Journal of Andrology, Vol. 26, No. 3, May/June 2005
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Cooling and Freezing of Boar Spermatozoa: Supplementation of the Freezing Media With Reduced Glutathione Preserves Sperm Function

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ABSTRACT: In this study, we evaluated the effects of glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) supplementation of the freezing extender on semen parameters during the cooling (2 hours at 5°C) and freezing phases of the cryopreservation process to compensate for the decrease in GSH content observed during sperm freezing. To fully address these questions, we incorporated a new set of functional sperm tests. These included tests of mitochondrial function, inducibility of the acrosome reaction, in vitro penetration (IVP) of oocytes, changes in sulfhydryl group content in membrane proteins, and capacitation status. The main findings emerging from this study were that the addition of GSH to the freezing media resulted in 1) an improvement in percent motility (%MOT) and motion parameters of thawed spermatozoa, as measured by both microscopic analysis and computer-assisted semen analysis (CASA); 2) a higher number of total viable spermatozoa; 3) a higher number of noncapacitated viable spermatozoa; and 4) a decrease in the number of spermatozoa with changes in the sulfhydryl groups in membrane proteins. This protective effect on sperm function was more pronounced with 1 mM of GSH than with 5 mM of GSH.

Key words: Pig spermatozoa, antioxidants, cryopreservation, in vitro fertilization, capacitation status.

J Androl 2005;26:396–404

The processes of cooling, freezing, and thawing produce physical and chemical stress on the sperm membrane that reduces sperm viability and their fertilizing ability. The cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species (ROS) generation (Chatterjee et al, 2001). Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is a tripeptide ubiquitously distributed in living cells, and it plays an important role as an intracellular defense mechanism against oxidative stress (Irvine, 1996). The process of freezing is associated with a significant reduction in GSH content in porcine (Gadea et al, 2004), bovine (Bilodeau et al, 2000), and human sperm (Molla et al, unpublished data). Sperm freezing has also been reported to result in a reduction in sperm viability; changes in sperm function, lipid composition, and organization of the sperm plasma membrane (Buhr et al, 1994); and changes in sulfhydryl group content in membrane proteins (Chatterjee et al, 2001).

We have previously reported the effect of adding GSH to the freezing and thawing extender on sperm cryosurvival. Surprisingly, the addition of 5 mM of GSH to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability, whereas the addition of 1 or 5 mM of GSH to the thawing extender resulted in a tendency to increase sperm fertilizing ability (Gadea et al, 2004). However, few studies have investigated the precise mechanism by which GSH mediates this effect (Nishimura and Morii, 1993). Therefore, more thorough studies are needed to elucidate what changes in sperm function take place during cryopreservation and the mechanism(s) by which GSH exerts its effect(s).

To answer these questions, we incorporated a new set of functional sperm tests. These included tests of mitochondrial function, inducibility of the acrosome reaction, in vitro penetration (IVP) of oocytes, changes in sulfhydryl group content in the membrane protein, and capacitation status. In addition, to better understand the protective potential of GSH, a greater number of ejaculates were evaluated, and different phases of the freezing process were studied.

Sperm capacitation and acrosome reaction are 2 key steps in the fertilization process. Thus, an evaluation of these processes would be of paramount importance in assessing sperm fertilizing ability (Harrison, 1997). The binding and penetration of the zona pellucida is one of

Supported by project AGL-2003-03144 from the Spanish Ministry Science and Technology.

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Received for publication September 30, 2004; accepted for publication January 5, 2005.

DOI: 10.2164/jandrol.04155

the most important barriers spermatozoa must overcome to fertilize the egg. Also, sperm interaction with the oocyte plasma membrane appears to explain much of the variability in sperm fertilizing potential observed among fertile boars (Berger et al, 1996). Therefore, tests that measure gamete interaction may be more predictive of male fertility than routine semen analysis (Rodriguez-Martinez, 2003; Gadea, 2005). Moreover, we have previously shown that in vitro fertilization (IVF) has a high predictive value in evaluating boar semen fertility in both refrigerated and frozen-thawed semen (Gadea et al, 1998; Sellés et al, 2003). Therefore, IVF could also be helpful in identifying changes in sperm function that standard assays fail to detect (Larsson et al, 2000; Rodriguez-Martinez, 2003).

Likewise, flow cytometry has been very helpful in evaluating sperm quality by providing a specific, objective, accurate, and reproducible method compared to traditional microscopy-based methods (Graham, 2001). Computer-assisted semen analysis (CASA) has also provided an objective and accurate means of evaluating overall sperm motility (MOT) (Verstegen et al, 2002).

