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ORIGINAL ARTICLE

Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa

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Summary

In this study, we evaluated the effects of glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) supplementation of the thawing extender on bull semen parameters to compensate for the decrease in GSH content observed during sperm freezing. To address these questions fully, we used a set of functional sperm tests. These included tests of sperm motility assayed by computer-assisted semen analysis, membrane lipid packing disorder, spontaneous acrosome reaction, free radical production [reactive oxygen species (ROS) generation], sperm chromatin condensation, DNA fragmentation by terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling and acridine orange staining measured by flow cytometry. Finally, the in vitro penetrability of in vitro matured oocytes and the in vitro production of embryos were evaluated. The main findings emerging from this study were that addition of GSH to the thawing medium resulted in: (i) a higher number of non-capacitated viable spermatozoa; (ii) a reduction in ROS generation; (iii) lower chromatin condensation; (iv) lower DNA fragmentation; (v) higher oocyte penetration rate in vitro and (vi) higher in vitro embryo production compared with control group. Nevertheless, GSH had no significant effect on motion parameters or the occurrence of the spontaneous acrosome reaction. Addition of GSH to the thawing extender could be of significant benefit in improving the function and fertilizing capacity of frozen bull spermatozoa.

Introduction

The processes of cooling, freezing and thawing produce physical and chemical stress on the sperm membrane that reduces their viability and fertilizing ability. The cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species (ROS) generation (Chatterjee *et al.*, 2001). Glutahione (L-γ-glutamyl-L-cysteinylglycine) (GSH) is a tripeptide ubiquitously distributed in living cells and it plays an important role as an intracellular defence mechanism against oxidative stress. 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GSH content in porcine (Gadea *et al.*, 2004), bovine (Bilodeau *et al.*, 2000) and human sperm (M. Molla and J. Gadea, unpublished observations). 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 (reviewed by Foote & Parks, 1993; Bailey *et al.*, 2000).

We have previously reported the effect of addition of GSH to the freezing and thawing extender on boar sperm cryosurvival (Gadea *et al.*, 2004, 2005a,b), as well as in human, ovine and goat spermatozoa (M. Molla, E. Sellés, J. C. Gardon and J. Gadea, unpublished observations). However, few studies have investigated the precise mechanism by which GSH could mediate this effect (Foote *et al.*, 2002). 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 used a set of functional sperm tests. These included tests of sperm motility assayed by computer-assisted semen

analysis (CASA), membrane lipid packing disorder, spontaneous acrosome reaction, free radical production (ROS generation), sperm chromatin condensation and DNA fragmentation by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and acridine orange (AO) staining measured by flow cytometry. Finally, the in vitro penetrability of in vitro matured oocytes and the in vitro production of embryos were evaluated. Therefore, in vitro fertilization (IVF) and in vitro embryo production could also be helpful in identifying changes in sperm function that standard assays fail to detect (Rodriguez-Martinez, 2003; Gadea, 2005; Graham & Moce, 2005).

With the increasing evidence that oxidative stress is a major cause of DNA damage in spermatozoa (Aitken *et al.*, 1998), it was important to determine if any improvement in survival and fertility of cryopreserved bull spermatozoa could be accomplished by treating semen and/or semen extender with various combinations of antioxidants (Foote *et al.*, 2002).

The main objective of this study was to evaluate the effect of GSH supplementation of the thawing extender on bull sperm function.

Materials and methods

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

Semen handling

Frozen bull spermatozoa from six fertile bulls of autochthonous Spanish breed 'Asturiana de Valles' generously provided by the Breeders Association (ASEAVA, Gijón, Asturias, Spain) were used. Straws (0.5 mL) were thawed in a water bath at 37 °C for 30 sec and the content was diluted in Tyrode's sperm medium (sperm-TALP; Parrish *et al.*, 1988) without addition of GSH (control), and with addition of 1 mM or 5 mM GSH to the sperm-TALP medium and maintained 30 min at 37 °C in this medium before assayed.

