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ORIGINAL ARTICLE

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The achievement of boar sperm *in vitro* capacitation is related to an increase of disrupted disulphide bonds and intracellular reactive oxygen species levels

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SUMMARY

The aim of this work was to determine the relationship of intracellular reactive oxygen species (ROS) and the disulphide bonds established between sperm proteins with the achievement of capacitation in boar spermatozoa. With this purpose, spermatozoa were incubated in a specifically designed *in vitro* capacitation medium (CM) in the presence or absence of reduced glutathione (GSH). Incubation of boar spermatozoa in CM for 4 h significantly ($p < 0.05$) increased free cysteine residues, which is a marker of disrupted disulphide bonds, and also intracellular ROS levels. The addition of GSH to the medium prevented most capacitation-like changes in sperm motility, membrane lipid disorder, mitochondrial membrane potential, intracellular calcium levels and localization of tyrosine-phosphorylated proteins (pTyr), but not in tyrosine phosphorylation of P32. These effects were accompanied by the inhibition of the ability of sperm cells to trigger the acrosome exocytosis in response to progesterone. When GSH was added together with progesterone after 4 h of incubation, acrosome exocytosis was not altered, but the subsequent decrease in intracellular calcium observed in controls cells was inhibited. Furthermore, co-incubation of oocytes with spermatozoa previously incubated in CM in the presence of GSH for 4 h significantly ($p < 0.05$) increased the number of spermatozoa attached to the oocyte surface but decreased normal fertilization rates. Our results suggest that boar sperm capacitation is related to an increase in disrupted disulphide bonds and intracellular ROS levels and that both events are related to the regulation of hyperactivated motility, intracellular calcium dynamics, sperm binding ability to the oocyte and achievement of proper nuclear decondensation upon oocyte penetration.

INTRODUCTION

Sperm capacitation is the series of events required for an ejaculated sperm cell to fertilize an oocyte and takes place within the female reproductive tract (Yeste *et al.*, 2013). This characteristic hampers the study of sperm capacitation *in situ*. However, with the advent of assisted reproductive technology (ART), a large amount of information has been gathered on how sperm cells achieve capacitation and acquire the ability to fertilize the oocytes. These modifications involve the activation of several signalling pathways, which are related to an increase in intracellular secondary messengers, such as cAMP and Ca^{2+}, changes in motility patterns and reorganization of plasma membrane. Specifically, the fusion of the outer acrosome membrane with the plasma membrane occurs during capacitation and allows the spermatozoa to trigger the acrosome reaction (see Kątska-Książkiewicz, 2007; Visconti, 2009, for review).

Antioxidant agents have been used to improve storage strategies for mammalian spermatozoa (Yeste, 2016). Particularly, antioxidants have been demonstrated to increase the percentage of intact sperm cells after freeze-thawing, which has raised the question on how these agents exert their positive effects. On this respect, it is worth mentioning that cryopreservation detrimentally affects sperm function and survival, decreasing their motility and increasing lipid packaging of plasma membrane (Cormier *et al.*, 1997; Bailey *et al.*, 2000; Yeste, 2015; Yeste *et al.*, 2017). Interestingly, the presence of antioxidants in

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cryopreservation media appears to stabilize better the sperm membrane (Yeste *et al.*, 2013; Giaretta *et al.*, 2015), and, particularly, reduced glutathione (GSH) improves the fertilizing ability of boar spermatozoa both *in vitro* and *in vivo* (Gadea *et al.*, 2004, 2005; Estrada *et al.*, 2014, 2017a). Notably, some of the positive effects of GSH on sperm function and survival (Yeste *et al.*, 2013; Estrada *et al.*, 2014, 2017b) appear to be related to a better maintenance of the intracellular redox balance (Jacob *et al.*, 2003). Indeed, GSH is known to decrease intracellular reactive oxygen species (ROS) levels (Gadea *et al.*, 2004, 2005) and stabilize the integrity of disulphide bridges between sperm proteins in cryopreserved spermatozoa (Chatterjee *et al.*, 2001; Yeste *et al.*, 2013; 2014). On the other hand, moderate levels of ROS have been suggested to be linked to sperm capacitation and mitochondria are known to be the main sources of ROS generation in mammalian spermatozoa (reviewed by Aitken, 2017). In spite of this, it remains to be elucidated to which extent antioxidants, such as GSH, modulate sperm capacitation and how their effects on this crucial physiological mechanism could underlie their positive impact observed in previous studies conducted with frozen-thawed boar spermatozoa.

Taking the aforementioned into account, the main aim of this study was to evaluate the role of intracellular ROS levels and maintenance of protein disulphide bridges in sperm proteins on the achievement of *in vitro* capacitation and subsequent *in vitro*, progesterone-induced acrosome exocytosis of boar spermatozoa. With this purpose, boar spermatozoa were subjected to *in vitro* capacitation and acrosome reaction in the presence or absence of GSH, a well-known protective agent against both high ROS levels and disulphide bonds disruption (Chatterjee *et al.*, 2001; Gadea *et al.*, 2004, 2005). The evaluation of the achievement of *in vitro* capacitation and acrosome exocytosis was carried out by the analysis of several capacitation markers and other sperm quality parameters, such as percentages of viable and motile spermatozoa, acrosome integrity, membrane lipid disorder, DNA integrity, intracellular levels of peroxides and superoxides, tyrosine phosphorylation P32 and localization of tyrosine-phosphorylated proteins (pTyr). Furthermore, free cysteine levels in both head and tail sperm extracts were evaluated to determine the degree of disrupted disulphide bonds. Finally, the sperm ability to penetrate *in vitro*-matured porcine oocytes was tested after *in vitro* capacitation in the presence or absence of GSH.

MATERIALS AND METHODS

Seminal samples

A total of 62 ejaculates collected from 35 healthy Pietrain boars aged between two and three years were used in this study. These animals were housed in climate-controlled buildings (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with an adjusted diet and provided with water *ad libitum*. Sperm-rich fractions were collected manually using the hand-gloved method, diluted to a final sperm concentration of 2×10^7 spermatozoa/mL in a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain) and cooled to 16 °C. Diluted semen was then split into 90-mL commercial AI doses and the resulting 90-mL doses were placed in a thermal packaging container at 16 °C for approximately 45 min, which was the time required to arrive to our laboratory.

Ethical approval

The ejaculates involved in this study were initially intended to artificial insemination and we just bought them for our experimental purposes. Therefore, we did not need any specific ethical approval to perform this work, as no animal was manipulated by us. In spite of this, the experimental protocol was approved by the Ethics Committee of our institution. This ethics committee was known as *Bioethics Commission of the Autonomous University of Barcelona* (Bellaterra, Cerdanyola del Vallès, Spain).

Experimental design, *in vitro* capacitation and subsequent acrosome exocytosis

The work consisted of two separate experiments. In the first one, we addressed how the addition of GSH to the capacitation medium (CM) at the beginning of the experiments affected the sperm ability to achieve the capacitated status. The second experiment aimed at determining the effects of GSH on the achievement of acrosome exocytosis in previously capacitated boar spermatozoa; with this purpose, GSH was added together with progesterone. In each experiment, three concentrations of GSH ($C_{10}H_{17}N_3O_6S$; GSH, Sigma-Aldrich®, St Louis, MO, USA) were tested (1, 2 and 5 mM). Therefore, there were five separate treatments: a positive control, in which cells were incubated in CM; three different GSH concentrations, where GSH was added at either 0 h (experiment 1) or 4 h, and a negative control, in which spermatozoa incubated in a non-capacitating medium (NCM). In each treatment and experiment, the following sperm parameters were evaluated: sperm motility; sperm viability; true acrosome exocytosis (i.e. percentages of viable, acrosome-exocytosed spermatozoa); membrane lipid disorder; mitochondrial membrane potential (MMP); intracellular levels of calcium, peroxides and superoxides; DNA fragmentation; free cysteine residues of sperm proteins; immunolocalization of tyrosine-phosphorylated sperm proteins; and tyrosine phosphorylation of P32, a specific capacitation marker of boar spermatozoa (Bravo *et al.*, 2005).

For the first experiment, 50 mL of an extended boar semen sample was centrifuged at 600 ***g*** for 10 min at 16 °C and then resuspended at a final concentration of 20–30 $\times$ 10^6 sperm/mL in NCM. NCM was made up of 20-mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (pH = 7.4), 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM $NaHPO_4$, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (osmolarity: 287 mOsm/kg $\pm$ 6 mOsm/kg). After resuspension, 40 mL were separated and added with 5 mg/mL BSA and 37.6 mM $NaHCO_3$ to obtain the CM (pH = 7.4; osmolarity = 304 mOsm/kg $\pm$ 5 mOsm/kg). The remaining 10 mL-aliquot (NCM) was the negative control. The 40-mL CM suspension cell was further divided into four 10-mL aliquots. One of these 10-mL aliquots was the positive control, whereas the other three were added with GSH at final concentrations of 1, 2 and 5 mM. All aliquots were incubated at 38.5 °C and 5% CO_2 for 4 h, as described in Ramio *et al.* (2008). Spermatozoa were evaluated at 0 and 4 h of incubation to evaluate the sperm parameters described below. After 4 h of incubation, sperm cells were added with progesterone to induce the acrosome reaction (Jimenez *et al.*, 2003; Wu *et al.*, 2006). In brief, 10 μg/mL progesterone was added and after thorough mixing, spermatozoa were

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further incubated at 38.5 °C and 5% CO_2 for an additional hour. Sperm parameters were evaluated after 1, 5 and 60 min of the addition of progesterone.

Although the design of the second experiment was similar to that of experiment 1, the main difference was that GSH was added after 4 h of incubation, together with progesterone. Again, three different GSH concentrations were tested and samples were evaluated before (0 and 4 h) and after (1, 5, 60 min) of adding GSH together with progesterone.

Determination of extracellular GSH concentration

Extracellular GSH levels were determined by centrifuging 1-mL aliquots at 2000 ***g*** and 4 °C for 30 s. The resultant supernatants were immediately frozen in liquid N_2 and stored at −80 °C until analysis, which was performed within the following 15 days. Concentration of GSH was determined using a spectrophotometer and following the protocol described by Rahman *et al.* (2006). Additionally, to determine whether there were non-sperm-related changes in extracellular GSH levels, GSH concentration was determined in CM without spermatozoa, added or not with GSH, after incubation at 38.5 °C and 5% CO_2 for 4 h.

Evaluation of sperm motility

Sperm motility was evaluated with a commercial computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain), following the settings and parameter ranges described in Ramio *et al.* (2008). Briefly, samples were previously incubated at 37 °C for 5 min in a water bath and a 5-μL drop was subsequently placed in a pre-warmed Neubauer chamber. Three replicates of 1000 spermatozoa each were counted prior to calculating the mean±standard error of the mean (SEM). A sperm cell was considered to be motile when its average path velocity (VAP) was higher than 10 μm/s.