The main objective of this study was to evaluate the effect of GSH supplementation of the freezing extender on semen parameters during the cooling (2 hours at 5°C) and freezing phases of the cryopreservation process.

Materials and Methods

All reagents were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain) unless otherwise indicated.

Semen Collection and Handling

Semen was routinely collected from mature fertile boars using the manual method and a dummy. The sperm-rich fraction was collected in a prewarmed thermo flask, and the gel fraction was held on a gauze tissue covering the thermo opening. The semen was then diluted with isothermal Beltsville Thawing Solution extender (BTS) (Pursel and Johnson, 1975).

Freezing and Thawing Protocol

Semen samples were processed using the straw freezing procedure described by Westendorf et al (1975) with minor modifications, as indicated below. Diluted semen was placed at 15°C for 2 hours and centrifuged at 800 × *g* for 10 minutes. The supernatant was discarded, and the semen pellet was resuspended with lactose egg yolk (LEY) extender (80 mL of 11% lactose and 20 mL of egg yolk) to provide 1.5×10^9 spermatozoa/mL. After further cooling to 5°C for 120 minutes, 2 parts of LEY-extender semen were mixed with LEY extender with 1.5% Orvus Es Paste (Equex-Paste; Minitüb, Tiefenbach, Germany) and 9% glycerol. The final concentration of semen to be frozen was 1×10^9 spermatozoa/mL and 3% glycerol. The diluted and cooled semen was loaded into 0.5-mL straws (Minitüb), sealed and transferred to a programmable freezer (Icecube 1800; Minitüb), and frozen horizontally in racks. The freezing rate was 1°C/min from 5°C to −4.5°C, 1 minute at −4.5°C, and then 30°C/min from −4.5°C to −180°C. The straws were then stored in liquid nitrogen until thawing.

Thawing was carried out by immersing the straws in a circulating water bath at 52°C for 12 seconds (Sellés et al, 2003). Immediately after thawing, the semen was diluted in BTS at 37°C.

Analysis of Seminal Parameters by Microscopy

The %MOT and percentage of progression were determined by placing 2 sample aliquots on warm glass slides (39°C) and examining them under light microscopy (magnification 100×). The percentage of motile sperm was estimated to the nearest 5% MOT, and the forward progressive MOT was estimated using an arbitrary scale from 0 to 5.

The proportion of spermatozoa with a normal apical ridge (NAR) was evaluated after fixation in a buffered 2% glutaraldehyde solution and examined under phase-contrast microscopy (magnification 1000×) to analyze acrosomes (Pursel et al, 1972). Sperm membrane integrity was also evaluated applying a combination of the fluorophores carboxyfluorescein diacetate and propidium iodide (PI) (Harrison and Vickers, 1990).

Changes in the mitochondrial membrane potential of spermatozoa (MIT) were examined by monitoring the fluorescence of Rhodamine-123 simultaneously with the DNA-specific fluorochrome PI, as described by Graham et al (1990).

The sulfhydryl group content of sperm surface proteins was measured using fluorescent staining with 5-iodoacetamidofluoresceine (5-IAF). Fresh, cooled (5°C), and frozen semen were washed in BTS, and 1 mL of semen at a concentration of 5 to 10×10^6 was incubated with 10 μL of 5-IAF stock solution (500 μM), with a final solution of 5 μM, at 37°C for 15 minutes. The supernatant was eliminated by centrifugation (1000 × *g* × 3 minutes), and the resulting pellet was resuspended with BTS. These parameters were determined on 2 slides per sample and a total of 200 spermatozoa per sample. Aliquots of 10 μL were observed under a DMR Leica microscope (Wetzler, Germany) equipped with phase-contrast and epifluorescence accessories.

Motion Parameters

Motion parameters were determined using the CASA system (Sperm Class Analyzer; Microptic, Barcelona, Spain). The CASA-derived MOT 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), and beat cross-frequency (BCF, Hz).

A 7-μL drop of the sample was placed on a warmed (37°C) slide and covered with a 24 × 24-mm coverslip. The setting parameters were as follows: 25 frames in which spermatozoa had to be present in at least 15 to be counted and images obtained at 200× magnification in a phase-contrast microscope. Spermatozoa with a VAP less than 20 μm/s were considered immotile. A minimum of 5 fields per sample were evaluated, counting a minimum of 200 spermatozoa per subsample.

Analysis of Seminal Parameters by Flow Cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc, Miami, Fla). A 15-mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10 000 events per sample were collected in list mode, and 4 measures per sample were recorded. Flow cytometric data were analyzed by the program Expo32ADC (Beckman Coulter) using a gate in forward and side scatter to exclude any remaining debris and aggregates from the analysis.