Analysis of the motion parameters

Motion parameters were determined using a CASA system (Sperm Class Analyzer, Microptic, Barcelona, Spain). The CASA-derived motility characteristics studied were total motility (%), progressive motility (%), 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, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), 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 cover slip. The setting parameters were: 25 frames in which spermatozoa had to be present in at least 15 in order to be counted, images were obtained at ×200 magnification in a contrast phase microscope. Spermatozoa with a VAP < 20 μm/s were considered immotile. A minimum of five fields per sample was evaluated, counting a minimum of 200 spermatozoa per sub-sample.

Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). 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 analysed 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 plasma membrane lipid packing disorder

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) and Yo-Pro 1 (25 μM; Molecular Probes, Eugene, OR, USA) 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 into three categories: low merocyanine fluorescence (viable, low disorder), high merocyanine fluorescence (viable, high disorder) or Yo-Pro-1 positive (dead).

Acrosome reaction

Seminal samples (1 mL of semen with 5–10 × 10^6 cells) were incubated with 2 μL of fluorescein-labelled lectin from the peanut plant, Arachis hypogaea (FITC-PNA, 100 mg/mL) and 5 μL of propidium iodide (PI) stock solution (500 mg/mL), at room temperature for 10 min. Fluorescence was measured using a FL-1 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm band-pass filter to detect PI. Three sperm subsets were detected: live acrosome intact,

live acrosome damaged and dead spermatozoa (with and without acrosome intact).

Production of reactive oxygen species

Production of ROS was measured by incubating the spermatozoa in thawing medium (sperm-TALP) with and without the addition of GSH in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (0.5 μM) for 60 min at 37 °C (Gadea *et al.*, 2005b). This dye is a fluorogenic probe commonly used to detect cellular ROS production. H_2DCFDA is a stable cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescin upon oxidation. Green 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 (Gadea *et al.*, 2005b). Thawed samples after 30 min of incubation in the experimental media were centrifuged (1200 $g \times 3$ min) and the pellet resuspended in a solution of ethanol and phosphate-buffered saline (PBS) (70/30 v/v) for 30 min for the sperm membranes permeabilization. After that the samples were centrifuged, the supernatant was discarded and the pellet was resuspended in a propidium iodide solution (PI, 10 mg/mL) in PBS. Samples were maintained in darkness for 1 h 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 it was used as an index of the state of the chromatin condensation, as this is directly related to the PI uptake by DNA.

Evaluation of sperm DNA fragmentation by TUNEL

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling staining was used to determine sperm DNA fragmentation according to the method described previously by Li *et al.*, 1995. In brief, the cells were concentrated by centrifugation, fixed in a solution of ethanol and PBS (70/30 v/v), and stored overnight at −20 °C. Cells (aprox. 10^6) were washed twice with PBS and resuspended in 50 μL of Terminal deoxynucleotidyltransferase (TdT) reaction buffer containing: 10 μL of 5× concentrated buffer solution (Invitrogen S. A, Barcelona, Spain), 1 μL (15 units) of TdT (Invitrogen), 0.25 nmoles of fluorescein-dUTP (BODIPY®-FL-X- 14-dUTP; Molecular Probes, C7614) (0.25 μL) and 39 μL distilled water.

Negative controls were incubated in fluorescein-dUTP in the absence of enzyme terminal transferase. The cells were incubated with the reaction buffer for 60 min at 37 °C, then rinsed twice and measured by flow cytometry. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter and two populations were determined. The cells with fragmented DNA presented an intense green nuclear fluorescence. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1) and it was used as an index of the DNA fragmentation, as this is directly related to the dUTP uptake by DNA.

Assessment of DNA fragmentation by the SCSA assay

Staining of single-stranded DNA in sperm samples was performed as previously described (Evenson *et al.*, 2002). Sperm samples were centrifuged and the pellet was washed once and resuspended in TNE buffer (containing 0.15 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.2) at 4 °C to a final concentration of 5×10^6 cells/mL. Two hundred microlitres of this sperm suspension was mixed with 400 μL of a detergent/acid solution of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 M HCl (pH 1.4). After 30 sec, cells were stained by adding 1.2 mL of a solution containing 6 mg/mL of AO in staining buffer [0.15 M NaCl, 1 mM EDTA, 10 mM Tris, 0.2 M Na2HPO4, 0.1 M citric acid (pH 6.0)].

