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Research Article
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Research Article

Depletion of thiols leads to redox deregulation, production of 4-hydroxinonenal and sperm senescence: a possible role for GSH regulation in spermatozoa†

Cristina Ortega-Ferrusola [1,‡], Patricia Martin Muñoz[2,‡], Jose Manuel Ortiz-Rodriguez [2,‡], Luis Anel-López [1], Carolina Balao da Silva [3], Mercedes Álvarez[1], Paulino de Paz [1], Jose Antonio Tapia [2], Luis Anel [1], Antonio Silva- Rodríguez[4], Robert J. Aitken[4], M. Cruz Gil [2], Zamira Gibb[5] and Fernando J. Peña [2,*]

[1]Reproduction and Obstetrics Department of Animal Medicine and Surgery, University of León, Spain; [2]Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain; [3]Escola Superior Agraria de Elvas, Elvas, Portugal; [4]Facility of Innovation and Analysis in Animal Source Foodstuffs, University of Extremadura, Cáceres, Spain and [5]Priority Research Center in Reproductive Science, School of Environmental and Life Sciences, University of Newcastle, Callaghan, New South Wales, Australia

*__Correspondence:__ Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine, University of Extremadura, Avd de la Universidad s/n, 10003 Cáceres, Spain. Tel: +34 927 257167; Fax: +34 927 257102; E-mail fjuanpvega@unex.es

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‡COF, PMM, and JMO-R contributed equally to this paper.
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Abstract

We hypothesized that thiols and particularly glutathione (GSH) are essential for the regulation of stallion sperm functionality. To test this hypothesis, we initially investigated the relationship between sperm function and GSH content, revealing highly significant correlations between GSH, sperm viability, motility, and velocity parameters ($P < 0.001$). Furthermore, the deleterious effects of GSH depletion using menadione and 1,3 dimethoxy 1,4, naphtoquinone (DMNQ) were able to be prevented by the addition of cysteine, but no other antioxidant. Pre-incubation with cysteine prevented menadione and DMNQ induced damage to sperm membranes after 1 h ($P < 0.001$; $P < 0.05$) and after 3 h of incubation ($P < 0.001$, $P < 0.05$). Pre-incubation with cysteine ameliorated both the menadione- and DMNQ-induced increase in 4-hydroxynonenal ($P < 0.001$). As cysteine is a precursor of GSH, we hypothesized that stallion spermatozoa are able to synthesize this tripeptide using

exogenous cysteine. To test this hypothesis, we investigated the presence of two enzymes required to synthesize GSH (GSH and GCLC) and using western blotting and immunocytochemistry we detected both enzymes in stallion spermatozoa. The inhibition of GCLC reduced the recovery of GSH by addition of cysteine after depletion, suggesting that stallion spermatozoa may use exogenous cysteine to regulate GSH. Other findings supporting this hypothesis were changes in sperm functionality after BSO treatment and changes in GSH and GSSG validated using HPLC-MS, showing that BSO prevented the increase in GSH in the presence of cysteine, although important stallion to stallion variability occurred and suggested differences in expression of glutamate cysteine ligase. Mean concentration of GSH in stallion spermatozoa was 8.2 $\pm$ 2.1 μM/10^9 spermatozoa, well above the nanomolar ranges per billion spermatozoa reported for other mammals.

Summary Sentence

Thiols regulate stallion sperm functionality.

Key words: stallion, sperm, GSH, flow cytometry, cysteine, ROS.

Introduction

The use of artificial insemination (AI) with transported semen plays an integral role to the equine breeding industry. Horses are selected on the basis of pedigree, conformation, and athletic performance, with little emphasis placed on the importance of reproductive fitness, as is the common practice in other domestic livestock species [1, 2]. The lack of pressure for the improvement of reproductive efficiency leads to a high male-to-male variability in sperm quality and high incidence of subfertility in stallions [3]. A better understanding of the molecular basis underpinning the reduced sperm quality of many stallions will expand the possibilities to treat stallion subfertility and improve the quality of semen for use in AI regimes, thereby paving the way for improved breeding efficiency and profitability [2]. Moreover, detailed molecular studies in stallion sperm constitute a good model for human spermatology since age-related sperm disfunctions also occur in horses [3]. Recent developments in our understanding of stallion sperm biology point to redox regulation and oxidative stress as areas of major interest [4–12]. Reactive oxygen species (ROS) were originally considered in view of their harmful effects on cells, but new evidences implicate ROS in cell fate decisions and signaling transduction pathways [13–17]. The mechanisms involved in ROS-dependent signaling include the reversible oxidation and reduction of specific amino acids, with crucial cysteine residues being the most frequent targets [13, 18, 19]. Stallion spermatozoa are particularly notable in this context because of their dependence on mitochondrial oxidative phosphorylation (OXPHOS) to meet the energy demands required by this highly specialized cell type [11, 20, 21]. Apparently paradoxical findings are explained by this dependence, including the observations that more fertile spermatozoa produce more ROS [11], that live highly motile spermatozoa produce high amounts of superoxide anion (O_2^-) [21], and that stallions showing good freezeability of their ejaculates also show more ROS activity [22]. Despite these beneficial associations with stallion sperm fertility and ROS production, it is clear that the unchecked production of ROS during sperm storage will eventually accelerate their demise [11]. In spite of the recent findings that challenge current dogma concerning the role of ROS in sperm biology, little is known of the molecular mechanisms ultimately involved in the redox regulation of this particular cell. While the concept of ROS as dangerous molecules for sperm function persists, redox homeostasis arises as the new paradigm, a phenomenon that depends on an adequate equilibrium between ROS production and ROS scavenging [14]. Sperm antioxidant defenses include enzymatic and nonenzymatic molecules that are present in both the seminal plasma and in the sperm cell itself. Arguably the most important of these nonenzymatic antioxidants is glutathione (GSH), a cysteine containing tripeptide thiol with well-demonstrated roles in the regulation of sperm function [12, 23, 24], with recent reports linking thiol content to improved stallion sperm functionality after cryopreservation [24]. GSH is considered as the major natural antioxidant protecting all cells from oxidative stress [25], and the availability of cysteine is the rate-limiting step in GSH synthesis [26–28]. In view of this evidence, we hypothesize that exhaustion of GSH occurs during the storage of stallion spermatozoa, leading to a state of oxidative stress and inevitable sperm demise. In addition, we hypothesize that stallion spermatozoa are able to synthesize GSH through the use of exogenous substrates which could prevent the loss of stallion sperm functionality during storage.

Material and methods

Reagents and media

LIVE/DEAD aqua [(Ex: 405 nm, Em: 525 nm), (Ref: L34957)], ThiolTracker Violet [(Ex: 404 nm, Em: 526 nm), (Ref: T10095)], and LIVE/DEAD Fixable Far Red Dead Cell Solution [(Ex: 633 nm, Em: 655 nm), (Ref: L10120)] were purchased from Molecular Probes (Leiden, The Netherlands). Anti-glutathione synthetase antibody [EPR6563] (Ref ab133592), anti-glutamate cysteine ligase GCLC antibody (Ref ab154770), anti-4 hydroxynonenal (4-HNE) antibody [HNEJ-2] (Ref: ab48506), goat anti-mouse IgG H&L (Alexa Fluor® 647) [(Ex: 652 nm, Em: 668 nm), (Ref: ab150115)], anti 8-iso-prostglandin F2α antibody (Ref: ab2280), goat anti-rabbit IgG H&L antibody (Alexa Fluor 405) (Ex 405, Em 488), and 1,3 dimethoxy 1,4, naphtoquinone (DMNQ ab145804) were purchased from Abcam (Cambridge, UK). L-Buthionine sulfoximide (BSO), L-cysteine, menadione, and all other chemicals were purchased from Sigma Aldrich (Madrid, Spain).

Semen collection and processing

Semen was collected from 11 Purebred Spanish horses (PRE) (at least three ejaculates each) individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. Stallions were maintained according to institutional and European animal care regulations (Law 6/2913 June 11 and European Directive 2010/63/EU), and semen was collected on a regular basis (two collections/week) throughout the 2015, 2016, and 2017 breeding seasons. All the procedures used in this study received the

approbation of the ethical committee of the University of Extremadura (Ref AGL2013–43211-R). Ejaculates were collected using a prewarmed, lubricated Missouri model artificial vagina with an in-line filter to eliminate the gel fraction and debris. After collection, the semen was immediately transported to the laboratory for evaluation and processing. The ejaculate was extended 1:2 in INRA-96 diluent (IMV L'Aigle, France), centrifuged at 600 × g for 10 min to remove the bulk of the seminal plasma and resuspended at 40 × 10^6 spermatozoa/mL in BWW medium [10] supplemented with 1% polyvinyl alcohol (instead of BSA). All of the experiments followed a split sample design, with each ejaculate divided to yield control and treatment groups. After centrifugation, aliquots of stallion spermatozoa extended in BWW were incubated at 37°C for up to 9 h. Additional aliquots were supplemented with menadione, DMNQ (0 or 200 μM) or menadione, or DMNQ plus antioxidants (cysteine, GSH, GLU, TAU, melatonin, and PEN) and incubated in a water bath at 37°C for up to 3 h. In an additional set of experiments, stallion spermatozoa were incubated in presence of inhibitors of glutamate cysteine ligase (GCLC).

Sperm motility

Sperm motility and kinematic parameters were assessed using a computer-assisted sperm analysis (CASA) system (ISAS V.1.2, Proiser, Valencia, Spain). Semen was loaded in a Leja® chamber with 20 μm of depth (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 38°C. The analysis was based on an evaluation of 60 consecutive digitalized images obtained using a 10X negative phase-contrast objective (Olympus CX 41). At least three different fields were recorded to ensure that at least 200 spermatozoa were analyzed per sample. Spermatozoa with a VAP (average velocity) < 15 μm/s were considered immotile, spermatozoa with a VAP > 15 μm/s and < 35 μm/s were considered local motile spermatozoa, while only spermatozoa with a VAP > 35 μm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

Flow cytometry

Flow cytometric analyses were conducted using a MACSQuant Analyzer 10 (Miltenyi Biotech) flow cytometer equipped with three lasers emitting at 405, 488, and 635 nm and 10 photomultiplier tubes (V1 (excitation 405 nm, emission 450/50 nm), V2 (excitation 405 nm, emission 525/50 nm), B1 (excitation 488 nm, emission 525/50 nm), B2 (excitation 488 nm, emission 585/40 nm), B3 (excitation 488 nm, emission 655–730 nm (655LP + split 730), B4 (excitation 499 nm, emission 750 LP), R1 (excitation 635 nm, emission 655–730 nm (655LP + split 730), and R2 (excitation 635 nm, emission filter 750 LP). The system was controlled using the MACSQuantify software. The quadrants or regions that were applied to quantify the frequency of each sperm subpopulation differed depending on the particular assay being run. Debris was gated out by applying a gate around the sperm population on the forward and sideways light scatter dot plot, and a total of 50 000 events were recorded per sample. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. Compensation overlap was performed before each experiment. The data were analyzed using Flowjo V 10 Software (Ashland, OR, USA). Unstained, single stained, and fluorescence minus one (FMO) controls were used to determine compensations, positive and negative events, and set regions of interest.

Determination of lipid peroxidation (4-HNE positive cells)

The samples (1 mL) containing 5 × 10^6 spermatozoa in PBS were stained with 1 μL of LIVE/DEAD Fixable Aqua Dead Cell Solution. After thorough mixing, the samples were incubated at room temperature (22°C) for 30 min in the dark. Spermatozoa were then washed with PBS and fixed with 900 μL of 2% paraformaldehyde in PBS for 15 min at 4°C, washed in PBS, and stored in 0.1 M glycine in PBS at 4°C overnight. Spermatozoa (5 × 10^6/mL) in 1 mL of PBS were stained with 2 μL/mL of a stock solution of 0.1 mg/mL of anti 4-HNE primary antibody and incubated at RT in the dark for 30 min. The cells were then washed with PBS and stained with 2 μL/mL of secondary anti-mouse Alexa Fluor 647 antibody for 30 min in the dark at RT. Cells were then washed in PBS, and the samples were immediately analyzed via flow cytometry. The controls consisted of unstained, single stained, secondary only antibody staining, and FMO controls to properly set gates and compensations. The positive controls for 4-HNE were samples incubated for 1 h at 37°C in the presence of 800 μM SO_4Fe and 1.7 M of H_2O_2 (Sigma) previous to fixation to induce the Fenton reaction.

