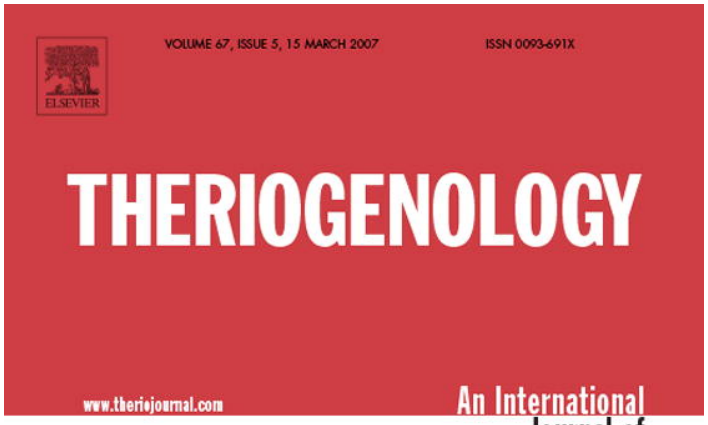

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Short communication

Evaluation of a cushioned method for centrifugation and processing for freezing boar semen

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Abstract

The purpose of this investigation was to evaluate the use of an iodixanol cushion during centrifugation on sperm recovery and yield after centrifugation (sperm recovery, sperm motility, viability, membrane lipid disorder, acrosome reaction and ROS generation); and to investigate how this procedure affects sperm function after freezing–thawing (sperm motility, membrane lipid disorder, acrosomal status and homologous in vitro penetration test). The sperm-rich fractions from fertile boars were centrifuged under two centrifugation régimes: 800 × *g* for 10 min (standard method) and 1000 × *g* for 20 min with an iodixanol (60% w/v) cushion at the bottom of the centrifuge tubes (Cushion method). The highest recovery was achieved using the cushion method (sperm loss for cushion method was 0.50% ± 0.18 *versus* 2.97% ± 0.43 for standard method, $P < 0.01$) and sperm quality was not significantly affected by the centrifugation régime. The motion parameters (% progressive motility, % motility, VCL, VSL, VAP, ALH, BCF, $P < 0.05$) of frozen–thawed samples showed higher values using the standard method. However, a higher number of viable spermatozoa with lower lipid disorders were found in spermatozoa processed with the cushion method. The in vitro penetration assay showed that the individual boar influenced the parameters studied but there were no differences between the two centrifugation régimes used. Our results support the hypothesis that the proportion of sperm loss in frozen–thawed semen was significantly influenced by the centrifugation régime. Therefore, the iodixanol cushion method is a suitable tool for cryopreservation of boar semen in order to reduce sperm loss without affecting sperm quality.

Keywords: Pig-spermatozoa; Cryopreservation; IVF; Cushion centrifugation; Iodioxanol

1. Introduction

Cryopreservation involves placing spermatozoa in special media (with the presence of cryoprotectants and high osmotic pressures) and returning them after treatment to a more physiological medium and conditions. The usual approach is to pellet the cells by centrifugation and to replace the supernatant with physiological saline. However, centrifugation and resuspension of the resulting pellet subject cells to mechanical forces due to close packing [1], and centrifugation could lead to structural damage of the sperm acrosome [2] and to substantial loss of motility [3] and enzymatic activity of the sperm cell [4]. Spermatozoa must be centrifuged under carefully defined conditions to minimize the damage and to maximize the recovery of viable cells. Revell et al. [5] developed a cushioned centrifugation technique for

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processing equine semen for freezing. They used a cushion of iodixanol (a non-ionic, polysucrose-based gradient) and high force centrifugation to ensure maximum sperm recovery while minimizing physical damage to sperm. A cushion technique is widely used with boar sperm to separate them from seminal plasma following incubation or IVF treatment, but there is a lack of studies on the use of a cushion prior to cryopreservation [6–8]. The objective of this study was to examine whether sperm centrifugation on a dense isotonic solution cushion before freezing semen affects the efficiency of sperm recovery and sperm quality. The cushion is an iodixanol isotonic solution (60%, w/v) that, placed at the bottom tube, could be used with higher centrifugal forces ($1000 \times g$) to minimize the damage and to maximize the recovery of viable cells.