Stained cells were analysed by flow cytometry. Under these experimental conditions, when excited with a blue-light source, AO intercalates with double-stranded DNA and emits green fluorescence (detected in a fluorescence detector FL1). AO associated with single-stranded DNA emits red fluorescence (detected in a fluorescence detector FL3). Equivalent instrument settings were used for all samples. By using a double scatter plot for forward and sideways scatter and based on different scatter properties, non-sperm events were gated out.

This shift is expressed as the function alpha t (αt), which is the ratio of red to the total (i.e. red and green) fluorescence intensity, thus representing the amount of denatured DNA relative to the total cellular DNA. In the analysis, αt was calculated for each spermatozoon in a sample and the results were expressed as the mean (Xαt), the standard deviation of the at distribution (SDαt), and the percentage of cells with high αt values, named 'cells outside the main population (% COMPαt)', representing the cells with an excess of denatured DNA. Recently, this terminology has been changed: 'the mean of αt' has been renamed 'x-DNA fragmentation index (X-DFI); 'SD αt' has become 'SD-DFI'; and 'COMP αt'

has become 'DFI' (Evenson *et al.*, 2002). In this study, we use the new nomenclature.

In vitro maturation, in vitro fertilization and in vitro culture of embryos.

In vitro maturation (IVM) and IVF, were performed as previously described by Coy *et al.* (2005). Oocyte–cumulus cell complexes (COC) were collected from antral follicles (3–8 mm) and then cultured for 24 h (30–40 COC in each well of a Nunclon 4-well plate containing 500 mL oocyte maturation medium (OMM; M199 with Earle's salts, 10% (v/v) Fetal bovine serum (FBS), 2 mmol/L L-glutamine, 0.2 mmol/L sodium pyruvate, 50 mg/mL gentamicin, 0.3 mg/mL LH and 5.0 mg/mL FSH) at 38.5 °C in 5% CO_2 and humidified air.

Oocytes presumed mature, were fertilized with frozen-thawed semen (10^6 total sperm/mL). The sperm samples (diluted in sperm-TALP with or without GSH) were maintained at 37 °C for 30 min, washed (1200 × g, 3 min), and then resuspended in the corresponding IVF-TALP medium (Parrish *et al.*, 1988). 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 10^6 cells/mL.

In experiment 2a, 18 h after IVF, putative zygotes were denuded of cumulus and fixed for penetration evaluation. The total number of pronuclei (female and male) within each oocyte was determined by staining the embryos with Hoechst 33342 (10 mg/mL; 20 min).

In experiment 2b, after 18 h of coincubation with spermatozoa, the presumptive zygotes were stripped of cumulus cells by repeated passage through a fine pipette, and then washed three times with culture medium. KSOM medium was used for the basal culture medium. It consisted of 94.97 mM NaCl, 2.50 mM KCl, 1.71 mM CaCl, 0.20 mM $MgSO_4$, 0.35 mM $KHPO_4$, 25.07 mM $NaHCO_3$, 21.09 mM sodium lactate, 0.6 mM sodium pyruvate, 73.1 mM L-glutamine, 3 mg/L EDTA, 0.2 mM D-glucose, 1% basal medium Eagles (BME) essential amino acids solution, 1% minimum essential medium (MEM) non-essential amino acids solution and 50 pg/mL gentamicin sulphate. The presumptive zygotes were cultured in 500 μL of KSOM supplemented with 3 mg/mL fatty acid-free BSA for 7 days at 39 °C in humidified air with 5% CO_2, 5% O_2 and 90% N_2. At 48 h post-insemination, cleavage rates were recorded and the embryos were transferred to newly prepared medium. Embryos were examined for developmental stages at intervals of 48 h until 168 h under a dissecting microscope. Finally the total number of nuclei within blastocyst was determined by staining the embryos with Hoechst 33342 (10 mg/mL; 20 min).