Flow cytometric determination of intracellular thiols in stallion spermatozoa

Intracellular free thiol content was determined using previously published protocols for somatic cells [29] and stallion spermatozoa [24]. According to the manufacturer ThiolTracker Violet is a brighter and robust intracellular thiol probe for reproducible GSH detection. Since reduced glutathione represents the majority of intracellular free thiols in the cell, ThiolTracker Violet can be used in estimating the cellular level of free reduced glutathione. The following stock solutions were prepared: LIVE/DEAD Fixable Far Red Dead Cell Stain kit (50 μL of DMSO in the LIVE/DEAD vial as per manufacturer's instructions) and ThiolTracker Violet (10 mM in DMSO). The samples (1 mL) containing 6–7 × 10^6 spermatozoa/mL in PBS were stained with 0.5 μL of LIVE/DEAD Fixable Far Red Dead Cell Solution (1:2000 final concentration) and 0.3 μL of ThiolTracker Violet (3 μM final concentration) at 38°C for 30 min in the dark. Spermatozoa were washed with PBS prior to flow cytometric analysis. The spermatozoa were gated out of debris based on forward and side scatter characteristics of the events and on the positivity of LIVE/DEAD staining. The positive controls were high-quality sperm obtained after single layer centrifugation treated with DTT 2 mM [30], and the negative controls were samples supplemented with 200 μM menadione. The spermatozoa were classified based in the intensity of the fluorescence signal (arbitrary fluorescence units) using histogram plots.

Detection of 8-ISO $PGF_{2\alpha}$

The following stock solutions were prepared: LIVE/DEAD Fixable Aqua Dead Cell Stain kit (50 μL of DMSO in the LIVE/DEAD vial) and MitoTracker Deep Red (500 μM in DMSO). The samples (1 mL) containing 6–7 × 10^6 spermatozoa/mL in PBS were stained with 1 μL of LIVE/DEAD Fixable Aqua Dead Cell Solution and 0.3 μL of MitoTracker Deep Red and incubated at RT for 30 min in the dark (final concentration 300 nM). Spermatozoa were then washed with PBS, fixed with 900 μL of 2% paraformaldehyde in PBS for 15 min at 4°C, and stored in 0.1M glycine in PBS at 4°C overnight. Spermatozoa were washed in PBS and incubated with 1 μL of anti-8 iso prostaglandin F2 alpha antibody (Ref: ab2280) for 30 min at

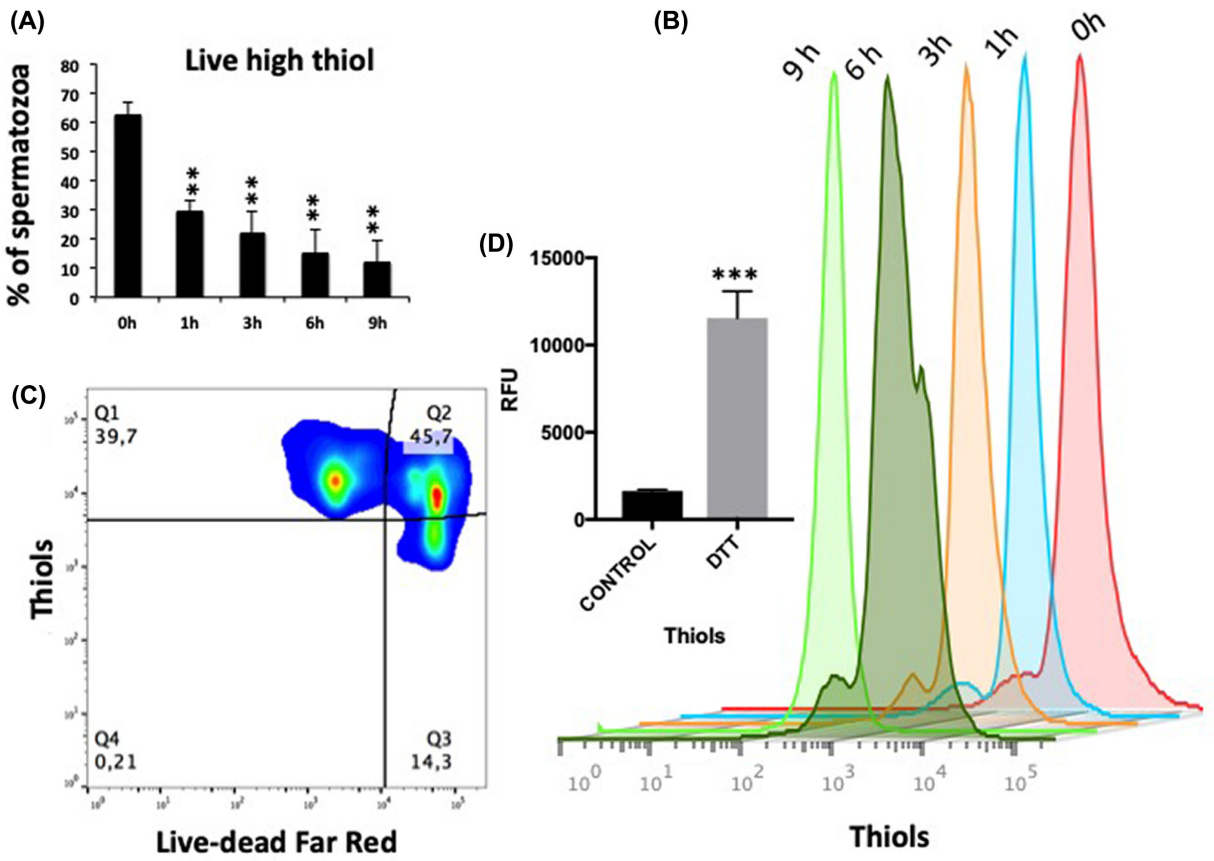

Figure 1. Changes in GSH content over the incubation period of stallion spermatozoa. Stallion sperm was incubated as described in the material and methods section up to 9 h. At the beginning of the incubation period and every hour, an aliquot was removed for GSH analysis. (A) Changes in the percentage of spermatozoa showing high GSH. (B) Representative overlay hystograms showing the loss of GSH over the incubation period. (C) Representative dot plot of the GSH determination, Q1 represents live spermatozoa with high GSH content, Q2 and Q3 represent dead spermatozoa. (D) Controls for the percentage of free thiols ** $P < 0.01$.

RT in the dark, washed in PBS, and stained with 4 μL/1 mL sperm suspension of goat anti-rabbit IgG H&L antibody (Alexa Fluor 405) at RT for 30 min in the dark. Finally, the samples were washed in PBS before flow cytometric analysis.

UHPLC-MS/MS

The reduced glutathione and oxidized glutathione were analyzed by UHPLC-MS/MS. A chromatography separation was performed using a Thermo Ultimate 3000 Ultra (Thermo Fisher Scientific, Waltham, MA, USA), and separations were carried out on a C18 column (100 × 21 mm; 1.7 μm, Bruker Intensity Solo, Billerica, MA, USA). Flow rate was 0.3 mL/min. The injection volume was 5 μL. For gradient elution, solvent A was 0.1% (v/v) formic acid in Milli Q water and solvent B was acetonitrile. At the beginning, 0% solvent B was maintained until 4 min. Solvent B was increased from 0 to 60% from 4 to 7 min and held at 80% for an additional 4 min. Then solvent B returned to the initial conditions up to 15 min.

The analytes eluted from UHPLC column were directly introduced in an ion trap mass spectrometer (Amazon SL, Bruker Daltonik GmbH, Bremen, Germany). This MS detector was equipped with an electrospray (ESI) source and operated in positive polarity; it was also equipped with duel ion funnel to increase the ion transmission to the trap. The optimization of the ionization source parameters and the MS/MS conditions was achieved by infusing standard solution (5 μg/mL) of reduced glutathione and oxidized glutathione at 4 μL/min. Operating conditions of the source were capillary voltage: 4500 V, nebulizer pressure 30 psi, drying gas flow 10 L/min, at a temperature of 220°C. The mass spectrometer was run in MS/MS mode, in which the protonated reduced glutathione (308 m/z) and protonated oxidized glutathione (613 m/z) were isolated and subjected to collision-induced dissociation, using helium gas and radio frequency (RF) with fragmentation amplitude of 0.45 and 0.65 V, respectively. In these conditions, the full scan MS/MS or MS2 spectra were obtained, in which the ion 178.6 m/z was selected as quantification ion for reduced glutathione and 484 + 355 m/z was selected as quantification ion for oxidized glutathione. The trap parameters were set in ion charge control with a target of 100 000 and a maximum accumulation time of 100 ms at m/z range from 200 to 700 u and working in ultrascan (32 000 u) mode.

Western blotting

To separate the proteins according to their apparent molecular masses, SDS-PAGE was performed as previously described [31]. In brief, proteins were extracted and denatured by boiling for 10 min at 70°C in a loading buffer supplemented with 5% mercaptoethanol.

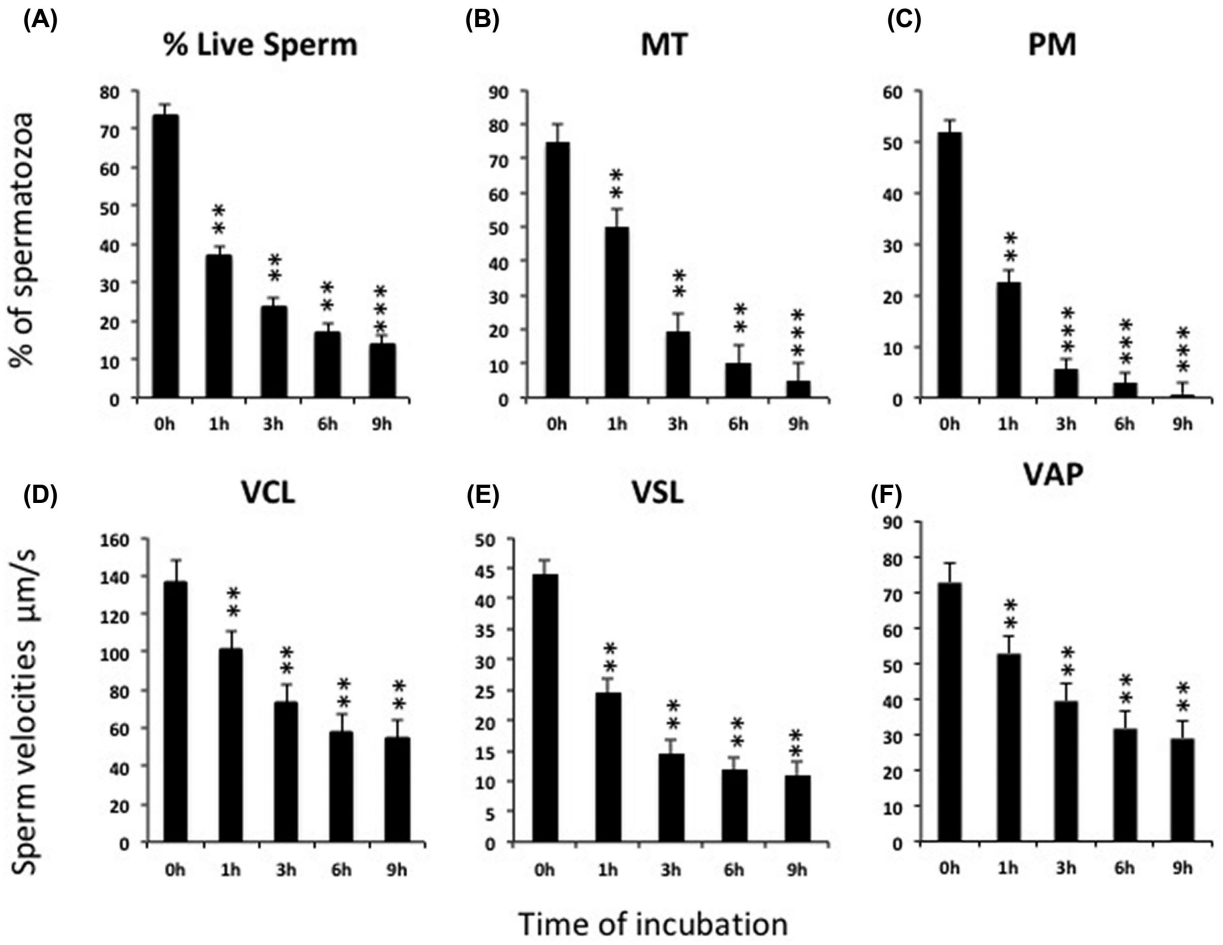

Figure 2. Assessment of sperm functionality of stallion spermatozoa incubated at 37°C up to 9 h. Stallion ejaculates were processed as described in the material and methods section and at the beginning and every hour aliquots were taken and the percentage of live sperm using flow cytometry and sperm motility and velocities of the spermatozoa using computerized analysis were evaluated. % live sperm, spermatozoa with intact membranes (A), MT, percentage of motile spermatozoa (B), PM, percentage of spermatozoa depicting linear movement (C), VCL, circular velocity (μm/s) (D), VSL straight line velocity (μm/s) (E), VAP average path velocity (μm/s) (F). ** $P < 0.01$.

