centrifugal forces and must manipulated under carefully defined conditions to reduce sperm damage. Spermatozoa from other species, such as equine and bovine (Picket et al, 1975; Katkov and Ostashko, 1996; Crockett et al, 2001), are somewhat less sensitive to centrifugation, which indicates that species specificity is very important with respect to spermatozoa injury caused by centrifugation.

With regard to boar spermatozoa, no studies have been made regarding the effect of centrifugation on spermatozoa viability, to our knowledge. In the present study, there were no differences between the percentages of viability of the population recovered by each one of the centrifugation regimes, including the reference treatment (data not shown), immediately after centrifugation. From the point of view of using frozen-thawed spermatozoa for AI, the most important objective is to achieve the maximum total number of viable sperm with intact acrosomes after the freezing-thawing process and, therefore, to provide an optimal number of doses per cryopreserved ejaculate. Consequently, a high percentage of viability may mean little if the population measured is a small subset of the original, as happened with those samples centrifuged using low *g*-forces and short times of centrifugation. Therefore, we focused on the effect of centrifugation on the sperm yield and not on the percentage of viability achieved. The centrifugation regime used influenced both sperm recovery and yield in a similar fashion. The poor sperm yield that occurred with the lowest *g*-forces combined with the shortest time was obviously due to the low sperm recovery values that were obtained using those regimes. Using the highest *g*-forces with the longest times of centrifugation (2400 and $1600 \times g$ for 5 minutes) or the highest *g*-force combined with the shortest centrifugation time ($2400 \times g$ for 3 minutes) provided the highest ($P < .05$) fraction of viable sperm from the initial sperm suspension. This suggests that we can use a relatively high *g*-force combined with a short centrifugation time without any negative effect on sperm yield, at least when viability is assessed immediately after centrifugation.

The influence of different centrifugation regimes on spermatozoa viability has been the objective of some research. The effect of different *g*-forces at a given centrifugation time on sperm motility and viability has been evaluated in humans (Fredricsson and Kinnari, 1979; Mack and Zaneveld, 1987) immediately after centrifugation and also in dogs (Rijsselaere et al, 2002), both immediately after centrifugation and daily, during 3 days of posterior storage at 4°C. These studies observed that high centrifugal forces depressed both sperm motility and viability; they therefore recommended the use of a medium *g*-force (in humans, $300–600 \times g$ for 10–20 minutes, and in dogs, $720 \times g$ for 5 minutes). Katkov and Mazur (1998), in a study of the effect of different centrifugation *g*-force/time combinations on mouse sperm motility (assessed after centrifugation and 2.5 hours later, stored at room temperature), recommend the use of $400 \times g$ for 10–12 minutes to achieve the maximum number of motile sperm or the use of $200 \times g$ for 5 minutes to maximize the percentage of motility. In any case, all of these studies recommend the use of *g*-forces below $800 \times g$. Data obtained in our study indicate that the highest *g*-force ($2400 \times g$) for any of the centrifugation times tested (3 or 5 minutes) achieved high sperm recovery and yield and did not reduce boar sperm viability, at least immediately after centrifugation. However, we must note that the potentially harmful effect of centrifugation on spermatozoa is not observed immediately after centrifugation but that it develops with the passage of time (Alvarez et al, 1993). Therefore, it could be that the potentially harmful effect

of centrifugation becomes evident with the passage of time, after the manipulation or processing (incubation, cool storage, or cryopreservation) of centrifuged boar sperm. For this reason, we designed the second experiment to evaluate the possible damage that the centrifugation step of the cryopreservation protocol had on frozen-thawed boar spermatozoa.

There is not much literature about the impact that centrifugation before freezing has on the quality of frozen-thawed spermatozoa. With regard to boar sperm, Salamon (1973) showed that centrifugation before freezing had an adverse effect on the motility of frozen-thawed spermatozoa. On the other hand, currently, centrifugation is the only technique used for separating boar spermatozoa from seminal plasma in cryopreservation protocols. In spite of this, to our knowledge, no study has been published concerning the effect of centrifugation regimes on the cryosurvival of boar sperm.