Experimental design

To examine the effect of GSH supplementation during the thawing process, spermatozoa from six different bulls were incubated without addition of GSH (control), and with addition of 1 or 5 mM GSH to the sperm-TALP thawing medium and maintained for 30 min at 37 °C in these media before assayed. Each assay was evaluated four straws per bull.

Effect of the addition of GSH to the thawing medium on sperm function.

Seminal samples were evaluated for:

1 Motion parameters by CASA.
2 Lipid membrane disorder status by merocyanine 540 and plasma membrane integrity by Yo-Pro 1.
3 Acrosome reaction by FITC-PNA and plasma membrane integrity by propidium iodide.
4 Reactive oxygen formation by H_2DCFDA staining.
5 Chromatin condensation by propidium iodide staining.
6 Evaluation of sperm DNA fragmentation by TUNEL.
7 Evaluation of the sperm DNA stability by the SCSA assay.

Effect of the addition of GSH to the thawing medium on sperm in vitro penetration ability of in vitro matured oocytes.

1 *In vitro penetration capacity*. The penetration rate, sperm number per penetrated oocyte and male pronuclear formation were evaluated after 18 h coculture of gametes.
2 *In vitro embryo production*. Ability of putative zygotes to cleave (assessed by recording the number of two- to four-cell embryos present at 72–75 h post-insemination) and to develop to the blastocyst stage was recorded on Day 3 and Day 8 post-IVF, respectively. The total number of nuclei within blastocysts (Day 8, 186–193 h post-insemination) was determined by staining with Hoechst 33342.

Statistical analysis

Data are expressed as the mean ± SEM and analysed by ANOVA, considering the specific sperm treatment (GSH addition) and the bulls as the main variables. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at $p < 0.05$.

In vitro penetration rate and cleavage data (categorical data) were modelled according to the binomial model of

parameters by arcsin transformation of the data and were analysed by ANOVA.

Results

Effect of the addition of GSH to the thawing medium on sperm function

Addition of GSH to the sperm-TALP medium for 30 min did not have a significant effect on the motility parameters evaluated (Table 1). Progressive motility (evaluated by CASA) ranged from 40.2% in the control group to 43.6% in the 5 mM GSH group. This difference was not statistically significant ($p > 0.05$). The same pattern was observed for sperm velocities. The control group had 115.8 μm/s for VCL and increased to 118.5 in the 5 mM GSH group. Only STR and BCF were affected by GSH addition, the 1 mM GSH group presented lower values for STR and BCF than control and 5 mM GSH group. For these parameters bull-GSH interaction was not significant.

The addition of GSH to the thawing medium increased both the percentage of viable low lipid disorder and of dead sperm in a dose-dependent manner ($p < 0.01$, Table 2). The viable low lipid disorder sperm increased from 58.8% in the control group to 61.0% in the 5 mM GSH group and reduced the percentage of dead spermatozoa ($p < 0.01$, Table 2) (ranged from 39.5% for control vs. 34.4% for 5 mM GSH). In both cases a significant bull effect was observed ($p < 0.01$, Table 2) but they presented a similar pattern under the same treatments (interaction between GSH treatment and bull ≥0.05).

The generation of ROS during the 60 min of incubation period was significantly reduced following the addition of GSH to the thawing medium ($p < 0.01$, Table 3). Mean values of ROS generation for the GSH groups were close to 50% of the values obtained in the control group (control: 13.0 vs. 1 mM GSH: 9.5 and 5 mM GSH: 7.8 arbitrary fluorescence units). A very low percentage of spermatozoa had a spontaneous acrosome reaction (spermatozoa acrosome reacted and viable were <0.5%) and no differences between experimental groups were observed (Table 4, $p > 0.05$).

