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Supplementation of the Thawing Media With Reduced Glutathione Improves Function and the In Vitro Fertilizing Ability of Boar Spermatozoa After Cryopreservation

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ABSTRACT: In this study, we evaluated the effects of glutathione (L-g-glutamyl-L-cysteinylglycine; GSH) supplementation of the thawing extender on semen parameters to compensate for the decrease in GSH content observed during sperm freezing. To fully address these questions, we used a set of functional sperm tests. These included tests of motility and motion parameters, changes in sulfhydryl group content in membrane proteins, capacitation status, measures of intracellular reactive oxygen species generation, sperm chromatin condensation, and in vitro penetration of immature oocytes. The main findings emerging from this study were that addition of GSH to the thawing media resulted in a lower number of capacitated viable spermatozoa, a decrease in the number of spermatozoa with changes in the sulfhydryl groups in membrane proteins, a reduction of the reactive oxygen species generation, a lower chromatin condensation, and a higher penetration ability of oocytes in vitro and a higher proportion of decondensated sperm heads. GSH appears to play an important role in sperm antioxidant defense strategy. Addition of GSH to the thawing extender could be of significant benefit in improving the function and fertilizing capacity of frozen boar spermatozoa.

Key words: Pig spermatozoa, oxidative stress, IVF, capacitation status.

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The processes of cooling, freezing, and thawing produce physical and chemical stress on the sperm membrane that reduce 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in GSH content in porcine (Gadea et al, 2004), bovine (Bilodeau et al, 2000) and human sperm (Molla and Gadea, unpublished data).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), as well as changes in sulfhydryl group content in membrane proteins (Chatterjee et al, 2001).

We have previously reported the effect of addition of GSH to the freezing and thawing extender on sperm cryosurvival (Gadea et al, 2004, 2005). However, few studies have investigated the precise mechanism by which GSH could mediate this effect (Nishimura and Morii, 1993; Funahashi and Sano, 2005). Therefore, more thorough studies are needed to elucidate what changes in sperm function take place during cryopreservation and the mechanisms by which GSH exerts its effects. To answer these questions, we incorporated a new set of functional sperm tests. These included tests of sperm motility assayed by computer-assisted semen analysis (CASA), changes in sulfhydryl group content in the membrane protein, capacitation status, free radical production (ROS generation) and sperm chromatin condensation by flow cytometry, and finally the in vitro penetrability of immature oocytes.

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

Sperm capacitation and acrosome reaction are two key steps in the fertilization process. Thus, evaluation of these processes would be of paramount importance in assessing sperm fertilizing ability (Harrison, 1997). The measurements of the ROS intracellular generation could be decisive when evaluating the balance between free-radical generating and scavenging systems. Although levels of ROS are commonly evaluated by chemiluminescence as-

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say (Sikka, 2004), it is possible to use flow cytometry for accurate and low-cost measurements, using fluorochromes for evaluating intracellular ROS production as the dichloro-dihydrofluorescein diacetate.

Previous studies have shown greater boar sperm chromatin condensation after freezing-thawing procedures (Hamamah et al, 1990; Cordova et al, 2002). In addition, changes in the status of nuclear chromatin structure have been proposed as a possible cause of infertility (Evenson et al, 1994; Fernandez et al, 2003; Alvarez et al, 2004). Thus, evaluation of chromatin condensation could be a valuable tool for evaluation of cryopreserved boar spermatozoa.

However, the binding and penetration of the zona pellucida is one of the most important barriers that 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 (Rodriguez-Martinez, 2003).

The main objective of this study was to evaluate the effect of GSH supplementation of the thawing extender on boar sperm functionality measured by different assays.

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 eight 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 egg yolk) to provide 1.5×10^9 spermatozoa/mL. After further cooling to 5°C for 120 minutes, two 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 the thawing media (BTS with or without GSH addition) at 37°C and maintained 30 minutes in these media before being assayed.

Analysis of Seminal Parameters by Microscopy

Percentage motility and progression were determined by placing two sample aliquots on warm glass slides (37°C) and examining them under light microscopy (magnification ×100). The percentage of motile sperm was estimated to the nearest 10% (MOT) and the forward progressive motility, using an arbitrary scale from 0 to 5.