Assessment of Capacitation Status

To detect an increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 (Harrison et al, 1996). Stock solutions of M540 (1 mM, Sigma-Aldrich) and Yo-Pro 1 (25 μM, Molecular Probes, Eugene, Oreg) in DMSO were prepared. For each 1 mL of diluted semen sample ($5–10 \times 10^6$), 2.7 μL of M540 stock solution (final concentration = 2.7 μM) and 1 μL of Yo-Pro (final concentration = 25 nM) were added. M540 fluorescence was collected with an FL2 sensor using a 575-nm band-pass filter, and Yo-Pro 1 was collected with an FL1 sensor using a 525-nm band-pass filter. Cells were classified in one of three ways: 1) low merocyanine fluorescence (viable, uncapacitated), 2) high merocyanine fluorescence (viable, capacitated), or 3) Yo-Pro 1 positive (dead).

Sulfhydryl Groups of Proteins From the Sperm Surface

The sulfhydryl groups of proteins from the sperm surface were evaluated by a fluorescent staining with 5-IAF. Seminal samples (1 mL of semen containing $5–10 \times 10^6$ cells) were incubated with 5 μL of 5-IAF stock solution (500 μM), with a final solution of 2.5 μM, at room temperature for 10 minutes. 5-IAF fluorescence data were collected with an FL1 sensor using a 525-nm band-pass filter. Cells were classified in 2 categories according to the intensity of fluorescence.

Plasma Membrane Integrity

Seminal samples (1 mL of semen with $5–10 \times 10^6$ spermatozoa) were incubated with 5 μL of PI stock solution (500 μg) at room temperature for 10 minutes (final concentration = 2.5 μg). PI fluorescence data were collected with an FL2 sensor using a 575-nm band-pass filter.

In Vitro Penetration

IVP ability was assessed using immature oocytes (Gadea et al, 1998) with minor modifications. In brief, porcine oocytes were collected just after slaughter at a local abattoir from the fresh ovaries of prepubertal gilts weighing approximately 95 kg and were transported to the laboratory within 30 minutes in saline (0.9% [wt/vol] NaCl) containing 100 mg of kanamycin/mL at 37°C. Cumulus–oocyte complexes were collected from nonatretic follicles (3–6 mm in diameter) by slicing, and they were washed twice in modified Dulbecco phosphate-buffered saline (PBS) supplemented with 1 mg of polyvinyl alcohol/mL. Only oocytes with a homogeneous cytoplasm and a complete and dense cumulus oophorus were used. The selected complexes were then washed twice again in fertilization medium, previously equilibrated for a minimum of 3 hours at 38.5°C under 5% CO_2 in air.

The sperm samples (diluted in BTS extender and kept at 15°C) were washed at $150 \times g$ for 10 minutes, and the pellet was placed on a 45/90 Percoll (Pharmacia, Upssala, Sweden) gradient and centrifuged again at $500 \times g$ for 30 minutes. The pellet was resuspended in the corresponding IVF medium (5 mL) and washed again to remove the Percoll at $150 \times g$ for 10 minutes. After resuspension in the IVF medium, 100 μL of diluted spermatozoa was added to the 35-mm plastic dish containing the oocytes, giving a final sperm concentration of 10×10^6 cells/mL.

Each group of 15 immature oocytes was coincubated with spermatozoa (10^7 cells/mL) for 18 hours in a petri dish containing 2 mL of modified Tyrode's albumin-lactate-pyruvate (TALP) fertilization medium (Rath et al, 1999) at 38.5°C under 5% CO_2 in air. At the end of the coincubation period, oocytes were stripped from cumulus cells and spermatozoa, mounted in slides, and fixed for a minimum of 24 hours with ethanol:acetic acid (3:1 [vol/vol]). They were later stained with 1% lacmoid and examined for evidence of sperm penetration under a phase-contrast microscope (magnification 400×). Immature oocytes were considered penetrated when spermatozoa with unswollen heads and their corresponding tails were found in the vitellus. The distinction between unswollen spermatozoa that had entered the oocyte cytoplasm and those remaining on the surface of the vitelline membrane was based on the facts that 1) the former had more intensive staining after lacmoid treatment than the latter, and 2) the sperm tails inside the vitellus were straight and slightly separated from the heads.

Acrosome Reaction Induction

Sperm samples after being processed through a Percoll gradient were resuspended in modified TALP to a final concentration of 10^7 spermatozoa/mL. The acrosome reaction was then induced by 1 μM of calcium ionophore A23187 or 10 μM of progesterone in modified TALP medium and incubated in 5% CO_2 at 38.5°C for 30 and 45 minutes, respectively.

