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Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze–thawing by stabilising disulfide bonds

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Abstract. One important change the head of boar spermatozoa during freeze–thawing is the destabilisation of its nucleoprotein structure due to a disruption of disulfide bonds. With the aim of better understanding these changes in frozen–thawed spermatozoa, two agents, namely reduced glutathione (GSH) and procaine hydrochloride (ProHCl), were added at different concentrations to the freezing media at different concentrations and combinations over the range 1–2 mM. Then, 30 and 240 min after thawing, cysteine-free residue levels of boar sperm nucleoproteins, DNA fragmentation and other sperm functional parameters were evaluated. Both GSH and ProHCl, at final concentrations of 2 mM, induced a significant ($P < 0.05$) increase in the number of non-disrupted sperm head disulfide bonds 30 and 240 min after thawing compared with the frozen–thawed control. This effect was accompanied by a significant ($P < 0.05$) decrease in DNA fragmentation 240 min after thawing. Concomitantly, 1 and 2 mM GSH, but not ProHCl at any of the concentrations tested, partially counteracted the detrimental effects caused by freeze–thawing on sperm peroxide levels, motility patterns and plasma membrane integrity. In conclusion, the results show that both GSH and ProHCl have a stabilising effect on the nucleoprotein structure of frozen–thawed spermatozoa, although only GSH exerts an appreciable effect on sperm viability.

Additional keywords: sperm cryopreservation.

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Introduction

Currently, the cryopreservation of boar spermatozoa in liquid nitrogen is the most efficient method for storing sperm samples for a long period of time, but this procedure induces a wide variety of cell alterations that can reduce sperm fertilising ability (for a review, see Watson 2000).

One of these changes concerns sperm chromatin. In some species, like humans and horses, this damage is related to increased DNA fragmentation immediately after thawing (Baumber *et al.* 2003; Silva and Gadella 2006), whereas in other species, such as the ovine, DNA fragmentation appears 2–6 h after incubation of frozen–thawed spermatozoa at 37°C (López-Fernández *et al.* 2010).

In boars, cryopreservation of the spermatozoa destabilises nucleoprotein structure, disrupting the disulfide bonds of sperm nucleoproteins (Flores *et al.* 2011). It is worth noting that cysteine residues of the nucleoproteins form inter- and intra-protamine cross-links via the formation of disulfide bonds (Fuentes-Mascorro *et al.* 2000) and these bonds are one of the most important stabilising mechanisms of sperm chromatin (Brewer *et al.* 2003). Conversely, there are inconsistent findings in the literature regarding the effects of cryopreservation on DNA fragmentation. Thus, some authors have observed that cryopreservation increases sperm DNA fragmentation (Fraser and Strezeżek 2005), whereas others have not (Hernández *et al.* 2006; Flores *et al.* 2008, 2011).

Because the integrity of sperm chromatin plays a critical role at the time of fertilisation (Didion *et al.* 2009; Oliva 2006; Tsakmakidis *et al.* 2010), the addition of agents to protect the disulfide bonds could counteract the destabilisation of sperm nucleoprotein structure linked to freeze–thawing procedures. Thus, the aim of the present study was to determine whether two different agents that can protect disulfide bonds, namely reduced glutathione (GSH) tripeptide and procaine hydrochloride (ProHCl), were able to protect boar sperm chromatin from the damage induced by cryopreservation.

The antioxidant GSH is the most abundant thiol in cells and, among other functions, is vital for the maintenance of the

intracellular redox balance (for a review, see Jacob *et al.* 2003). Although sperm cryopreservation induces changes in the quantity and distribution of sulfhydryl groups in sperm membrane proteins, the addition of GSH to freezing media has been reported to maintain a normal distribution of these sulfhydryl groups (Chatterjee *et al.* 2001). Furthermore, the addition of GSH to freeze–thawing media has been reported to increase sperm motility, reduce levels of reactive oxygen species (ROS) and increase the ability of spermatozoa to penetrate the oocyte (Gadea *et al.* 2004, 2005, 2011) for spermatozoa from the boar and other mammalian species.

The second agent evaluated in the present study, ProHCl, also protects disulfide bonds (Zhang *et al.* 1992) and exhibits antioxidant activity (Lee *et al.* 2010). It is a well-known local anaesthetic that increases the antitumoural activity of cisplatin (Fenoglio *et al.* 2002) and induces sperm capacitation and hyperactivation in some species (i.e. bull, stallion and guinea-pig; Mújica *et al.* 1994; Márquez and Suárez 2004; McPartlin *et al.* 2009).

Thus, the main aim of the present study was to determine whether the addition of GSH and/or ProHCl to freezing media could protect boar sperm chromatin from the damage induced by cryopreservation without affecting other functional parameters. To this end, different concentrations and combinations of these two agents were added to the freezing extenders used in the present study, namely one containing lactose and egg yolk (LEY) and another (LEYGO) containing LEY with 6% glycerol and 1.5% Orvus ES Paste (OEP; Equex STM; Nova Chemical Sales, Scituate, MA, USA). These two extenders are the most commonly used extenders for the cryopreservation of boar spermatozoa according to the Westendorf method and its modifications (Westendorf *et al.* 1975; Casas *et al.* 2009). After cryopreservation of boar spermatozoa, we assessed the number of free cysteine residues in sperm nucleoproteins (as a direct indication of disulfide bond levels), sperm DNA fragmentation and several functional parameters, such as computer-assisted motility analysis (CASA), ROS and plasma membrane integrity.

Materials and methods

Sperm samples

The experimental protocol was designed according to the guidelines established by the Animal Welfare Directive of the Autonomous Government of Catalonia (Spain) and the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Spain).

Twenty ejaculates from different healthy and adult boars (ages range 18 months–3 years) were used in the present study. Each ejaculate came from a different boar. Boars were housed in climate-controlled buildings, fed an adjusted diet (2.3 kg day^{-1}) consisting of basal diet plus 1% premix for boars (P174N; TecnoVit, Tarragona, Spain) and were provided with water *ad libitum*.