Analysis of sperm parameters with flow cytometry

Flow cytometry was used to determine sperm viability, capacitation-like membrane lipid changes, acrosome integrity and intracellular levels of calcium, peroxides and superoxides following the recommendations detailed in Lee *et al.* (2008). In all assessments, sperm concentration was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL. After staining, samples were evaluated through a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA), which was calibrated periodically. Sheath flow rate was set at 4.17 μL/min in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EV vs. SS dot plots) for a minimum of 10,000 events per replicate. Each parameter was evaluated in triplicate in independent tubes. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 μm) and cell aggregates (particle diameter > 12 μm). Compensation was used to minimize fluorescence spill-over into a different channel.

Information on the events was collected in List-mode Data files and the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter) was used to analyse cytometric histograms and dot plots. In all assessments except SYBR14/PI, data were corrected with the procedure described by Petrunkina *et al.* (2010).

Unless otherwise stated, all fluorochromes were purchased from Invitrogen™ Molecular Probes™ Molecular Probes® (Thermo Fisher Scientific, Waltham, MA, USA) and diluted with dimethyl sulphoxide (DMSO; Sigma-Aldrich).

Evaluation of sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/PI), according to the protocol described by Garner & Johnson (1995). Following staining, three separate sperm populations were identified: (i) viable, green-stained spermatozoa (SYBR-14^+/PI^-); (ii) non-viable, red-stained spermatozoa (SYBR-14^-/PI^+); and (iii) non-viable spermatozoa that were stained both green and red (SYBR-14^+/PI^+). Non-sperm particles (debris) were found in the SYBR-14^-/PI^- quadrant.

Single-stained samples were used to set EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the FL-3 channel (2.45%).

Evaluation of acrosome exocytosis

True acrosome exocytosis (percentages of viable spermatozoa with non-intact acrosome) was determined by co-staining of spermatozoa with ethidium homodimer (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1) and peanut agglutinin (from *Arachis hypogaea*) conjugated with fluorescein isothiocyanate (FITC-PNA). This protocol was originally described by Cooper & Yeung (1998) and was adapted to boar spermatozoa in our laboratory. Briefly, samples were incubated with EthD-1 (final concentration: 2.5 μg/mL) at 38 °C for 5 min in the dark. Following this step, samples were washed by centrifugation at 2000 ***g*** for 30 s and then resuspended with PBS containing 4 mg/mL bovine serum albumin (BSA) to remove free dye. Thereafter, samples were again centrifuged and fixed and permeabilized by adding 100 μL of ice-cold methanol (100%) for 30 s. Methanol was removed by centrifugation at 2000 ***g*** for 30 s and resuspension with PBS containing 4 mg/mL BSA. Following this, 0.8 μL PNA-FITC (final concentration: 2.5 μM) was added and samples were incubated at room temperature in the dark for 15 min. Thereafter, samples were washed twice with PBS at 2000 ***g*** for 30 s and finally resuspended in 250 μl BTS.

Four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1^-); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1^-); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1^+); (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1^+).

Evaluation of capacitation-like sperm membrane lipids changes

Capacitation-like membrane lipid changes of boar sperm membrane was evaluated by merocyanine-540 (M540) and YO-PRO-1 co-staining, as described by Rathi *et al.* (2001) and Yeste *et al.* (2015). A total of four sperm populations were distinguished: (i) viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO-1^-); (ii) viable spermatozoa with high, capacitation-like membrane lipid changes (M540$^+$/YO-PRO-1^-); (iii) non-viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO-1^+) and (iv) non-viable spermatozoa with high membrane lipid changes (M540$^+$/YO-PRO-1^+).

Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was analysed with tetraethylbenzimidazolcarbocyanine iodide (JC-1), as described by Gillan *et al.* (2005). Two sperm subpopulations were

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determined: the first subpopulation was formed by spermatozoa with high MMP that showed orange staining (JC-1 aggregates), whereas the second one contained the sperm cells with low MMP, which were stained in green (JC-1 monomers).

Evaluation of intracellular levels of peroxides and superoxides

Intracellular superoxide ($O_2^{-\cdot}$) and peroxide (H_2O_2) levels were determined using two different oxidation-sensitive fluorescent probes: hydroethidine (HE) for $O_2^{-\cdot}$ and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for H_2O_2, following the protocol described by Guthrie & Welch (2006). Sperm membrane integrity was simultaneously evaluated with YO-PRO-1 and PI, respectively. Following staining, percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence) and percentages of viable spermatozoa with high $O_2^{-\cdot}$ levels (high ethidium fluorescence; E^+) were recorded.

Evaluation of intracellular calcium levels

Intracellular calcium levels were determined through two separate, specific stains: Rhod-5N-AM (Rhod5) and Fluo-3-AM (Fluo3), following the protocols described previously (Harrison *et al.*, 1993; Kadirvel *et al.*, 2009; Yeste *et al.*, 2015). Rhod5 mainly stains the calcium of sperm head, whereas Fluo3 mainly stains that of the mitochondrial piece (Yeste *et al.*, 2015).

In the case of Rhod5, where sperm membrane integrity was simultaneously evaluated with YO-PRO-1 staining, the following four sperm populations were identified: (i) viable spermatozoa with low levels of intracellular calcium ($Rhod5^-$/$YO\text{-}PRO1^-$); (ii) viable spermatozoa with high levels of intracellular calcium ($Rhod5^+$/$YO\text{-}PRO\text{-}1^-$); (iii) non-viable spermatozoa with low levels of intracellular calcium ($Rhod5^-$/$YO\text{-}PRO\text{-}1^+$); and (iv) non-viable spermatozoa with high levels of intracellular calcium ($Rhod5^+$/$YO\text{-}PRO\text{-}1^+$).

In the case of Fluo3, sperm membrane integrity was simultaneously evaluated with PI and the following four sperm populations were identified: (i) viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^-); (ii) viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^-); (iii) non-viable spermatozoa with low levels of intracellular calcium ($Fluo3^-$/PI^+); and (iv) non-viable spermatozoa with high levels of intracellular calcium ($Fluo3^+$/PI^+).

Evaluation of free cysteine residues in sperm proteins

Free cysteine residues in sperm head and tail proteins were determined following the protocol described by Flores *et al.* (2011) with minor modifications. Samples were centrifuged at 600 ***g*** and 16 °C for 10 min and resuspended in ice-cold 50 mM Tris buffer (pH = 7.4) containing 150 mM NaCl, 1% (v:v) nonidet, 0.5% (w:v) sodium deoxycholate, 1 mM benzamidine, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator; Ika Labortechnik, Staufen, Germany). Obtained homogenates were centrifuged at 850 ***g*** and 4 °C for 20 min. Whereas the resulting supernatants were used to measure free cysteine residues in sperm tail proteins, the pellets were resuspended in 300 μL of Tris buffer to measure free cysteine residues in sperm head proteins. The presence of tails in supernatants and heads in pellets were determined by previous observation under a phase-contrast microscope at 20× and 40× magnification. We found that the percentages of tails in supernatants and heads in pellets were higher than 85% (data not shown).

Levels of free cysteine residues in both tail and head fractions were determined with 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma-Aldrich) as described by Brocklehurst *et al.* (1979). Five replicates per sample and treatment were evaluated, and the corresponding mean ± SEM was calculated.

Evaluation of DNA fragmentation

DNA fragmentation was assessed using a commercial sperm chromatin dispersion test (SCDt) kit specifically designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain). This test is based on the different response that both intact and fragmented DNA show after deproteinization (Enciso *et al.*, 2005). Thus, the procedure is based on the incubation with a commercial proteinase K solution included in the kit of spermatozoa previously embedded in agarose for 10 min at room temperature. Subsequently, samples were subjected to specific DNA staining through incubation with 12 μM PI in PBS at room temperature for 15 min. Afterwards, samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss, Jena, Germany) at 1000× magnification. Three counts of 250 spermatozoa each using three different slides were evaluated per sample and treatment, prior to calculating the corresponding mean ± SEM.

Immunoblotting

A total of seven ejaculates were selected for Western blot assays. For this purpose, 1 mL-aliquot belonging to each experimental point was centrifuged at 1000 ***g*** for 30 s and pellets were stored at −80 °C until use. Pellets were resuspended and sonicated in 300 μL ice-cold lysis buffer (pH = 7.4), containing 50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 25 mM dithiothreitol, 1.5% (v:v) Triton® X-100, 1 mM PMSF, 10 μg/mL leupeptin, 1 mM orthovanadate and 1 mM benzamidine. After 30 min on ice, the homogenized suspensions were centrifuged at 10,000 ***g*** and 4 °C for 20 min and total protein content in supernatants was calculated through the Bradford (1976) using a commercial kit (Bio-Rad Laboratories; Fremont, CA, USA). Afterwards, samples were added with a loading buffer (1 : 5; v : v) containing 250 mM Tris-HCl (pH = 6.8), 50 mM dithiothreitol, 10% (w : v) SDS, 0.5% (v : v) bromophenol blue and 50% (v : v) glycerol and stored at −20 °C until their subsequent assay.

Samples were loaded onto 10% (w : v) acrylamide gels to perform SDS-PAGE (Laemmli, 1970). Pre-stained protein standards with a molecular mass range of approximately 250 kDa to 10 kDa were added to another lane. Separated proteins were transferred onto a low-fluorescence polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot® Turbo Transfer System (Bio-Rad). Membranes were subsequently immersed in blocking solution for 60 min; blocking solution consisted of a Tris-buffered saline solution added with 5% (w : v) BSA and 0.1% (v : v) Tween-20. Subsequently, membranes were submerged in blocking solution containing the appropriate concentration of the primary antibody. Membranes were incubated with the antibody at 4 °C for 8 h. Primary antibodies were mouse PY20 clone anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich) and mouse monoclonal anti-β-tubulin (ref. T5201; Sigma-Aldrich). In both cases, the dilution factor was 1 : 1000 (v : v). After three washes, membranes were

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incubated with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Dako; Glostrup, Denmark) at a dilution of 1 : 5000 (v : v) in blocking solution for 60 min. Membranes were washed six times and were revealed using a chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Dallas, TX, USA). β-tubulin was used as an internal standard to normalize the volume of protein bands, after stripping and reprobing.

Analysis of membrane images was performed with ImageJ 1.49 (National Institute of Health, USA) software. The intensity of each band was quantified and the background, defined as the surrounding area of the band with a width of 1 mm, was utilized to adjust the value of intensity of each band. Ratios between the intensity values of pTyr-P32 and their corresponding β-tubulin bands were calculated using the following formula: (P-BP)/(T-BT), where P was the intensity value in arbitrary units obtained from the pTyr-P32 band, BP was the intensity of the background of the pTyr-P32 band, T was the intensity of the β-tubulin band, and BT was the intensity of the background of the β-tubulin band. Data were corrected to a basal arbitrary value of 100 for the control point, which corresponded to the incubation in standard CM at 0 h.