Membrane integrity was evaluated by PI, and acrosomal status was monitored by means of fluorescein isothiocyanate labeled peanut agglutinin (FITC-PNA). A 100-μL aliquot of the sperm suspension from each treatment group was supplemented with 5 μL of FITC-PNA (200 μg/mL) and 5 μL of PI (200 μg/mL), kept at 38°C for 5 minutes, and finally fixed in 10 μL of paraformaldehyde (1% [vol/vol]) in saline solution). Spermatozoa were then examined under an epifluorescence microscope and divided into 3 categories according to their FITC-PNA-PI staining pattern (Coy et al, 2002): 1) spermatozoa with no FITC-PNA or PI staining were considered live and acrosome intact; 2) spermatozoa with no PI staining but with acrosomal areas stained with FITC-PNA were considered live and acrosome reacted; and 3) spermatozoa stained with PI were believed to have damaged membranes (irrespective of the degree of FITC-PNA staining).

Experimental Design

Experiment 1: Effect of the Addition of GSH to the Freezing Media on Sperm Function During the Cooling Process—To ex-

Table 1a. *Boar seminal parameters before and after cooling at 5°C in freezing media (lactose egg yolk) supplemented (1 and 5 mM) or not with reduced glutathione**†

Freezing Media	Time, h	MOT	FPM	CFD	NAR	5-IAF	MIT
Fresh semen before cooling		84.06 ± 1.65	3.50 ± 0.11	88.06 ± 1.09	97.31 ± 0.33	16.63 ± 2.03	82.03 ± 3.45
Control	0	69.37 ± 3.05	2.69 ± 0.16	86.86 ± 1.24	95.87 ± 0.65	17.36 ± 1.16	80.14 ± 2.65
	1	58.50 ± 2.59	2.41 ± 0.16	85.68 ± 1.40	94.31 ± 0.64	19.12 ± 1.38	76.93 ± 3.86
	2	51.87 ± 1.87	2.12 ± 0.16	82.37 ± 2.39	95.31 ± 0.42	18.81 ± 1.47	71.35 ± 2.43
1 mM GSH	0	69.06 ± 2.99	2.69 ± 0.16	83.50 ± 1.29	95.69 ± 0.35	16.50 ± 1.04	77.79 ± 3.69
	1	56.75 ± 2.35	2.37 ± 0.15	81.25 ± 1.87	95.56 ± 0.56	15.81 ± 1.72	75.28 ± 3.17
	2	50.62 ± 2.20	2.12 ± 0.16	80.64 ± 1.98	94.75 ± 0.59	17.50 ± 1.64	69.28 ± 4.12
5 mM GSH	0	68.12 ± 2.98	2.69 ± 0.16	81.69 ± 1.88	95.81 ± 0.34	17.37 ± 1.29	80.54 ± 3.01
	1	57.22 ± 2.37	2.31 ± 0.13	80.31 ± 1.85	94.75 ± 0.54	19.13 ± 1.76	75.50 ± 1.73
	2	50.50 ± 2.03	2.09 ± 0.16	80.50 ± 2.52	94.12 ± 0.46	20.44 ± 1.21	71.92 ± 4.19

* GSH indicates glutathione; MOT, motility; FPM, forward progressive motility; CFD, carboxyfluorescein diacetate and propidium iodide staining; NAR, normal apical ridge; 5-IAF, 5-iodoacetamidofluoresceine; and MIT, mitochondrial membrane potential.
† Averages data from 8 cooling batches.

amine the effect of GSH supplementation during the cooling process, the spermatozoa of 8 cooling batches from the pooled ejaculates of 3 boars were processed without the addition of GSH (control) and with the addition of 1 or 5 mM of GSH to the freezing extender. Seminal samples were evaluated for 1) %MOT, viability, NAR, mitochondria activity, and sulfhydryl group content in membrane proteins by microscopic analysis in fresh and cooled semen at 5°C for 0, 1, and 2 hours; 2) calcium ionophore and progesterone-induced acrosome reaction in both fresh and cooled semen at 5°C for 2 hours; and 3) IVP of oocytes in fresh and cooled semen at 5°C for 2 hours.