Ejaculates were collected twice a week by the gloved-hand technique with an interval of at least 3 days between collections. After removing the gelatinous fraction by filtration through gauze, the total volume of the sperm-rich fraction was diluted 1 : 5 (v/v) in a long-term extender (Duragen; Magapor, Zaragoza, Spain). These diluted sperm-rich fractions were transported within to the laboratory within 4 h of extraction in an insulated container before being stored at 17°C for 24 h. The quality of the sperm samples was then evaluated to confirm that they satisfied the quality standard (i.e. total sperm motility >80%, morphologically normal spermatozoa; sperm viability >85%; see Casas *et al.* 2009). Because the quality of the 20 ejaculates used in the present study was over the set thresholds, they were frozen according to the experimental design described below.

Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method adapted by Casas *et al.* (2009). All ejaculates diluted in long-term extender were centrifuged at 400*g* for 5 min at 17°C. The pellets were then resuspended in 3–4 mL of the remaining supernatant and diluted to a concentration of 1.5×10^9 spermatozoa mL^{-1} in LEY using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). All spermatozoa diluted in LEY were then cooled down to 5°C for 150 min before being subsequently diluted to 1×10^9 spermatozoa mL^{-1} in LEYGO. The final concentration of glycerol and OEP in LEYGO was 2% and 0.5%, respectively. Spermatozoa were finally packed into 0.5-mL plastic straws (Minitub Ibérica, Tarragona, Spain) and transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica). The freezing program (SY-LABORATORY software; Minitub Ibérica) consisted of 313 s of cooling at: -6°C min^{-1} from 5°C to -5°C for 100 s; -39.82°C min^{-1} from -5°C to -80°C for 113 s; 30 s at -80°C; and -60°C min^{-1} from -80°C to -150°C for 70 s. The straws were then plunged into liquid nitrogen (-196°C) for further storage.

After at least 2 months storage in liquid nitrogen, four straws per ejaculate and treatment were thawed and diluted with three volumes of warmed Beltsville Thawing Solution (BTS) at 37°C (at a final dilution of 1 : 4). To thaw the samples, each straw was shaken for 20 s in a 37°C waterbath.

Experimental design

Each ejaculate was split into 10 fractions consisting of two controls (extended control and frozen–thawed control [FT-C]), and eight treatments in which both freezing media (LEY and LEYGO) were supplemented with different combinations of GSH and ProHCl. The extended control, diluted in a long-term extender, was incubated at 37°C for 30 or 240 min before levels of free cysteine radicals and sperm DNA fragmentation were determined, and sperm motility and other functional parameters were assessed by flow cytometry.

The remaining nine fractions were used in the cryopreservation study. As mentioned above, the eight treatments consisted of supplementation with both LEY and LEYGO cryopreservation extenders with reduced L-glutathione ($C_{10}H_{17}N_3O_6S$; Sigma-Aldrich, St Louis, MO, USA) and/or ProHCl ($C_{13}H_{20}N_2O_2 \cdot HCl$; Fluka; Sigma-Aldrich) and the following concentrations and combinations of test drugs: 1 mM GSH (G1); 2 mM GSH (G2); 1 mM ProHCl (P1); 2 mM ProHCl (P2); 1 mM GSH + 1 mM ProHCl (G1P1); 2 mM GSH + 1 mM ProHCl

(G2P1); 1 mM GSH + 2 mM ProHCl (G1P2); and 2 mM GSH + 2 mM ProHCl (G2P2).

Samples were cryopreserved and stored in liquid nitrogen at −196°C for at least 2 months, for methodological purposes only. After thawing, samples were incubated for 30 or 240 min at 37°C before evaluation of sperm functional parameters and levels of free cysteine radicals and sperm DNA fragmentation. Thus, two time-points (30 and 240 min) were chosen to evaluate spermatozoa after freeze–thawing, the last being set to ensure the survival of frozen–thawed spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses (Casas *et al.* 2010).

The experiments were replicated 20 times, using 20 different ejaculates, each from a different boar.

Determination of free cysteine radicals in the sperm head

The determination of free cysteine radicals in sperm nucleoproteins was performed according to the protocol adapted to boar spermatozoa and described by Flores *et al.* (2011). Briefly, samples were centrifuged at 600*g* for 20 min at 17°C and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1 mM benzamidine, 10 μg mL^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). The homogenates were then centrifuged at 850*g* for 20 min at 4°C. Both the supernatant and the upper layer of the pellet were discarded, and the lower layer of the pellet was resuspended in 500 μL phosphate-buffered saline (PBS). The purity of this separation was determined by observation under a phase contrast microscope (Zeiss Primo Star; Carl Zeiss, Jena, Germany) at ×40 magnifications (Zeiss Plan-Achromat 40×/0.65; Carl Zeiss). The purity of the samples is given as the percentage of loose heads compared with the presence of whole, non-fractioned spermatozoa and separated tails in each sample. In all cases, the mean purity was >95% loose heads compared with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separation of the heads from their respective mid-pieces.

Levels of free cysteine radicals in sperm nucleoproteins were determined using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulfide; Sigma, St Louis, MO, USA), as described by Brocklehurst *et al.* (1979). Briefly, 10-μL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 μL of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine and the mixture was incubated at 37°C for 1 h. Then, levels of free cysteine radicals were determined using spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalised against the total protein content of the samples, determined in parallel by the Bradford method (Bradford 1976) using a commercially available kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules, CA, USA).

Sperm chromatin dispersion test

We assessed DNA fragmentation in the present study using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm-Halomax-Sus for fluorescence microscopy; ChromaCell, Madrid, Spain) according to the manufacturer's instructions. This test is based on the different responses exhibited by intact and fragmented DNA a deproteinisation treatment, and previous studies have reported that the results obtained using the SCDt are strongly correlated with those obtained using other tests, such as the neutral comet assay (Enciso *et al.* 2006).

Briefly, the lysis buffer included in the kit was incubated to 22°C and vials containing low-melting agarose were heated at 100°C for 5 min in a waterbath. Vials were then left in another waterbath at 37°C for 5 min to equilibrate the agarose temperature. Then, 25 μL of each sperm sample (at a final concentration of 10^7 spermatozoa mL^{-1}) was added to a vial and mixed thoroughly. One drop of the 25-μL solution containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid the formation of air bubbles.