2. Materials and methods

2.1. Sperm collection and handling

Semen was routinely collected from fertile boars using the manual method and a dummy; it was diluted 1:2 with isothermal Beltsville Thawing Solution extender (BTS [9]). The diluted sperm-rich fractions were divided into two aliquots (50 mL) and transferred to 50 mL-Corning centrifugation tubes and placed at 15 °C for 2 h. After that, seminal samples were centrifuged under two centrifugation régimes: (a) a standard method (SM): $800 \times g$ for 10 min usually applied in boar semen freezing procedures [10] and (b) a cushioned method (CM): $1000 \times g$ for 20 min adding to the bottom of the centrifugation tube 5 ml of an iodixanol isotonic solution (60%, w/v) (Maxifreeze®; IMV Technologies, L'Aigle, France). To evaluate the spermatozoa number, before centrifugation and after the two centrifugation régimes, a Thoma counting chamber was used under the 20 × bright-field objective. The concentration of spermatozoa recovered from the supernatant and percentage of spermatozoa lost were assessed. Semen samples were processed using the straw freezing procedure described by Westendorf et al. [10]. Thawing was carried out by immersing the straws in a circulating water bath at 37 °C for 30 s. Immediately after thawing, the sperm suspension was diluted in the thawing medium (BTS) at 37 °C and maintained 20 min in this medium before being assayed.

2.2. Analysis of seminal parameters

To test the hypothesis that a cushioned technique provided higher sperm recovery without increasing the sperm injuries caused by centrifugal forces, we incorporated a set of functional sperm tests including: (1) computer-assisted semen analysis (CASA; sperm motility); (2) sperm membrane lipid packing disorder, free radical production (ROS generation) acrosome reaction and viability (by flow cytometry) and finally 3) the in vitro penetrability of immature oocytes as described previously Gadea et al. 2005 [11].

2.3. Experimental design

2.3.1. Evaluation of a cushioned method for centrifugation boar semen on sperm function

To evaluate the effect of the centrifugation method on the sperm quality and loss of spermatozoa in the process, 21 ejaculates from 12 fertile boars were diluted in BTS (pre-centrifuged spermatozoa) and centrifuged at 15 °C under two centrifugation régimes described previously (SM and CM). After centrifugation, the supernatants were discarded and the number of spermatozoa determined. The pelleted spermatozoa were gently rediluted with BTS to the initial volume (50 mL) and samples were evaluated.

The seminal parameters assessed were: spermatozoa concentration, motility parameters by CASA, membrane lipid disorder by staining with merocyanine 540 and Yo-Pro 1, reactive oxygen formation by staining with H_2DCFDA, and acrosome reaction staining with PNA-FITC and PI examined by flow cytometry.

2.3.2. Evaluation of a cushioned method for centrifugation on frozen–thawed sperm function

To perform this experiment, 10 ejaculates from 5 boars were frozen using two centrifugation regimes (SM and CM). Thawed samples were assessed to evaluate the effect of the centrifugation process on sperm function. The parameters assessed were: motility parameters by CASA, membrane lipid disorder staining with merocyanine 540 and Yo-Pro 1 examined by flow cytometry after thawing in BTS and after incubation in capacitation medium (TALP) for 45 min. Finally, the sperm in vitro penetration ability of immature oocytes was evaluated.

2.4. Statistical analysis

Data are expressed as the mean ± S.E.M. and analyzed by ANOVA, considering the specific centrifugation régime (before freezing) and boar (after thawing) as the main variable. When ANOVA revealed a significant effect, values were compared by the LSD post hoc test. Differences were considered statistically

significant at $P < 0.05$. In vitro penetration data (categorical data) were modelled according to the binomial model of parameters and were analyzed by ANOVA.

