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Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders ☆

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ABSTRACT

In this study, total glutathione content was determined in human spermatozoa before and after cryopreservation. Total GSH in fresh semen was 4.47 ± 0.46 nmol/10^8 cells. Following semen cryopreservation, GSH decreased to 1.62 ± 0.13 nmol/10^8 cells, a 64% reduction ($p < 0.01$). This decrease in GSH content was associated with a decrease in sperm progressive motility (68% of reduction, $p < 0.01$). Addition of 1 mM GSH to the freezing extender increased the percentage of total motility and sperm viability. It also modified the motility pattern measured by CASA with changes in the straight-line and average path velocities and wobble of the curvilinear trajectory. Addition of GSH to the freezing media reduced spermatozoa ROS levels and increased the level of sulfhydryl groups on membrane proteins. Nevertheless, no effect of GSH addition on lipid membrane disorder or chromatin condensation was detected. Addition of 1 or 5 mM GSH to the thawing media increased the percentage of motile and progressively motile spermatozoa, but no effect on viability was detected. In conclusion, the antioxidant defensive capacity of the GSH is severely altered by the freeze–thawing process. The addition of GSH to the freezing and thawing extender could be of partial and limited benefit in improving the function of frozen human spermatozoa.

Introduction

Cryopreservation of human semen represents a useful therapeutic option in the management of infertility with several indications [13,44,9]. However, during cryopreservation spermatozoa are exposed to physical and chemical stress that results in adverse changes in membrane lipid composition, sperm motility, viability and acrosome status [43,7,48,29]. All these changes reduce the fertilizing ability of human spermatozoa after cryopreservation [13,44].

Mechanisms of cryodamage to human spermatozoa are thought to be multifactorial. Some authors have been reported direct physical damage to sperm structure or function during cell freezing related to ice formation and high osmotic pressure during freezing [13,39]. Cold shock during sperm cryopreservation is associated with oxidative stress and reactive oxygen species (ROS) generation [55]. ROS-induced damage to spermatozoa is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids (PUFAs), leading to lipid peroxidation [3]. To counteract the harmful effects of ROS, spermatozoa and seminal plasma possess a number of antioxidant systems that scavenge ROS and prevent internal cellular damage. Enzymatic antioxidant defence mechanisms in seminal plasma and spermatozoa include the glutathione peroxidase/reductase system, superoxide dismutase, and catalase [32,8,5]. Non-enzymatic anti-oxidants include reduced glutathione (GSH), urate, ascorbic acid, vitamin E, carotenoids, ubiquinones, taurine and hypotaurine [5,35,52].

Oxidative stress may be defined as any imbalance between pro-oxidants and anti-oxidants in which the former prevail and produce a free radical cascade leading to a lipoperoxidative process. The effects of lipid peroxidation include irreversible loss of motility, leakage of intracellular enzymes, damage to sperm DNA [56], and deficiencies in oocyte penetration and sperm-oocyte fusion [4].

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide ubiquitously distributed in living cells. It plays an important role in the intracellular defence mechanism against oxidative stress. Glutathione peroxidase uses GSH to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor. GSH content has been reported in mammalian spermatozoa [5,35,20]. Previous studies have analyzed the relationship between GSH content in human spermatozoa and seminal plasma and infertility [42,46,25,10]. However, to our knowledge, this is the first report where a reduction in GSH

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content in human spermatozoa induced by the freezing procedures is described, with similar findings previously reported in domestic animals such as bull [11,50] or boar [20].

Once it is known that freezing induces a decrease in the antioxidant system and that increased ROS levels are present after cryopreservation [55,6,34], it is reasonable to explore the use of antioxidant strategies in an attempt to overcome cryodamage related to this oxidative stress. However, limited information exists about the use of antioxidant in the cryoprotection of human spermatozoa. Some antioxidant compounds (ascorbate, genistein, catalase and vitamin E) have been used for improving semen freezing protocols [37,53,51].