Slides were placed on a cooled plate within a fridge and left at 4°C for 5 min. The coverslip was then removed and 50 μL lysis solution was added to each slide. Slides were then incubated at 22°C for 5 min before being washing for 5 min with MilliQ water. The slides were subsequently dehydrated by three steps of 2 min each with ethanol at 70%, 90% and 100%. Finally, sperm samples were stained with propidium iodide (PI; 2.5 μg mL^{-1}) and mounted in DABCO anti-fading medium (Sigma-Aldrich, St Louis, MO, USA). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at ×100 magnification.

Three counts of 250 spermatozoa each using three different slides were carried out per sample, prior to calculating the corresponding mean ± s.e.m. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only a small halo.

Flow cytometric analyses

Flow cytometry analyses were performed according to the recommendations of the International Society for Advancement of Cytometry (ISAC), as described previously (Lee *et al.* 2008). These analyses were conducted to evaluate certain parameters of sperm function, namely sperm viability and membrane permeability, acrosome integrity, disordering of membrane lipids, and ROS, in all treatment groups. After either 30 or 240 min incubation at 37°C after thawing, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa mL^{-1} in a final volume of 0.5 mL. The spermatozoa were then stained with the appropriate combinations of fluorochromes according to the protocols described in Annex A available as Supplementary Material to this paper (i.e. SYBR-14/PI, YO-PRO-1/PI, peanut agglutinin (PNA)-fluorescein isothiocyanate [FITC]/PI, M540/YO-PRO-1, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DFCDA)/PI, hydroethidine (HE)/YO-PRO-1, PI after hypotonic treatment to correct raw data).

Samples were evaluated using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA). This instrument, which had not been altered from the original configuration

provided by the manufacturer (see http://www.beckmancoulter.com, accessed 5 July 2012), was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter and volume was measured directly using a Cell Laboratory Quanta SC cytometer (Beckman Coulter) and the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. Thus, in this system, forward scatter (FS) is replaced by electronic volume (EV). The EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning the beads in Channel 200 on the volume scale.

The optical filters used were also the original ones supplied (FL1, FL2 and FL3). The optical characteristics for these filters were as follows: for FL1 (green fluorescence), Dichroic/Splitter, dichroic longpass (DRLP) 550 nm, band pass (BP) filter 525 nm, detection width 505–545 nm; for FL2 (orange fluorescence), DRLP 600 nm, BP filter 575 nm, detection width: 560–590 nm; and for FL3 (red fluorescence), long pass (LP) filter 670 nm. Signals were amplified logarithmically and photomultiplier settings were adjusted to particular staining methods. The FL1 filter was used to detect green fluorescence (SYBR14, YO-PRO-1, PNA-FITC and dichlorfluorescein-positive (DCF^+)), whereas the FL3 filter was used to detect PI and ethidium-positive (E^+).

Sheath flow rate was set at 4.17 μL min^{-1} in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE vs SS dot plots) for a minimum of 10 000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particles with a diameter <7 μm) and cell aggregates (particles with a diameter >12 μm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described in Annex A, compensation was used to minimise spill-over of green fluorescence into the red channel.

Information on the events was collected in list-mode data files (.LMD). These files were then analysed using Cell Laboratory Quanta SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 *v.* FL3) and to analyse the cytometric histograms. Data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina and Harrison (2010) and Petrunkina *et al.* (2010). (See Annex A for more detailed information regarding the protocol used.) Each assessment for each sample and parameter was repeated three times in independent tubes before calculation of the mean ± s.e.m.

Sperm motility

Sperm motility was analysed using a commercially available CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain) on 15-μL sperm samples placed in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Total and progressive motility, together with other kinetic parameters, were recorded. A more detailed description is available in Annex B available as Supplementary Material to this paper.

Statistical analyses

The present study was developed with 20 ejaculates from 20 different boars. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and data are presented as the mean ± s.e.m. Data obtained from the analysis of all sperm parameters were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. When necessary, data were transformed using arcsine square root before a generalised estimating equation (GEE), an extension of generalised linear model (GLM) for repeated-measures, was performed. Characteristics of the GEE were normal distribution and identity link function. The inter-subject factor was treatment, with an intrasubject factor of incubation time after thawing (i.e. 30 or 240 min). In all cases, each functional parameter was the dependent variable and multiple post hoc comparisons were calculated using Sidak's test.

When transformation did not result in normal data distribution (i.e. geometric mean of fluorescence intensity (GMFI) of viable spermatozoa with a high H_2O_2 content, GMFI of DCF^+-stained spermatozoa, GMFI of viable spermatozoa with a high $\cdot O_2^-$ content, GMFI of E^+-stained spermatozoa, and in the case of three kinetic parameters, namely straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP)), non-parametric procedures were used with raw data. Friedman's test was performed as a non-parametric alternative to the GEE, and the Wilcoxon matched-pairs test was used to evaluate differences among treatments, as well as the effects of thawing time.

In all statistical analyses, the minimal level of significance was set at $P < 0.05$.

Results

Effects of GSH and ProHCl on levels of free cysteine radicals in sperm nucleoproteins after freeze–thawing

Sperm cryopreservation significantly increased ($P < 0.001$) levels of free cysteine radicals, from 3.11 ± 0.32 nmol μg^{-1} protein in extended refrigerated samples to 6.92 ± 0.55 nmol μg^{-1} protein in FT-C after 30 min incubation after thawing, and from 3.29 ± 0.34 nmol μg^{-1} protein in the extended control to 8.41 ± 0.63 nmol μg^{-1} protein in FT-C after 240 min incubation after thawing (Fig. 1).