Immunolocalization of tyrosine-phosphorylated sperm proteins

Three treatments (NCM, CM and GSH at 2 mM) were evaluated and a total of seven ejaculates were used. Immunolocalization of tyrosine-phosphorylated proteins in boar spermatozoa was carried out as described in Medrano *et al.* (2006), using a mouse PY20 clone anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich) at a final dilution of 1 : 200 (v : v) in PBS, and an Alexa Fluor® 488 donkey anti-mouse secondary antibody (final dilution: 1 : 500, v : v). Slides were carefully washed with PBS at 4 °C three times and a drop of anti-fade mounting medium containing 4,6-diamidino-2-phenylindole hydrochloride (DAPI, 125 ng/mL; Vysis Inc., Downers Grove, IL, USA) was placed onto the specimen. Fluorescent images were obtained with a laser confocal scanning microscope (Leica TCS 4D; Leica, Heidelberg, Germany). Successive image slices (0.5 μm) were integrated into three-dimensional reconstructions, which were further saved as TIFF-format images.

In vitro fertilization

The ability of boar spermatozoa to adhere and fertilize *in vitro*-matured pig oocytes was evaluated following the protocol described by Matás *et al.* (2003) with minor modifications. Briefly, ovaries were obtained from a local slaughterhouse (Frigorífics Costa Brava; Riudellots de la Selva, Spain) and brought to the laboratory in saline solution (0.9% (w/v) NaCl) containing 100 μg/mL kanamycin sulphate at 37 °C. Upon arrival, ovaries were washed twice with the same saline solution. Oocyte-cumulus cell complexes (COCs) were subsequently collected from follicles of 3–6 mm diameter and washed two times in Dulbecco's PBS medium supplemented with 4 mg/mL polyvinyl alcohol (PVA). Thereafter, COCs were washed in maturation medium previously equilibrated at 38.5 °C and 5% CO_2 in 100% humidified air for 3 h. This maturation medium consisted of NCSU-37 medium (Petters & Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotrophin (eCG, Folligon, Intervet International BV; Boxmeer, The Netherlands), 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, Barcelona, Spain) and 10% (v/v) pig follicular fluid. Only oocytes harvested within 2 h of slaughtering with a complete, dense *cumulus oophorus* were *in vitro*-matured (Matás *et al.*, 1996). After washing with maturation medium, COCs were pooled into groups of 50 oocytes with 500 μL maturation medium and ten incubated at 38.5 °C and 5% CO_2 for 22 h. After 22 h, COCs were transferred to fresh maturation medium without hormones or dibutyryl cAMP, and incubated for a further 22-h period.

After maturation, oocytes were mechanically stripped of *cumulus* cells by carefully aspiration with a pipette. Denuded oocytes were then washed with TALP medium (Castillo-Martín *et al.*, 2014) and each group of 50 oocytes was transferred to a well of a four-well Nunc multidish (Nunc, Roskilde, Denmark) containing 250 μL TALP medium previously equilibrated at 38.5 °C and 5% CO_2 for 3 h (Matás *et al.*, 2010). Two hundred fifty μL of sperm suspensions from each treatment group were added to the fertilization wells to obtain a final concentration of 1×10^5 cells/mL. Three treatments were tested. In the first treatment (identified as GSH), spermatozoa were previously incubated in CM containing 2 mM GSH at 38.5 °C and 5% CO_2 for 4 h and then added to the *in vitro*-matured oocytes. In the second treatment (identified as GSH+PG), spermatozoa were previously incubated in CM without GSH at 38.5 °C and 5% CO_2 for 4 h and then added together with 2 mM GSH to the *in vitro*-matured oocytes. This treatment was included to reach a final GSH concentration of 2 mM in the oocyte-sperm co-incubation medium, without a previous exposure of spermatozoa to GSH. Finally, in the third treatment (identified as control), oocytes were added with spermatozoa previously incubated with CM at 38.5 °C and 5% CO_2 for 4 h. Capacitation medium were not supplemented with GSH.

In all treatments, spermatozoa and oocytes were co-incubated for 1 h. Non-attached spermatozoa were removed by gentle aspiration with a pipette and subsequent washing with TALP medium. Oocyte-sperm complexes were transferred to a new well containing 500 μL TALP and cultured at 38.5 °C and 5% CO_2 for 18 h. After 7 h and 18 h of culture, cells were collected to perform nuclear staining. With this purpose, cells were washed in warmed PBS and subsequently fixed in 4% (w : v) paraformaldehyde in PBS at 38 °C for 30 min. After fixation, sperm cells were washed twice with PBS and stained with 1% (v : v) Hoechst® 33342 (Sigma-Aldrich) in PBS at room temperature for 25 min. Following this, cells were washed twice with PBS, mounted on glass slides and examined under a TCS 4D confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) for evidence of sperm penetration.

In the oocyte-sperm complexes cultured for 7 h, we evaluated the number of spermatozoa adhered to the external surface of oocytes and the number of oocyte-sperm complexes that showed normal fertilization. Normal fertilization was only considered when a unique sperm nucleus was observed within the oocyte and was expressed as a rate against the total number of oocyte-sperm complexes. In the oocyte-sperm complexes cultured for 18 h, we also evaluated normal fertilization rates. In this case, normal fertilization was considered when the nuclei observed inside the fertilized oocyte showed evident signs of

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decondensation; again, normal fertilization rates were calculated against the total number of oocyte-sperm complexes.

Statistical analyses

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 23.0; IBM Corp., Armonk, NY, USA). Data are shown as mean ± SEM and the minimal level of significance was set at $p < 0.05$ in all tests.

Data were first tested for normality and homoscedasticity through Shapiro–Wilk and Levene tests. When required, data (x) were transformed using arcsine square root (arcsin $\sqrt{x}$) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (NCM, CM, GSH concentrations) and the intrasubject factor was the incubation time (i.e. 0, 4, 4 h 1 min, 4 h 5 min, 4 h 60 min). In all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were worked out by using Sidak's test.

When no transformation remedied the normality, non-parametric procedures were used with raw data. Friedman's test and Wilcoxon matched-pairs test were used as non-parametric alternatives to mixed models. Percentages of normally fertilized oocytes were determined through a chi-square test (χ^2).

RESULTS

Extracellular GSH concentration during *in vitro* capacitation and acrosome exocytosis

As expected, GSH was absent from both freshly made CM and NCM before its addition. Extracellular concentration of GSH decreased after 4 h of incubating spermatozoa at 38.5 °C and 5% CO_2 with a medium containing 2 mM GSH (174.4 ± 12.4 μM, Table 1). The addition of progesterone after 4 h of incubation did not have any immediate effect on extracellular concentration of GSH, but a further decrease was observed after 60 min of progesterone addition (58.9 ± 11.7 μM, Table 1). A decrease in the extracellular concentration of GSH was also observed when GSH was added together with progesterone and samples were examined after 60 min of that addition (975.8 ± 62.1 μM Table 1).

Effects of GSH on sperm viability and acrosome integrity during *in vitro* capacitation and acrosome reaction

Incubation of sperm cells in both CM and NCM showed a progressive drop in viability (Figure S1). The addition of GSH to CM at 0 h had no remarkable effect on sperm viability, but this parameter decreased at the end of the experimental period (60 min after progesterone addition; 60.5% ± 3.8% in control vs. 54.2% ± 3.3% in 1 mM GSH, Fig. 1A). In contrast, sperm viability was not affected when GSH was added together with progesterone (Figure S1B).

Percentages of viable spermatozoa with an exocytosed acrosome (true acrosome exocytosis) after incubation in CM for 4 h were low before the addition of progesterone (Fig. 1). The addition of progesterone progressively increased the percentages of viable spermatozoa with an exocytosed acrosome, which reached maximum values after 60 min of the addition of the hormone (59.0% ± 2.9%, Fig. 1). This phenomenon was not observed when spermatozoa were incubated in NCM. The presence of GSH since 0 h counteracted the progesterone-induced increase in true acrosome exocytosis in a concentration-dependent manner, the percentages of viable spermatozoa with an exocytosed acrosome being the lowest in 5 mM GSH treatment (46.1% ± 2.0%, see Fig. 1A). In contrast, the addition of GSH together with that of progesterone after 4 h of incubation in CM did not significantly affect the percentages of viable spermatozoa with an exocytosed acrosome in the spermatozoa incubated in CM (Fig. 1B).

Effects of GSH on sperm capacitation-like membrane lipid changes during *in vitro* capacitation and acrosome exocytosis

Incubation of boar spermatozoa with CM significantly ($p < 0.05$) increased the percentage of viable spermatozoa with high membrane lipid disorder, which were compatible with the achievement of the capacitation status (from 5.9% ± 1.4% at 0 h of incubation to 38.5% ± 2.9% after 4 h of incubation, see Fig. 2). The subsequent addition of progesterone decreased this percentage, reaching values of 27.9% ± 2.1% at end of the experimental period (Fig. 2). The addition of GSH at 0 h counteracted the observed increase in the percentage of viable spermatozoa with high membrane lipid disorder (Fig. 2A). On the contrary, percentages of viable spermatozoa with high membrane lipid disorder did not differ from those observed in CM when GSH was added together with progesterone (Fig. 2B).

Effects of GSH on sperm motility during *in vitro* capacitation and acrosome exocytosis

Incubation in CM significantly ($p < 0.05$) decreased percentages of total sperm motility (55.1% ± 2.9% at 4 h. Fig. 3) and increased the number of agglutinated spermatozoa (data not shown). While total motility of spermatozoa incubated in NCM was even lower than that of CM along the incubation period (Fig. 3), the degree of agglutination was lower. When GSH (2 mM and 5 mM) was added at 0 h, sperm motility was significantly ($p < 0.05$) lower than in CM (5Mm GSH at 4 h: 13.4% ± 1.6%,

Table 1 GSH concentrations in capacitation medium with or without GSH and with or without spermatozoa

	Treatment	0 h	4 h	1 min	5 min	60 min
Without spermatozoa	CM	N.D.	N.D.	N.D.	N.D.	N.D.
	GSH	2047.4 ± 78.9^a	503.3 ± 29.8^b	511.5 ± 31.6^b	501.3 ± 32.9^b	274.8.±23.7^c
	GSH+PG	N.D.	N.D.	2098.1 ± 79.0^a	2098.1 ± 79.0^a	1710.6 ± 42.0^b
With spermatozoa	CM	N.D.	N.D.	N.D.	N.D.	N.D.
	GSH	1987.4 ± 75.8^a	174.4 ± 12.4^b	160.5 ± 14.8^b	164.4 ± 16.1^b	58.9 ± 11.7^c
	GSH+PG	N.D.	N.D.	1885.9 ± 78.8^a	1953.6 ± 77.0^a	975.8 ± 62.1^b

CM, capacitation medium; GSH, reduced glutathione (2 mM) added at the beginning of the experiment; GSH+PG, reduced glutathione (2 mM) added together with progesterone after 4 h of incubation in CM; N.D., not detectable. Different superscripts indicate significant differences ($p < 0.05$) between columns within a given row. Results are shown as mean ± SEM of seven experiments.