Motion Parameters

Motion parameters were determined using a CASA system (Sperm Class Analyzer, Microptic, Barcelona, Spain). The 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), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), 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 cover slip. The setting parameters were: 25 frames, of which spermatozoa had to be present in at least 15 to be counted; images were obtained at ×200 magnification in a contrast phase microscope. Spermatozoa with a VAP less than 20 μm/s were considered immotile. A minimum of five fields per sample was 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 four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

Assessment of Sperm Capacitation

To detect 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) and Yo-Pro 1 (25 μM, Molecular Probes, Eugene, Ore) in

DMSO were prepared. For each 1 mL diluted semen sample (containing 5–10 × 10^6 cells), 2.7 μL M540 stock solution (final concentration of 2.7 μM) and 1 μL of Yo-Pro (25 nM final concentration) were added. M540 fluorescence was collected with a FL2 sensor, using a 575-nm band-pass filter, and Yo-Pro 1 with a FL1 sensor, using a 525-nm band-pass filter. Cells were classified in three categories: low merocyanine fluorescence (viable, incapacitated), high merocyanine fluorescence (viable, capacitated), or Yo-Pro-1 positive (dead).

Sulfhydryl Group Content of Proteins from the Sperm Surface

The sulfhydryl group content of proteins from the sperm surface were evaluated with a fluorescent staining 5-iodoacetamidofluorescein (5-IAF). Seminal samples (1 mL of semen with 5–10 × 10^6 cells) were incubated with 5 μL of 5-IAF stock solution (500 μM), final solution 2.5 μM, and 5 μL propidium iodide (PI) stock solution (500 mg/mL) at room temperature for 10 minutes. Green 5-IAF fluorescence was collected with a FL1 sensor using a 525-nm band-pass filter, and red PI fluorescence was collected with a FL3 sensor using a 650-nm band-pass filter. Cells were classified in three categories: low 5-IAF fluorescence (viable, intact proteins), high 5-IAF fluorescence (viable, altered proteins), or PI positive (dead).

Production of Reactive Oxygen Species

Production of ROS was measured by incubating the spermatozoa in thawing media (BTS with and without the addition of GSH) in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (0.5 μM) during 30, 60, and 90 minutes at 37°C. This dye is a fluorogenic probe commonly used to detect cellular ROS production. H_2DCFDA is a stable cell-permeable nonfluorescent probe. It is deesterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescin on oxidation. Green 5-IAF fluorescence was collected with a FL1 sensor using a 525-nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1), and it was used as index of ROS generation.

Determination of Chromatin Condensation

Sperm chromatin was stained with propidium iodide for the determination of sperm chromatin condensation (Molina et al, 1995; Cordova et al, 2002).

Thawed samples were centrifuged (1200 × *g* for 3 minutes), and the pellet resuspended in a solution of ethanol and Dulbecco's phosphate-buffered saline (PBSD) (70/30 v/v) for 30 minutes for the sperm membranes' permeabilization. After that, the samples were centrifuged, the supernatant discarded, and the pellet resuspended in a propidium iodide solution (PI, 10 mg/mL) in PBSD. Samples were maintained in the darkness for 1 hour before flow cytometric analysis. Red PI fluorescence was collected with a FL3 sensor using a 650-nm band-pass filter. Measurements were expressed as the mean red intensity fluorescence units (mean channel in the FL3), and they were used as an index of the state of the chromatin condensation, as this is directly related to the PI uptake by DNA.

In Vitro Penetration

Sperm in vitro penetration ability was assessed using immature oocytes, as previously described (Gadea et al, 1998) with minor modifications. In brief, porcine oocytes were collected from fresh ovaries from prepubertal gilts weighing approximately 95 kg, just after slaughter at a local abattoir, and transported to the laboratory within 30 minutes in saline (0.9% w/v NaCl) containing 100 mg mL^{-1} kanamycin at 37°C. Cumulus–oocyte complexes were collected from nonatretic follicles (3–6 mm in diameter) by slicing and were washed twice in modified Dulbecco's phosphate-buffered saline supplemented with 1 mg mL^{-1} polyvinyl alcohol. Only oocytes with a homogeneous cytoplasm and a complete and dense cumulus oophorus were used. The selected complexes were then again washed twice 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 with or without GSH) were maintained at 37°C for 30 minutes, washed (1200 × *g*, 3 minutes), and then resuspended in the corresponding IVF medium. 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 fertilization medium modified Tyrode's Albumin-Lactate-Pyruvate (Rath et al, 1999) at 38.5°C under 5% CO2 in air. At the end of the coincubation period, oocytes were stripped of cumulus cells and attached spermatozoa, mounted on slides, and fixed for a minimum of 24 hours with ethanol:acetic acid (3:1 v/v). 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 to be penetrated when spermatozoa heads and their corresponding tails were found in the vitellus.