Table 2. Membrane lipid packing disorder. Sub-populations of spermatozoa after staining with merocyanine 540 and Yo-Pro 1 and examined by flow cytometry. Bull frozen spermatozoa thawed in sperm-TALP medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)

	Viable high lipid disorder	Viable low lipid disorder	Dead
Thawing media			
Control	4.7 ± 0.2	58.8 ± 0.8[a]	39.5 ± 0.7[a]
1 mM GSH	4.9 ± 0.2	59.5 ± 0.8[a]	35.6 ± 0.7[a]
5 mM GSH	4.6 ± 0.3	61.0 ± 0.9[b]	34.4 ± 0.7[b]
Source variation *p*-values			
GSH	0.16	<0.01	<0.01
Bull	<0.01	<0.01	<0.01
Interaction	0.05	0.05	0.08

[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 3. Reactive oxygen species (ROS) generation (mean channel of fluorescence). Bull frozen spermatozoa thawed in sperm-TALP medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)

	ROS
Thawing media	
Control	13.0 ± 0.8[a]
1 mM GSH	9.5 ± 0.5[b]
5 mM GSH	7.8 ± 0.4[c]
Source variation *p*-values	
GSH	<0.01
Bull	<0.01
Interaction	0.48

[a,b,c]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 1. Motility parameters measured by computer-assisted semen analysis. Frozen bull spermatozoa thawed in sperm-TALP medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)

	% Progressive motility	% Motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Thawing media										
Control	40.2 ± 1.2	59.0 ± 1.4	115.8 ± 2.0	87.1 ± 2.2	98.3 ± 2.1	74.0 ± 0.7	87.1 ± 0.4[a]	83.9 ± 0.5	2.7 ± 0.16	6.1 ± 0.1[a]
1 mM GSH	41.00 ± 1.4	61.8 ± 1.6	114.0 ± 2.1	84.2 ± 2.0	96.9 ± 2.1	72.3 ± 0.7	85.4 ± 0.5[b]	83.7 ± 0.4	2.87 ± 0.0	5.8 ± 0.1[b]
5 mM GSH	43.6 ± 2.0	59.3 ± 1.3	118.5 ± 2.00	88.7 ± 2.1	100.3 ± 2.1	73.6 ± 0.7	87.1 ± 0.4[a]	83.5 ± 0.5	2.8 ± 0.0	6.1 ± 0.1[a]
Source variation *p*-values										
GSH	0.19	0.35	0.16	0.18	0.34	0.16	<0.01	0.79	0.60	0.04
Bull	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Interaction	0.15	0.02	<0.01	0.01	0.01	0.50	0.30	0.07	0.04	0.47

VCL, 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); BCF, beat cross-frequency.
[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 4. Acrosome reaction. Sub-populations of spermatozoa after staining with FITC-PNA and PI and examined by flow cytometry. Bull frozen spermatozoa thawed in sperm-TALP medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)

	Intact acrosome and viable	Acrosome reacted and viable	Dead
Thawing media			
Control	55.7 ± 0.6	0.3 ± 0.0	44.0 ± 0.6
1 mM GSH	55.8 ± 0.6	0.3 ± 0.0	43.9 ± 0.6
5 mM GSH	56.0 ± 0.6	0.3 ± 0.0	43.7 ± 0.6
Source variation *p*-values			
GSH	0.56	0.93	0.57
Bull	<0.01	<0.01	<0.01
Interaction	0.66	1.00	0.61

Chromatin condensation and stability were equally affected by the addition of GSH ($p < 0.01$; Table 5). When GSH was added, a lower chromatin condensation was observed, as reflected by the higher red fluorescence intensity and higher PI uptake (control: 22.1 vs. 1 mM GSH: 25.2 and 5 mM GSH: 27.5 fluorescence units). The evaluation of DNA fragmentation by TUNEL showed a significant decrease from 22.3 (arbitrary units) for control group to 17.5 and 17.9 for 1 and 5 mM GSH respectively ($p < 0.01$, Table 5). These results were consistent with the data obtained from the AO staining expressed as the percent cells outside the main population of αt (DNA fragmentation index DFI) and SD-DFI were significantly higher in the control group than in the GSH group ($p < 0.01$, Table 5).

Effect of the addition of GSH to the thawing medium on sperm in vitro penetration ability and embryo production

The data from the in vitro penetration assays showed that addition of GSH to the thawing medium had a positive effect on the penetration rate. The penetration rate was higher when GSH was used (50.8% from control to 62.8% and 66.8% for 1 and 5 mM GSH respectively, $p < 0.01$, Table 6). No differences were found for either sperm per penetrated oocyte, monospermy rate or male nuclear formation. The male nuclear formation was very high (more than 90%) in all the IVF replicates. The bull affected the parameters studied significantly with the exception of the monospermy rate.