Our group has previously reported the effect of addition of GSH to the freezing and thawing extender on boar sperm cryosurvival [20,22,23], as well as on bovine, ovine and goat spermatozoa cryosurvival [24]. The only reference to this approach in human spermatozoa [54] reported that the addition of 5 mM of GSH to a glycerol-based cryoprotectant freezing media increased the DNA integrity of cryopreserved human spermatozoa, but failed to reduce the lipid peroxidation or to increase motility. Therefore, more thorough studies are needed to elucidate what changes in human sperm function take place during cryopreservation and the mechanism(s) by which GSH exerts its effect(s).

The main objectives of this study were (i) to determine GSH content in human spermatozoa before and after cryopreservation; and (ii) to assess the effect of GSH supplementation of freezing and thawing extender on semen parameters.

Material and methods

Sampling collection and preparation

This study was developed following institutional approval from the Instituto Valenciano de Infertilidad (IVI) and after obtaining informed consent from men who visited our centre for infertility screening.

Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction at 37 °C with 5% CO_2 in air, semen samples were examined for volume, sperm concentration, pH, morphology and motility according to the World Health Organization guidelines [57].

Cryopreservation of semen samples

Semen samples were frozen in pellets on the surface of dry ice using a glycerol-based cryoprotectant (Sperm Freezing Medium, MediCult, Jyllinsge, Denmark) with or without the addition of GSH (final concentration 1 mM GSH, experiment 2) as previously described [26,40] with slight modifications. Briefly, seminal plasma was removed by centrifugation (10 min at 400*g*) and spermatozoa were diluted (1:1 v/v) by slow addition of a glycerol-based cryoprotectant medium with continuous shaking. The diluted sperm samples were cooled to 4 °C over a 90 min period and the sample was transferred to a dry ice surface, where small dimplets or dents (approximately 50 μL) were engraved. After 2 min (when semen was totally frozen), all the pellets formed were transferred into a cryotube immersed into liquid nitrogen and then immediately stored in semen banks for long-term storage.

The sperm freezing protocol has been employed since 1996, when the IVI clinic group reported the first full-term pregnancy achieved with frozen sperm obtained by testicular sperm extraction (TESE). To date thousands of live births have been achieved in IVI facilities [41]. Cryopreservation in pellets offers some advantages over other packaging systems. These pellets have a higher surface-to-volume ratio with important implications for cooling, freezing and thawing rates of the semen [13]. Pellet-freezing of domestic animal semen generally produces the best results, but there are commercial pressures to use other methods of packaging [38].

Thawing process

In experiment 1 and 2, aliquots of the samples were thawed by transferring 3 pellets per sample (total 150 μL) to new tubes that were immersed in room temperature water and thawing for 10 min, after which the samples were treated for 10 min at 37 °C. The samples were then washed and resuspended in phosphate buffered saline (PBS) medium for analysis.

In experiment 3, aliquots of the samples were thawed in PBS medium supplemented with or without GSH (final concentration 0, 1 and 5 μM GSH). Briefly, 3 pellets per sample (total 150 μL) were transferred to new tubes containing 300 μL of the thawing media (PBS supplemented with GSH or not) and maintained at 37 °C for 30 min before analysis. The samples were then washed and resuspended in phosphate buffered saline (PBS) medium for analysis.

Assessment of seminal parameters

Motion parameters

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, Microptic, Barcelona, Spain). The CASA-derived motility characteristics studied were percentage of motility and 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 aliquot of the sperm sample was placed on a warmed (37 °C) slide (Superfrost Menzel-Glaser, Braunschweig, Germany) 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, imagines obtained at 200× magnification in a contrast phase microscope. Spermatozoa with a VAP < 10 μm/s were considered immotile. A minimum of 5 fields per sample were evaluated, counting a minimum of 200 spermatozoa per sub-sample.