The protein content was calculated using the Bradford assay [32]. Ten micrograms of sperm protein extract was loaded and resolved by SDS PAGE on a 10% polyacrylamide gel. Immunoblotting was performed by incubating the membranes in blocking buffer overnight at 4°C with primary antibodies (GSS and GCLC, 1/1000, 1/500, respectively). Proteins from whole rat kidney cells lysates were used as positive control for both proteins GSS and GCLC.

Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described [33]. After blocking, cells were incubated with primary antibodies GSS and GCLC, overnight at 4°C, and diluted 1/200 in PBS containing 5% BSA (w/v). The following day, cells were washed with PBS and further incubated for 45 min at RT with goat anti-rabbit IgG antibody conjugated with the Alexa Fluor 647 diluted to 1/500 in PBS containing 5% BSA (w/v). Finally, cells were thoroughly washed with PBS. A total of 5000 cells were analyzed in the ImageStream X Mark II Imaging Flow Cytometer (Merck Millipore) using a laser of 642 nm line with intensity set to 100 mW, at ×60 of magnification. Data analysis of the raw images was accomplished using IDEAS1software (Version 6.0.309). Absence of nonspecific staining was assessed by processing the samples without primary antibody (secondary antibody only).

Statistical analyses

All experiments were repeated at least three times with independent samples (three independent ejaculates from each of the 11 stallions). The normality of the data was assessed using the Kolmogorov–Smirnov test. Because the data show equivalence of variance, the results were analyzed by ANOVA followed by the Dunnett test for multiple comparisons using SPSS 19.0 software for Mac. $P < 0.05$ was regarded as significant. Correlations were investigated by the Pearson correlation test using the same software.

Results

In vitro incubation of stallion spermatozoa leads to depletion of intracellular thiols

Stallion spermatozoa were incubated in BWW media at 37°C over a 9-h period, with aliquots removed at 0 h and every hour thereafter for measurement of intracellular thiol content. The percentage of

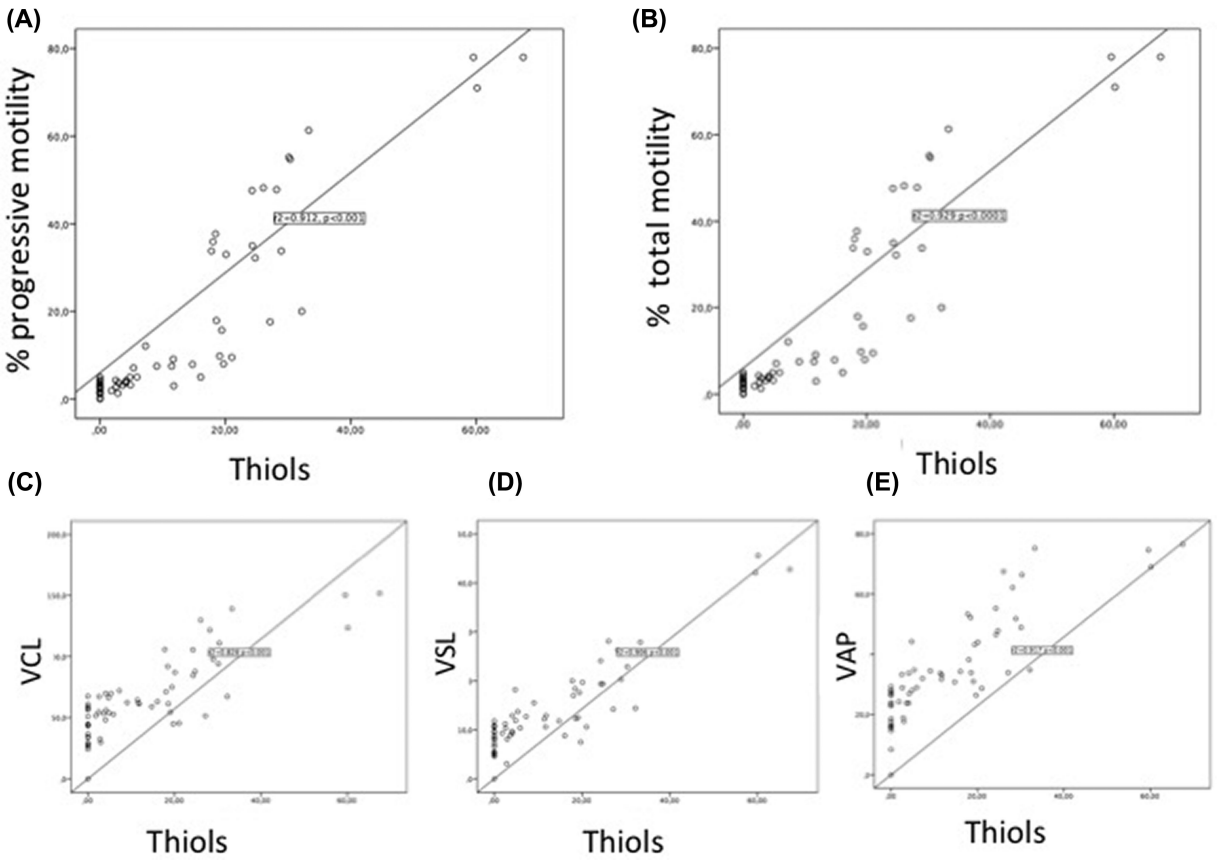

Figure 3. Significant correlations ($P < 0.001$) among GSH and sperm motility and velocities. (A) Progressive motility, (B) % of total motile sperm, (C) circular velocity (μm/s), (D) straight line velocity (μm/s), (E) average path velocity.

live spermatozoa with high GSH content dropped significantly after 1 h of incubation to around half of the initial value (62.4 ± 4.4 to 29.6 ± 3.5% between 0 and 1 h, respectively; $P < 0.001$; Figure 1) and spermatozoa with high thiol content further decreased to reach minimum levels at the end of the incubation period, from 62.4 ± 4.4% of the spermatozoa showing high thiol content to 12.2 ± 7.3% after 9 h of incubation.

In vitro incubation of stallion spermatozoa leads to reduced functionality

Incubation of stallion spermatozoa at 37°C caused a time-dependent reduction in sperm functionality starting after 1 h of incubation. All parameters studied showed the same trend, with parallel reductions in membrane integrity (Figure 2A), percent total (Figure 2B) and progressive motility (Figure 2C), and all velocity parameters (Figure 2D and E). The magnitude of decreased functionally became increasingly prevalent and by the end of the 9-h incubation period, the proportion of live spermatozoa was only 18.4% of the initial values (73.2 ± 2.8 to 13.5 ± 2.6%), total motility 6.5% (74.5 ± 5.7 to 4.9 ± 5.2%), and progressive motility dropping to nearly 0% (51.7 ± 2.4 to 0.7 ± 2.2%). The same trend was observed for all sperm velocity parameters (Figure 2D–F).

Sperm functionality correlates with intracellular thiols

To study the relationship between sperm functionality and GSH content, correlations between GSH content and motility parameters (total and progressive motility and average straight line and circular velocities) were studied. Highly significant positive correlations between GSH content progressive motility ($r^2 = 0.912$ $P < 0.001$), total motility ($r^2 = 0.929$, $P < 0.0001$), VCL ($r^2 = 0.828$ $P < 0.001$), VSL ($r^2 = 0.906$, $P < 0.001$), and VAP ($r^2 = 0.917$, $P < 0.001$) were observed (Figure 3A–E).

Stallion spermatozoa experience significant increases of 4-hydroxynonenal only after long incubation periods, while no changes occur in 8-iso $PGF_{2\alpha}$

In order to further investigate the role of oxidative and electrophilic stress on sperm senescence during in vitro incubation, we monitored changes in the adduction of 4 hydroxynonenal (4-HNE) and 8 iso-PGF2α during the incubation period. The percentage of cells with 4-HNE adducts increased by 55.3% after 9 h of incubation ($P < 0.01$, Figure 4A and B). On the other hand, no significant changes in 8 iso-PGF2α production were observed during the incubation period (Figure 4C and D).

Depletion of intracellular thiols by menadione can be prevented by cysteine

In order to determine the effect of depleted intracellular thiols on sperm functionality, stallion spermatozoa were incubated for up to 3 h in the presence of 200 μM menadione (Figure 5A and B). Menadione rapidly reduced intracellular thiol content to less than of 10% the initial value after 1 h of incubation (Figure 5A) and less than 6%