Both GSH and ProHCl counteracted the increase in free cysteine radicals due to the freeze–thawing protocols. This counteraction was dependent on the concentration of both GSH and ProHCl, the incubation time (i.e. 30 or 240 min at 37°C) after thawing and even on the combined effect of concentration × thawing time ($P < 0.01$ for all factors). In five of the treatments tested (G2, P2, G1P2, G2P1 and G2P2), levels of free cysteine radicals after 30 min thawing were similar to those observed in the extended control. After 240 min incubation after thawing, the addition of 2 mM GSH + 2 mM ProHCl (G2P2) completely abolished the effects of freeze–thawing on levels of free cysteine radicals. In contrast, for all other

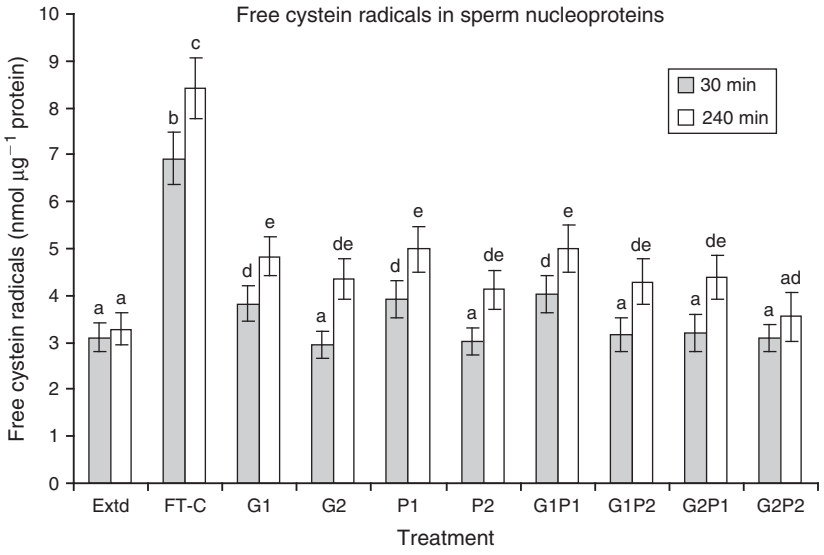

Fig. 1. Free cysteine radicals in sperm head proteins in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing. Extd, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl. Data are the mean ± s.e.m. Columns with different superscript letters differ significantly ($P < 0.05$).

Table 1. Percentage of spermatozoa exhibiting DNA fragmentation, sperm viability and acrosome integrity in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl; PI, propidium iodide; PNA, peanut agglutinin

	% Spermatozoa with fragmented DNA		% Viable spermatozoa ($SYBR14^+/PI^-$)		% Acrosome-intact spermatozoa (PNA^-)	
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	1.4 ± 0.2^{a}	2.7 ± 0.3^{b}	86.1 ± 3.2^{a}	52.6 ± 2.5^{b}	87.0 ± 4.0^{a}	64.7 ± 3.2^{b}
FT-C	1.8 ± 0.3^{a}	6.2 ± 0.8^{c}	46.8 ± 2.0^{c}	32.5 ± 1.4^{d}	47.7 ± 2.2^{c}	21.3 ± 1.0^{d}
G1	1.6 ± 0.3^{a}	4.7 ± 0.7^{d}	50.6 ± 2.3^{bc}	36.7 ± 1.6^{ef}	51.5 ± 2.4^{ch}	26.1 ± 1.2^{i}
G2	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	60.5 ± 2.5^{d}	46.9 ± 2.0^{c}	61.2 ± 2.9^{b}	38.3 ± 1.8^{eg}
P1	1.7 ± 0.3^{a}	4.9 ± 0.6^{d}	48.3 ± 2.2^{bc}	35.0 ± 1.5^{df}	49.8 ± 2.4^{ch}	24.1 ± 1.1^{di}
P2	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	49.5 ± 2.2^{bc}	39.3 ± 1.7^{e}	48.4 ± 2.3^{ch}	29.5 ± 1.3^{f}
G1P1	1.7 ± 0.3^{a}	4.8 ± 0.7^{d}	48.1 ± 2.1^{bc}	35.8 ± 1.6^{ef}	49.3 ± 2.4^{ch}	24.7 ± 1.2^{di}
G1P2	1.6 ± 0.2^{a}	4.1 ± 0.6^{d}	51.1 ± 2.2^{b}	39.1 ± 1.7^{e}	52.9 ± 2.5^{h}	28.8 ± 1.3^{fi}
G2P1	1.5 ± 0.2^{a}	2.9 ± 0.4^{b}	59.1 ± 2.5^{d}	46.2 ± 2.0^{c}	61.1 ± 2.9^{b}	37.0 ± 1.8^{e}
G2P2	1.5 ± 0.2^{a}	2.7 ± 0.4^{b}	61.9 ± 2.6^{d}	49.4 ± 2.3^{bc}	62.6 ± 3.0^{b}	41.2 ± 2.0^{g}

conditions tested, these levels remained significantly higher ($P < 0.001$) than in the extended control (e.g. 4.35 ± 0.45 nmol μg^{-1} protein in the G2 group vs 3.29 ± 0.34 nmol μg^{-1} protein in the extended control; see Fig. 1).

Effects of GSH and ProHCl on DNA fragmentation of boar spermatozoa subjected to freeze–thawing

Freshly obtained and diluted boar spermatozoa exhibited very low levels of DNA fragmentation (1.4% ± 0.2%; Table 1). Furthermore, under the present conditions, freeze–thawing did not modify sperm DNA fragmentation when determined after 30 min incubation at 37°C after thawing (Table 1), nor did the addition of either GSH or ProHCl modify this result at any of the concentrations and combinations tested.

In contrast, sperm DNA fragmentation was significantly higher in the FT-C group after 240 min incubation after thawing ($P < 0.05$) compared with levels in extended samples, although the levels remained relatively low in both groups (6.2 ± 0.8% vs 2.7 ± 0.3%, respectively; Table 1). Supplementation of the freezing medium with G2, P2, G2P1 and G2P2 completely

counteracted the increase in DNA fragmentation observed in the FT-C group after 240 min incubation after thawing (e.g. $2.7 \pm 0.3\%$ in the extended control vs $2.9 \pm 0.4\%$ in the G2 group; $P > 0.05$).

Effects of GSH and ProHCl on viability of boar spermatozoa subjected to freeze–thawing (SYBR-14/PI)

Treatment ($P < 0.001$), incubation time ($P < 0.001$) and treatment × incubation time ($P < 0.05$) significantly affected the viability of spermatozoa (SYBR-14^+/PI^-), with extended semen having significantly higher viability than the spermatozoa from the other treatment groups (Table 1). Furthermore, in frozen–thawed samples analysed after 30 min incubation after thawing, the percentage of viable spermatozoa in the G2, G2P1 and G2P2 groups was significantly higher than in the FT-C group. Sperm viability after 240 min incubation after thawing decreased in all treatment groups, but although sperm viability in the FT-C group was significantly lower than in the extended control, that in the G2P2 group was similar to that in the extended control ($49.4 \pm 2.4\%$ vs $52.6 \pm 2.5\%$, respectively; $P > 0.05$; Fig. 2).