When embryo production was evaluated, the percentage of cleaved embryos on day 2 (2–4 cells), day 3 (8–16 cells) and morulae and blastocysts at day 7 was higher in the 5 mM GSH group than in control, with the 1 mM group showing intermediate values (Table 7). The number of nuclei within the blastocysts ranged from 39.9 to 47.7 with a high standard error of the mean (SEM). No differences were observed between experimental groups (Table 7).

Discussion

Freezing is associated with damage to 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: (i) production of ROS (Bilodeau *et al.*, 2000; Ball *et al.*, 2001; Chatterjee *et al.*, 2001) that can induce changes in membrane function and structure and (ii) an alteration in antioxidant defence systems (Bilodeau *et al.*, 2000), including a decrease in intracellular GSH content (Bilodeau *et al.*, 2000; Gadea *et al.*, 2004). Therefore, the supplementation of the freezing and thawing medium with antioxidants could potentially be used to improve the viability and subsequent fertilizing capacity of frozen-thawed bull spermatozoa.

In this study, we evaluated the effects of GSH supplementation of the thawing extender on sperm function in

Table 5. Chromatin condensation (mean red intensity fluorescence units), DNA fragmentation by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) (mean green intensity fluorescence units) and SCSA parameters of bull frozen spermatozoa thawed in sperm-TALP medium supplemented (1 and 5 mM) or not with reduced glutathione (GSH)

	Chromatin condensation (PI uptake)	TUNEL	DFI (%)	x-DFI	SD-DFI
Thawing media					
Control	22.1 ± 1.1^{a}	22.3 ± 0.8^{a}	1.2 ± 0.1^{a}	404.8 ± 1.1^{a}	21.4 ± 0.5^{a}
1 mM GSH	25.2 ± 1.1^{b}	17.5 ± 0.8^{b}	0.8 ± 0.1^{b}	410.0 ± 0.9^{ab}	18.1 ± 1.1^{b}
5 mM GSH	27.5 ± 1.4^{b}	17.9 ± 0.8^{b}	0.8 ± 0.2^{b}	413.1 ± 0.5^{b}	17.6 ± 0.6^{b}
Source variation *p*-values					
GSH	<0.01	<0.01	0.01	<0.01	0.01
Bull	<0.01	0.39	0.01	0.24	0.28
Interaction	0.45	0.01	0.02	<0.01	0.08

DFI, DNA fragmentation index; X-DFI, mean value-DFI; SD-DFI, standard deviation-DFI (Evenson *et al.*, 2002).
^{a,b}Numbers within columns with different superscripts differ ($p < 0.05$).

Table 6. In vitro penetration ability of bull frozen spermatozoa thawed in sperm-TALP medium with or without the addition of reduced glutathione (GSH)

	N oocytes	Penetration rate (%)	Sperm per penetrated oocyte*	Male pronuclear formation (%)*	Monospermy (%)*
Thawing media					
Control	244	50.8 ± 3.2[a]	1.3 ± 0.1	96.8 ± 1.69	80.4 ± 3.4
1 mM GSH	234	62.8 ± 3.2[b]	1.3 ± 0.0	94.5 ± 1.9	74.2 ± 3.4
5 mM GSH	244	66.8 ± 3.0[b]	1.3 ± 0.1	92.6 ± 2.0	76.8 ± 3.1
Source variation *p*-values					
GSH		<0.01	0.73	0.21	0.52
Bull		<0.01	<0.01	0.04	0.12
Interaction		0.05	0.97	0.53	0.65

Pool data from seven IVF replicates.
*Related to penetrated oocytes.
[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 7. In vitro embryo production using bull frozen spermatozoa thawed in sperm-TALP medium with or without the addition of reduced glutathione (GSH)