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Figure 1 Effects of GSH on percentages of true acrosome exocytosis of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

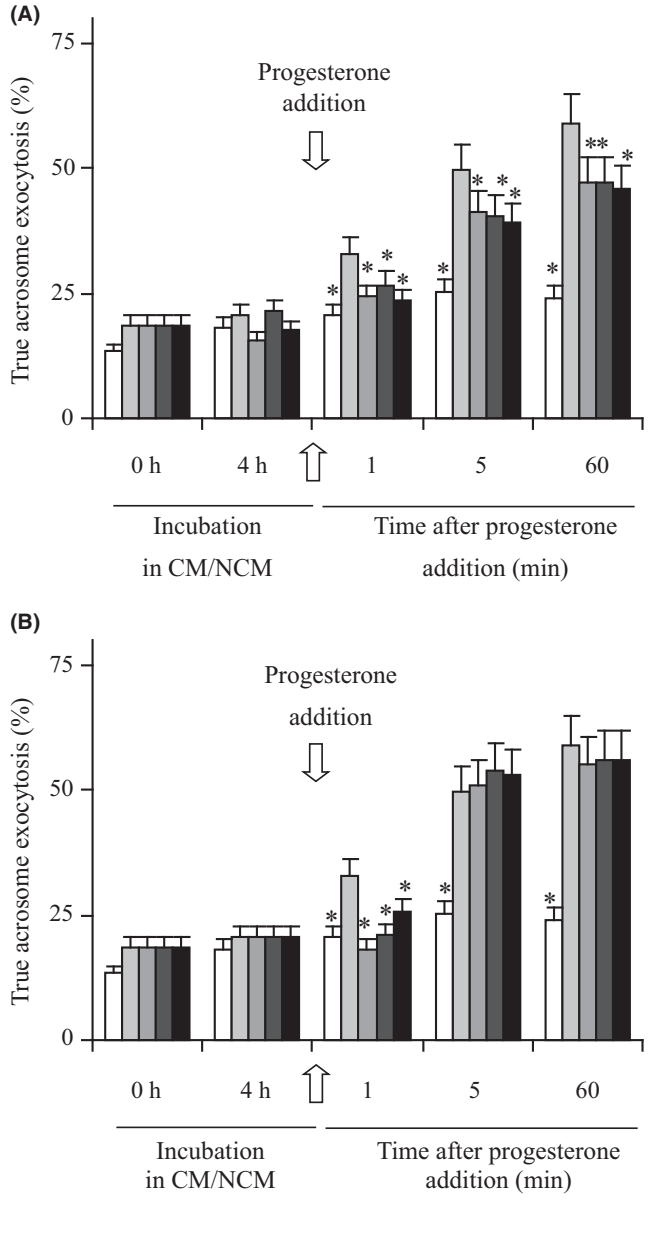
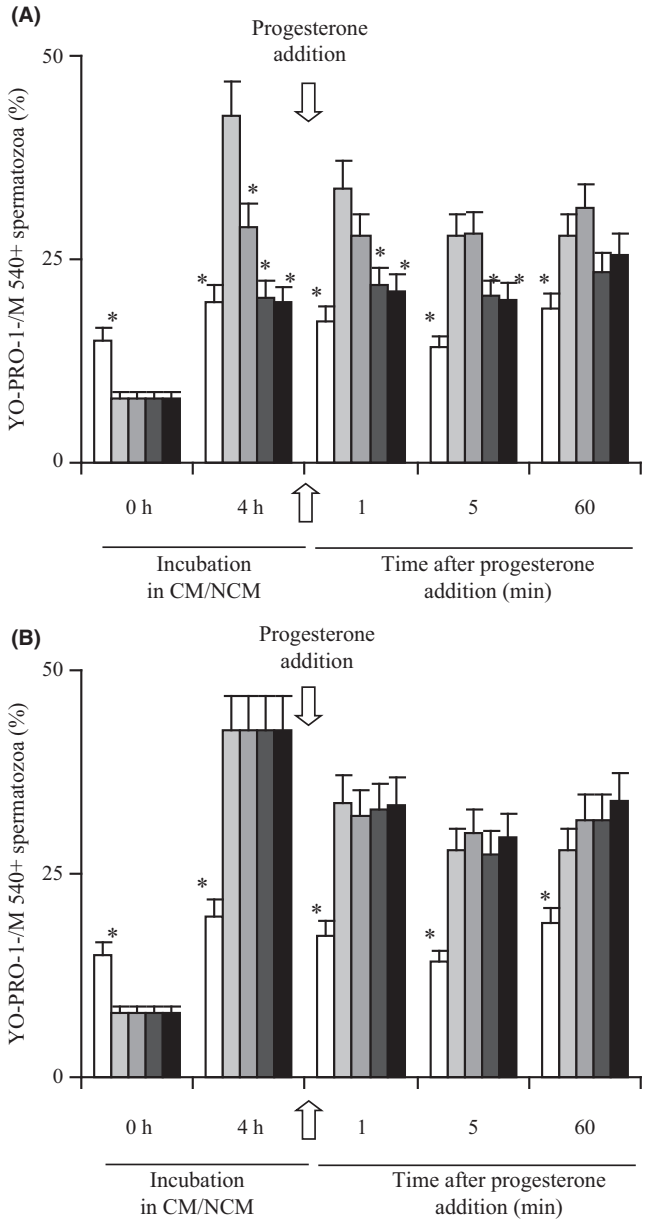


Figure 2 Effects of GSH on percentages of viable cells with capacitation-like lipid membrane disorder of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



see Fig. 3A). However, this GSH-induced decrease in total sperm motility did not seem to be related with a noticeable increase in sperm agglutination (data not shown). Similarly, when GSH was added together with progesterone at 4 h, sperm motility also decreased, reaching minimal values at the end of the experimental period (Fig. 3B).

Incubation in CM and NCM increased VCL, VAP, LIN and STR (Tables 2–4). In the case of CM, but not in that of NCM, ALH decreased. These changes in sperm kinematic parameters are related to the achievement of capacitation status and are similar to those published previously in the same conditions (García-Herreros *et al.*, 2005; Yeste *et al.*, 2013, 2015). The addition of progesterone after 4 h of incubation in CM induced a rapid increase in VCL, VAP and ALH that was concomitant with a decrease in LIN and STR (Tables 2–4). Incubation of spermatozoa in a medium containing GSH since the beginning of the experiment had slight effects on kinematic parameters. In

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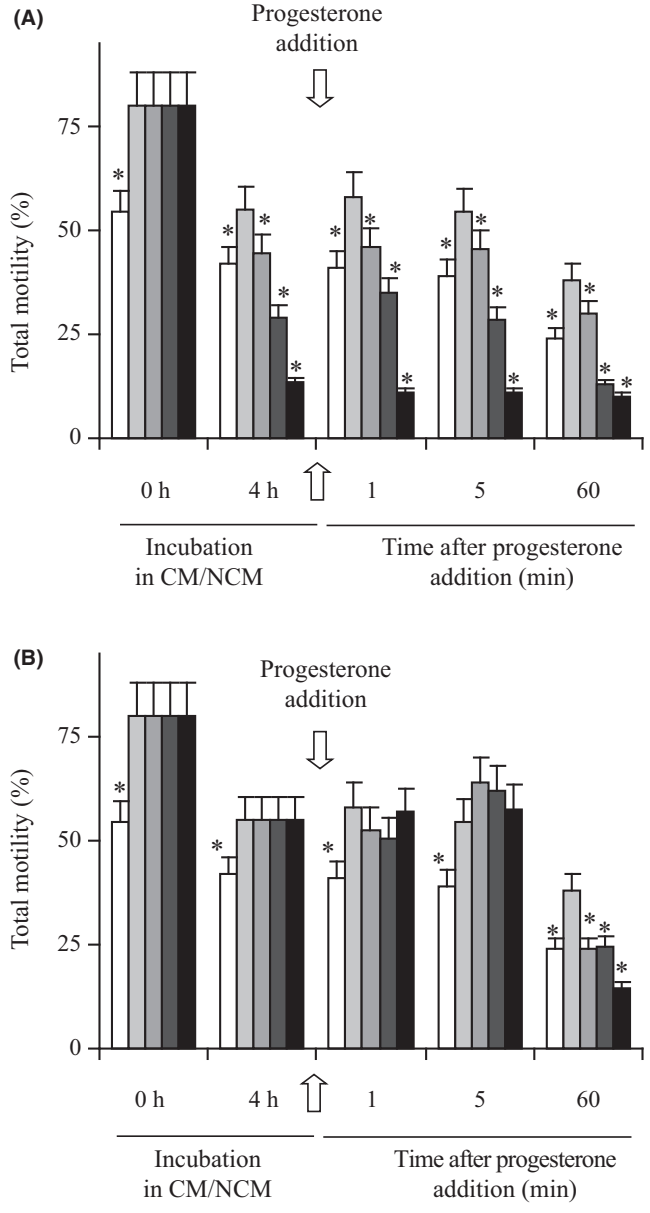
contrast, the addition of GSH together with progesterone at 4 h increased VCL and BCF, but counteracted the progesterone-induced increase of LIN and STR observed in CM (2Mm GSH and 5Mm GSH, Tables 2–4).

Effects of GSH on tyrosine phosphorylation of P32 protein during *in vitro* capacitation and acrosome exocytosis

Incubation of boar spermatozoa in CM for 4 h significantly ($p < 0.05$) increased the intensity of pTyr signal in P32 protein (Figure S2). This increase was maintained after the addition of progesterone. When GSH was added at 0 h, there were no differences in the intensity of P32 pTyr signal during the first 4 h of incubation, but that signal decreased after 5 min (5 mM GSH) and 60 min (2 mM GSH and 5 mM GSH) of progesterone addition (Figure S2A,B). In contrast, the addition of GSH together with progesterone had no effects on the intensity of pTyr-P32 signal (Figure S2C,D).

Figure 3 Effects of GSH on total motility of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione in added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



Effects of GSH on the localization of tyrosine-phosphorylated proteins during *in vitro* capacitation and acrosome exocytosis

At 0 h of incubation in CM, tyrosine-phosphorylated proteins were mainly located at the equatorial and post-acrosomal regions of sperm head (Figure S3). Incubation in CM for 4 h increased the intensity of the post-acrosomal signal, which was maintained after progesterone addition. Furthermore, there was an apparent marking in the spermatozoa tail after progesterone addition (Figure S3). Although the increase of both post-acrosomal and tail pTyr signal was maintained and even increased after 5 min of progesterone addition (Figure S3), a clear reduction of its intensity was observed after 60 min of progesterone addition (data not shown). Addition of 2 mM GSH at 0 h counteracted the increase in the post-acrosomal signal observed in CM after 4 h of incubation and that of the tail observed after progesterone addition. In contrast, the addition of GSH together with progesterone did not differ from the incubation in CM (Figure S3 and data not shown).