Determination of GSH content of spermatozoa

Fresh and frozen–thawed semen samples were centrifuged at 1000*g* for 5 min at room temperature and the resulting pellet resuspended in saline solution and centrifuged again. The supernatant was discarded, the pellet resuspended in saline solution, and sperm concentration adjusted to $1–5 \times 10^8$ spermatozoa/mL. To release intracellular GSH, the sperm cells were lysed following three cycles of rapid cooling in liquid nitrogen and thawing at 37 °C. The resulting suspensions were centrifuged at 7000*g* for 10 min in order to remove membrane fragments.

Glutathione content was determined using a modified coupled optical test system [20]. In this system glutathione is oxidized by 5,5-dithiobis-(2-nitrobenzoic acid (DTNB) and then reduced by glutathione-reductase from *Saccharomyces* cerevisiae (Sigma G-3664) with NADPH as the hydrogen donor. During the oxidation of glutathione by DTNB 2-nitro-5 thiobenzoeic acid is formed, which can be detected photometrically by a change of absorption at 412 nm. The total glutathione content (oxidized glutathione (GSSG) and reduced glutathione (GSH) is calculated according to a standard curve constructed using glutathione (0.1–10 mM) in

physiologic saline. Results were expressed as GSH equivalents (nmol GSH/10^8 cells) as previously reported [42].

Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. Data from 10,000 events per sample were collected in list mode, and 4 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 viability. Sperm viability was evaluated by staining with a fluorescent propidium iodide (PI). Seminal samples (1 mL of sperm suspension with 10^6 cells) were incubated with 5 μL of PI stock solution (500 mg/mL) at room temperature for 10 min in the dark. Red PI fluorescence was collected with a FL3 sensor using a 650-nm band-pass filter. Cells were classified into two categories: low PI fluorescence (viable) and high PI (unviable cells).

Assessment of lipid packing disorder. To detect increases in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 [30]. Stock solution of M-540 (1 mM, Sigma) and Yo-Pro 1 (25 μM, Molecular Probes) in DMSO, were prepared. For each 1 ml diluted semen sample, 2.7 μl M540 stock solution was added (final concentration of 2.7 μM) and 1 μl of Yo-Pro (25 nM final concentration). M 540 fluorescence was collected with a FL2 sensor using a 575 nm band-pass filter and YO-PRO 1 with a FL-1 sensor using a 525 nm band-pass filter. Cells were classified in three categories: low merocyanine fluorescence (viable, low disorder), high merocyanine fluorescence (viable, high disorder) or Yo-Pro-1 positive (dead).

Levels of reactive oxygen species. Levels of reactive oxygen species (ROS) were measured by incubating the spermatozoa in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (0.5 μM in PBS) for 30, 60, 90 and 120 min at 37 °C stored in the dark prior to flow cytometric analysis [23]. 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′-dichlorofluorescein 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 this was used as index of ROS generation.

Sulfhydryl groups of proteins on the sperm surface. The sulfhydryl groups of proteins on the sperm surface were evaluated by staining with fluorescent 5-iodoacetamidofluoresceine (5-IAF) [23]. Seminal samples were incubated in the presence of 5-IAF (final solution 2.5 μM in PBS), at room temperature for 10 min. 5 IAF fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter. Cells were classified into two categories according to the intensity of green fluorescence.

Determination of chromatin condensation. Sperm chromatin was stained with propidium iodide for the determination of sperm chromatin condensation [22]. Thawed samples were centrifuged (1200 g × 3 min) and the pellet resuspended in a solution of ethanol and PBS (70/30 v/v) for 30 min for sperm membrane permeabilization before being stored at -80C until analysis. The samples were then centrifuged, the supernatant discarded and the pellet resuspended in a propidium iodide solution (PI, 10 mg/ml) in PBS. Samples were maintained in the dark 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 this was used as index of the state of chromatin condensation, as this is directly related to PI uptake by DNA.

Statistical analysis

Data are expressed as the mean ± SEM and analysed by ANOVA, considering the specific sperm treatment (Freezing procedure in experiment 1 and GSH addition to media in experiments 2 and 3) and donors as the main variables. When ANOVA revealed a significant effect, values were compared by the least significant difference pair wise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at $p < 0.05$.