Effects of GSH and ProHCl on acrosome integrity of boar spermatozoa subjected to freeze–thawing (PNA-FITC/PI)

The proportion of acrosome-intact spermatozoa (PNA-$FITC^-$) was significantly higher ($P < 0.001$) in the extended control than in all of the frozen–thawed groups after 30 min incubation at 37°C after thawing (Table 1). Three treatments (G2, G2P1 and G2P2) resulted in a significantly higher percentage ($P < 0.01$) of acrosome-intact spermatozoa compared with the FT-C group after 30 min incubation after thawing (e.g. $47.7 \pm 2.2\%$ vs $61.2 \pm 2.9\%$ in the FT-C and G2 groups, respectively) and after 240 min incubation after thawing. In contrast, no significant differences were observed between the FT-C, P1 and G1P1 groups after 240 min incubation after thawing.

Effects of GSH and ProHCl on the plasma membrane of boar spermatozoa subjected to freeze–thawing (YO-PRO-1/PI)

In all cases, freeze–thawing increased the percentage of viable spermatozoa with early changes in membrane permeability (YO-PRO-1^+/PI^-), as well as that of non-viable spermatozoa (PI^+), after both 30 and 240 min incubation after thawing (Table 2). Conversely, the percentage of viable spermatozoa without changes in membrane permeability (YO-PRO-1^-/PI^-) were significantly ($P < 0.05$) higher in the G2, P2, G2P1 and G2P2 groups than in the FT-C group after both 30 and 240 min incubation after thawing, whereas the percentage of viable spermatozoa with early changes in membrane permeability (YO-PRO-1^+/PI^-) was significantly higher in the latter groups than in the former group (Fig. 3).

Effects of GSH and ProHCl on membrane lipids in boar spermatozoa subjected to freeze–thawing

Freeze–thawing significantly ($P < 0.001$) increased the percentage of both viable and non-viable spermatozoa exhibiting increased levels of disordered membrane lipid organisation ($M540^+$; Table 3) after 30 min incubation after thawing. Incubating samples at 37°C for 240 min after thawing also increased the percentage of non-viable spermatozoa with increased membrane disorder in both the extended and frozen–thawed groups. However, the increase in the percentage of non-viable spermatozoa with high membrane disorder after 30 min incubation after thawing was significantly lower after the addition of G2, G2P1 and G2P2 to the freezing extenders (e.g. $41.0 \pm 2.2\%$ vs $24.5 \pm 1.6\%$ in the FT-C and G2P2 groups, respectively; $P < 0.01$). After 240 min incubation after thawing, the percentage of viable spermatozoa exhibiting low levels of disordered membrane lipid organisation was significantly higher ($P < 0.05$) in the G1, G2, P2, G1P1, G1P2, G2P1 and G2P2 groups compared with the FT-C group (Fig. 4).

Effects of GSH and ProHCl on intracellular peroxide and superoxide levels in boar spermatozoa subjected to freeze–thawing

Table 4 lists peroxide levels in the different treatment groups. The percentage of viable spermatozoa with high levels of peroxides (DCF$^+$/PI$^-$) was significantly higher ($P < 0.05$) in the FT-C group after 30 min incubation after thawing than in the extended control and G2, G2P1 and G2P2 groups (e.g. $3.5 \pm 0.3\%$, $2.4 \pm 0.2\%$ and $2.5 \pm 0.2\%$ in the FT-C, extended control and G2P2 groups, respectively; Fig. 5). Concomitantly, after 30 min incubation after thawing, the GMFI was significantly higher ($P < 0.01$) for both total and viable spermatozoa with high levels of H_2O_2 in the FT-C group after 30 min incubation after thawing compared with the G2, G2P1 and G2P2 groups.

After 240 min incubation after thawing, significantly lower ($P < 0.01$) GMFI was observed for viable spermatozoa with high levels of peroxides in the G2, G2P1, and G2P2 groups compared with the extended control and FT-C groups (e.g. 19.2 ± 1.1, 68.6 ± 3.8 and 45.3 ± 2.5 a.u. in the G2, extended control and FT-C groups, respectively). The GMFI of DCF$^+$ in the G2, G2P1 and G2P2 groups was similar to that in the extended control group after 240 min incubation after thawing.

In contrast with peroxide levels, no significant differences were observed in superoxide levels among any of the groups, including the extended control and FT-C groups; specifically, there were no differences in the percentage of viable spermatozoa with high superoxide levels (E^+/YOPRO-1) after either 30 min incubation after thawing (e.g. $3.3 \pm 0.3\%$, $3.4 \pm 0.3\%$ and $3.3 \pm 0.3\%$ in the extended control, FT-C and G2P2 groups, respectively) or after 240 min incubation after thawing (e.g. $3.4 \pm 0.3\%$, $3.5 \pm 0.3\%$ and $3.5 \pm 0.3\%$ in the extended control, FT-C and G2P2 groups, respectively). There were no significant differences in the GMFI of total (E^+) and viable (E^+/YOPRO-1-) spermatozoa with high superoxide levels.

Effects of GSH and ProHCl on motility parameters of boar spermatozoa subjected to freeze–thawing

As expected, the percentage of total motile spermatozoa (TMOT) decreased after cryopreservation in all treatment groups (Table 5). This reduction was observed after both 30 and 240 min incubation after thawing (e.g. $58.0 \pm 2.9\%$ and