Effects of GSH on mitochondrial membrane potential during *in vitro* capacitation and acrosome exocytosis

Incubation of spermatozoa in CM for 4 h increased the percentage of spermatozoa with high MMP, which went from 23.0% ± 3.8% at 0 h to 54.6% ± 4.9% at 4 h (Fig. 4). The extent of that increase was higher ($p < 0.05$) than that observed in NCM. The addition of progesterone to CM induced a further increase in this percentage, reaching peak values after 1 min of that addition. Thereafter, percentages of spermatozoa with high MMP in CM decreased, reaching values of 57.7% ± 5.2% after 60 min of progesterone addition (Fig. 4).

When GSH was present in the CM since 0 h, the aforementioned increases in the percentages of spermatozoa with high MMP were counteracted (Fig. 4A). Likewise, adding GSH (2 mM and 5 mM) together with progesterone prevented the progesterone-induced increase in the percentage of spermatozoa with high MMP observed in CM at 1 min (Fig. 4B).

Effects of GSH on intracellular calcium levels during *in vitro* capacitation and acrosome exocytosis

Percentages of viable boar spermatozoa with high intracellular calcium levels stained by Rhod5 (mainly sperm head) progressively increased after incubation in CM, reaching values of 38.5% ± 3.9% after 4 h of incubation (Figure S4). Subsequent addition of progesterone induced a transient increase in Rhod5-stained calcium (65.6% ± 5.1% after 1 min; Figure S4). The addition of 5 mM GSH at 0 h significantly ($p < 0.05$) decreased the transient increase of calcium observed after 1 min of progesterone addition (Figure S4A). This transient increase of calcium observed after 1 min was also counteracted when 5 mM GSH was added together with progesterone (56.0% ± 4.9% vs. CM: 65.6% ± 5.1%, Figure 4B).

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Table 2 Effects of GSH on curvilinear velocity (VCL) and average path velocity (VAP) of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
VCL (μm/s)					
NCM	52.4 ± 1.1^{a}	61.8 ± 1.7^{b}	101.6 ± 3.5^{c}	94.0 ± 2.9^{c}	112.2 ± 4.4^{d}
CM	62.6 ± 1.8^{a}	54.7 ± 1.9^{b}	70.5 ± 2.8^{c}	67.5 ± 3.1^{ac}	70.8 ± 5.3^{c}
1 mM GSH	62.6 ± 1.8^{a}	60.1 ± 5.0^{ab}	60.2 ± 4.3^{ab}	56.3 ± 2.8^{b*}	78.0 ± 6.2^{c}
2 mM GSH	62.6 ± 1.8^{a}	37.1 ± 1.5^{b*}	58.0 ± 3.6^{a*}	60.6 ± 4.2^{a}	64.5 ± 4.0^{a}
5 mM GSH	62.6 ± 1.8^{a}	41.7 ± 3.0^{b*}	58.0 ± 5.2^{a*}	47.2 ± 5.6^{b*}	39.0 ± 3.4^{b*}
1 mM GSH+PG	62.6 ± 1.8^{a}	54.7 ± 1.9^{b}	75.1 ± 4.7^{b*}	68.0 ± 4.8^{b}	91.8 ± 6.9^{c*}
2 mM GSH+PG	62.6 ± 1.8^{a}	54.7 ± 1.9^{b}	83.8 ± 5.6^{a}	77.4 ± 5.2^{a}	84.4 ± 6.0^{a}
5 mM GSH+PG	62.6 ± 1.8^{a}	54.7 ± 1.9^{b}	78.2 ± 5.2^{a}	70.7 ± 5.6^{b*}	86.9 ± 6.4^{b*}
VAP (μm/s)					
NCM	23.8 ± 0.9^{a}	47.8 ± 4.9^{b}	47.4 ± 5.0^{b}	55.3 ± 5.9^{b}	78.6 ± 7.0^{c}
CM	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	38.8 ± 2.2^{b}	39.1 ± 2.3^{b}	44.3 ± 2.7^{b}
1 mM GSH	31.4 ± 1.0^{a}	33.5 ± 3.0^{a}	45.3 ± 5.0^{b}	37.1 ± 2.5^{ab}	40.6 ± 5.0^{ab}
2 mM GSH	31.4 ± 1.0^{a}	22.2 ± 1.4^{b*}	37.5 ± 4.2^{a}	36.9 ± 4.6^{a}	42.7 ± 4.1^{b}
5 mM GSH	31.4 ± 1.0^{a}	23.3 ± 1.8^{b*}	36.9 ± 6.5^{a}	31.4 ± 3.1^{a*}	19.2 ± 2.1^{b*}
1 mM GSH+PG	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	30.1 ± 1.0^{a}	36.4 ± 2.1^{b}	46.2 ± 3.2^{c}
2 mM GSH+PG	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	37.9 ± 2.0^{b}	44.9 ± 2.9^{b}	44.5 ± 3.1^{b}
5 mM GSH+PG	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	45.4 ± 2.8^{b*}	50.7 ± 3.3b^{c*}	59.8 ± 3.9^{c*}

NCM, non-capacitation medium; CM, capacitation medium; GSH, reduced glutathione (at 1, 2 or 5 mM) added at the beginning of the experiment; GSH+PG, reduced glutathione (at 1, 2 or 5 mM) added together with progesterone after 4 h of incubation in CM; N.D., not detectable. Different superscripts indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks mean significant differences ($p < 0.05$) between a given treatment and CM at the same time of incubation. Results are shown as mean ± SEM of seven experiments.

Table 3 Effects of GSH on percentages of linearity (%LIN) and straightness (%STR) of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
LIN (%)					
NCM	24.0 ± 1.1^{a}	74.1 ± 6.3^{b}	76.6 ± 6.9^{b}	75.7 ± 6.5^{b}	81.6 ± 7.2^{b}
CM	34.2 ± 1.4^{a}	50.1 ± 2.9^{b}	33.9 ± 2.5^{a}	41.8 ± 2.1^{c}	50.5 ± 3.0^{b}
1 mM GSH	34.2 ± 1.4^{a}	37.8 ± 4.8^{a*}	58.9 ± 5.5^{b*}	59.3 ± 5.2^{b*}	37.1 ± 4.0^{a*}
2 mM GSH	34.2 ± 1.4^{a}	38.6 ± 2.0^{b*}	45.6 ± 4.9^{b*}	45.8 ± 4.6^{b}	49.4 ± 2.2^{b}
5 mM GSH	34.2 ± 1.4^{a}	40.1 ± 4.2^{b*}	63.3 ± 6.2^{c*}	37.2 ± 5.6^{b}	43.5 ± 1.4^{b*}
1 mM GSH+PG	34.2 ± 1.4^{a}	50.1 ± 2.9^{b}	40.5 ± 4.4^{b}	39.1 ± 4.6^{b}	58.2 ± 6.0^{c}
2 mM GSH+PG	34.2 ± 1.4^{a}	501 ± 2.9^{b}	39.1 ± 4.6^{b}	42.0 ± 4.9^{b}	60.6 ± 7.8^{c}
5 mM GSH+PG	34.2 ± 1.4^{a}	50.1 ± 2.9^{b}	38.3 ± 5.2^{b}	40.4 ± 4.9^{b}	70.8 ± 7.3^{c*}
STR (%)					
NCM	59.6 ± 2.2^{a}	83.0 ± 5.4^{b}	82.8 ± 5.5^{b}	84.4 ± 5.9^{b}	83.9 ± 5.1^{b}
CM	64.9 ± 1.2^{a}	74.2 ± 3.5^{b}	59.9 ± 2.2^{c}	63.3 ± 3.9^{a}	71.6 ± 3.7^{b}
1 mM GSH	64.9 ± 1.2^{a}	45.5 ± 5.6^{b*}	79.1 ± 4.0^{c*}	79.8 ± 4.1^{c*}	56.9 ± 5.5^{b*}
2 mM GSH	64.9 ± 1.2^{a}	61.4 ± 2.3^{a*}	64.4 ± 4.8^{a}	76.0 ± 2.3^{b*}	68.6 ± 4.6^{a}
5 mM GSH	64.9 ± 1.2^{a}	58.7 ± 5.5^{a*}	85.2 ± 3.0^{b*}	65.6 ± 6.2^{a}	75.9 ± 2.5^{a}
1 mM GSH+PG	64.9 ± 1.2^{a}	74.2 ± 3.5^{b}	58.1 ± 2.7^{c}	64.1 ± 4.2^{c}	71.1 ± 4.2^{c}
2 mM GSH+PG	64.9 ± 1.2^{a}	74.2 ± 3.5^{b}	76.5 ± 4.9^{b*}	79.1 ± 5.4^{b*}	74.1 ± 4.8^{b}
5 mM GSH+PG	64.9 ± 1.2^{a}	74.2 ± 3.5^{b}	87.0 ± 6.1^{b*}	87.6 ± 6.7^{b*}	88.3 ± 7.1^{b*}

NCM, non-capacitation medium; CM, capacitation medium; GSH, reduced glutathione (at 1, 2 or 5 mM) added at the beginning of the experiment; GSH+PG, reduced glutathione (at 1, 2 or 5 mM) added together with progesterone after 4 h of incubation in CM; N.D., not detectable. Different superscripts indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks mean significant differences ($p < 0.05$) between a given treatment and CM at the same time of incubation. Results are shown as mean ± SEM of seven experiments.

Percentages of viable boar spermatozoa with high intracellular calcium stained by Fluo3 (mainly located in the mid-piece) progressively increased during incubation with CM, reaching values of 14.1% ± 2.2% after 4 h of incubation (Figure S5). In a similar fashion to that observed for Rhod5-staining, addition of progesterone induced a transient increase in the percentage of viable spermatozoa with high intracellular calcium levels stained with Fluo3, reaching values of 38.2% ± 3.9% after 1 min (Figure S5). Subsequently, this percentage decreased rapidly (19.1% ± 2.6% after 5 min) and was maintained until the end of the experiment. The addition of GSH at 0 h significantly ($p < 0.05$) decreased the percentages of viable spermatozoa with Fluo3-marked calcium after 4 h of incubation and also reduced the transient increase observed after progesterone addition. Interestingly, not only did the addition of GSH together with progesterone not affect the peak in Fluo3-calcium observed in CM, but this calcium-increase was maintained at 5 min and 60 min (60 min: 1 mM GSH added with progesterone: 33.9% ± 4.2% vs. 19.2% ± 2.3% in CM, Figure S5B).