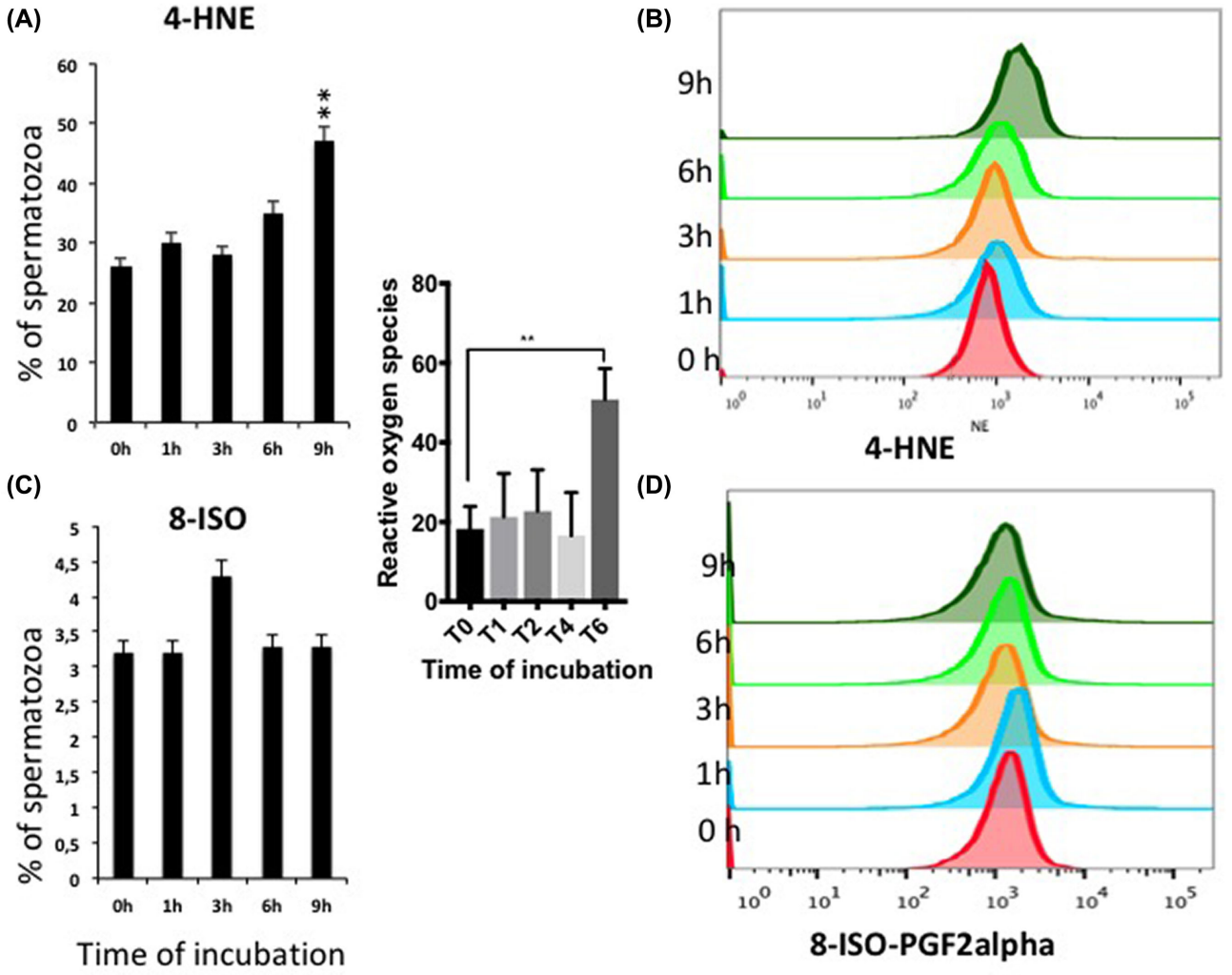

Figure 4. Changes in 4-HNE and 8-iso-$PGF_{2\alpha}$ during incubation of stallion spermatozoa at 37°C. Stallion ejaculates were processed as described in the material and methods section and these two markers of oxidative stress evaluated at the beginning of the incubation period and every hour. (A) Percentage of spermatozoa depicting high level of 4-HNE. (B) Representative histograms overlays of changes in 4-HNE over the incubation period. (C) Percentage of spermatozoa depicting high level of 8-iso-$PGF_{2\alpha}$. (D) Representative overlay histograms of changes in 8-iso-$PGF_{2\alpha}$. ** $P < 0.01$.

of the initial value after 3 h of incubation (Figure 5B). The effect of pre-incubation with melatonin 1 μM and 100 pM, taurine 1 mM, penicillamine 1 mM, glutathione 1 mM, and cysteine 2 mM on the prevention of GSH depletion was evaluated. Cysteine and penicillamine alone were able to partially rescue intracellular GSH after 1 h of incubation (Figure 5A), and only cysteine was effective after 3 h (Figure 5B). All other antioxidants tested had no effect.

Cysteine rescues the loss of membrane integrity induced by menadione

Loss of thiols is linked to compromised functionality in stallion spermatozoa. Given that cysteine was able to prevent menadione-induced thiol depletion, we evaluated the effect of cysteine supplementation in the loss of viability induced by menadione. The percentage of live sperm was reduced from 79.92 ± 4.5 in controls to 55.9 ± 6.5% in samples treated with menadione ($P < 0.01$). Samples treated with menadione but at the same time supplemented with cysteine maintained the same percentage of live spermatozoa as the controls after 1 h of incubation (Figure 6A and C). The same trend was observed after 3 h of incubation, but in this case menadione reduced membrane integrity from 78.49 ± 4.12 to 29.51 ± 8.58% ($P < 0.001$), a loss that was completely prevented by the addition of cysteine (Figure 6B and D). The nucleophile penicilamine was ineffective at preventing the effects of menadione, and in fact exacerbated the deleterious effect of menadione on membrane integrity (3.16 ± 2.16)

Cysteine prevented menadione-induced increases in 4-HNE

Prevention of membrane damage by cysteine was likely to be related to the prevention of a total loss of intracellular GSH that in turn may prevent lipid peroxidation. To test this hypothesis, 4-HNE levels were monitored over the incubation period. Treatment with menadione increased 4-HNE after 3 h of incubation. This increase in 4-HNE was prevented by pre-incubation with cysteine (Figure 7B and D)

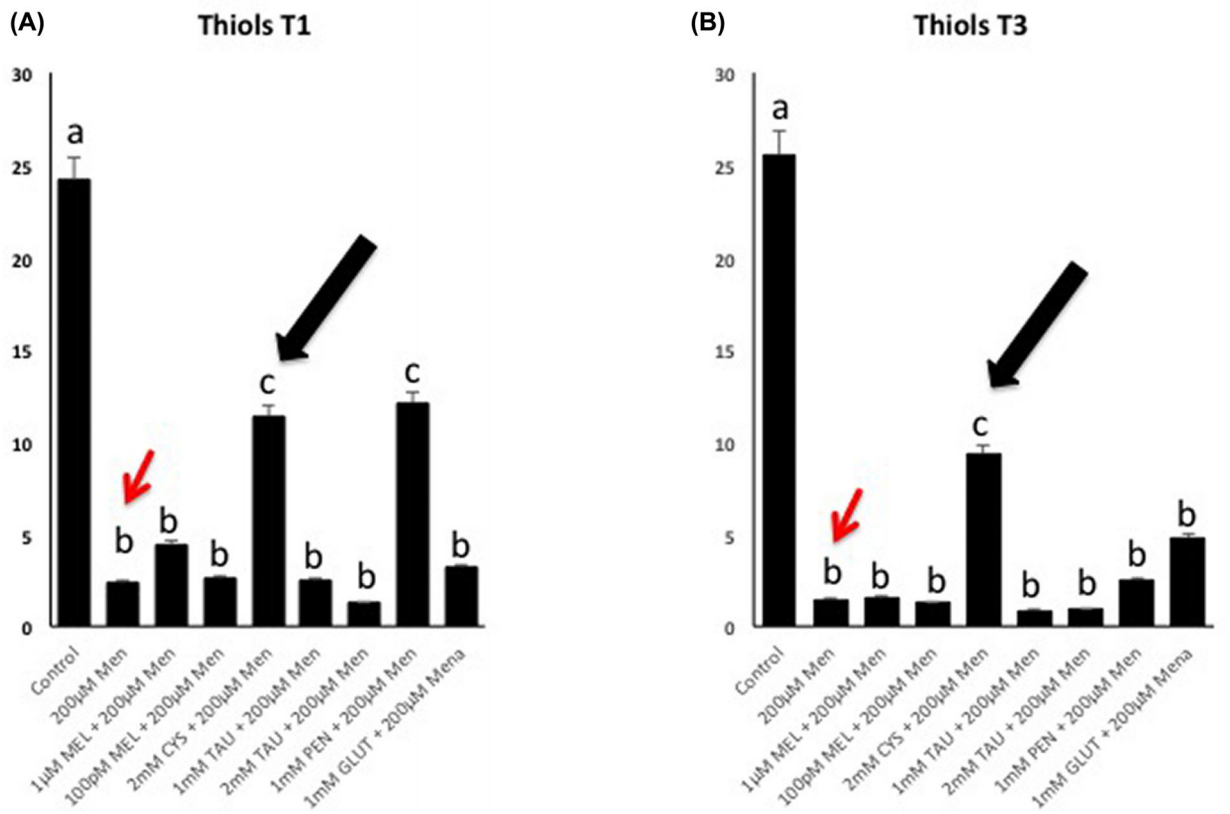

Figure 5. Effect of menadione in GSH content of stallion spermatozoa and effect of pre-incubation with cysteine and selected antioxidants. Stallion ejaculates were processed as described in the material and methods section, aliquots were obtained and used as controls, medadione was used to deplete GSH and additional aliquots were supplemented with 200 μM menadione, and 200 μM menadione and 1μM melatonine, 200 μM menadione and 100 pM melatonine, 200 μM menadione and 2 mM cysteine, 200 μM menadione and 1 mM taurine, 2 mM taurine and 200 μM menadione, 200 μM menadione and 1 mM penicilamine, and 200 μM menadione and 1 mM glutathione for 1 (A) and 3 h (B). Menadione depleted GSH content (red arrow) and cysteine was able to reduce this effect both after 1 and 3 h of incubation. Penicilamine was also effective only after 1 h of incubation values with different superscript differ statistically a,b, c,b $P < 0.01$, a–c $P < 0.05$.

Stallion spermatozoa express glutathione syntethase and glutamate cysteine ligase (GCLC)

If cysteine is able to restore glutathione reserves of the spermatozoa better than glutathione itself (Figure 5), one possible explanation is that stallion spermatozoa harbor the machinery necessary to synthetize glutathione. To test this hypothesis, sperm lysates from seven different stallions were investigated for the presence of GSS and GCLC using specific monoclonal antibodies and western blotting. The subcellular distribution of these enzymes was evaluated using imaging flow cytometry. Western blotting revealed the presence of GSS and GCLC in the spermatozoa of all seven stallions (Figure 8). The subcellular distribution of GSS was in the mid-piece, while GCLC was identified in the postacrosomal region, mid piece, and principle piece.

Inhibition of gamma-glutamylcysteine synthetase (glutamate cysteine ligase, GCLC) reduces the restoration of thiols in the presence of cysteine

If glutamate cysteine ligase is active in stallion spermatozoa, its inhibition should reduce GSH levels [25]. To further evaluate the presence of an active GSH synthesis pathway by stallion spermatozoa, semen samples were incubated in the presence of BSO (50 μM), a specific inhibitor of GCLC which is the rate-limiting enzyme in the synthesis of GSH [25]. GSH content was then evaluated using flow cytometry. As in previous treatments, menadione significantly depleted intracellular thiols both after 1 and 3 h of incubation (Figure 9A and B). To provide further evidence that stallion spermatozoa are able to use exogenous cysteine to synthetize GSH, spermatozoa were incubated in the presence of BSO alone, cysteine plus BSO, menadione plus cysteine, or menadione plus cysteine and BSO. Although after 1 h of incubation no differences were observed between cys and cys plus inhibitor, Figure 9A shows significant differences among various treatments; after 3 h, BSO treatment resulted in significantly lower thiol levels in samples supplemented with cysteine (80 RFU in samples supplemented with cysteine to 24.8 RFU in samples supplemented with cysteine and BSO, $P < 0.01$; Figure 9B).