Variation in sperm parameters in samples from different donors, and in samples from the same donor collected over time, has long been known to be considerable [8]. Because of that, the model used in this study was a two-way ANOVA, considering the specific sperm treatment and the donor as the main variables (two fully factorial). We select this two-factorial model because it is important to see the effect of the treatment, but also whether most of the donors have the same pattern in the different treatment groups, and this is easy to check by the evaluation of the interaction between treatment and donor.

Linear regression analysis was used to further investigate relationships between semen parameters. Pearson correlation was calculated for motility and GSH content before and after freezing ($n = 34$). Later, Pearson correlation was evaluated between GSH content in fresh semen and other seminal parameters in fresh semen ($n = 34$).

Results

Experiment 1: determination of GSH content of fresh and cryopreserved spermatozoa and the relationship with progressive motility

Sperm motility and GSH content (GSH equivalents) were determined in spermatozoa from 34 seminal samples before and after cryopreservation. Data for the sperm parameters before freezing are shown in Table 1.

In all the samples the values for GSH and motility was lower in frozen semen than fresh semen, however, there was great variation between samples in the degree of reduction of GSH and progressive motility (Fig 1a and b). GSH content (GSH equivalent) in fresh semen was 4.47 ± 0.46 nmol GSH/10^8 cells. After freezing, there was a significant reduction in GSH content to 1.62 ± 0.13 nmol GSH/10^8 cells ($p < 0.01$), representing a 64% of reduction in GSH content. Also, a reduction in sperm progressive motility in the frozen–thawed samples was observed compared with the fresh samples (motility decreased from 52.41 ± 2.98% to 16.41 ± 1.60%, $p < 0.01$) a reduction in progressive motility of almost 68%.

Table 1
Semen parameters of the cases studied in experiment 1.

Parameter	Mean ± SEM
Progressive motility (%)	52.41 ± 2.98
Sperm concentration (10^6/ml)	72.07 ± 6.28
Volume (ml)	3.65 ± 0.23
Total cells in ejaculate (10^6)	263.78 ± 31.05
pH	8.20 ± 0.05
Normal morphology (%)	10.76 ± 1.17
GSH nm/ml	2.56 ± 0.23
GSH nm/10^8	4.47 ± 0.46