3. Results

3.1. Evaluation of a cushioned method for centrifugation before freezing boar semen on sperm function

The centrifugation régime had a significant influence on sperm recovery, with a lower proportion of sperm loss in the cushioned than standard method (CM 0.50% ± 0.18 *versus* SM 2.97% ± 0.43, $P < 0.01$). In relation to the quality and functionality of the spermatozoa recovered, motility parameters measured by CASA for two centrifugation régimes are shown in Table 1. No significant differences were found using either centrifugation régime. Nevertheless, some differences in the pattern of motility were found between pre-centrifuged ejaculates and centrifuged samples, with a higher percentage of motile cells, higher average path velocity (VAP), linearity of the curvilinear trajectory (LIN), Wobble (WOB) and beat cross-frequency (BCF). The membrane lipid packing disorder was not affected by the centrifugation regimes and no differences were found between control and cushioned spermatozoa (viable spermatozoa with low lipid disorder SM: 83.47 ± 0.67 *versus* CM: 82.45 ± 0.86). Nevertheless, the pre-centrifuged group presented a higher number of viable spermatozoa with low membrane lipid disorder (87.80% ± 0.50 *versus* 83 7 and 82.45% ± 0.86 SM, CM, respectively $P < 0.01$). In relation to the ROS generation, no differences were found for the groups studied (CM: 5.60 *versus* SM: 5.48). Similarly, the acrosome reaction pattern after staining with PNA-FITC and PI and evaluated by flow cytometry was not affected by the centrifugation régime (SM 2.49 ± 0.15 *versus* CM: 2.28 ± 0.13).

3.2. Evaluation of a cushioned method for centrifugation after freezing–thawing on sperm function

The motility parameters of the frozen–thawed samples showed higher values for SM in some characteristics than the cushioned method (progressive motility, total motility, VCL, VSL, VAP, ALH, BCF; Table 2, $P < 0.05$). Nevertheless, the motion parameters were influenced by the boar effect and the interaction between treatment and boar. In the study of membrane

Table 1
Motility parameters measured by CASA from spermatozoa before and after centrifugation by two different regimes: SM: 800 × *g* 10 min, CM: 1000 × *g* 20 min with a cushion at the bottom of the centrifugation tubes

Treatment	Progressive motility (%)	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Pre-centrifuged	63.80 ± 2.27	88.40 ± 1.92^{a}	74.83 ± 2.01	29.34 ± 0.41	53.44 ± 1.64^{s}	38.29 ± 0.97^{a}	53.13 ± 1.16	71.41 ± 0.93^{a}	2.69 ± 0.07	6.31 ± 0.13^{a}
Standard	58.37 ± 1.54	81.68 ± 1.61^{b}	75.27 ± 1.53	30.71 ± 0.88	48.82 ± 1.43^{b}	34.98 ± 0.85^{b}	54.33 ± 0.80	64.08 ± 0.96^{b}	2.97 ± 0.07	6.15 ± 0.14^{b}
Cushion	60.02 ± 1.63	83.62 ± 1.52^{b}	72.77 ± 2.12	31.05 ± 2.23	47.15 ± 1.76^{b}	34.47 ± 1.01^{b}	54.04 ± 1.09	63.40 ± 1.28^{b}	3.11 ± 0.18	6.15 ± 0.14^{b}
Source variation *P*-values	Progressive motility	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Treatment	0.12	0.01	0.46	0.61	0.01	0.01	0.51	0.01	0.09	0.06

VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity of the curvilinear trajectory, STR: straightness, WOB: Wobble (VAP/VCL), ALH: amplitude of lateral head displacement, BCF: beat cross-frequency. ^{a,b}Numbers within columns with different superscripts differ ($P < 0.05$).

Table 2
Motility parameters measured by CASA. Frozen samples processed after centrifugation by two different regimes: SM: 800 × *g* 10 min, CM 1000 × *g* 20 min with a cushion at the bottom of the centrifugation tubes

Treatment	Progressive motility (%)	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Standard	38.58 ± 1.58^{a}	45.73 ± 1.72^{a}	76.04 ± 1.88^{a}	37.33 ± 1.53^{a}	52.44 ± 1.52^{a}	48.01 ± 1.14	67.96 ± 0.99	68.00 ± 0.88	3.01 ± 0.07^{a}	6.21 ± 0.12^{a}
Cushion	33.18 ± 1.51^{b}	41.54 ± 1.70^{b}	70.35 ± 1.69^{b}	33.11 ± 1.02^{b}	47.51 ± 1.42^{b}	46.92 ± 1.03	67.58 ± 0.87	66.98 ± 0.98	2.87 ± 0.07^{b}	5.82 ± 0.15^{b}
Source variation *P* -values	Progressive motility	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Treatment	<0.01	0.03	<0.01	0.01	0.01	0.58	0.84	0.50	0.03	0.03
Boar	<0.01	0.02	0.01	0.22	0.31	0.08	0.02	<0.01	<0.01	0.92
Treatment* Boar	<0.01	0.03	0.04	0.29	0.04	0.22	0.94	<0.01	0.01	0.03

VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity of the curvilinear trajectory, STR: straightness, WOB: Wobble (VAP/VCL), ALH: amplitude of lateral head displacement, BCF: beat cross-frequency. [a,b]Numbers within columns with different superscripts differ ($P < 0.05$).

lipid disorder, a significant effect ($P < 0.01$) of the centrifugation régime was found in the number of intact viable spermatozoa with higher values for the cushioned method (SM: 51.30% ± 1.19 *versus* CM: 54.67% ± 0.92) and a significantly lower proportion of dead sperm (SM: 46.25 ± 1.17 *versus* CM: 43.07% ± 0.96). When spermatozoa were incubated for 45 min in a capacitating medium (TALP), these differences appeared in the same way (viable sperm with low membrane lipid packing disorder SM: 19.33 ± 0.76 *versus* CM: 22.80 ± 0.76; and dead SM: 74.85 ± 1.02 *versus* CM: 70.96 ± 1.00, $P < 0.01$). The data from the in vitro penetration assay showed that the boar used had a significant effect on the parameters studied ($P < 0.01$) and that there were no differences between two centrifugation regimes used in the penetration rate (SM: 36.7% ± 1.71 *versus* CM: 38.47% ± 1.78).

4. Discussion

In this study, we used a higher centrifugation force (1000 × *g*) and a longer period of time (20 min) on a cushion of iodixanol than in the standard method. This cushioned technique allows maximal recovery rates and avoids cells being packed in a pellet at the bottom of the tube. However, this procedure could affect sperm functionality. For this reason we decided to evaluate sperm function with a set of tests after using the centrifugation cushioned technique in two important steps of the freezing process: immediately after centrifugation at 15 °C (before freezing) and after thawing the samples.

Progressive motility of spermatozoa is an indicator of both unimpaired metabolism and integrity of membranes and Computer-assisted semen analysis (CASA) has provided an objective and accurate means of evaluating overall sperm motility [12]. Our study shows that the motility parameters were comparable whichever centrifugation régime was used, but lower than those of sperm before centrifugation. These data confirm that centrifugation procedures induce damage in sperm functionality. When we analyzed the motility parameters after thawing, we found that progressive motility, motility, VCL, VSL, VAP, ALH and BCF were higher using the standard centrifugation régime. However, it is known that the ''capacitation-like'' process [13,14] occurs during sperm freezing step; and this is related with a change in the motility pattern. This hypothesis is consistent with the results obtained after staining by merocyanine 540 and Yo-Pro, where the higher number of viable low lipid membrane disorders and lower number of dead cells were in spermatozoa

from cushion centrifugation. The centrifugation régime employed affected the sperm membrane because when we analyzed the membrane lipid disorder in spermatozoa after thawing, the higher values were obtained in the control group. This effect was more apparent when we incubated the spermatozoa into TALP medium for 45 min, when we found the lowest lipid disorder with the highest viable sperm centrifuged on cushion.

Centrifugation has been shown to be critical for the human sperm plasma membrane as it may induce peroxidation [15]. In human, Shekarriz et al. [16] concluded that the time of centrifugation is more important than the *g*-force for inducing ROS formation in semen. However, the ROS generation was neither affected by time centrifugation nor by *g*-force. We tried using a cushion in a higher centrifugation regime, avoiding the injuries produced in the pellet formation. In human sperm, Jeulin et al. [17] showed that centrifugation forces greater than $100 \times g$ must not be used because centrifugation per se may mechanically damage spermatozoa, and the damage can be sublethal. However, before freezing, we found neither difference in the viability between two-centrifugation protocols used nor with the precentrifuged sperm and the acrosome reaction was also similar between groups.

The IVF tests are the most suitable for assessing overall sperm function during fertilization [18]. To accomplish fertilization, spermatozoa must bind to and penetrate the zona pellucida, the extracellular matrix that surrounds the oocyte. Results obtained in the homologous in vitro test showed both penetration rates and mean numbers of sperm per oocyte to be different for individual boars, but there were no differences between two centrifugation methods.

We conclude that sperm loss in frozen–thawed semen was significantly influenced by the centrifugation régime, and that the_iodixanol cushion method is a suitable tool for cryopreservation of boar semen in order to reduce sperm loss without affecting sperm quality.

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