Inhibition of glutamate cysteine ligase negates the restoration of viability and motility in the presence of cysteine after depletion of GSH with menadione

Menadione treatment reduced GSH content, the percentage of viable spermatozoa (Figure 9C and D), and motility (Figure 10), the latter of which was rescued by pre-incubation with cysteine. When BSO was added to the system, the ability of cysteine to prevent a loss of sperm viability in the presence of menadione over a 3-h

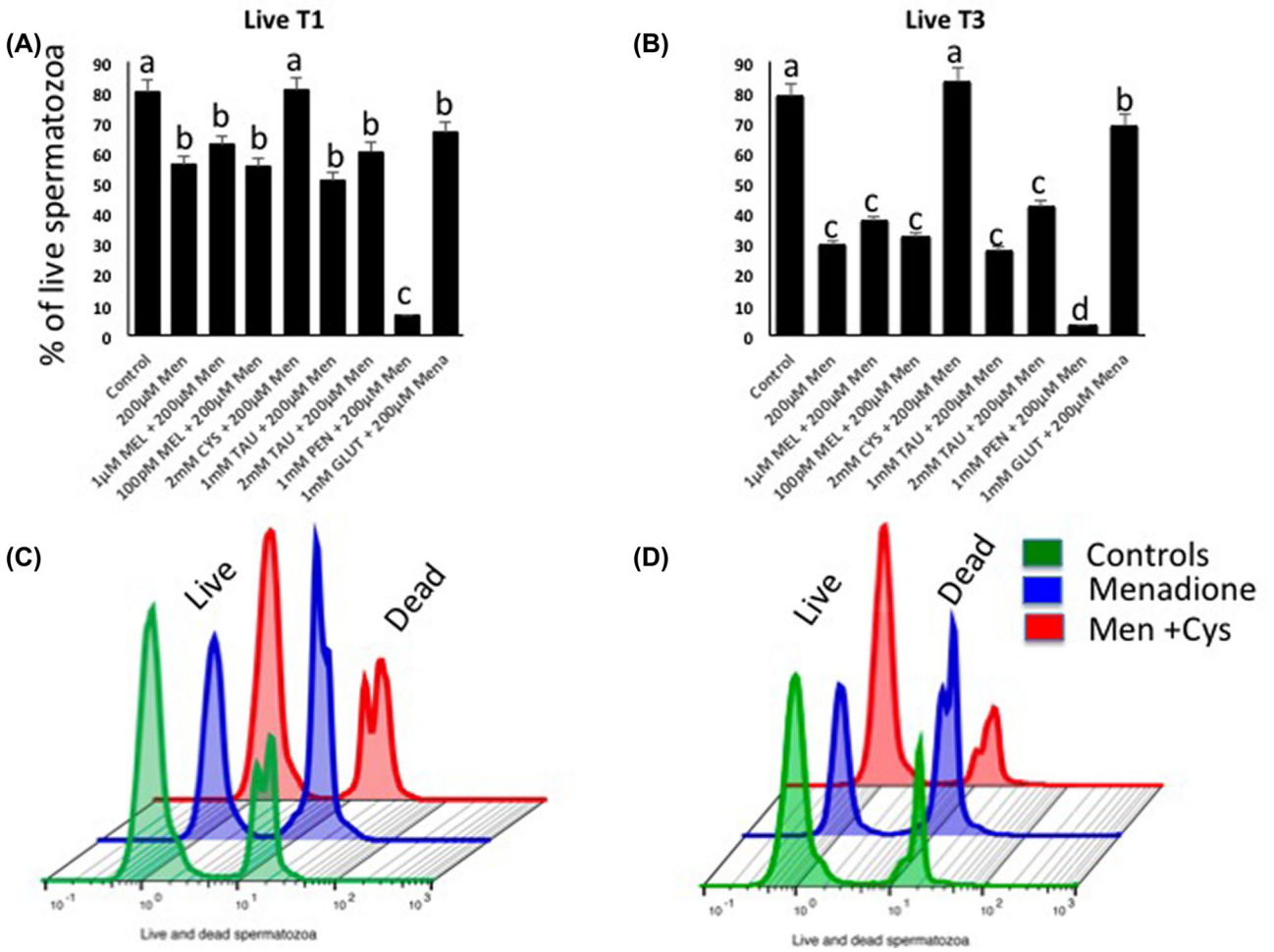

Figure 6. Effect of menadione in the percentage of live stallion spermatozoa and effect of pre-incubation with cysteine and selected antioxidants. Stallion ejaculates were processed as described in the material and methods section, aliquots were obtained and used as controls, medadione was used to deplete GSH and additional aliquots were supplemented with 200 μM menadione, and 200 μM menadione and 1 μM melatonine, 200 μM menadione and 100 pM melatonine, 200 μM menadione and 2 mM cysteine, 200 μM menadione and 1 mM taurine, 2 mM taurine and 200 μM menadione, 200 μM menadione and 1 mM penicilamine, and 200 μM menadione and 1 mM glutathione for 1 (A) and 3 h (B). (C, D) Representative histogram overlay of the assay. Menadione reduced the percentage of live spermatozoa and cysteine was able to prevent sperm death both after 1 and 3 h of incubation. Values with different superscript differ statistically a–d $P < 0.01$.

incubation period was reduced (Figure 9D). Surprisingly, the combination of menadione and cysteine resulted in more viable sperm compared to the control after 3 h of incubation (Figure 9D). The ability of cysteine to prevent the menadione-induced loss of motility was also impeded by GCLC inhibition with BSO after 1 h of incubation (Figure 10A).

Effect of incubation of stallion spermatozoa in the presence of DMNQ

Menadione has redox cycling activity and is also a potential alkylating agent capable of alkylating the nucleophilic centers of proteins associated with motility such as AKAP3 and 4V [34]. Moreover, cysteine and menadione may have reacted together (cysteine is a potent nucleophile) reducing the latter bioavailability. To test this hypothesis and try to clearly define if an alkylating effect was present, stallion spermatozoa were incubated in the presence of DMNQ, a compound with reduced capacity to bind to cysteine, but still presenting redox cycling activity [34]. The percentage of live spermatozoa with high thiol content was reduced after 1 h of incubation in the presence of DMNQ, and this reduction was prevented by pre-incubation with cysteine (Figure 11B); however, when the total free thiols were examined the picture was different, DMNQ also caused a significant reduction, as did cysteine but this amino acid was not able to revert thiol depletion induced by DMNQ (Figure 11A and C). Interestingly, and as occurred in the previous experiment after 3 h of incubation, inhibition of GCLC further reduced intracellular thiol content in the presence of cysteine compared to DMNQ alone (Figure 11C)

Cysteine rescues the reduced motility of spermatozoa incubated in the presence of DMNQ

Stallion sperm motility was measured using CASA. Incubation in the presence of DMNQ reduced sperm total and progressive motility (Figure 12A and B). Pre-incubation of spermatozoa in the presence

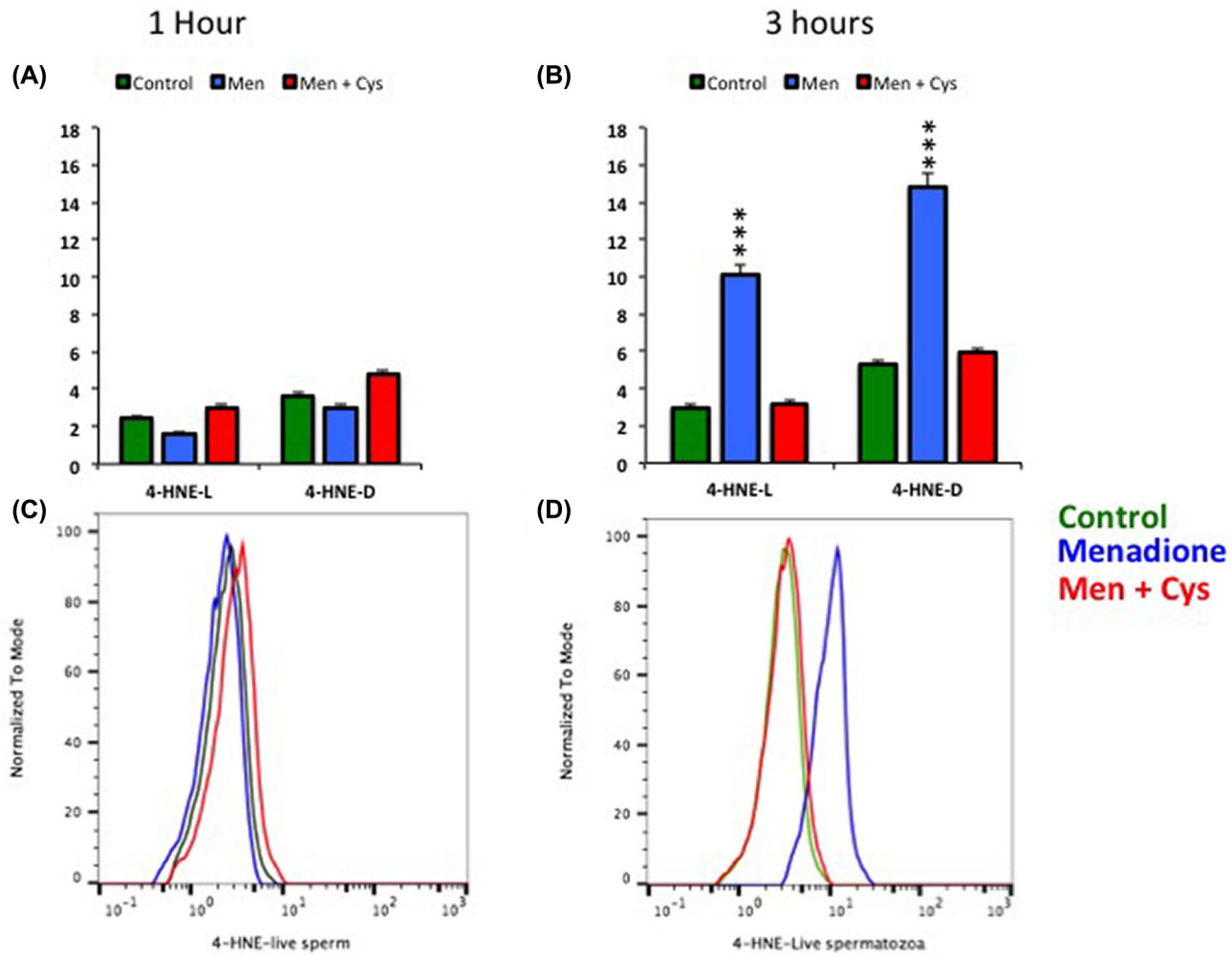

Figure 7. Effect of menadione on 4-HNE levels in stallion spermatozoa and prevention of menadione induced lipid peroxidation with cysteine. Stallion ejaculates were processed as described in the material and methods section. Semen samples were incubated in the presence of menadione (0 and 200 μM) and in the presence of menadione (200 μM) and cysteine (2 mM). 4 HNE levels were monitored after 1 and 3 h of incubation at 37°C. *** $P < 0.001$.

of 0.5 mM cysteine prevented this reduction in sperm motility after 1 h, but this effect disappeared after 3 h of incubation (Figure 12B).

Cysteine prevented increases in 4-HNE induced by DMNQ

Incubation of stallion spermatozoa in the presence of DMNQ increased 4-HNE in stallion spermatozoa after 3 h of incubation, particularly in the subpopulation of dead cells (Figure 13B). Pre-incubation with cysteine prevented DMNQ-induced increase in 4-HNE adduction.

HPLC-MS/MS monitoring of changes in GSH and GSSG

Since the probe used in the flow cytometry assay may detect reduced thiols others than GSH, specific changes in GSG and GSSG were monitored using HPLC/MS/MS analysis. Using this assay we were able to confirm depletion of GSH during the incubation period from 8.2 ± 2.9 nM/10^6 spmtz after 1 h of incubation to 6. 2 ± 0.8 nM/10^6 spmtz after 3 h of incubation ($P < 0.05$). Moreover, we observed similar results to that obtained after flow cytometry analysis (Figure 9A and B), with reduction in GSH after incubation in the presence of Cys plus BSO after 1 (4.9 ± 1.8 vs 3.8 ± 1.2 nM/10^6 spmtz; $P < 0.05$) and 3 h of incubation (4.5 ± 0.8 vs 3.7 ± 0.7 nM/10^6 spmtz; $P < 0.01$). After 3 h of incubation, BSO also reduced GSH concentration compared to the control sample (4.7 ± 0.8 vs 4.1 ± 0.7 nM/10^6 spmtz; $P < 0.01$) ((Figure 14A and B). Moreover, after 6 h of incubation in the presence of 0.5 mM cysteine GSH levels increased (4.6 ± 0.9 to 12.4 ± 5.1 nM/10^6 spmtz; $P < 0.05$), which were reduced in the presence of BSO (Figure 14C).