Experimental Design

To examine the effect of GSH supplementation during the thawing process, spermatozoa from five freezing batches of pooled ejaculates from three boars were processed without addition of GSH (control) and with addition of 1 or 5 mM GSH to the BTS thawing extender, and maintained 30 minutes in these media before assayed.

Effect of the Addition of GSH to the Thawing Media on Sperm Function

Seminal samples were evaluated for percentage motility and forward progressive motility by microscopic observation, motion parameters by CASA, capacitation status by merocyanine 540 and plasma membrane integrity by Yo-Pro 1, sulfhydryl group content in membrane protein by 5-IAF and plasma membrane integrity by propidium iodide, reactive oxygen formation by H_2DCFDA staining, and chromatin condensation by propidium iodide staining.

Effect of the Addition of GSH to the Thawing Media on Sperm In Vitro Penetration Ability of Immature Oocytes

Statistical Analysis

Data are expressed as the mean ± SEM and analyzed by ANOVA, considering the specific sperm treatment (GSH addition)

Table 1. *Motility parameters measured by CASA. Frozen boar spermatozoa thawed in BTS medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)**

	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	ALH (μm/s)	LIN (%)	STR (%)	WOB (%)	BCF (Hz)
Thawing media									
Control	33.08 ± 1.79	77.03 ± 1.67	25.93 ± 1.41	42.93 ± 1.58	2.75 ± 0.13	32.75 ± 1.40	59.03 ± 1.44	54.10 ± 1.19	6.77 ± 0.39
1 mM GSH	33.31 ± 1.86	77.50 ± 1.86	25.93 ± 1.59	42.34 ± 1.76	2.95 ± 0.20	32.41 ± 1.32	59.43 ± 1.48	52.88 ± 1.31	6.66 ± 0.37
5 mM GSH	37.98 ± 2.09	78.22 ± 1.63	26.67 ± 1.63	42.16 ± 1.26	2.95 ± 0.16	31.66 ± 1.16	59.93 ± 1.38	52.95 ± 0.89	6.40 ± 0.38
Source variation *P* values									
GSH	0.158	0.831	0.540	0.319	0.158	0.652	0.849	0.159	0.776
Batch	0.000	0.704	0.026	0.070	0.861	0.000	0.000	0.000	0.006
Interaction	0.545	0.306	0.661	0.108	0.438	0.737	0.830	0.106	0.587

* 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; WOB, Wobble (VAP/VCL); and BCF, beat cross-frequency.

Table 2. *Motility parameters measured by microscopic analysis; frozen boar spermatozoa thawed in BTS medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)**

	Motility (%)	FPM* (%)
Thawing media		
Control	26.39 ± 2.13	2.58 ± 0.11
1 mM GSH	28.61 ± 2.28	2.61 ± 0.11
5 mM GSH	30.83 ± 2.53	2.64 ± 0.11
Source variation *P* values		
GSH	0.112	0.959
Batch	0.000	0.000
Interaction	0.670	0.623

* BTS indicates Beltsville Thawing Solution extender; FPM, forward progressive motility.

and the freezing batches as the main variables. When ANOVA revealed a significant effect, values were compared using the Fisher's least significant difference post hoc test. Differences were considered statistically significant at $P < .05$.

In vitro penetration rate data (categorical data) were modeled according to the binomial model of parameters and were analyzed by ANOVA.

Results

Effect of the Addition of GSH to the Thawing Media on Sperm Function

The addition of GSH to the thawing extender for 30 minutes did not have a significant effect on the motility parameters evaluated. The motion parameters monitored by CASA were similar to those evaluated by direct microscopic evaluation (Tables 1 and 2). Nevertheless, a higher motility score was recorded by CASA than by microscopic observation. Percentage motility (by CASA) ranged from 33.08 in the control group to 37.98 in the 5-mM GSH group. However, this difference did not reach statistical significance ($P > .05$). Most of the motion parameters were affected by the freezing batch, but they presented a similar pattern under the same treatments. Although no significant differences were found for percentage of motility (both CASA and microscopic analysis), a slight tendency toward an increase in percentage motility was observed when GSH is added.