Experiment 2: Effect of the Addition of GSH to the Freezing Media on Sperm Function During the Freezing Process—To examine the effect of GSH supplementation during the freezing process, the spermatozoa of 6 freezing batches from the pooled ejaculates of 3 boars were processed without the addition of GSH (control) and with the addition of 1 or 5 mM of GSH to the freezing extender. Seminal samples were evaluated for 1) %MOT, viability, NAR, mitochondria activity, and sulfhydryl group content in membrane proteins by microscopic analysis in fresh and cooled semen at 5°C for 0, 1, and 2 hours; 2) motion parameters by CASA; 3) plasma membrane integrity by PI and sulfhydryl group content in membrane protein using 5-IAF by flow cytometry; and 4) capacitation status using merocyanine and Yo-Pro 1 by flow cytometry.

Table 1b. *Analysis of variance**

Source of Variation	*P* Values					
	MOT	FPM	CFD	NAR	5-IAF	MIT
Freezing media	.794	.945	.286	.582	.107	.700
Time	<.001	<.001	.022	.027	.299	.013
Interaction	.998	.998	.726	.337	.842	.824

* MOT indicates motility; FPM, forward progressive motility; CFD, carboxyfluorescein diacetate and propidium iodide staining; NAR, normal apical ridge; 5-IAF, 5-iodoacetamidofluoresceine; and MIT, mitochondrial membrane potential.
† Averages data from 8 cooling batches.

Statistical Analysis

Data are expressed as the mean ± SEM and were analyzed by analysis of variance (ANOVA), with the specific sperm treatment (GSH addition) as the main variable. When ANOVA detected a significant effect, values were compared by the least significant difference post hoc test. Differences were considered statistically significant at $P < .05$.

In experiment 1a for microscopic evaluation, a 2-way ANOVA was used, with time of cooling and GSH addition as the main variables. In experiment 1c, in vitro PEN data (categorical data) were modeled according to the binomial model of parameters and were analyzed by ANOVA.

Results

Experiment 1: Effect of the Addition of GSH to the Freezing Media on Sperm Functionality During the Cooling Process

Microscopic evaluation of MOT, viability, NAR, mitochondria activity, and sulfhydryl group content in membrane proteins was performed in semen cooled at 5°C (0, 1, and 2 hours). The addition of GSH to the freezing extender did not result in any effect on these parameters (Table 1a and b). All of the seminal parameters studied, except when using 5-IAF, were significantly affected by the time of cooling at 5°C ($P < .001$), with a decrease in quality observed during the time of cooling at the same velocity for the 3 freezing media studied (interaction $P > .05$).

The induction of the acrosome reaction by calcium ionophore was affected by the addition of GSH to the freezing media (Table 2a). The percentage of viable spermatozoa with the acrosome intact was significantly lower in the 5-mM GSH group than in the others ($P = .045$). However, no differences were found between the percentage

Table 2a. *Acrosome reaction induced by calcium ionophore in boar spermatozoa before and after cooling at 5°C in freezing media (lactose egg yolk) with or without the addition of reduced glutathione**†

Freezing Media	Intact Acrosome and Viable	Acrosome Reacted and Viable	Died
Fresh semen before cooling	19.00 ± 1.45 A	20.20 ± 4.29	62.80 ± 3.84
Control	15.00 ± 3.89 A	26.20 ± 8.05	58.80 ± 10.35
1 mM GSH‡	17.20 ± 3.65 A	22.20 ± 8.11	60.60 ± 10.21
5 mM GSH	7.42 ± 1.48 B	32.22 ± 11.76	60.36 ± 11.78
ANOVA, *P* values‡	.045	.761	.993

* Numbers within columns with different letters differ ($P < .05$).
† Averages data from 8 cooling batches.
‡ GSH indicates glutathione; ANOVA, analysis of variance.

of spermatozoa with acrosome reaction and nonviable sperm. The induction of acrosome reaction by progesterone showed no significant differences between the 3 freezing media studied (Table 2b).

The results of the IVP assay showed that the cooling process affects the penetration rate (%PEN) and the number of sperm per oocyte penetrated (S/O). Both parameters, %PEN and S/O, were significantly lower in spermatozoa after the cooling process (5°C for 2 hours) than in fresh semen, irrespective of the addition of GSH, with a reduction of ca 20% in PEN and a decrease in the mean number of S/O to nearly half. The addition of GSH to the cooling process had no protective effect (Table 3).

Experiment 2: Effect of the Addition of GSH to the Freezing Media on Sperm Functionality During the Freezing Process

A microscopic evaluation of MOT, viability, NAR, mitochondria activity, and sulfhydryl group content in membrane proteins was carried out in frozen-thawed samples. The addition of 1 mM of GSH to the freezing media improved MOT (total and progressive) compared to the control. No differences between groups were found for the other seminal parameters (Table 4).