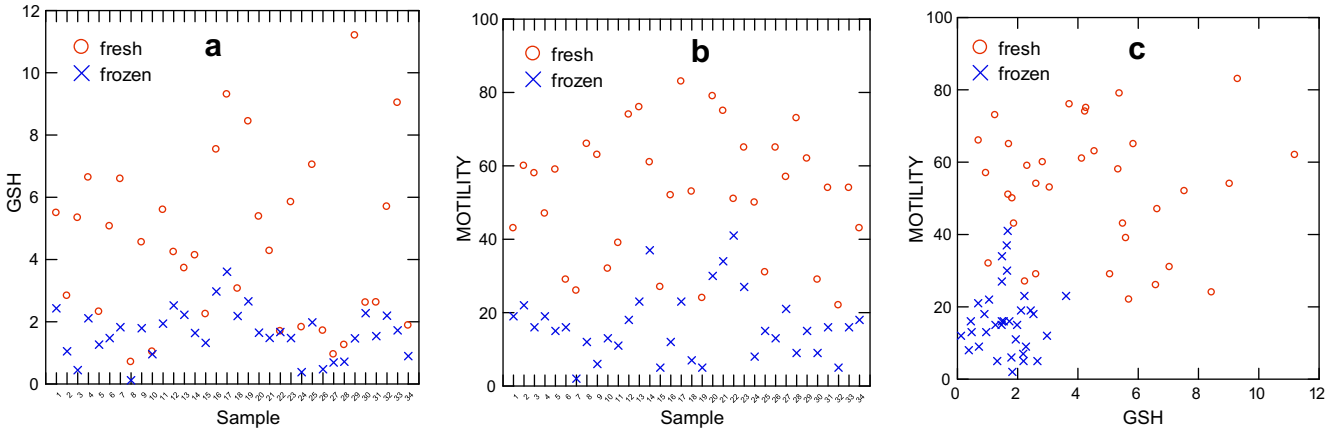

Fig. 1. Effect of the freezing–thawing procedure on the GSH content (nmol/10^8 spermatozoa) and motility in 34 human semen samples. (a) GSH content in fresh (4.47 ± 0.46 nmol GSH/10^8 cells) and frozen–thawed samples (1.62 ± 0.13 nmol GSH/10^8 cells, $p < 0.01$). (b) Progressive Motility (% grade a + b) in fresh (52.41 ± 2.98%) and frozen–thawed samples (16.41 ± 1.60%, $p < 0.01$). (c) Relationship between GSH content (nmol/10^8 spermatozoa) and progressive motility (%).

Nevertheless, the GSH content was not directly related to motility. No significant correlation was found between GSH content and motility in fresh or frozen–thawed samples ($p > 0.05$, Fig 1c) nor was any relationship found between the reduction of GSH content and reduction of motility induced by freezing ($p > 0.05$).

No significant relationship ($p > 0.05$) was found between GSH content in fresh semen and the seminal parameters evaluated (concentration, volume, pH, motility and morphology).

Experiment 2: effect of GSH supplementation of the freezing extender on the viability and functionality of cryopreserved spermatozoa

Ejaculates from 24 men were processed with or without addition of 1 mM GSH to the freezing extender. We selected this concentration of GSH based in our previous experience with boar and bull spermatozoa [24,21]. Different sperm parameters were evaluated: (i) Motility and motion parameters by CASA; (ii) Viability; (iii) Lipid membrane disorder status (iv) Reactive oxygen formation; (v) Sulfhydryl groups of proteins from the sperm surface and (vi) Chromatin condensation.

Effect of the addition of GSH to the freezing media on motility and sperm viability

The addition of GSH to the freezing extender has a significant positive effect on the motility parameters evaluated. The total percentage of motile spermatozoa, measured by CASA was higher in the GSH group than in the control group ($p < 0.01$, Table 2). The pattern of movement was also slightly modified by GSH addition to the freezing media with lower VSL, VAP and WOB than control ($p < 0.05$, Table 2). The addition of GSH to the freezing media had a limited effect on the sperm viability with higher values in GSH than in control group (Table 1, $p < 0.05$).

Effect of the addition of GSH to the freezing media on membrane lipid disorder, sulfhydryl groups of proteins, ROS generation and chromatin condensation

The addition of GSH (1 mM) to the freezing media had a significant effect on the reducing conditions of the thiol groups in the membrane proteins. With the use of 5-IAF we detected in the GSH group a higher proportion of cells with sulphydryl groups in the membrane proteins than in the control group (61.49 ± 1.35 vs. 48.36 ± 1.06%, $p < 0.01$). However, we did not observe any effect on membrane lipid disorder. In the control group 43.79 ± 1.17% of the sperm were viable with low lipid disorder vs. 46.09 ± 1.23 in the GSH group ($p = 0.11$), and 9.08 ± 0.62% in the control were viable sperm with high lipid membrane disorder vs. 8.45 ± 0.66% in the GSH group ($p = 0.49$).

The level of ROS during the 120 min incubation period was significantly reduced following the addition of GSH to the freezing medium ($p < 0.01$, Fig. 2). Mean values of ROS levels for the GSH groups were reduced to close to 30% of the values obtained in the control group (control: 23.37 ± 1.85 vs. GSH: 15.93 ± 0.95 arbitrary fluorescence units).

Table 2
Motility parameters measured by CASA. Human spermatozoa from 24 men frozen in freezing medium without GSH (control) or supplemented with 1 mM GSH′. Pooled data from 24 seminal samples, 3 replicates.