Effects of GSH on free cysteine residues of sperm proteins during *in vitro* capacitation and acrosome exocytosis

Incubation of spermatozoa in CM progressively increased the levels of free cysteine residues in sperm head proteins during the

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Table 4 Effects of GSH on the amplitude of lateral head displacement (ALH) and frequency of head displacement (BCF) of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
ALH (μm)					
NCM	3.01 ± 0.09^{a}	2.41 ± 0.15^{b}	2.24 ± 0.16^{b}	3.02 ± 0.20^{b}	4.17 ± 0.35^{d}
CM	3.07 ± 0.08^{a}	2.29 ± 0.12^{b}	2.93 ± 0.13^{ac}	2.64 ± 0.16^{c}	2.71 ± 0.14^{ac}
1 mM GSH	3.07 ± 0.08^{a}	2.14 ± 0.13^{b}	2.00 ± 0.19^{b*}	2.52 ± 4.8^{b}	2.95 ± 0.22^{a}
2 mM GSH	3.07 ± 0.08^{a}	1.96 ± 0.15^{b}	2.18 ± 0.14^{c*}	2.77 ± 0.16^{ad}	2.47 ± 0.06^{d*}
5 mM GSH	3.07 ± 0.08^{a}	1.89 ± 0.10^{b}	1.71 ± 0.19^{b*}	2.56 ± 0.14^{c}	1.81 ± 0.10^{b*}
1 mM GSH+PG	3.07 ± 0.08^{a}	2.29 ± 0.12^{b}	3.12 ± 0.21^{ac}	2.61 ± 0.21^{abc}	2.57 ± 0.29^{abc}
2 mM GSH+PG	3.07 ± 0.08^{a}	2.29 ± 0.12^{b}	2.74 ± 0.19^{ac}	3.36 ± 0.31^{c}	3.01 ± 0.22^{c}
5 mM GSH+PG	3.07 ± 0.08^{a}	2.29 ± 0.12^{b}	3.49 ± 0.26^{c*}	3.28 ± 0.27^{c*}	3.09 ± 0.25^{c}
BCF (Hz)					
NCM	6.63 ± 0.10^{a}	10.88 ± 0.41^{b}	11.24 ± 0.45^{b}	11.98 ± 0.63^{b}	14.18 ± 0.74^{c}
CM	7.76 ± 0.13^{a}	8.17 ± 0.23^{a}	7.88 ± 0.24^{a}	8.00 ± 0.22^{a}	7.78 ± 0.24^{a}
1 mM GSH	7.76 ± 0.13^{a}	7.91 ± 0.22^{a}	7.28 ± 0.10^{b*}	10.17 ± 0.37^{c*}	9.25 ± 0.36^{c*}
2 mM GSH	7.76 ± 0.13^{a}	6.49 ± 0.22^{b*}	8.13 ± 0.25^{a}	8.87 ± 0.24^{b*}	7.60 ± 0.24^{a}
5 mM GSH	7.76 ± 0.13^{a}	8.05 ± 0.24^{a}	7.58 ± 0.24^{a}	8.47 ± 0.32^{b}	6.70 ± 0.18^{c*}
1 mM GSH+PG	7.76 ± 0.13^{a}	8.17 ± 0.23^{a}	8.88 ± 0.37^{b*}	10.29 ± 0.49^{b*}	10.86 ± 0.55b*
2 mM GSH+PG	7.76 ± 0.13^{a}	8.17 ± 0.23^{a}	8.63 ± 0.29^{b*}	10.59 ± 0.58^{b*}	10.34 ± 0.47^{b*}
5 mM GSH+PG	7.76 ± 0.13^{a}	8.17 ± 0.23^{a}	11.42 ± 0.38^{b*}	11.68 ± 0.47^{b*}	12.29 ± 0.53^{b*}

NCM, non-capacitation medium; CM, capacitation medium; GSH, reduced glutathione (at 1, 2 or 5 mM) added at the beginning of the experiment; GSH+PG, reduced glutathione (at 1, 2 or 5 mM) added together with progesterone after 4 h of incubation in CM; N.D., not detectable. Different superscripts indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks mean significant differences ($p < 0.05$) between a given treatment and CM at the same time of incubation. Results are shown as mean ± SEM of seven experiments.

first 4 h (from 3.0 nmol/μg protein ± 0.2 nmol/μg protein at 0 h to 17.4 nmol/μg protein ± 1.9 nmol/μg protein at 4 h, Fig. 5); this was followed by a decrease after progesterone addition. When GSH was added at the beginning of the experiment, the increase in the free cysteine residues of sperm head proteins observed in CM was inhibited in a dose-dependent manner (Fig. 5A). When GSH was added together with progesterone, the observed decrease in CM was accentuated (Fig. 5B).

Incubation of boar spermatozoa in CM also increased the levels of free cysteine residues in sperm tail proteins and this increase was maintained after progesterone addition (Fig. 6). Whereas the addition of GSH at 0 h inhibited the aforementioned increase, the addition of GSH together with progesterone did not significantly differ from CM (Fig. 6).

Effects of GSH on DNA fragmentation during *in vitro* capacitation and acrosome exocytosis

In all cases, percentages of boar spermatozoa with fragmented DNA were very low. There was only a very slight increase at the end of the experimental period in spermatozoa incubated in NCM (Figure S6). The addition of GSH either at 0 h or at 4 h had no effect on this parameter.

Effects of GSH on intracellular peroxide levels during *in vitro* capacitation and acrosome exocytosis

Percentages of viable spermatozoa with high intracellular peroxide levels significantly ($p < 0.05$) increased after 4 h of incubation in CM (0 h: 2.4% ± 0.5% vs. 4 h: 18.6% ± 2.0%, Fig. 7). This percentage was roughly maintained after the addition of progesterone.

When GSH was added at 0 h, the increase observed in CM was counteracted in a dose-dependent manner throughout all the experimental period (Fig. 7A). In contrast, adding GSH together with progesterone had no effect on the percentage of viable spermatozoa with high peroxide levels (Fig. 7B).

Effects of GSH on intracellular superoxide levels during *in vitro* capacitation and acrosome exocytosis

Percentages of viable spermatozoa with high intracellular superoxide levels underwent a slight, but significant ($p < 0.05$) increase after 4 h of incubation in CM (0 h: 11.6% ± 1.3% vs. 4 h: 17.2% ± 1.9%, see Fig. 8). The subsequent addition of progesterone induced a transient decrease in this percentage, as it recovered and reached maximal values at the end of the experimental period.

The addition of GSH at 0 h counteracted the observed increase in the percentage of spermatozoa with high superoxide levels in all GSH concentrations (Fig. 8A). This counteracting effect of GSH progressively diminished upon addition of progesterone, observing no significant differences from CM at the end of the experiment (Fig. 8A). No significant differences between CM and GSH treatments were observed when this antioxidant was added together with progesterone (Fig. 8B).

Effects of GSH during *in vitro* fertilization

As shown in Table 5, the number of spermatozoa adhered to ZP after 7 h was 78.2 ± 4.7 in CM, whereas that observed in spermatozoa previously incubated with 2 mM GSH was 96.4 ± 5.9 ($p < 0.05$). In contrast, when spermatozoa were not previously incubated with 2 mM GSH but the antioxidant was added together with the oocytes, no significant differences were observed in the number of spermatozoa adhered to the ZP (Table 5).

Although no significant differences in the percentages of normal fertilization were observed at 7 h, normal fertilization rates after 18 h of sperm–oocytes co-incubation were significantly ($p < 0.05$) lower when spermatozoa had previously been capacitated in the presence of 2 mM GSH (Table 6). When spermatozoa were capacitated in CM and GSH was added together with the oocytes, normal fertilization rates were significantly lower than those observed in the control, but significantly ($p < 0.05$) higher

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Figure 4 Effects of GSH on mitochondrial membrane potential of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

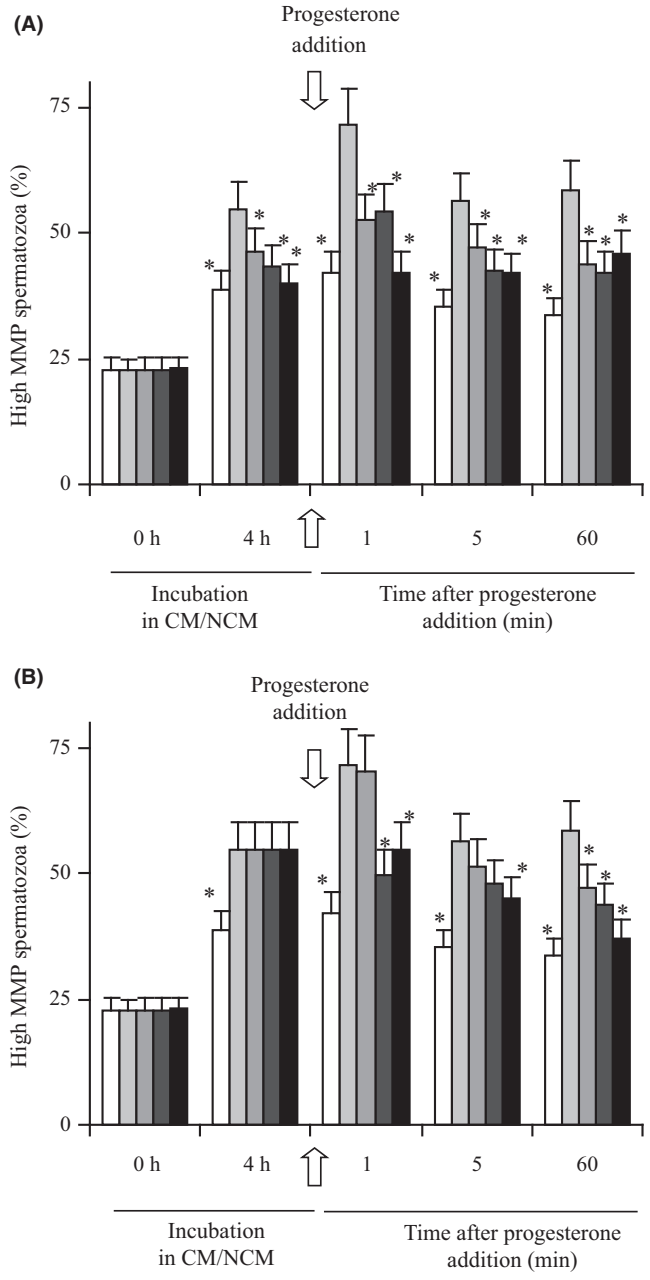
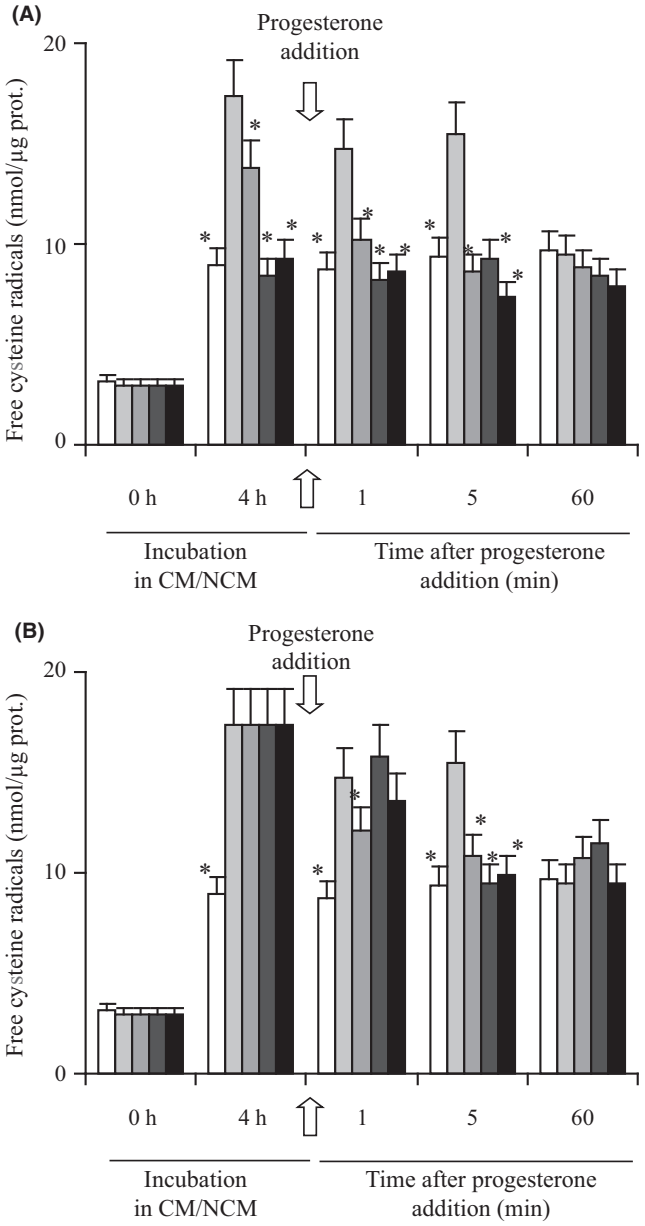


Figure 5 Effects of GSH on sperm head proteins and free cysteine residues of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



than those observed in spermatozoa previously capacitated in the presence of GSH (Table 6).