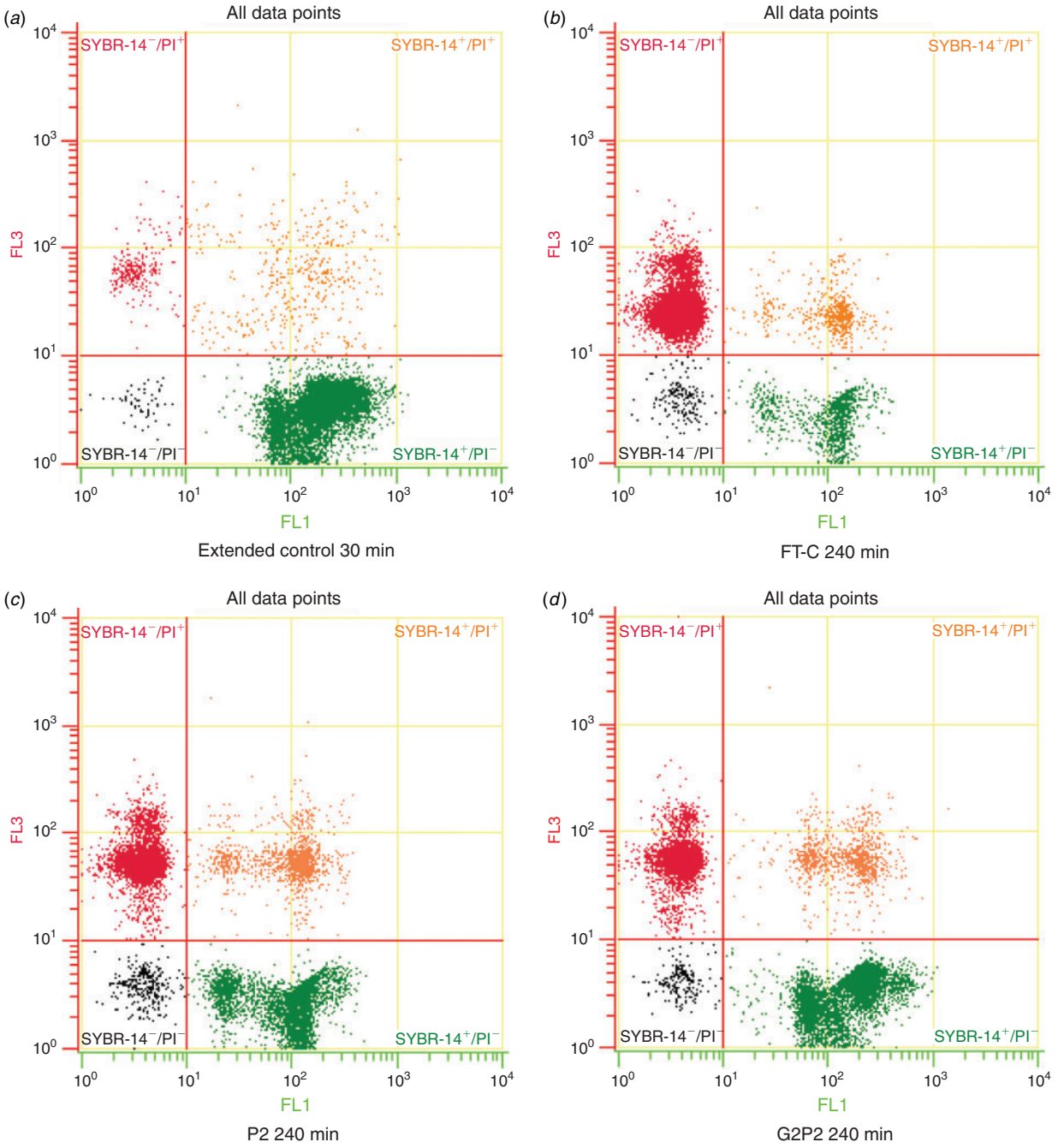

Fig. 2. Representative dot plots from SYBR-14/propidium iodide (PI) staining obtained (*a*) before or (*b–d*) after boar spermatozoa were subjected to different treatments. Samples were incubated at 37°C for 30 or 240 min after thawing, as indicated. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G2, 2 mM reduced glutathione; P2, 2 mM procaine hydrochloride. The upper left quadrants ($SYBR\text{-}14^-/PI^+$) contain non-viable red-stained spermatozoa, the upper right quadrants ($SYBR\text{-}14^+/PI^+$) contain non-viable spermatozoa stained both green and red, the lower left quadrants ($SYBR\text{-}14^-/PI^-$) contain alien particles (debris) and the lower right quadrants ($SYBR\text{-}14^+/PI^-$) contain viable green-stained spermatozoa.

38.7 ± 2.3% in the extended control and FT-C groups after 240 min incubation after thawing).

A significant ($P < 0.01$) increase in TMOT was observed in the G1, G2, G2P1 and G2P2 groups compared with the FT-C group after 30 and 240 min incubation after thawing (e.g. 50.4 ± 2.9% vs 38.7 ± 2.3% in the G2 and FT-C groups after 240 min incubation after thawing). Supplementation of the freezing medium with G1, G2, P2, G1P1, G1P2, G2P1 and

Table 2. Percentage of spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing during the YO-PRO-1/ propidium iodide assay

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. PI, propidium iodide; MP, membrane permeability; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	Viable spermatozoa				Non-viable spermatozoa (PI^+)	
	No changes in MP ($YO\text{-}PRO\text{-}1^-/PI^-$)		Early changes in MP ($YO\text{-}PRO\text{-}1^+/PI^-$)			
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	83.0 ± 4.2^{a}	45.9 ± 2.4^{b}	3.1 ± 0.2^{a}	5.0 ± 0.4^{b}	13.3 ± 0.9^{a}	47.3 ± 2.7^{b}
FT-C	37.0 ± 1.8^{c}	8.9 ± 0.8^{f}	12.9 ± 1.1^{c}	11.7 ± 1.0^{cd}	40.5 ± 2.1^{c}	66.1 ± 3.5^{d}
G1	42.1 ± 2.2^{bc}	16.0 ± 1.0^{e}	11.5 ± 1.0^{cd}	10.6 ± 0.9^{de}	37.7 ± 1.9^{c}	60.7 ± 3.0^{d}
G2	57.3 ± 2.9^{e}	28.3 ± 1.5^{d}	8.9 ± 0.8^{efg}	8.4 ± 0.7^{fg}	27.4 ± 1.5^{e}	52.4 ± 2.5^{b}
P1	39.7 ± 2.0^{bc}	12.6 ± 0.9^{f}	12.2 ± 1.1^{cd}	10.8 ± 1.0^{cd}	39.0 ± 2.0^{c}	63.3 ± 3.3^{d}
P2	45.2 ± 2.3^{b}	18.9 ± 1.1^{e}	10.5 ± 1.0^{de}	9.7 ± 0.9^{eg}	36.0 ± 1.8^{c}	59.2 ± 2.9^{df}
G1P1	40.7 ± 2.0^{bc}	12.5 ± 0.9^{f}	12.1 ± 1.1^{cd}	10.3 ± 1.0^{de}	38.3 ± 1.9^{c}	64.0 ± 3.3^{d}
G1P2	44.1 ± 2.3^{b}	17.6 ± 1.0^{e}	10.9 ± 1.0^{cd}	9.5 ± 0.9^{eg}	36.6 ± 1.8^{c}	60.6 ± 2.9^{d}
G2P1	55.8 ± 2.8^{e}	28.2 ± 1.5^{d}	9.4 ± 0.9^{eg}	8.0 ± 0.8^{fg}	28.1 ± 1.5^{e}	53.1 ± 2.6^{bf}
G2P2	59.5 ± 3.0^{e}	30.7 ± 1.6^{d}	8.1 ± 0.8^{fg}	7.7 ± 0.7^{f}	26.3 ± 1.5^{e}	51.2 ± 2.5^{b}