Discussion

Stallion spermatozoa heavily depend on OXPHOS for the production of ATP [4, 11, 21], and for this reason they produce large amounts of mitochondrial ROS. From an evolutionary perspective, it is reasonable to argue that intrinsic and sophisticated antioxidant defenses shall be present to counteract oxidative damage in the interest of preserving sperm function. With this hypothesis in mind, we proposed that total thiol content, and particularly GSH, is a key element in the functionality of stallion spermatozoa after ejaculation. In the present study, for the first time we provide evidences that stallion spermatozoa may be able to regulate their intracellular thiol content. Although mechanisms for reducing oxidized glutathione (GSSG) and thus increasing GSH in spermatozoa have been previously described

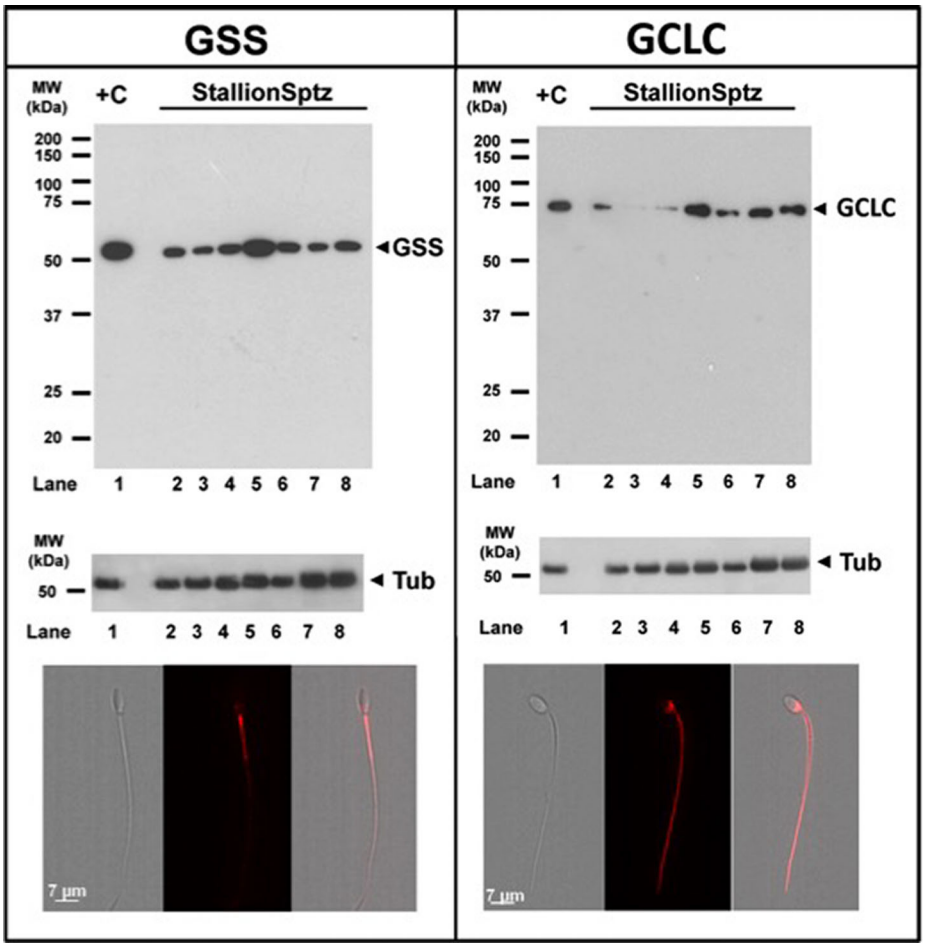

Figure 8. Identification of glutathione syntethase (GSS) and gamma-glutamylcysteine synthetase (glutamate cysteine ligase, GCLC). Sperm lysates were processed as indicated in the material and methods section and processed using specific monoclonal antibodies. (A) Identification of GSS. (B) Identification of GCLC: Line 1 controls, Lanes 2–8 stallion sperm lysates. Lower panels, subcelullar distribution of GSS and GCLC in stallion spermatozoa. The subcellular distribution of these enzymes in fixed and permeabilized stallion sperm was assessed by immunocytochemistry as described in the material and methods section. Immunoreactivity with GSS antibodies indicated that this was distributed in the mid piece. GCLC was distributed in the postacrosomal region, mid piece, and rest of the tail. Magnification ×60.

[35], we have provided the first evidences of potential GSH synthesis by stallion spermatozoa, also for the first time we reported data on GSH content of stallion spermatozoa, and interestingly GSH is present at higher concentrations in stallions (8 μM/10^9 spmtz) compared with other species, in which nanomolar ranges per billion spermatozoa have been reported, and even in boars was undetectable [36]. This point supports the hypothesis that stallion spermatozoa have developed a sophisticated antioxidant defense in relation to its intense mitochondrial activity, and may also explain why the outcome of antioxidant supplementation to improve cryopreservation varies greatly among species. This is a remarkable finding since it is accepted that mature spermatozoa are unable to undertake de novo synthesis of peptides, and the dynamic change in protein profile of the spermatozoa is attributed either to the acquisition of new proteins via vesicular transport or to several post-translational modifications occurring on an already expressed protein complement [37]. The finding that thiols are critical for sperm function and that thiol levels could be modulated by ejaculated sperm paves the way to develop new methods of sperm conservation and treatments for male factor infertility.

In eukaryotic cells GSH is the principal intracellular free thiol; it is predominantly present in the cytosol (80%), with about 10–15% in the mitochondria [25, 38]. Spermatozoa have very limited cytoplasm, so this may explain the rapid exhaustion of GSH in these cells, and secondly may also explain why stallion spermatozoa have developed a method to use external sources of cysteine to synthetize GSH for the maintenance of sperm functionality. Moreover, the intense mitochondrial activity of stallion spermatozoa [9, 21] may lead to rapid depletion of mitochondrial GSH, necessitating the continuous synthesis of GSH for sperm functionality.

To test this hypothesis, we monitored changes in thiols during incubation of stallion spermatozoa while simultaneously monitoring sperm functionality. Additionally, we studied the effects of depleting and restoring intracellular thiols on stallion sperm viability. We also correlated intracellular thiol content with markers of oxidative stress, namely 8-iso PGF2α and 4-HNE. As thiol content decreased, the sperm functionality parameters of viability, total and progressive motility, and velocity all decreased in parallel. Moreover, highly significant correlations were found between GSH/thiols and sperm functionality. In order to provide further evidence linking

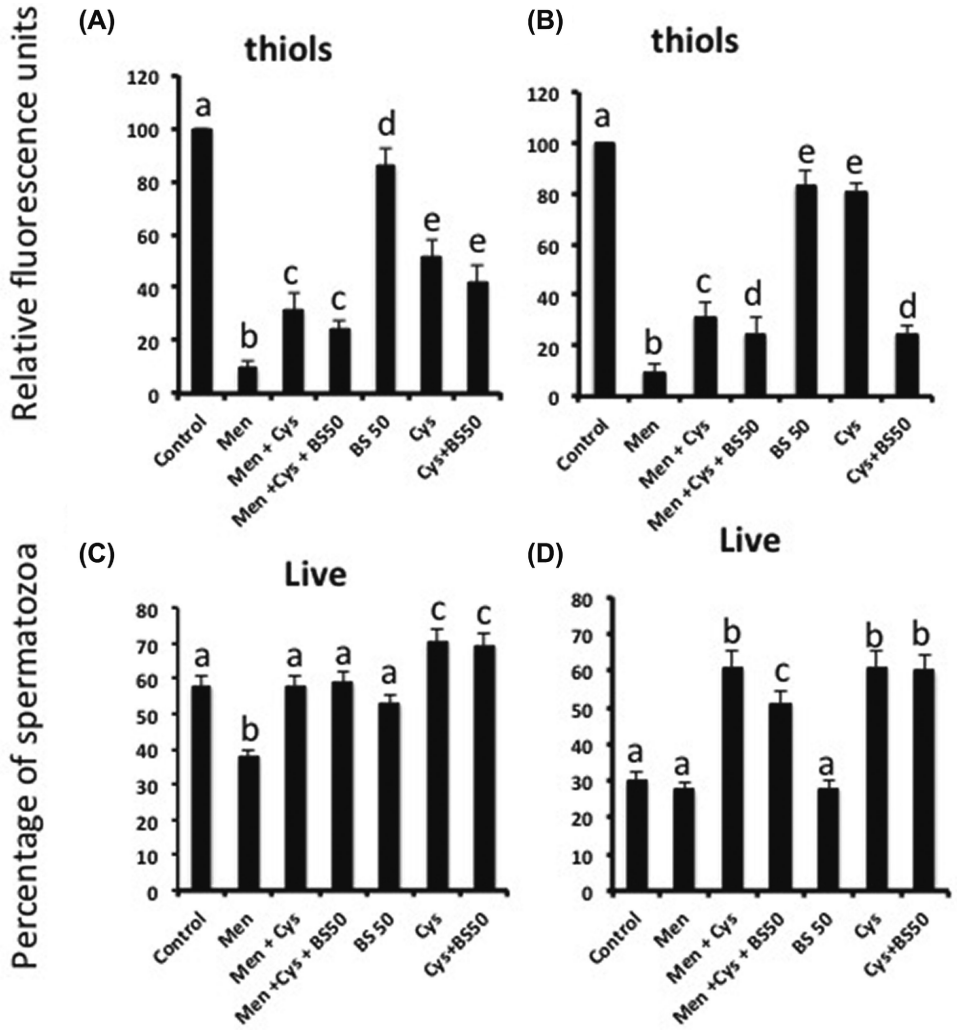

Figure 9. Effect of the CGLC inhibitor BSO in the recovery of glutathione induced by cysteine after menadione treatment of stallion spermatozoa. GSH is determined using a flow cytometry-based assay (A) after 1 h (B) and 3 h of incubation. Effect on the percentage of live spermatozoa (C) after 1 (D) and 3 h of incubation. Values with different superscripts differ statistically a–d $P < 0.05$. In A and B, controls represent spermatozoa in medium alone after 1 and 3 h and effects of different treatments are reported as % changes respect these controls.

GSH/thiols and sperm functionality, GSH was effectively depleted with menadione, and restored with cysteine, suggesting that stallion spermatozoa are capable of synthesizing GSH de novo.

Menadione generates ROS through redox cycling, and at high concentrations it triggers death in somatic cells by inducing rapid oxidation in the cytosol and mitochondrial matrix [39]. Our results indicate a similar effect in stallion spermatozoa, with menadione treatment leading to rapid oxidation as indicated by increased 4-HNE and rapid depletion of GSH. However, menadione is also an alkylating agent and as such, some of the menadione toxicity observed in the current study may be attributed to alkylation of sperm proteins. To distinguish alkylating effects from pure redox cycling, we conducted further experiments using DMNQ instead of menadione. This compound has pure redox cycling activity and does not have the capacity to react with GSH that menadione has [34], since it does not have the electrophilic centers capable of forming covalent links with cysteine. Menadione was more efficacious at depleting thiols than DMNQ, suggesting that this compound exerted its effect both through redox cycling and covalently binding with thiols. Thus, the effect of cysteine can be at least partially explained by simple chemistry; the covalent binding of menadione in solution may have reduced its bioavailability as a ROS-inducing agent. Furthermore, cysteine was not able to prevent depletion of thiols induced by DMNQ, and interestingly an additive deleterious effect of cysteine and DMNQ was observed, with cysteine being able to counter the toxic effects of DMNQ only during short incubation periods (Figure 12). One possible explanation for the findings is that cysteine may have begun redox cycling in the presence of DMNQ [40], with the fact that reducing the concentration of cysteine reduced the deleterious effect of DMNQ on motility supporting this hypothesis. Additionally, it is known that cysteine oxidizes spontaneously to cystine generating H_2O_2, O_2^-, and $OH^\bullet$ during the process [41], providing an explanation for the above-mentioned phenomenon.