	N oocytes	Embryos 2–4 cells	Embryos 8–16 cells	Morulae + blastocysts	No cells
Thawing media					
Control	233	47.6 ± 3.3[a]	42.2 ± 3.5[a]	22.3 ± 2.8[a]	47.7 ± 8.4
1 mM GSH	246	54.9 ± 3.2[ab]	49.6 ± 3.3[ab]	29.0 ± 3.4[ab]	42.6 ± 5.8
5 mM GSH	229	64.6 ± 3.2[b]	56.9 ± 3.6[b]	31.3 ± 3.3[b]	39.9 ± 3.1
Source variation *p*-values					
GSH		0.04	0.03	0.02	0.26
Bull		<0.01	<0.01	<0.01	0.30
Interaction		0.01	0.05	<0.01	0.13

[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

order to compensate for the observed decrease in GSH content produced during the sperm freezing. The main findings emerging from this study are that addition of GSH to the thawing medium resulted in: (i) a higher number of non-capacitated viable spermatozoa; (ii) a reduction in ROS generation; (iii) lower chromatin condensation; (iv) lower DNA fragmentation; (v) higher oocyte penetration rate in vitro and (vi) higher in vitro embryo production. Nevertheless, no significant effect was observed on motion parameters and the occurrence of the spontaneous acrosome reaction.

Addition of GSH has been shown to help maintain bull sperm motility (Lindemann *et al.*, 1988; Bilodeau *et al.*, 2001; Foote *et al.*, 2002) and to protect sperm against oxidative damage (Alvarez & Storey, 1989). In this study, no significant effect on per cent motility and motion parameters was found after the addition of GSH to the thawing medium, as previously observed under similar experimental conditions in bull (Kim *et al.*, 1999) and frozen-thawed boar spermatozoa (Gadea *et al.*, 2005b). However, the addition of GSH to the thawing extender significantly increased the motility parameters in ram and caprine frozen-thawed spermatozoa (J.C. Gardón and J. Gadea, unpublished data). This apparent discrepancy could be related to the contact time of GSH with the sperm cells which was 30 min in the present study compared with 6 h (Bilodeau *et al.*, 2001) or 12 h (Foote *et al.*, 2002) of incubation in the other studies. However, these latter studies were conducted using a disrupted membrane model (reactivated) (Lindemann *et al.*, 1988). Another possible explanation could be the variability between the bulls used in the present study that could have masked the GSH effect.

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 defence mechanisms (Aitken *et al.*, 1989; Griveau & Le Lannou, 1997). GSH could be an important regulator of the scavenging system and one of the most important non-enzymatic antioxidants in sperm. In this study, a decrease in ROS generation was found when GSH was present in the thawing medium, indicating that it may be responsible, at least in part, for the lower disruption of lipid packing. Nevertheless, in this study no differences were

found when the spontaneous acrosome reaction was evaluated. It is possible that the changes in the redox balance only affected the first steps of the capacitation process, and did not lead to the spontaneous acrosome reaction process. Previously some authors related the redox balance and acrosome reaction induced by the calcium ionophore A23187 or lysophosphatidylcholine (de Lamirande *et al.*, 1998; O'Flaherty *et al.*, 2005).

Previous studies in human and porcine spermatozoa have shown that freezing and thawing induce important changes in the sperm chromatin resulting in greater compactness (Hamamah *et al.*, 1990; Cordova *et al.*, 2002; Gadea *et al.*, 2005b). Thus, chromatin condensation and stability may be critical factors to consider when using frozen semen (Madrid-Bury *et al.*, 2005). In this study, we observed a lower condensation of the nucleus of the spermatozoa treated by GSH. This fact would be related to a lower condensation in the thawing process, because of the antioxidant effect of GSH against the high level of ROS generated during the thawing process and a direct decondensation effect induced by GSH as previously demonstrated in the bull (Delgado *et al.*, 2001). Concerning this mechanism, interaction of sperm chromatin with ROS such as H_2O_2 could lead to sulphydryl group oxidation (Pirie, 1931) and to an increase in disulfide bond cross linking and higher chromatin compactness. GSH would block this putative effect of H_2O_2 leading to a lower degree of compactness.