Freezing media	Viability (%)	Motility (%)	Progressive motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Control	33.15 ± 1.38[a]	38.53 ± 1.40[a]	21.87 ± 0.83	49.06 ± 1.12	24.57 ± 0.67[a]	32.48 ± 0.78[a]	49.48 ± 0.74	73.30 ± 0.61	65.33 ± 0.55[a]	2.09 ± 0.06	6.58 ± 0.12
1 mM GSH	35.51 ± 1.57[b]	40.07 ± 1.47[b]	22.01±0.77	46.46 ± 1.09	22.93 ± 0.66[b]	30.63 ± 0.76[b]	48.36 ± 0.75	72.01 ± 0.66	64.74 ± 0.58[b]	2.08 ± 0.06	6.54 ± 0.13

p-values	Viability DCF (%)	Motility (%)	Progressive motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
Source variation											
GSH	0.03	0.01	0.69	0.10	0.03	0.03	0.07	0.10	0.04	0.54	0.41
Man	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
GSH ∗ man	0.04	<0.01	0.10	0.52	0.06	0.36	<0.01	<0.01	<0.01	0.38	<0.01

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; BCF, beat cross-frequency.
[a,b]Significant difference vs. control ($p < 0.05$).

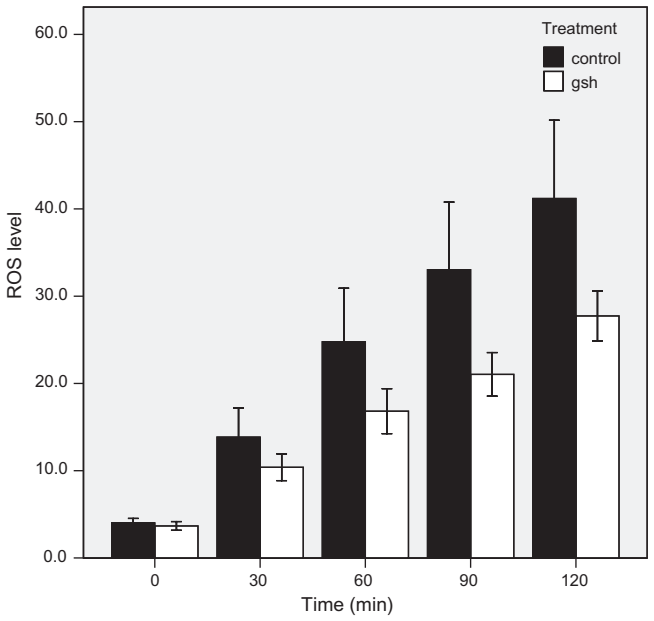

Fig. 2. ROS level (mean channel of fluorescence). Human spermatozoa from 24 men frozen in freezing medium without GSH (control) or supplemented with 1 mM GSH′. $p < 0.05$.

Chromatin condensation was evaluated and the addition of GSH had no effect on this parameter, presenting values of 20.78 ± 1.52 (mean channel of fluorescence) for control vs. 19.70 ± 1.39 for GSH group ($p = 0.88$).

Experiment 3: effect of GSH supplementation of the thawing extender on the motility parameters and viability of cryopreserved spermatozoa

Ejaculates from 8 men were processed with or without addition of 1 and 5 mM GSH to the freezing extender. Motion parameters and viability were evaluated.

The addition of GSH (1 or 5 mM) to the thawing extender has a significant effect on the motility parameters evaluated with significantly higher total and progressive motility measured by CASA than control (Table 3, $p < 0.01$). However, the addition of GSH has no effect on viability or on the pattern of motility ($p > 0.05$, table 3). A dose effect was found for VAP, only with higher values in the 1 mM than in the 5 mM GSH group (Table 3).

Discussion

Freezing is associated with damage to sperm function affecting those processes required for successful in vivo and in vitro fertilization of the oocyte. Mechanisms for cryodamage to human spermatozoa are thought to be multifactorial, but excessive ROS production during freezing and thawing and oxidative stress have been previously demonstrated to be significant contributing factors [55,53]. During freezing, two important processes have been reported: (i) production of reactive oxygen species (ROS) [55,14] that can induce changes in membrane function and structure; and (ii) an alteration in antioxidant defence systems [11,34].