The effect of the freezing media on motion parameters measured by CASA was consistent with data obtained previously by direct microscopic observation, since the %MOT, VSL, and VAP were significantly higher in the group supplemented with 1 mM of GSH than in the control (Table 5). Linearity was higher in the GSH-supplemented groups than in the control.

When flow cytometry was used to measure plasma membrane integrity and changes in the sulfhydryl group content in membrane proteins, higher numbers of viable sperm were found when GSH was added to freezing media (Table 6). Finally, the evaluation of the capacitation status showed that the group supplemented with 1 mM of GSH had the highest number of viable noncapacitated spermatozoa and the lowest number of nonviable sperm, with a similar number of viable capacitated sperm (Table 7).

Discussion

Freezing is associated with damage of sperm function affecting those processes required for the successful in vivo fertilization of an oocyte (Bailey et al, 2000). During freezing, 2 important processes have been reported. First is the production of ROS (Bilodeau et al, 2000; Ball et al, 2001), which can induce changes in membrane function and structure. Because the most likely mechanism of redox regulation is the posttranslational modification of

Table 2b. *Acrosome reaction induced by progesterone in boar spermatozoa before and after cooling at 5°C in freezing media (lactose egg yolk) with or without the addition of reduced glutathione**

Freezing Media	Intact Acrosome and Viable	Acrosome Reacted and Viable	Died
Fresh semen	34.57 ± 2.36	3.92 ± 1.04	61.50 ± 2.32
Control	27.95 ± 1.64	5.12 ± 1.17	66.93 ± 2.09
1 mM GSH†	33.91 ± 2.74	4.61 ± 0.76	61.48 ± 2.71
5 mM GSH	30.75 ± 3.31	6.25 ± 1.88	63.00 ± 2.97
ANOVA, *P* values†	.260	.645	.392

* Averages data from 8 cooling batches.
† GSH indicates glutathione; ANOVA, analysis of variance.

Table 3. *In vitro penetration ability of the boar spermatozoa before and after cooling at 5°C in freezing media (lactose egg yolk) with or without the addition of reduced glutathione**

Freezing Media	N†	Penetration Rate, %	Sperm per Penetrated Oocyte
Fresh semen before cooling	283	62.19 ± 2.89 A	4.14 ± 0.34 A
Control	269	44.98 ± 3.04 B	2.39 ± 0.21 B
1 mM GSH†	270	47.78 ± 3.05 B	2.19 ± 0.21 B
5 mM GSH	270	45.93 ± 3.04 B	2.42 ± 0.20 B
ANOVA, *P* values†		.0001	<.0001

* Numbers within columns with different letters differ ($P < .05$).
† N indicates number of immature oocytes inseminated; GSH, glutathione; and ANOVA, analysis of variance.

Table 4. *Boar seminal parameters after freezing-thawing in freezing media (lactose egg yolk) supplemented (1 and 5 mM) or not with reduced glutathione**†‡

Freezing Media	MOT	FPM	CFD	NAR	5-IAF	MIT
Control	26.11 ± 2.90 A	1.77 ± 0.14 A	51.53 ± 2.04	51.21 ± 2.33	51.32 ± 1.67	44.19 ± 1.74
1 mM GSH	37.50 ± 3.54 B	2.26 ± 0.15 B	44.80 ± 3.46	49.93 ± 3.09	52.94 ± 2.66	45.63 ± 2.73
5 mM GSH	32.61 ± 3.57 AB	2.02 ± 0.17 AB	47.33 ± 2.48	48.17 ± 3.38	49.66 ± 2.05	41.79 ± 1.93
ANOVA, *P* values	.053	.072	.205	.772	.292	.478

* MOT indicates motility; FPM, forward progressive motility; CFD, carboxyfluorescein diacetate and propidium iodide staining; NAR, normal apical ridge; 5-IAF, 5-iodoacetamidofluoresceine; MIT, mitochondrial membrane potential; GSH, glutathione; and ANOVA, analysis of variance.
† Numbers within columns with different letters differ ($P < .05$).
‡ Averages data from 6 freezing batches.

protein thiols, the detrimental effect of freezing could be blocked, at least in part, by the addition of exogenous GSH, since the cell employs GSH and thioredoxin systems to reverse oxidative stress. However, thiols can also be irreversibly oxidized to form sulfinic or sulfonic acids (Baty et al, 2002). Second is an alteration in antioxidant defense systems (Bilodeau et al, 2000), including a decrease in intracellular GSH content (Bilodeau et al, 2000; Gadea et al, 2004). So, one obvious way to improve the viability and subsequent fertilizing capacity of frozen-thawed boar sperm would be the addition of antioxidants to the freezing media. For example, vitamin E has been used with relative success in refrigerated and frozen boar semen (Cerolini et al, 2000; Pena et al, 2003).