G2P2 had a significant ($P < 0.05$) positive effect on VCL, VSL, VAP and percentage of linearity (LIN) after 30 and 240 min incubation after thawing compared with values in the FT-C group at the same time-points (see Annex C available as Supplementary Material to this paper).

Discussion

Cryopreservation of mammalian spermatozoa damages sperm chromatin (Flores *et al.* 2011). This damage is related to destabilisation of the nucleoprotein structure, as well as defective packaging, activity of sperm nucleases and/or ROS generation (Agarwal and Said 2003; Chapman and Michael 2003). Previous studies have reported a strong relationship between DNA integrity and the fertilising ability of mammalian spermatozoa (Silva and Gadella 2006). Indeed, although chromatin-damaged spermatozoa can fertilise oocytes (Tesarik *et al.* 2004), this may lead to early embryo death and can affect implantation and post-implantation development (Sakkas *et al.* 1998; Fatehi *et al.* 2006).

In the present study, we showed that GSH and ProHCl protect boar sperm chromatin against damage induced by freeze–thawing because they protect the disulfide bonds between the cysteine residues of protamines, reduce peroxide levels in the case of GSH and diminish DNA fragmentation after thawing. These three effects are discussed separately below.

The addition of GSH and/or ProHCl at final concentrations of 2 mM had a protective effect on the disulfide bonds of sperm nucleoproteins. This is even more evident after 240 min incubation after thawing, when the levels of free cysteine radicals in the G2P2 group were similar to those in the extended control. According to previous reports, freeze–thawing affects the sperm nucleus by inducing changes in the structural interaction between nucleoproteins (protamine 1 and histone 1) and DNA (Flores *et al.* 2008, 2011). These changes are related to the disruption of disulfide bonds between cysteines of the nucleoproteins analysed (Flores *et al.* 2011). Although the mechanism responsible for this disruption remains unknown, these bonds are responsible for the proper packaging, compaction and stabilisation of sperm chromatin (Nasr-Esfahani *et al.* 2004; Balhorn 2007). In addition, protamines protect DNA from nucleases and ROS, and remove transcription factors and proteins to help reset the imprinting code in the oocyte (Oliva 2006).

Disulfide bonds can be weakened and disrupted when osmotic conditions are greatly modified, as is the case during the cryopreservation of boar spermatozoa, or unspecifically formed in the cytosol under oxidising conditions, thereby causing irreversible damage to proteins (Cumming *et al.* 2004; Yang *et al.* 2007). Thus, the changes observed to boar sperm head disulfide bonds may be caused by a combined affect of oxidative and osmotic changes related to freeze–thawing. In the present study, GSH and ProHCl stabilisation of the nucleoprotein structure and, hence, of the disulfide bonds, could be explained by their role in the maintenance of intracellular redox balance (Jacob *et al.* 2003). Glutathione can exist in a reduced (GSH) or oxidised (GSSG) state and the thiol group of cysteine in GSH can donate an electron to unstable molecules, such as ROS. In somatic cells, ProHCl has an antioxidant effect against ROS-induced endothelial damage in the rabbit aorta (Lee *et al.* 2010). Thus, the beneficial effects of GSH and ProHCl in protecting disulfide bonds could be related to their antioxidant activity.

In the present study, ROS levels were also assessed. Freeze–thawing appeared to slightly increase the percentage of viable spermatozoa with high levels of H_2O_2, whereas the extended control and treatments containing 2 mM GSH had similar low percentages of viable spermatozoa with high levels of H_2O_2 after both 30 and 240 min incubation after thawing. In a previous study, Awda *et al.* (2009) did not