Glutathione is the major non-protein thiol compound in mammalian cells. GSH participates in a number of cell functions including amino acid transport, DNA and protein synthesis, reduction of disulfide bonds and protection against oxidative stress. The sulphydryl groups of GSH have been shown to confer protection against cell damage by oxidants, electrophiles and free radicals [1]. The values obtained in fresh human spermatozoa for GSH content, expressed as GSH equivalents, semen in this study (4.77 ± 0.68 nmol/10^8 cells) are in good agreement with previous reports ranging from 3.49 to 6.7 ± 0.4 nmol GSH/10^8 cells [5,42]. Nevertheless, it is noteworthy that comparisons between different studies are often difficult to interpret due to differences in the method of analysis and sperm processing protocols [17].

In our results we detected a lack of significant correlation between GSH and seminal parameters in fresh semen as has been previously reported [25]. Some authors have suggested that ROS generation is an independent marker of male factor infertility, irrespective of whether these patients have normal or abnormal semen parameters [2], and this concept could be extended to spermatozoa antioxidant defense system.

This is the first study, to our knowledge, to describe a reduction in GSH content in human sperm induced by freezing procedures. In this study the percentage decrease in GSH content of human spermatozoa was 64%, while in boar the decrease was 32% [20] and in bull it was 58% or 78% [11,50]. Similarly, cryopreservation of human spermatozoa is associated with a reduction in sperm motility of close to 68%. These data confirm previous studies that reported damage components of the antioxidant system such as SOD during cryopreservation [34].

During thawing, there is an increase in the levels of ROS, with an increase in superoxide anion production due to a decrease in SOD activity and a decrease in GSH content due to a decrease in GRD activity and to an increase in GSH oxidation by hydrogen peroxide. A decrease in intracellular GSH and an increase in ROS production during cryopreservation could be one of the concomitant causes of low viability in thawed spermatozoa. Previously Lasso et al. [34] showed that the reduction in superoxide dismutase (SOD) activity in human sperm cells as a result of cryopreservation was primarily due to membrane damage, such that cytosolic SOD leaked from the cells. There is a high probability that GSH loss occurs by the same mechanism, but further studies must be carried out.

Table 3
Motility parameters measured by CASA. Frozen human spermatozoa thawed in HTF-BSA medium without GSH (control) or supplemented with 1 mM or 5 mM GSH′.

Treatment	Viability (%)	Motility (%)	Progressive Motility (%)	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
0 mM	39.42 ± 0.78	39.68 ± 0.76[a]	25.45 ± 0.75[a]	49.76 ± 1.09	20.00 ± 0.53	29.93 ± 0.65[ab]	40.56 ± 0.80	65.60 ± 0.73	59.50 ± 0.60	2.01 ± 0.09	5.19 ± 0.16
1 mM	39.27 ± 1.05	47.10 ± 0.74[b]	32.22 ± 0.76[b]	50.62 ± 1.00	20.80 ± 0.49	30.27 ± 0.58[a]	41.61 ± 0.82	66.97 ± 0.70	60.27 ± 0.63	2.13 ± 0.09	5.17 ± 0.14
5 mM	39.11 ± 0.92	48.44 ± 0.70[b]	33.05 ± 0.74[b]	49.60 ± 1.02	19.28 ± 0.41	28.24 ± 0.54[b]	40.20 ± 0.77	66.81 ± 0.63	58.66 ± 0.61	1.91 ± 0.07	5.07 ± 0.13

p values	Viability (%)	Motility (%)	Progressive Motility (%)	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Source variation											
GSH	0.87	<0.01	<0.01	0.65	0.08	0.02	0.45	0.21	0.18	0.15	0.85
Man	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.08	<0.01
GSH ∗ man	<0.01	0.03	0.03	0.18	0.86	0.16	0.20	0.11	0.53	0.54	0.10

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; BCF, beat cross-frequency.
[a,b]Significant difference vs. control ($p < 0.05$).