DISCUSSION

Our results indicate that *in vitro* capacitation of boar spermatozoa is related with a disruption of disulphide bonds of both spermatozoa head and tail proteins, as well as with a slight but significant increase in intracellular ROS levels. Interestingly, both observations are counteracted when GSH is present, which occurs together with changes in other sperm capacitation markers, such as motility, membrane lipid disorder and tyrosine phosphorylation. As a consequence, incubating boar spermatozoa with a capacitating medium containing GSH also alters the acrosome exocytosis induced by progesterone. Furthermore, our

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Figure 6 Effects of GSH on sperm tail proteins and free cysteine residues of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

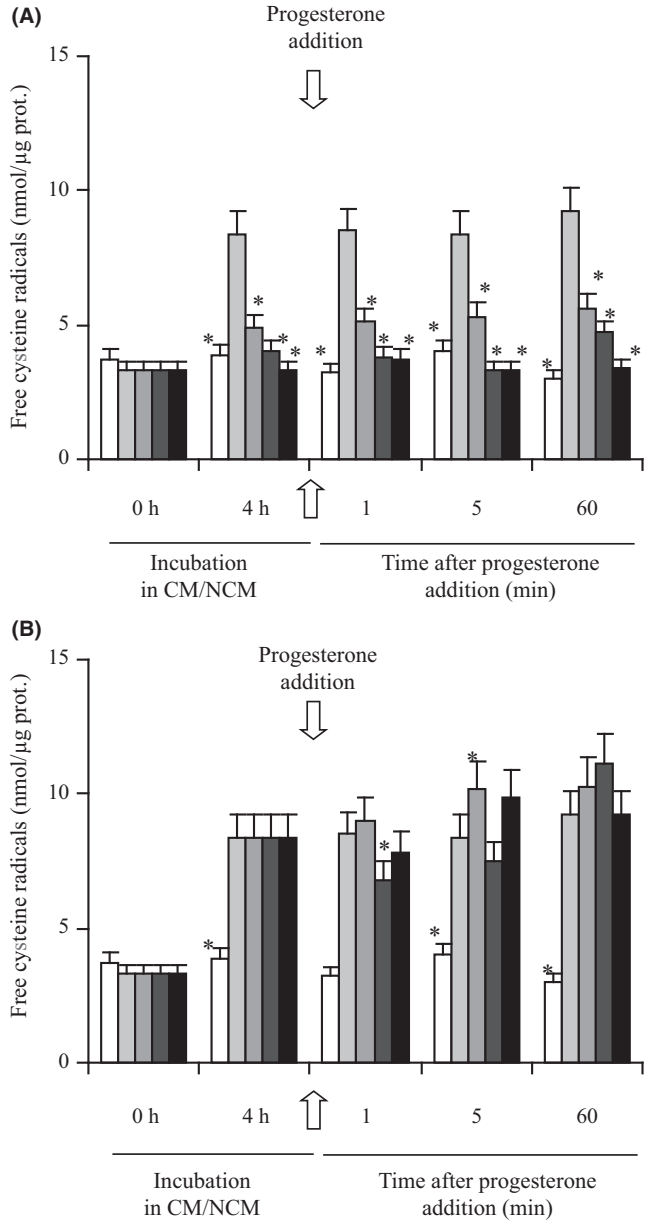
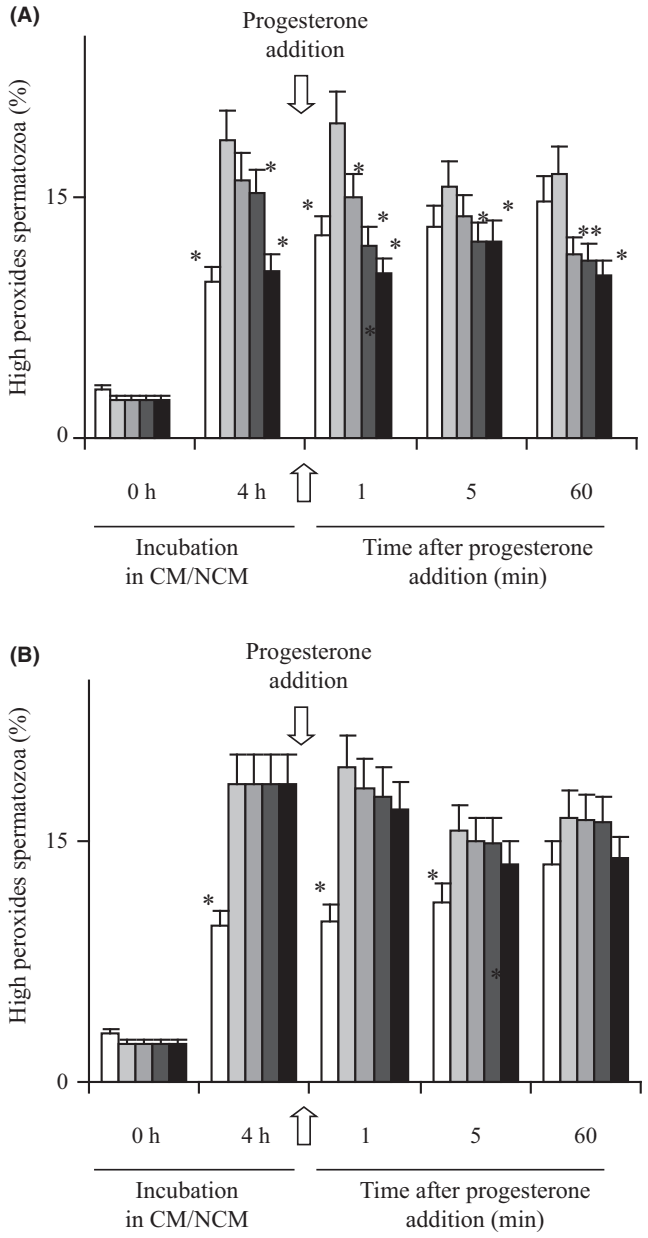


Figure 7 Effects of GSH on percentages of viable cells with high peroxide levels of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione is added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



results also suggest that while the presence of GSH largely prevents boar spermatozoa to achieve the capacitated status, its direct effect upon the acrosome exocytosis induced by progesterone is less apparent.

The effects of GSH on specific parameters, such as motility, membrane lipid disorder, mitochondrial membrane potential and intracellular calcium levels, also support the modulating role of GSH on sperm capacitation. In this regard, it is worth noting that calcium plays a crucial role during both sperm capacitation and acrosome reaction (Belmonte *et al.*, 2016; Vicente-Carrillo *et al.*, 2017), and that we previously identified two storage calcium sites (head and mid-piece) in boar spermatozoa (Yeste *et al.*, 2015). In this study, we observed that the effects of GSH on intracellular calcium levels were more apparent in Fluo3-stained calcium (mainly located in the mid-piece) than in the

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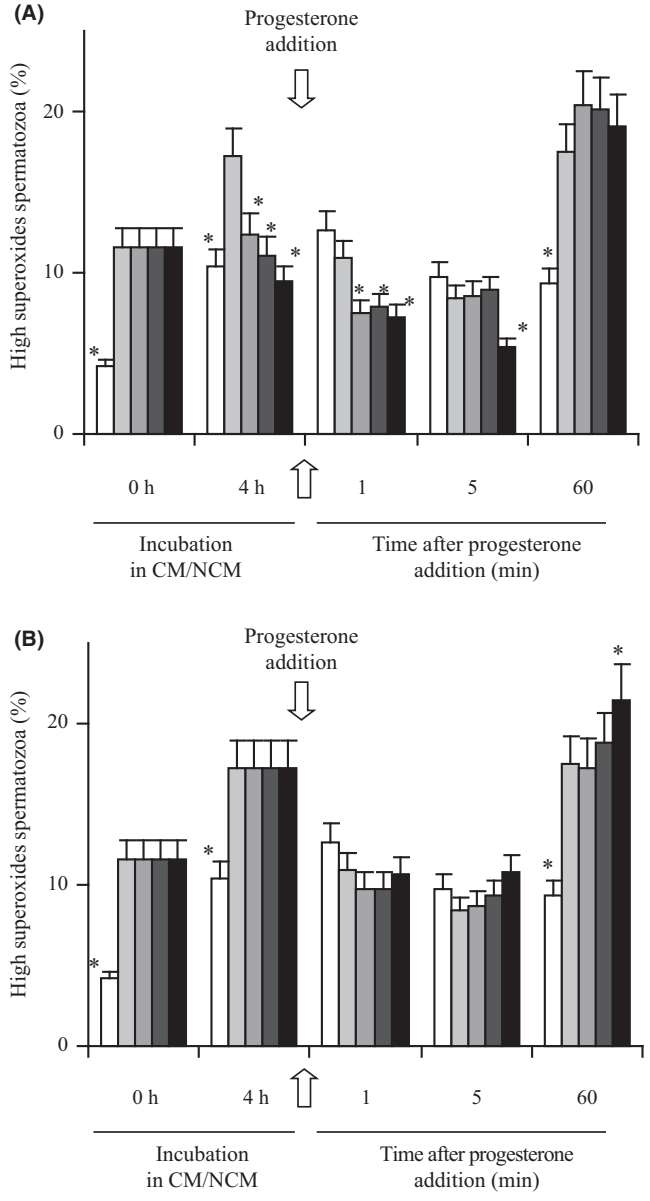
Figure 8 Effects of GSH on percentages of viable cells with high superoxide levels of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (A) Reduced glutathione in added at 0 h. (B) Reduced glutathione added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM (C−). Light grey bars: spermatozoa incubated in CM (C+). Medium grey bars: spermatozoa incubated in CM added with 1 mM reduced glutathione. Dark green bars: spermatozoa incubated in CM added with 2 mM reduced glutathione. Black bars: spermatozoa incubated in CM added with 5 mM reduced glutathione. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



Rhod5-stained calcium (mainly located in the head). These results resemble to those previously observed in boar spermatozoa when incubated in a capacitating medium without calcium (Yeste *et al.*, 2015). Therefore, one could suggest that the interfering GSH-effect on sperm capacitation could be related to the lack of sperm ability to increase mitochondrial calcium. If this was the case, there would still be some points to address, as while GSH has been found to decreased sperm motility in this work, the lack of external calcium induced the opposite effect (Yeste *et al.*, 2015). Thus, although GSH-influence on sperm capacitation can be partially explained through its action on the calcium found in the mid-piece, there are other mechanisms through which GSH exerts its effects.