The lower DNA damage observed following supplementation of the thawing medium with GSH suggests that ROS generation and redox balance are the most important factors responsible for the disruption of the condensation and stability of sperm chromatin after cryopreservation. Muratori *et al.* (2003) showed that inhibition of the activity of the enzymatic ROS scavenger, GPX, markedly increased DNA fragmentation. Recently, Arabi (2005) also reported a decrease in GSH content and an increase in lipid peroxidation and DNA damage (measured by COMET) in bull spermatozoa exposed to mercury-induced oxidative stress. The increase in DNA fragmentation associated with ROS generation in vitro by xanthine–xanthine oxidase (X-XO) in equine spermatozoa was prevented by the addition of GSH (10 mM) (Baumber *et al.*, 2003).

The relationship between chromatin stability and the fertility has already been reported in the bull. Ballachey *et al.* (1987) showed a significant relationship between deviation of αt and fertility rankings in bulls, while Januskauskas *et al.* (2003) found a significant inverse relationship between COMPTαt and fertility. Both parameters, now named SD DFI and DFI, were significantly reduced in this study when GSH was added to the thawing medium.

With regard to the capacity for penetration and the in vitro production of embryos, Kim *et al.* (1999) demonstrated a dose-dependent effect of GSH and other thiol group-containing compounds on oocyte penetration and embryo production in IVF when added to the fertilization medium. Addition of 1 mM GSH during IVF enhanced the blastocyst rate but had no effect on the rate of penetration, polyspermy, male pronuclear formation or embryo cleavage rate. On the other hand, when 10 mM GSH was used, the cleavage and blastocysts rates decreased. Luvoni *et al.* (1996) demonstrated a positive effect on blastocyst development when 1 mM GSH was added during the in vitro embryo culture, while addition of GSH during IVM and IVF had no effect.

Nevertheless, the effect of supplementation of the thawing medium with GSH in IVF and embryo production has not been addressed before. In this study we showed that incubation of spermatozoa with GSH for 30 min resulted in an improvement in sperm function as measured by the in vitro penetration of oocytes and embryo number and quality. Two main hypotheses can be provided to explain this improvement. (i) GSH supplementation increases the number of viable, low lipid disorder (non-capacitated) spermatozoa that have the ability to penetrate the oocyte; (ii) GSH supplementation improves embryo and blastocyst cleavage rate. In vitro culture results in an increased level of ROS production (Luvoni *et al.*, 1996) that can cause a disruption of normal cell function by inactivation of proteins, peroxidation of cell membranes and DNA damage. It has been shown that high ROS levels exert a negative effect on bovine embryo development up to the blastocyst stage (Hashimoto *et al.*, 2000).

The results emerging from this study also show that GSH supplementation results in a decrease in chromatin condensation. This could be mediated by the reduction of disulfide bonds by GSH in the sperm nucleus. An increase in disulfide bonds in sperm chromatin results in hypercondensation and packing of the sperm DNA (Calvin & Bedford, 1971). In addition to being a reducing agent, GSH is also important for sperm chromatin decondensation following sperm penetration of the oocyte, the destabilization and the replacement of the protamines by the oocyte-derived histones and the sperm nucleus develop into the male pronucleus (Sutovsky & Schatten, 1997). The DNA damage induced by ROS generation could negatively affect the fertilization rate and early embryonic development. Recently, Fatehi *et al.* (2006) have shown that although DNA damage in bovine sperm does not block fertilization or early embryo development, it induces apoptosis after the first embryo cleavage.

Finally, we would like to point out that the type of bull from which the sperm was obtained affected most of the

parameters assessed in this study. This could be related to differences in the levels of ROS produced by the spermatozoa of particular bulls, as previously suggested by Kim *et al.* (1999) and as also shown in this study. Therefore, it can be concluded that the overall effect of GSH supplementation of the thawing extender depends mainly on the balance between free radical generating and scavenging systems.

In conclusion, GSH appears to play an important role in the sperm antioxidant defence strategy. Addition of GSH to the thawing extender could be of significant benefit in improving the function and fertilizing ability of frozen bull spermatozoa.

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