The depletion of thiols leads to sperm death, which can be prevented by supplementation with cysteine, but not by other antioxidants investigated in the present study. This finding provides strong evidence linking GSH to sperm functionality. Further supporting this statement is the fact that cysteine was also able to prevent increases in 4-HNE induced by both menadione and DMNQ. These results suggest that thiols are critical for sperm function; in

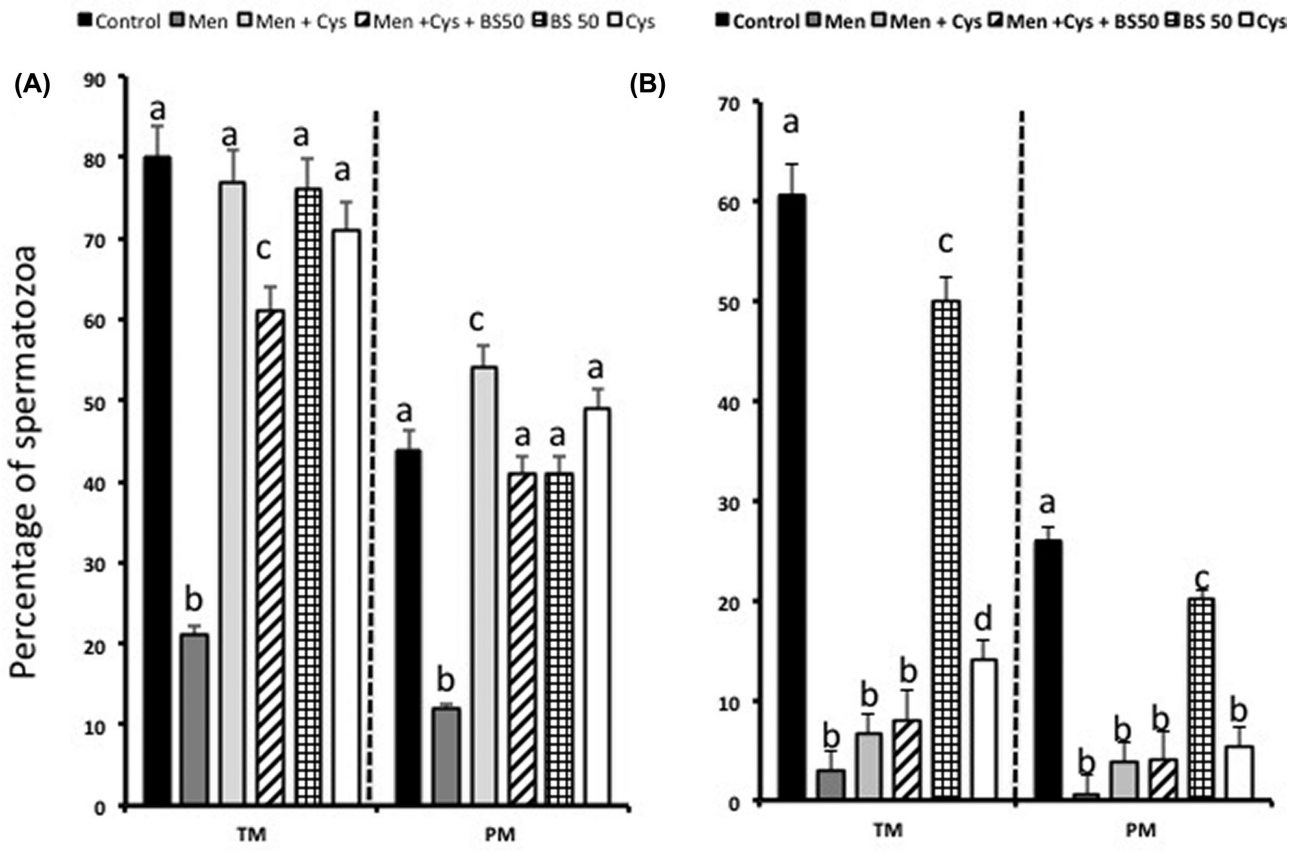

Figure 10. Effect of the CGLC inhibitor BSO in the recovery of motility induced by cysteine after menadione treatment of stallion spermatozoa (A) after 1 and (B) 3 h of incubation. %TM of motile spermatozoa, %PM percentage of linear motile spermatozoa. Values with different superscripts differ statistically a–c $P < 0.05$

addition to being the key antioxidant defense, these molecules probably play a key role in the regulation of redox signaling [42]. The fact is that cysteine may provide protection while other potent antioxidants such as melatonin do not support this hypothesis. While melatonin has been demonstrated to be a potent antioxidant for stallion spermatozoa [43], it was ineffective at ameliorating menadione-induced oxidative stress in the present study, suggesting additional mechanisms of GSH in sperm function. ROS are chemical species formed upon incomplete reduction of molecular oxygen, and include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO•). These compounds also operate as intracellular signaling molecules within spermatozoa [15–17]. This dual role of ROS in sperm function is an apparent paradox that highlights the significance of ROS homeostasis [24]. A large number of thiols including cysteine are amino acids. This amino acid is associated with different thiol-related functions [42, 44]. The reduced cysteine/oxidized cysteine (Cys/CysSS) pool has important regulatory functions through discrete redox pathways, rather than a global thiol/disulfide balance as in the traditional definition of oxidative stress [42]. Improvements in sperm functionality after cysteine addition in the presence of the low GSH levels found in our study may indicate the presence of an active Cys/CysSS pool in stallion spermatozoa or the use of cysteine for activation of thioredoxin antioxidant pathways [45, 46]. Glutathione serves as a continuous source of cysteine through the gamma-glutamyl cycle [25]. Glutathione is synthetized via a two-step energy-dependent enzymatic process in which the first step conjugates cysteine with glutamate via the glutamate cysteine ligase (GCLC), and the second step, catalyzed by GSH synthase, involves the addition of glycine to ϒ-glutamilcysteine to form glutathione [25]. We found that these enzymes were present in stallion spermatozoa and through the use of specific inhibitors we showed that their activity was necessary to maintain the glutathione pool of stallion spermatozoa. Evidence presented here suggests for the first time that stallion spermatozoa can synthetize GSH using exogenous cysteine, a mechanism that allows them to maintain their functionality despite producing large amounts of ROS. Further evidence of GSH synthesis in stallion spermatozoa emerged following experiments using specific inhibitors of gamma-glutamylcysteine ligase in which incubation of stallion spermatozoa in the presence of cysteine and the inhibitor BSO resulted in reduced thiol content compared to cysteine alone. Interestingly, expression of this enzyme varied among stallions (Figure 8), providing an explanation for different responses observed depending of each stallion. Moreover, determination of the expression of GCLC may be a potential approach to select stallions with better fertility and freezeability of their sperm.

Initially, we reported changes in GSH/thiols using flow cytometry; however, interaction of the probe with free thiols that are not GSH is a possibility, although most of non-GSH thiols of spermatozoa are linked to proteins [36]. To validate our results, we measured GSH and GSSG using HPLC/MS/MS. Interestingly, this technique validated our results, and showed that most of the GSH in

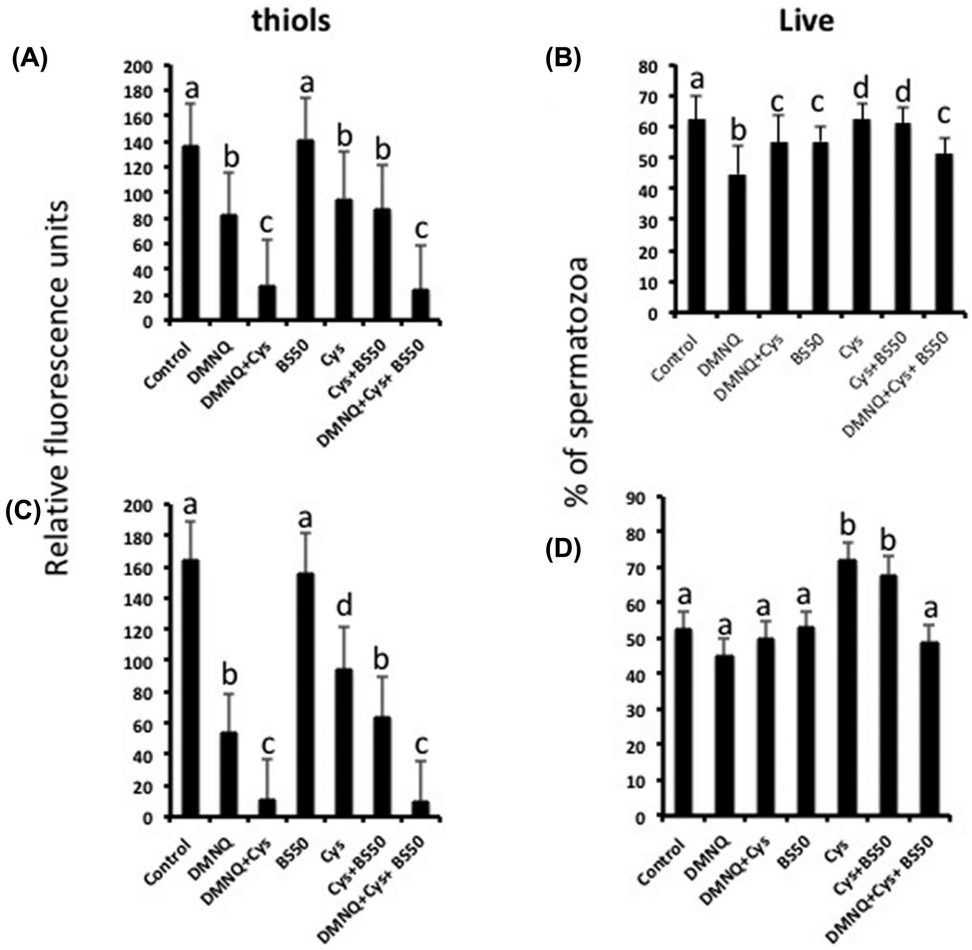

Figure 11. Effect of 1,3 dimethoxy 1,4, naphtoquinone (DMNQ) in stallion spermatozoa; samples were incubated as described in the material and methods section and incubated up to 3 h in the presence of DMNQ, DMNQ + cysteine, Cys + BS50, and DMNQ + Cys + BS50. Thiol content and the percentages of live spermatozoa were determined using flow cytometry. (A) Thiol content after 1 h of incubation. (C) Thiol content after 3 h of incubation. (B) Live high thiol spermatozoa after 1 h of incubation. (D) Live high thiol spermatozoa after 3 h of incubation. Values with different superscripts differ statistically a–d $P < 0.05$.

spermatozoa was in its reduced form, being GSSG below 0.5% of total glutathione in most of the samples. These facts point to the presence of efficient GSH reduction systems in spermatozoa involving enzymes such as glutathion reductase and thioredoxin reductases [47, 48]. In addition to the validation of findings of the flow cytometry assays, these results also point to a potential GSH synthesis by the spermatozoa, such as the GCLC inhibition experiments, and HPLC/MS/MS also provided further evidences showing increased GSH after 6 h of incubation in the presence of cysteine, which was reduced in the presence of the GLCL inhibitor BSO. When both cysteine and BSO were present in the media, the level of GSH dramatically dropped (Figures 9B and 14B) compared to controls and cysteine supplemented samples. The fact that the effect of BSO is more intense in the presence of cysteine may related to low cysteine content in BWW media, in which BSA is substituted by PVA; thus, when a source of cysteine is present, the inhibitory effect of BSO is clearly manifested. Interestingly, changes in total glutathione were not explained by oxidation to GSSG and may indicate the presence of an active gamma-glutamyl cycle [25] in spermatozoa. Although it is known that a great percentage of antioxidant defenses come from seminal plasma, once spermatozoa are deposited in the female genital tract loses contact with seminal plasma, being at this moment when intrinsic antioxidant systems may play a major role in sperm functionality. Elevated concentrations of thiol reducing agents including cysteine [49–51] around ovulation in the oviduct support this line of reasoning [23].