In this study, we evaluated the effects of GSH supplementation of the freezing extender on sperm function to compensate for the reported decrease in GSH content during the sperm freezing. The main findings emerging from this study were that the addition of GSH to the freezing media resulted in 1) an improvement in %MOT and motion parameters of thawed spermatozoa, as measured by both microscopic analysis and CASA; 2) a higher number of total viable spermatozoa; 3) a higher number of non-capacitated viable spermatozoa; and 4) a decrease in the number of spermatozoa with changes in the sulfhydryl groups in membrane proteins. This protective effect on sperm function was dose dependent, and it was more pronounced with 1 mM of GSH than with 5 mM of GSH.

In the current study and during the cooling phase (previous to the freezing phase), the addition of GSH to the media did not have any significant effects on sperm function, as measured by standard parameters or the IVP assay (Gadea and Matas, 2000). These results are in good agreement with previous studies (Gadea et al, 2004), in which a decrease in intracellular GSH content during the freezing step was reported. However, this was not observed during the cooling step (24 hours at 15°C). Therefore, the addition of exogenous GSH would be expected to have a beneficial effect only when endogenous GSH levels are decreased. Nevertheless, to confirm this hypothesis, the use of more accurate methodology to measure GSH content, such as high-performance liquid chromatography with fluorescence detection, would be required (Cereser et al, 2001).

During the cooling phase of the freezing procedure (2 hours, 5°C), a difference in the percentage of the calcium ionophore–induced acrosome reaction was observed. Different physiological and nonphysiological stimuli have been applied to induce the acrosome reaction in boar spermatozoa (revised by Flesch and Gadella, 2000). A short exposure (30 vs 45 minutes) of sperm to the calcium ionophore resulted in a higher percentage of viable acrosome-reacted sperm than that obtained when using progesterone, whereas sperm viability was similar after both treatments. When the calcium ionophore was used, the spermatozoa cooled in media with 5 mM of GSH showed a lower percentage of viable and acrosome-intact spermatozoa. This could be related to the lower membrane stability caused by a high concentration of exogenous GSH when endogenous GSH levels are within normal limits, as has been previously described in studies of bulls (Foote et al, 2002).

During the freezing phase, the addition of GSH to the freezing extender would be expected to improve the quality and fertilizing ability of frozen-thawed boar spermatozoa (Nishimura and Morii, 1993), since the addition of GSH has been shown to help to maintain bull sperm MOT (Lindemann et al, 1988; Bilodeau et al, 2001; Foote et al, 2002) and to protect sperm against oxidative damage (Alvarez and Storey, 1989). However, fertility trials further demonstrated the lack of a positive effect of antioxidants on frozen bull semen (Foote et al, 2002). In this study, a significant effect from the addition of GSH in seminal functionality was detected. For the experimental groups, the addition of GSH resulted in better MOT when it was measured by either direct observation or CASA.

GSH supplementation, compared to control media, appears to preserve sperm viability by protecting membrane structure and function. To address this issue, viability and sulfhydryl groups of proteins from the sperm surface were measured by 5-IAF fluorescent staining and flow cytometry. The results indicated that GSH supplementation resulted in fewer changes in these groups than in the

Table 5. *Motility parameters measured by CASA after freezing-thawing in freezing media (lactose egg yolk) supplemented (1 and 5 mM) or not with reduced glutathione**†‡

Freezing Media	Motility, %	VCL, μm/s	VSL, μm/s	VAP, μm/s	STR, %	LIN, %	ALH, μm/s	BCF, Hz
Control	42.12 ± 3.56 A	70.52 ± 3.01	28.57 ± 1.74 A	42.48 ± 2.04 A	65.07 ± 1.50	40.29 ± 1.73 A	2.84 ± 0.18	6.15 ± 0.36
1 mMGSH	50.62 ± 2.57 B	73.76 ± 2.51	41.96 ± 3.20 B	46.50 ± 2.00 AB	66.71 ± 1.34	45.80 ± 1.43 B	3.02 ± 0.11	5.96 ± 0.27
5 mMGSH	47.29 ± 2.15 AB	75.18 ± 2.94	32.89 ± 1.56 A	49.75 ± 2.33 B	67.61 ± 1.19	46.55 ± 1.71 B	3.20 ± 0.15	5.58 ± 0.22
Anova, *P* values	.014	.509	.001	.037	.817	.167	.681	.715

* VCL indicates curvilinear velocity; VSl, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency; CASA, computer-assisted semen analysis; GSH, glutathione; and ANOVA, analysis of variance.