In this paper, we evaluated the effect of 3 centrifugation regimes: 2 were experimental ($2400 \times g$ for 3 minutes and $1600 \times g$ for 5 minutes), and 1 was normally used in cryopreservation protocols ($800 \times g$ for 10 minutes) (Westendorf et al, 1975) as a reference. Both of the tested centrifugation regimes showed better postthaw sperm quality than the reference treatment. Because the procedure followed for cryopreservation was the same for all of the centrifugation regimes, the reason for these differences must lie in the centrifugation step itself. Therefore, our results suggest that the cryopreservation process facilitated the observation of presumed sublethal damage caused by centrifugation prior to freezing. The less harmful treatment appeared to be $2400 \times g$ for 3 minutes, because it yielded the highest oocyte penetrating ability. By contrast, the reference treatment ($800 \times g$ for 10 minutes) appeared to be more damaging than $2400 \times g$ for 3 minutes and $1600 \times g$ for 5 minutes. These findings seem to prove that the time of centrifugation is more critical than the g-force for inducing boar sperm damage. Shekarriz et al (1995) reached a similar conclusion for human sperm and recommended the use of short-term centrifugation.

There is still no complete or accepted explanation of how centrifugation induces sperm damage. However, it has been hypothesized that the damage to the spermatozoa is due to a direct mechanical effect on the sperm membranes (Alvarez et al, 1993) as well as to an indirect adverse effect caused by excessive ROS formation (Aitken and Clarkson, 1988; Mortimer, 1991). High levels of ROS are associated with sperm membrane damage through lipid peroxidation (Shekarriz et al, 1995), which may alter sperm function, leading to loss in motility and viability and, in the end, reduced fertility of spermatozoa. Since no differences in MDA production (an indicator of lipid peroxidation) were observed between the centrifugation regimes tested in the second experiment, it could be assumed that the sperm sublethal damage was caused by the mechanical effect of centrifugation. Abidor et al (1994) postulated that the cell damage is not a consequence of higher accelerations or higher sedimentation rates per se, but is a result of the cells being packed in a pellet at the tube bottom. The closer and the longer the cells stand packed, the more damaged they become (Katkov and Mazur, 1998). These affirmations would explain our results: higher postthaw sperm quality was achieved when using 3 and 5 minutes than when using 10 minutes, because cells centrifuged for 3 and 5 minutes spent less time in the pelleted state. Moreover, the centrifugation for 3 minutes achieved better postthaw oocyte penetrating ability than did the other centrifugations, because the time that the sperm remained packed was the lowest.

In conclusion, using relatively high g-forces combined with short centrifugation times has no negative effect on sperm recovery and yield, at least when viability is assessed immediately after centrifugation. Moreover, the centrifugation regime used in the centrifugation step of the cryopreservation process influences boar sperm cryosurvival; hence, we recommend the use of short-term centrifugation with a relatively high g-force ($2400 \times g$ for 3 minutes) to minimize the sublethal damage of boar spermatozoa, which will thus enhance postthawing sperm quality.

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References

Abidor IG, Li LH, Hui SW. Study of cell pellets. I. Electrical properties and porosity. *Biophys J.* 1994;67:418–426.

Agarwal A, Ikemoto I, Loughlin KR. Effect of sperm washing on levels of reactive oxygen species in semen. *Arch Androl.* 1994;33:157–162.

Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining of the efficiency of sperm preparation technique. *J Androl.* 1988;9:367–376.

Almlid T, Hofmo PO. A brief review of frozen semen applications under Norwegian AI swine conditions. *Reprod Domest Anim.* 1996;31:169–173.

Almlid T, Stavne SE, Johnson LA. Fertility evaluation of the straw freezing technique for boar semen under artificial insemination conditions. *Zuchthygiene (Berl).* 1987;22:193–202.

Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero P, Storey BT. 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 life. *Hum Reprod.* 1993;8:1087–1092.

Bwanga CO. *Cryopreservation of Boar Semen: Studies on Freezing,*

Packaging and Fertilizing Capacity [doctoral thesis]. Uppsala: Swedish University of Agricultural Sciences; 1990.

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

Comaschi V, Lindner L, Farruggia G, Gesmundo N, Colombi L, Masotti L. An investigation on lipoperoxidation mechanisms in boar spermatozoa. *Biochem Biophys Res Commun.* 1989;158:769–775.