The addition of GSH to the thawing media reduced the percentage of capacitated viable sperm in a dose-dependent manner ($P = .003$, Table 3) and reduced the percentage of viable spermatozoa with changes in the sulfhydryl groups in membrane proteins ($P < .04$, Table 4). In both cases, no significant differences were observed with regard to the percentage of dead spermatozoa, and a significant batch effect was observed ($P < .01$, Tables 3 and 4).

The generation of ROS increased as a function of in-

Table 3. *Capacitation status; subpopulations of spermatozoa after staining with merocyanine 540 and Yo-Pro 1 and examined by flow cytometry; boar frozen spermatozoa thawed in BTS medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)**

	Viable Capacitated†	Viable Noncapacitated	Dead
Thawing media			
Control	16.44 ± 0.77[a]	43.60 ± 1.39	39.96 ± 1.62
1 mM GSH	15.07 ± 0.59[b]	45.91 ± 1.59	39.02 ± 1.65
5 mM GSH	14.35 ± 0.50[c]	43.52 ± 1.68	42.13 ± 1.74
Source variation *P* values			
GSH	0.003	0.361	0.307
Batch	0.000	0.000	0.000
Interaction	0.080	0.263	0.376

* BTS indicates Beltsville Thawing Solution extender.
† Numbers with different superscript letters differ ($P < .05$).

cubation time (30, 60, and 90 minutes), and ROS levels were significantly reduced following addition of GSH to the thawing media ($P < .0001$, Table 5). Mean values of ROS generation for the GSH groups were close to 50% of the values of the control group (control: 140.45 vs. 1 mM GSH: 74.64 and 5 mM GSH: 66.32 fluorescence units).

Chromatin condensation was equally affected by the addition of GSH ($P = .0013$). When GSH was added, a lower chromatin condensation was observed, as reflected by higher red fluorescence intensity and higher PI uptake (control: 78.11 ± 2.32 vs. 1 mM GSH: 89.24 ± 2.28 and 5 mM GSH: 92.85 ± 3.42 fluorescence units).

Effect of the Addition of GSH to the Thawing Media on Sperm In Vitro Penetration Ability

The data from the in vitro penetration assays showed that addition of GSH to the thawing media had a positive effect on the parameters studied in a dose-dependent manner ($P < .001$, Table 6). For the penetration rate and mean number of sperm per penetrated oocyte, a significant effect of the interaction between GSH addition and batches was detected ($P < .05$). When GSH was added to the thawing extender, a higher proportion of decondensed sperm heads was observed inside the oocyte (18.52 vs 24.20 and 34.52%; $P = .002$, Table 6).

Discussion

Freezing is associated with damage of sperm function, affecting those processes required for successful in vivo fertilization of the oocyte (Bailey et al, 2000). During freezing, two important processes have been reported: first, production of ROS (Bilodeau et al, 2000; Ball et al, 2001) that can induce changes in membrane function and structure. Because the posttranslational modification of protein thiols is one of the major mechanisms of redox regulation, and freezing could affect this process, this detrimental effect of freezing could be prevented, at least in part, by addition of exogenous GSH because the cell employs GSH and thioredoxin systems to reverse oxidative stress. 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 and thawing media.

In this study, we evaluated the effects of GSH supplementation of the thawing extender on sperm function to compensate the observed decrease in GSH content pro-

Table 4. *Alteration of sulfhydryl group on the sperm membrane protein; subpopulations of spermatozoa after staining with 5-IAF and propidium iodide and examined by flow cytometry; boar frozen spermatozoa thawed in BTS medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)**

	Viable Altered SH†	Viable Nonaltered SH	Dead
Thawing media			
Control	7.24 ± 0.31[a]	60.14 ± 0.95	32.61 ± 0.95
1 mM GSH	6.83 ± 0.29[ab]	60.79 ± 1.06	32.38 ± 1.08
5 mM GSH	6.41 ± 0.21[b]	60.85 ± 1.04	32.73 ± 1.06
Source variation *P* values			
GSH	0.004	0.868	0.340
Batch	0.002	0.000	0.000
Interaction	0.001	0.946	0.486

* BTS indicates Beltsville Thawing Solution extender; SH, sulfhydryl.
† Numbers with different superscript letters differ ($P < .05$).