Once we detected a decrease in the GSH content of human frozen–thawed spermatozoa associated with a decrease in sperm functionality, we assessed the effect of GSH supplementation of freezing and thawing extenders on avoiding oxidative stress and protecting the spermatozoa against damage. GSH provided exogenously is used by glutathione S-transferases to provide protection to sperm during exposure to H_2O_2 or to products of lipid peroxidation [31]. Addition of GSH to the freezing and thawing extenders would be expected to improve the quality and fertilizing ability of frozen–thawed spermatozoa, since addition of GSH has been shown to help maintain sperm motility in some domestic animals [22,24,12,18,19] and to protect sperm against oxidative damage [5].

To our knowledge, limited information is available about the GSH addition to the extender for fresh semen and sperm preparation techniques and the results are still controversial. One report has shown that addition of GSH to the extender had no effect on progressive motility in fresh spermatozoa [16], on the contrary other studies reported that the addition of GSH prevented the fall in sperm motility and acrosome reaction after 24 h incubation or increased the recovery of motile spermatozoa when it is added during the centrifugation process [27,45]. There are also few studies concerning the addition of GSH to human freezing extender. Varghese et al. [54] reported that the addition of 5 mM of GSH to a glycerol-based cryoprotectant freezing media increased the DNA integrity of cryopreserved human spermatozoa, but failed to reduce lipid peroxidation or to increase motility.

The results of the present study show that addition of 1 mM GSH to the freezing media significantly improves sperm motility, viability and also results in changes in the pattern of sperm movement with a decrease in the straight-line and average path velocities, and also decreases in the values for wobble of the curvilinear trajectory and amplitude of lateral head displacement. In this study, a significant decrease in ROS levels was also found when GSH was present in the freezing medium. GSH could be an important regulator of the scavenging system and one of the most important non-enzymatic anti-oxidants in spermatozoa [5]. If GSH is acting in concert with GPX to decrease the steady-state level of hydrogen peroxide and membrane lipid peroxides in human sperm during thawing, an increase in sperm motility and viability should also be expected.

Diverse studies have shown the importance of surface thiol groups in maintaining motility in the sperm [49] and that blocking surface thiol groups with cobaltous results in a severe loss of motility in the spermatozoa [33]. Cryopreservation of the spermatozoa has also an effect on the surface thiol groups, reducing the percentage of cells with sulfhydryl groups [15]. In our study, the addition of GSH increased the percentage of cells with sulfhydryl groups, and this could be related to an increase in motility.

Previous studies have shown that freezing and thawing induce important changes in the sperm membrane lipid disorder and in chromatin resulting in greater compactness [23,24,28]. In this study, we did not detect differences between experimental and control groups for these sperm parameters. The use of techniques for studying sperm DNA fragmentation (TUNEL, COMET, SCSA, etc.) could help increase understanding of the possible protective role of GSH in nuclear structure and function. Recently, we have reported that the addition of anti-oxidants (genistein) to the cryoprotectant had a significant protective effect on sperm DNA as a biomarker of oxidative stress and resulted in a significant increase in motility and viability [37]. This result was confirmed by other authors who concluded that human sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation, rather than the activation of caspases and apoptosis [53].

In this study, no significant effect on viability was found after addition of GSH to the thawing medium. In contrast, viability significantly improved when GSH was added to the freezing media. 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 min) than when it was added to the thawing media (30 min). Perhaps 30 min was an insufficient contact time to produce a significant effect on the viability or it is possible that the damaging effect on sperm membrane is produced mainly before the dilution in the thawing extender. 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. Further studies must be done to explore the effect of GSH on the thawing extender.

In conclusion, GSH appears to play a role in the sperm antioxidant defence strategy. The GSH content is altered by the freezing–thawing process, and the addition of GSH to the freezing extender could be of benefit in controlling the ROS and improving the function of frozen human spermatozoa. However, only a partial aspect of the antioxidant system has been analyzed and further studies must be done to evaluate the clinical application of anti-oxidants for improving the cryopreservation process and investigate the effect of combinations of antioxidant compounds [47,36].

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