Table 5 Number of spermatozoa adhered to ZP and normal and abnormal fertilization rates at 7 h of post-insemination, with spermatozoa previously subjected to *in vitro* capacitation in the presence (GSH) or absence of reduced glutathione (control and GSH+PG)

Treatment	Number of fertilized oocytes evaluated	Number of spermatozoa adhered to ZP	Fertilization rate	
			Normal	Abnormal
Control	60	78.2 ± 4.7[a]	71.7[a]	28.3[a]
GSH	63	96.4 ± 5.9[b]	66.7[a]	33.3[a]
GSH+O	59	83.4 ± 4.9[a]	76.3[a]	23.7[a]

GSH, spermatozoa previously capacitated with reduced glutathione (2 mM); GSH+O: spermatozoa previously incubated in capacitation medium and GSH (2 mM) added together with oocytes. Different superscripts indicate significant differences ($p < 0.05$) between treatments. Fertilization rates are calculated on the basis of the number of fertilized oocytes evaluated at 7 h post-insemination. Results are shown as mean ± SEM of five experiments.

Table 6 Number of spermatozoa adhered to ZP and normal and abnormal fertilization rates at 18 h of post-insemination, with spermatozoa previously subjected to *in vitro* capacitation in the presence (GSH) or absence of reduced glutathione (control and GSH+PG)

Treatment	Number of fertilized oocytes evaluated	Fertilization rate	
		Normal	Abnormal
Control	51	58.8[a]	41.2[a]
GSH	48	8.3[b]	91.7[b]
GSH+O	55	38.2[c]	61.8[c]

GSH, spermatozoa previously capacitated with reduced glutathione (2 mM); GSH+O: spermatozoa previously incubated in capacitation medium and GSH (2 mM) added together with oocytes. Different superscripts indicate significant differences ($p < 0.05$) between treatments. Fertilization rates are calculated on the basis of the number of fertilized oocytes evaluated at 18 h post-insemination. Results are shown as mean ± SEM of five experiments.

Another point that merits discussion is in regards to the effects of GSH on free cysteine residues of sperm head and tail proteins. In this work, we analyse free cysteine residues through the utilization of the 2,2′-dithiodipyridine technique method. The advantage of this method compared to others that involve thioglycolate and bibromobimane (Hartley *et al.*, 2016) is that it provides a more quantitative way due to its spectrophotometric nature, whereas the optical character of the other techniques only allows for a less sensitive, semi-quantitative approach. Free cysteine residues are an indicator of intact disulphide bonds between proteins (Reyes *et al.*, 1989; Jager *et al.*, 1990; Perreault, 1990, 1992; Chatterjee *et al.*, 2001; Cheng *et al.*, 2009). Centring on sperm head, the maintenance of a tight nucleoprotein structure in mammalian spermatozoa is, at least partially, controlled by the number of disulphide bonds between nucleoproteins (Reyes *et al.*, 1989; Jager *et al.*, 1990; Perreault, 1990, 1992; Chatterjee *et al.*, 2001; Cheng *et al.*, 2009). This relationship is especially apparent in frozen-thawed boar spermatozoa, as freeze-thawing disrupts disulphide bonds between sperm nucleoproteins (Flores *et al.*, 2011) and the presence of GSH in cryopreservation media counteracts that disruption (Yeste *et al.*, 2013). Additionally, our results from co-incubation of spermatozoa

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with *in vitro*-matured oocytes suggests that disruption of disulphide bonds of sperm head proteins during the achievement of capacitation could be involved in the decondensation of sperm nucleus that occurs upon fertilization (Perreault *et al.*, 1984). In fact, GSH secreted by cumulus cells is taken by the oocyte and participates in the regulation of nuclear decondensation upon fertilization (Perreault *et al.*, 1984; Zuelke *et al.*, 2003; Caglar *et al.*, 2005; Maedomari *et al.*, 2007). In accordance with this, we observed a noticeable decrease of nuclear decondensation in oocytes co-incubated with spermatozoa previously capacitated in the presence of GSH. Such decrease could affect the fertilization process, as GSH would prevent the disruption of disulphide bonds between sperm head proteins which would ultimately reduce the sperm fertilizing ability. Of course, this hypothesis should be taken with caution, as the presence of GSH in the co-incubation medium, albeit at micromolar concentrations, could also affect nuclear decondensation of oocytes and spermatozoa, thereby mimicking the effects of GSH observed *in vivo* following secretion by cumulus cells (Maedomari *et al.*, 2007). Nevertheless, the intrinsic effect of GSH would not be the unique one responsible for our observations, as normal fertilization rates were higher when GSH was added at the time of co-incubation (with higher levels of extracellular GSH, millimolar order) than when spermatozoa had previously been capacitated in the presence of GSH. Therefore, the main effects of GSH would result from its impact on sperm nuclear condensation during capacitation, rather than from its direct addition at the time of fertilization.

We also observed that incubation of spermatozoa with CM increased the disrupted disulphide bonds of the tail. In this context, it is worth remembering that disulphide bonds are instrumental in maintaining the structure of sperm tail proteins (flagellum, fibrous sheath and longitudinal columns; Su *et al.*, 2005; Buffone *et al.*, 2012). In addition, sperm tail proteins have strong influence on motion patterns (Luconi & Baldi, 2003). Thus, it is reasonable to suggest that changes in the number of disrupted disulphide bonds of sperm tail proteins result in changes of motility patterns, including those linked to capacitated spermatozoa.

The effects of GSH on ROS were of lesser extent than those observed in free cysteine residues and the increase of intracellular ROS following incubation of spermatozoa in CM was moderate. While this moderate increase appeared to have no deleterious effects, it could be, as previously suggested (O'Flaherty, 2015), related to the achievement of sperm capacitation. In this regard, it is worth noting that moderate changes in intracellular ROS levels modulate the activity of protein kinases and phosphatases that are involved in sperm capacitation (e.g. protein kinase C, microtubule-associated protein kinases, PI3 kinase. . .; Wright *et al.*, 2009; Corcoran & Cotter, 2013). Remarkably, GSH inhibited the increase of ROS observed during sperm capacitation and also altered tyrosine phosphorylation of P32 and tyrosine-phosphorylated proteins evaluated through immunolocalization. Therefore, our results match with previous studies conducted in other species, which reported that ROS partially modulates protein kinases and phosphatases involved in sperm capacitation (Wright *et al.*, 2009; Corcoran & Cotter, 2013).

When added together with progesterone, GSH had little effect. This suggests that capacitated spermatozoa are less sensitive to GSH because, after 4 h of incubation with CM, spermatozoa have underwent the majority of changes that are modulated by GSH. Furthermore, the fact that GSH has less influence when added together with progesterone indicates that the events related to acrosome exocytosis do not depend on ROS. In fact, a sudden peak of O_2 consumption and ATP synthesis has been described immediately after the induction of acrosome exocytosis with progesterone (Ramió-Lluch *et al.*, 2013). This peak seems to be related to a coupled status of boar sperm mitochondria upon acrosome reaction, whereas mitochondria are uncoupled before and after acrosome reaction (Ramió-Lluch *et al.*, 2013; Yeste *et al.*, 2015).

GSH decreases sperm motility either when added at 0 h or when added at 4 h. These results suggest that the effects of GSH on boar sperm motility do not entirely rely on disrupted disulphide bond of tail proteins and intracellular ROS levels. While it is not possible, at this moment, to ascertain which this mechanism is, we could hypothesize a putative one that would be related to the control of redox-sensitive enzymes regulating tail contractibility. In fact, proteins linked to the maintenance of cell redox status, such as the Na^+/K^+-dependent ATPase (Liu *et al.*, 2012), are regulators of sperm motility (Koçak-Toker *et al.*, 2002). On the basis of our results, we suggest that the anti-oxidative action of GSH would act on the sperm redox status, modulating the activities of specific proteins, such as the Na^+/K^+-dependent ATPase. This fact would explain why GSH modifies sperm motility, both in capacitated and frozen-thawed spermatozoa (Yeste *et al.*, 2013). Thus, the different impact of GSH on the motility of capacitated and frozen-thawed boar spermatozoa would result from the existence of a different redox status in each cell. More research is warranted to elucidate why GSH exerts a dual effect on sperm motility.

In conclusion, *in vitro* capacitation of boar spermatozoa is related to a partial disruption of disulphide bonds of sperm proteins and an increase in intracellular ROS levels. These two phenomena could also be involved in the decondensation of sperm pronucleus after fertilization. In contrast, neither the increase in ROS levels nor the disruption of disulphide bonds in sperm proteins appear to play an instrumental role in the induction of acrosome exocytosis induced by progesterone. Finally, the effects of GSH on sperm motility suggest that this functional parameter is partially controlled by ionic and redox mechanisms.

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DISCLOSURES

None of the authors has anything to disclose.

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AUTHORS' CONTRIBUTIONS

R.P.B. and M.R. equally contributed to the work and were involved in all experiments. M.Y. significantly contributed to design the experiments, funded the work and was involved in drafting and correcting the final manuscript. J.M.N. was involved in confocal microscopy analysis and immunological techniques and critically revised the manuscript. A.Pl., B.A.P., M.C.M., E.E. and A.Pe. helped conduct laboratory analyses. M.G.Z. contributed to design the experiments, funded the work and critically revised the final manuscript. J.E.R.G. designed the experiments, funded the work and wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Figure S1 Effects of GSH on percentages of viability of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S2 Effects of GSH on tyrosine phosphorylation levels of P32 protein of boar spermatozoa subjected to *in vitro* capacitation and subsequent acrosome exocytosis induced with progesterone.
Figure S3 Effects of GSH on the localization of tyrosine-phosphorylated proteins of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.

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Figure S4 Effects of GSH on percentages of viable cells with high levels of Rhod5-marked intracellular calcium of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.

Figure S5 Effects of GSH on percentages of viable cells with high levels of Fluo3-marked intracellular calcium of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.

Figure S6 Effects of GSH on percentages of DNA fragmentation of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.

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