† Numbers within columns with different letters differ ($P < .05$).

‡ Averages data from 6 freezing batches.

Table 6. *Plasma membrane integrity assessed by propidium iodide and changes in the sulfhydryl groups in membrane protein assessed by 5-IAF by flow cytometry in boar spermatozoa frozen in freezing media (lactose egg yolk) supplemented (1 and 5 mM) or not with reduced glutathione**†

Freezing Media	Membrane Integrity	5-IAF
Control	42.45 ± 2.00 A	54.22 ± 1.76 A
1 mM GSH‡	53.32 ± 1.87 B	41.40 ± 1.60 B
5 mM GSH	51.24 ± 1.46 B	40.21 ± 1.46 B
ANOVA, *P* values‡	.0001	.0001

* Numbers within columns with different letters differ ($P < .05$).

† Averages data from 6 freezing batches.

‡ GSH indicates glutathione; ANOVA, analysis of variance.

control, as previously reported in frozen bull spermatozoa (Chatterjee et al, 2001).

In relation to membrane functionally, Watson (1995) suggested that cryopreservation-induced modifications in sperm membranes make them more prone to capacitation, so that cryopreserved sperm have been thought to be in a partially capacitated state (Bailey et al, 2000). In the present study, the addition of GSH affected the percentage of sperm with low plasma membrane lipid packing disorder and the percentage of viable spermatozoa presenting a higher number of noncapacitated sperm when GSH was added to the media. We have obtained similar results when 5 mM of GSH was added to human sperm (Sellés et al, unpublished data).

We have previously reported the effect of the addition of GSH to the freezing and thawing extender (Gadea et al, 2004). It did not result in any improvement in either standard semen parameters or sperm fertilizing ability in vitro. In contrast, a significant correlation between GSH supplementation to the thawing extender and IVP was observed. To explain the apparent paradoxical effect on sperm function observed with different GSH concentrations in different studies, several hypotheses can be postulated. First, there are individual differences in the response of sperm to exogenous GSH levels (Foote et al, 2002). In addition, spermatozoa GSH content before and after freezing may show variation between boars (Gadea et al, 2004). Furthermore, a dose-response study should be conducted, since a paradoxical effect of GSH on sulfhydryl and disulfide groups on human sperm has been previously described (de Lamirande and Gagnon, 1998). Second, the concentration of 1 mM of GSH tends to be more adequate in preserving boar sperm function. Therefore, additional dose-response studies are required to determine the optimal concentration of GSH. Finally, the results may be highly dependent on the methodology used to evaluate sperm function. The use of microscopic analysis to evaluate sperm function is less accurate than other techniques. IVF may be one of the best tests, since several different steps in the fertilization process are assessed in

Table 7. *Capacitation status; subpopulations of spermatozoa after staining with merocyanine 540 and Yo-Pro 1 and examined by flow cytometry; boar frozen spermatozoa with freezing media (lactose egg yolk) supplemented (1 and 5 mM) or not with reduced glutathione**†

Freezing Media	Viable Capacitated	Viable Capacitated	Not Dead
Control	8.66 ± 0.99	35.27 ± 2.64 A	56.06 ± 2.99 A
1 mM GSH‡	9.06 ± 0.53	48.63 ± 2.27 B	42.30 ± 2.00 B
5 mM GSH	8.97 ± 0.71	40.28 ± 2.17 AB	50.74 ± 1.85 AB
ANOVA, *P* values‡	.9181	.003	.001

* Numbers within columns with different letters differ ($P < .05$).
† Averages data from 6 freezing batches.
‡ GSH indicates glutathione; ANOVA, analysis of variance.

a single assay, and flow cytometry offers the possibility of measuring a high number of cells, the use of different fluorochromes, and the possibility of higher reproducibility (Graham, 2001; Gadea, 2005). In conclusion, the addition of GSH to the freezing media showed a protective effect on sperm function because it 1) improved MOT and motion parameters of thawed spermatozoa; 2) increased the proportion of noncapacitated viable spermatozoa; and 3) decreased the percentage of spermatozoa with changes in the sulfhydryl group content in membrane proteins. Additional experiments are required to understand changes in the redox system that take place in boar spermatozoa caused by freezing and the potentially beneficial effects of antioxidants in improving the fertility outcome in artificial insemination using boar frozen-thawed spermatozoa.

Acknowledgment

We acknowledge with gratitude Dr Juan G. Alvarez for a critical review and editing of the manuscript.

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