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

Davis RO, Siemers RJ. Derivation and reliability of kinematics measures of sperm motion. *Reprod Fertil Dev.* 1995;7:857–869.

Eriksson BM, Petersson H, Rodríguez-Martínez H. Field fertility with exported boar semen frozen in the new flatpack container. *Theriogenology.* 2002;58:1065–1079.

Fraser LR, Abeydeera LR, Niwa K. Ca^{2+}-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Mol Reprod Dev.* 1995;40:233–241.

Fredricsson B, Kinnari S. Vitality and morphology of human spermatozoa studies on the resistance to storage and centrifugation and on the removal of dead spermatozoa. *Andrologia.* 1979;11:135–141.

Graham EF, Rajamannan AHJ, Schmehl MKL, Maki-Laurila M, Bower RE. Preliminary report on procedure and rationale for freezing boar spermatozoa. *AI Digest.* 1971;19:12–14.

Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. *Biol Reprod.* 1990;43:55–64.

Holt WV. Basic aspects on frozen storage of semen. *Anim Reprod Sci.* 2000;62:3–22.

Johnson LA, Weitze KF, Fiser P, Maxwell WMC. Storage of boar semen. *Anim Reprod Sci.* 2000;62:143–172.

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

Katkov II, Ostashko FI. Correlation between electromechanical stability of cytoplasm membrane and cryoresistivity of bovine spermatozoa. *Cryobiology.* 1996;33:680–687.

Larsson K, Einarsson S, Swensson T. The development of a practicable method for deep freezing of boar spermatozoa. *Nord Vet Med.* 1977; 29:112–118.

Mack SR, Zaneveld LJ. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res.* 1987;18: 375–383.

Makler A, Jakobi P. Effects of shaking and centrifugation on human sperm motility. *Arch Androl.* 1981;7:21–26.

Martín Rillo S, Martínez EA, García Artiga C, De Alba C. Boar semen evaluation in practise. *Reprod Domest Anim.* 1996;31:519–526.

Martínez EA, Vázquez JM, Matas C, Gadea J, Alonso MI, Roca J. Oocyte penetration by fresh or stored diluted boar spermatozoa before and after in vitro capacitation treatments. *Biol Reprod.* 1996;55:134–140.

Maxwell WMC, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling or cryopreservation. *Mol Reprod Dev.* 1997;46:408–418.

Mortimer D. Sperm preparation techniques and iatrogenic failures of in vitro fertilization. *Hum Reprod.* 1991;6:173–176.

Ng SC, Bongso TA, Sathananthan H, Tok VCN, Ratnam SS. Microcentrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. *Hum Reprod.* 1990;5:209–211.

Paquignon M, Courot M. Fertilizing capacity of frozen boar spermatozoa. *8th International Congress on Animal Reproductive Artificial Insemination, Cracow, Poland.* 1976;4:1041–1044.

Pickett BW, Sullivan JJ, Byers MS, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. *Fertil Steril.* 1975;26:167–173.

Pursel VG, Johnson LA. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J Anim Sci.* 1975;40:99–102.

Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of centrifugation on in vivo survival of fresh diluted canine spermatozoa. *Theriogenology.* 2002;57:1669–1681.

Salamon S. Effects of centrifugation, diluent and dilution rate, pellet volume and method of thawing on survival of spermatozoa. *Aust J Biol Sci.* 1973;26:239–247.

Schreuders PD, Jetton AE, Baker JL, Critser JK, Mazur P. Mechanical and chill sensitivity of mouse sperm. *Cryobiology.* 1996;33:676–677.

Shekarriz M, De Wire DM, Thomas AJ Jr, Agarwal A. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol.* 1995;28:31–35.

Thurston LM, Watson PF, Holt WV. Sources of variation in the morphological characteristics of sperm sub-populations objectively assessed by a novel automated sperm morphology analysis system. *J Reprod Fertil.* 1999;117:271–280.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60–61:481–492.

Westendorf P, Ritcher L, Treu H. Zur Tiefgefrierung von Ebersperma. Labor-und Besamungsergebnisse mit dem Hülsenberger Pailletten-verfahren. *DtschTierärztl Wschr.* 1975;82:261–267.