This finding challenges the currently accepted paradigm that spermatozoa have a limited capacity to self-repair and also establishes new pathways in the study of sperm biotechnology and clinical andrology. Moreover, the results of the present study are well in line with previous reports challenging the current assumptions on oxidative stress in spermatozoa [11], and support the idea that balanced redox regulation is necessary for stallion sperm function, and that oxidative stress should be redefined as the disruption of redox signaling and control [42]. This seems especially important in stallion spermatozoa that are highly sensitive to oxidative stress due to the lipid composition of the plasma membrane and intense ROS production due to high rates of OXPHOS in the mitochondria [21, 52]. These findings also support the idea that the assessment of oxidative stress should be re-evaluated. This field of research has traditionally focused on the measurement of the increased presence of oxidants or oxidative compounds. Other recent reports have also

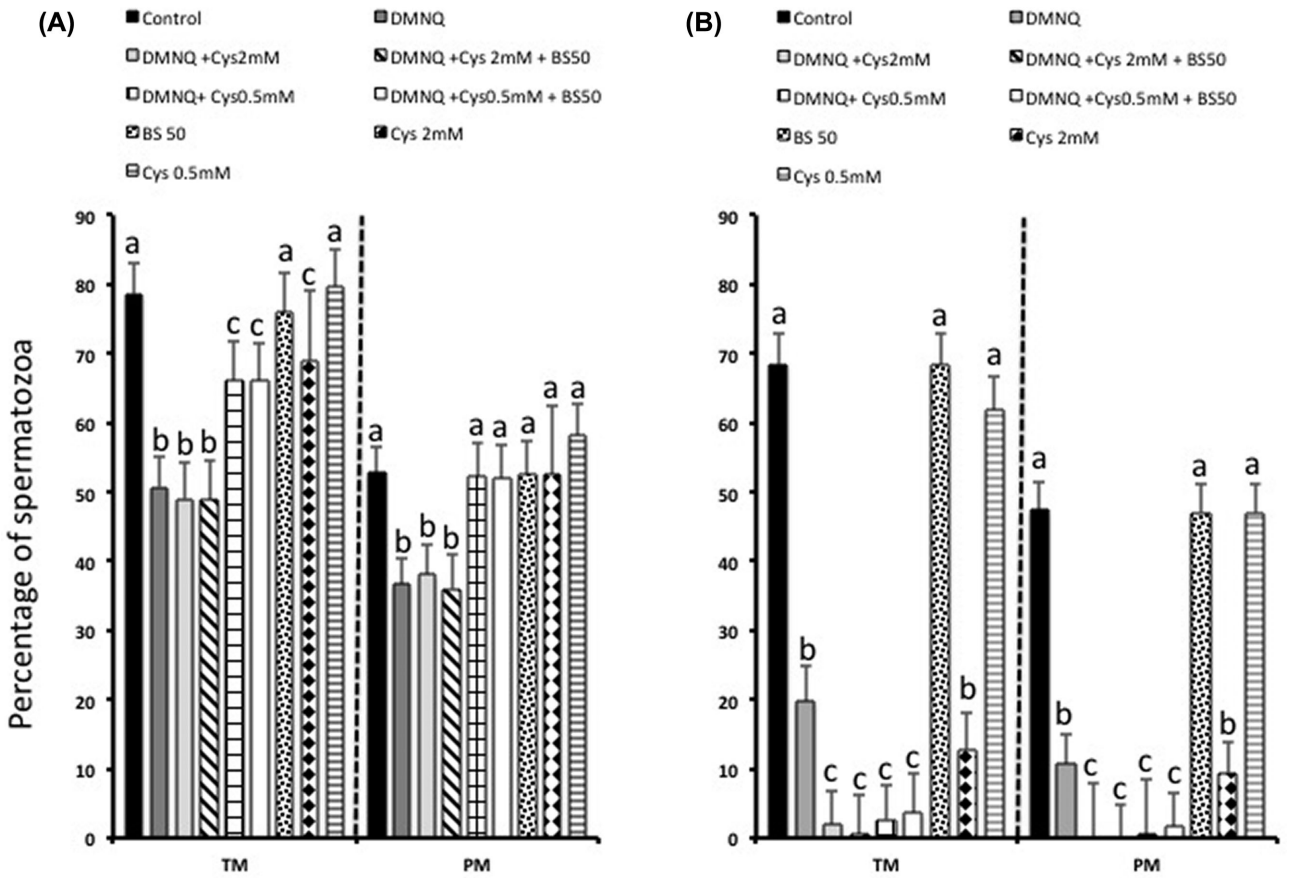

Figure 12. Effect of 1,3 dimethoxy 1,4, naphtoquinone (DMNQ) in stallion spermatozoa; samples were incubated as described in the material and methods section and incubated up to 3 h in the presence of DMNQ, DMNQ + cysteine, Cys + BS50, and DMNQ + Cys + BS50. Percentages of total motile and linearly motile spermatozoa were determined after 1 (A) and 3 h (B) of incubation at 37°C using computer-assisted sperm analysis (CASA). a–c, values with different superscripts differ statistically ($P < 0.05$).

challenged this approach, as it is apparent that more robust and more fertile spermatozoa produce more oxidation products as a result of high metabolic activity [11, 22]. We observed that the combined presence of menadione and cysteine improved sperm function compared with untreated spermatozoa. This apparently paradoxical finding can be explained by the phenomenon of hormesis [13]. A similar observation has been reported in human spermatozoa, whereby the addition of H_20_2 to human sperm actually increased the content of GSH, due to the activation of the pentose phosphate pathway resulting in NADH synthesis and the reduction of oxidized glutathione [35].

During this study two markers of oxidative stress were evaluated in relation to sperm senescence, with only 4-HNE showing increases during incubation, supporting previous reports from our laboratory and others [12, 24, 53, 54], and stressing the need to re-evaluate the concept of oxidative stress in stallion spermatozoa as a tightly regulated redox signaling mechanism in which thiols seem to play a major role. This approach also provides an explanation to the disappointing and contradictory results observed with the empirical use of antioxidants to improve the long-term conservation of stallion spermatozoa [55]. Moreover, our findings may pave the way to a more science-based approach to improve sperm survival during long-term preservation through the protection of the redox signaling system of this cell. The ability of cysteine to reduce the loss of glutathione after an oxidative insult together with the prevention of menadione-induced cell death provides strong support for this assumption and is supported by the findings of others who have demonstrated important regulatory functions of thiols on sperm function [23].

In conclusion, this study demonstrates relationship between intracellular glutathione and sperm functionality, and presents evidences of potential GSH synthesis by stallion spermatozoa, however, varying from stallion to stallion and also probably presenting a seasonal component, an observation which necessitates the re-thinking of the paradigm that this cell type contains a finite and nonrenewable cache of antioxidant defenses. Furthermore, this study adds weight to the emerging body of work, which emphasizes the importance of re-defining the mechanisms underpinning oxidative stress in spermatozoa. Collectively, the findings of this study are of practical relevance suggesting an important role of cysteine or other thiols as a potential therapeutic agent and a subject of future biotechnological interest.

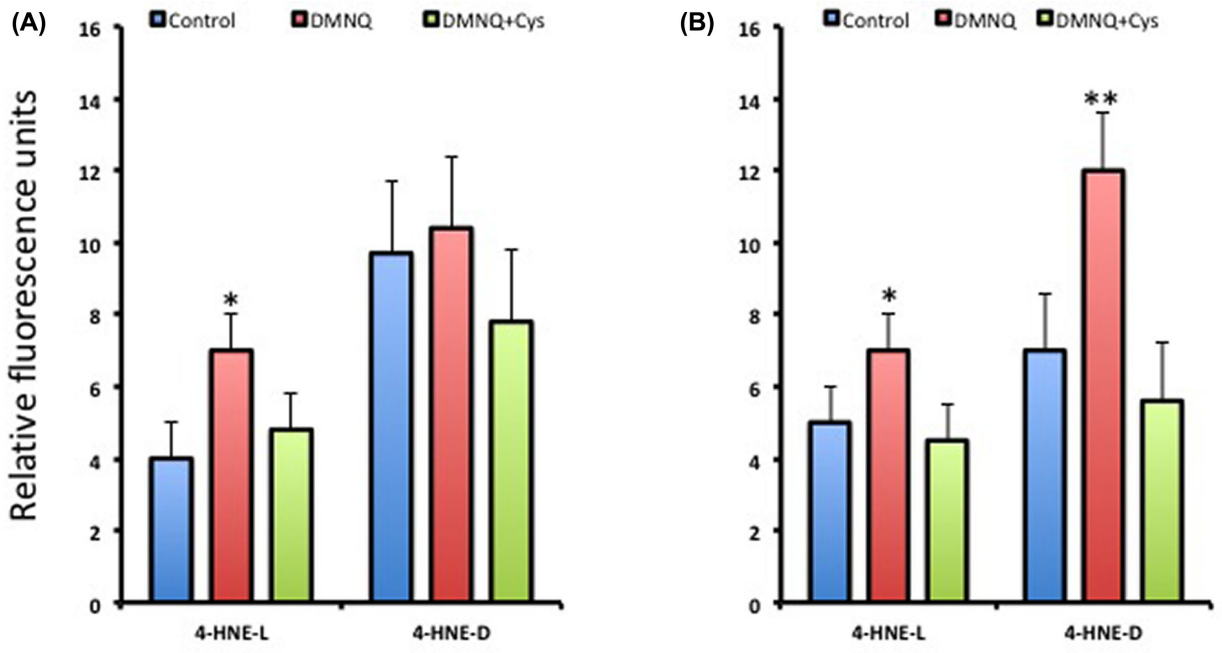

Figure 13. Effect of 1,3 dimethoxy 1,4, naphtoquinone (DMNQ) on 4-HNE levels in stallion spermatozoa and prevention of menadione induced lipid peroxidation with cysteine. Stallion ejaculates were processed as described in the material and methods section. Semen samples were incubated in the presence of DMNQ and in the presence of DMNQ and cysteine. 4 HNE levels were monitored after 1 (A) and 3 h (B) of incubation at 37°C. $^{*}P < 0.05$ $^{**}P < 0.001$.

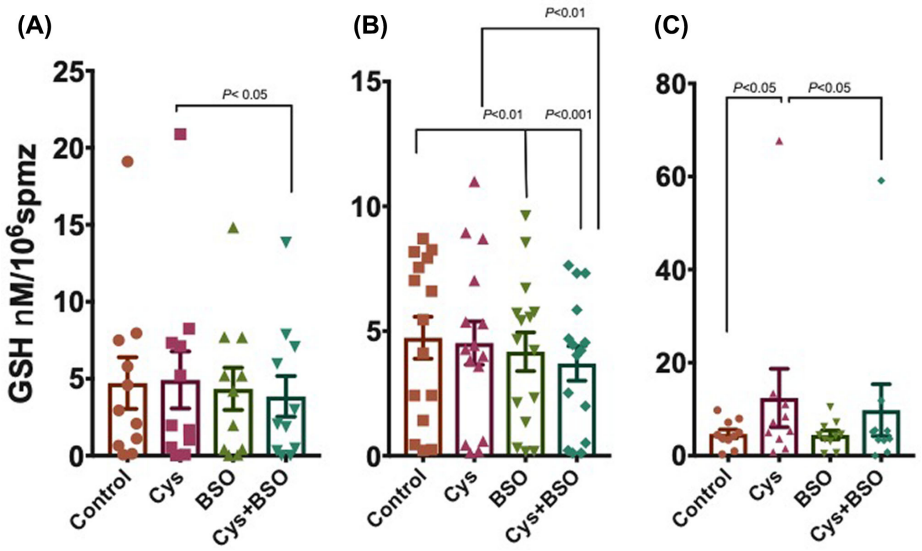

Figure 14. Changes in GSH determined after HPLC/MS/MS analysis of stallion spermatozoa incubated up to 6 h at 37°C, in the presence of cysteine and the GCLC inhibitor BSO (50 μM). (A) Changes after 1 h of incubation, (B) after 3 h of incubation, (C) after 6 h of incubation. Results are expressed as nM/10^6 spermatozoa. Results are derived from six different stallions, with three replicates each.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Conflict of Interest: The authors have declared that no conflict of interest exists.

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