Table 5. *ROS generation (mean channel of fluorescence) by boar frozen spermatozoa thawed in BTS medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH) during different time of incubation**

	30 min	60 min	90 min	Mean Value†	Source Variation *P* Values	ROS Generation
Thawing media						
Control	127.85 ± 6.39	142.11 ± 3.29	149.19 ± 3.21	140.45 ± 3.75[a]	GSH	< .0001
1 mM GSH	69.12 ± 1.69	74.50 ± 2.70	79.17 ± 2.86	74.64 ± 1.64[b]	Time	.0004
5 mM GSH	61.91 ± 0.87	66.90 ± 0.77	69.39 ± 2.09	66.32 ± 1.05[c]	Interaction	.4244

* BTS indicates Beltsville Thawing Solution extender; ROS, reactive oxygen species.
† Numbers with different superscript letters differ ($P < .05$).

duced during the sperm freezing. The main findings emerging from this study indicate that addition of GSH to the thawing media resulted in a lower number of capacitated viable spermatozoa, a decrease in the number of spermatozoa with changes in sulfhydryl groups in membrane proteins, a reduction in ROS generation, lower chromatin condensation, and a higher oocyte penetration rate in vitro and a higher proportion of decondensated sperm heads inside the oocyte. This protective effect on sperm function was more pronounced with 5 mM GSH than with 1 mM GSH.

Addition of GSH to the freezing and thawing extenders would be expected to improve the quality and fertilizing ability of frozen-thawed boar spermatozoa (Nishimura and Morii, 1993; Gadea et al, 2004, 2005), as addition of GSH has been shown to help to maintain sperm motility (Lindemann et al, 1988; Bilodeau et al, 2001, Foote et al, 2002; Funahashi and Sano, 2005) and to protect sperm against oxidative damage (Alvarez and Storey, 1989). In this study, no significant effect on percentage motility and motion parameters was found after addition of GSH to the thawing medium. In contrast, motility significantly improved when GSH was added to the freezing media (Gadea et al, 2005). This observation could be related to the contact time of GSH with the sperm cells. When GSH was added to the freezing media, exposure time was longer (at least 90 minutes) than when it was added to the thawing media (30 minutes). Perhaps 30 minutes was an insufficient contact time to produce a significant effect on the motility pattern. The other possible explanation could be related to the variability associated to the different batches used in this study that could mask the GSH effect.

The results of this study indicate that GSH probably affects plasma membrane lipid packing and sulfhydryl group content in membrane proteins in sperm, as reflected by the lower percentage of capacitated sperm and the lower number of crosslinked proteins observed when GSH was added to the thawing media. Similar results were found when GSH was added to the freezing media (Gadea et al, 2005).

The initiation of the sperm capacitation process is related to an alteration in the redox balance between ROS generation and the activity of the antioxidant defense mechanisms (Aitken et al, 1989; Griveau and Le Lannou, 1997). GSH could be an important regulator of the scavenging system and one of the most important nonenzymatic antioxidants in sperm. In this study, a decreased ROS generation was found when GSH was present in the thawing media, indicating that it may be responsible, at least in part, for the lower disruption of lipid packing and the lower changes in membrane proteins. Nevertheless, studies on these cellular changes affect sperm function,

Table 6. *In vitro penetration ability of boar frozen spermatozoa thawed in BTS medium with or without the addition of reduced glutathione (GSH)*†*

	N Oocytes	Penetration Rate (%)	Sperm per Penetrated Oocyte‡	Decondensed Sperm (%)
Thawing media				
Control	307	35.18 ± 2.73[a]	1.34 ± 0.06[a]	18.52 ± 3.76[a]
1 mM GSH	308	51.94 ± 2.85[b]	1.66 ± 0.07[b]	24.20 ± 3.43[b]
5 mM GSH	285	58.95 ± 2.92[b]	1.92 ± 0.08[c]	34.52 ± 3.68[c]
Source variation *P* values				
GSH		0.000	0.000	0.002
Batch		0.000	0.001	0.000
Interaction		0.026	0.039	0.831

* BTS indicates Beltsville Thawing Solution extender; N, number of immature oocyte inseminated.
† Numbers with different superscript letters differ ($P < .05$).
‡ Related to penetrated oocytes.

and whether these changes could be reversed by antioxidants is open to further investigations (reviewed by Sikka, 2004).

One of the main findings emerging from this study is that the addition of GSH to the thawing extender significantly improves sperm's in vitro oocyte penetration ability, as we previously hypothesized (Gadea et al, 2004). Therefore, cryopreservation-induced oxidative stress in boar sperm affects a sperm property that is related to both sperm binding and penetration of the zona pellucida, sperm–oocyte membrane fusion, oocyte activation, or pronuclear formation. In this sense, the membrane fusion events involved in binding with the oolema and the acrosome reaction appear to be more vulnerable to ROS-induced damage than overall motility (Aitken et al, 1989). Also, we have previously shown that IVF is a highly valuable tool to assess boar semen fertilizing ability in both refrigerated and frozen-thawed semen (Selles et al, 2003; Gadea et al, 1998).

Another important observation is the higher proportion of decondensed sperm heads found inside the oocytes when GSH was added to the thawing extender. We had previously reported improved male pronuclear formation ratios in an IVM/IVF system when oocytes matured in vitro were used (Gadea et al, 2004). In this way, up to now, the main factors associated with sperm head decondensation were related to the enzymatic mechanisms present in the oocyte cytosol (Funahashi et al, 1995). However, GSH also participates in the decondensation of the paternal genome after oocyte penetration (Calvin et al, 1986; Perreault et al, 1988) and may alter spindle microtubule formation in the ovum (Sikka, 2004). These results indicate that pretreatment of sperm with GSH could have an additional effect on sperm decondensation and male pronuclear formation inside the oocyte. Also, Boquest et al (1999) reported a higher embryo development rate when GSH was present during the gamete incubation. Further studies are needed to clarify these observations.

With regard to the sperm chromatin condensation/decondensation status, it is well known that sperm freezing induces chromatin hypercondensation (Hamamah et al, 1990; Cordova et al, 2002). In this study, a lower level of sperm chromatin condensation was observed when GSH was added. This protective effect of GSH could be the result of two different mechanisms: an indirect one, by reducing oxidative stress, thiol oxidation, and hypercondensation of sperm chromatin, and a direct one, by inducing sperm decondensation, as it normally occurs in the cytosol of the oocyte after sperm penetration. In addition, GSH may also be acting by reducing oxidative stress-induced DNA oxidation and DNA fragmentation (reviewed by Agarwal and Said, 2003).

In any case, important questions still remain to be solved. Is GSH content in raw semen a good predictor of semen quality following the freeze–thaw cycle? In a recent paper Meseguer et al (2004) found a negative correlation between postthaw motile sperm recovery rate and GSH concentration in raw human semen. In boar sperm, a direct correlation was found between acrosome status in frozen-thawed spermatozoa and GSH content in fresh semen ($r = 0.36$, $P = .02$), whereas no significant correlation was found for motility and viability (Gadea, unpublished data).

Other interesting questions are: How is GSH produced by the sperm cell? How is GSH metabolized in this highly specialized cell? In other type of cells, intracellular GSH levels are maintained indirectly by two tightly coupled enzymatic processes involving γ-glutamyl transpeptidase and membrane-bound dipeptidases that supply amino acids for GSH and protein biosynthesis. γ-glutamyl transpeptidase is widely expressed in many mammalian tissues, and it is essential for catalyzing secreted GSH into cysteinyl-glycine and -glutamic acid. After cleavage of cysteinyl-glycine by a dipeptidase, the amino acids are reabsorbed and used to synthesize GSH, a process known as the γ-glutamyl cycle. The generation of GSH is crucial for the protection of cells against oxidative stress and other forms of cellular injury. Although GSH metabolic pathways are known, little is known about GSH metabolism in sperm. Glutamyl transpeptidase is present in the midpiece and acrosomal regions of spermatozoa (Funahashi et al, 1996; Boilart et al, 2002), and it also has been detected in seminal fluid (Tate and Meister, 1981; Zalata et al, 1995). In fact, it has been used for semen identification in forensic samples (Abe et al, 1998).

In conclusion, GSH appears to play an important role in sperm antioxidant defense strategy. The addition of GSH to the thawing extender could be of significant benefit in improving the function and fertilizing capacity of frozen boar spermatozoa.

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