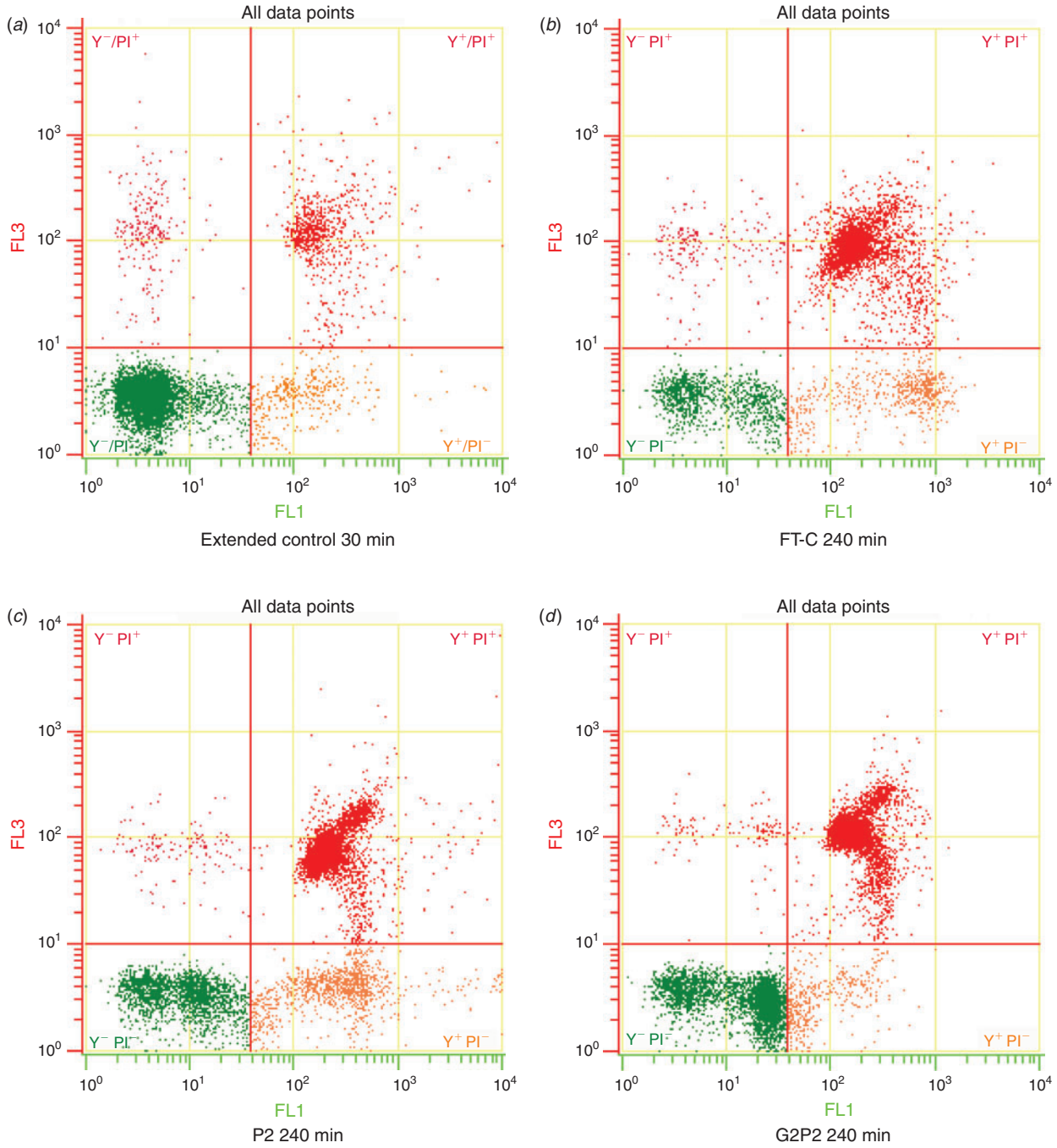

Fig. 3. Representative dot plots from YO-PRO-1/propidium iodide (PI) staining obtained (*a*) before or (*b–d*) after boar spermatozoa were subjected to different treatments. Samples were incubated at 37°C for 30 or 240 min after thawing, as indicated. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G2, 2 mM reduced glutathione; P2, 2 mM procaine hydrochloride. The upper left quadrants (YO-PRO-1^{-}/PI^{+} and YO-PRO-1^{-}/PI^{+}) contain non-viable spermatozoa, the lower left quadrants (YO-PRO-1^{-}/PI^{-}) contain viable spermatozoa without changes in membrane permeability and the lower right quadrants (YO-PRO-1^{+}/PI^{-}) contain viable spermatozoa with early changes in membrane permeability.

observe changes in H_2O_2 levels in viable boar spermatozoa after freeze–thawing protocols, whereas Kim *et al.* (2011) reported a slight increase in the percentage of viable spermatozoa with a high level of H_2O_2.

There were no changes in intracellular levels of $\cdot O_2^-$ in viable spermatozoa either in the FT-C group or after the addition of GSH and ProHCl. This could be related to the chemical properties of $\cdot O_2^-$, which has a very short half-life and is too polar to

Table 3. Levels of disordered membrane lipid organisation (high or low) in sperm plasma membrane after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	% Viable spermatozoa				% Non-viable spermatozoa			
	Highly disordered membrane lipids ($M540^+/YO\text{-}PRO\text{-}1^-$)		Low disordered membrane lipids ($M540^-/YO\text{-}PRO\text{-}1^-$)		Highly disordered membrane lipids ($M540^+/YO\text{-}PRO\text{-}1^+$)		Low disordered membrane lipids ($M540^-/YO\text{-}PRO\text{-}1^+$)	
	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min
Extended	5.0 ± 0.3^{a}	11.1 ± 0.6^{bc}	82.2 ± 4.5^{a}	47.7 ± 2.6^{b}	6.7 ± 0.4^{a}	24.2 ± 1.3^{b}	5.2 ± 0.3^{a}	15.0 ± 1.0^{b}
FT-C	13.8 ± 0.8^{b}	9.2 ± 0.5^{c}	32.2 ± 1.9^{c}	16.9 ± 0.9^{d}	41.0 ± 2.2^{ce}	59.6 ± 3.3^{d}	2.9 ± 0.2^{c}	1.8 ± 0.2^{c}
G1	13.6 ± 0.7^{b}	9.7 ± 0.5^{c}	37.8 ± 2.1^{ef}	21.0 ± 1.2^{gh}	36.5 ± 2.0^{eh}	55.9 ± 3.0^{df}	2.7 ± 0.2^{c}	1.6 ± 0.1^{c}
G2	13.4 ± 0.7^{b}	9.3 ± 0.5^{c}	49.9 ± 2.7^{b}	33.1 ± 2.0^{ce}	26.6 ± 1.6^{bi}	46.2 ± 2.6^{cg}	2.6 ± 0.1^{c}	1.5 ± 0.1^{c}
P1	13.9 ± 0.7^{b}	9.5 ± 0.5^{c}	37.0 ± 2.0^{ef}	18.7 ± 1.1^{dh}	36.0 ± 2.1^{eh}	57.6 ± 3.3^{df}	2.8 ± 0.2^{c}	1.6 ± 0.1^{c}
P2	14.2 ± 0.8^{b}	9.8 ± 0.5^{c}	40.6 ± 2.2^{f}	25.3 ± 1.4^{i}	33.5 ± 2.1^{h}	52.3 ± 2.8^{f}	2.7 ± 0.2^{c}	1.7 ± 0.1^{c}
G1P1	13.5 ± 0.7^{b}	9.5 ± 0.5^{c}	35.6 ± 2.0^{ce}	19.6 ± 1.2^{h}	38.2 ± 2.3^{eh}	57.5 ± 3.1^{df}	2.8 ± 0.2^{c}	1.6 ± 0.1^{c}
G1P2	14.1 ± 0.8^{b}	9.6 ± 0.5^{c}	37.7 ± 2.1^{ef}	23.7 ± 1.3^{gi}	36.2 ± 2.2^{eh}	53.6 ± 3.0^{df}	2.7 ± 0.2^{c}	1.5 ± 0.1^{c}
G2P1	13.5 ± 0.7^{b}	9.7 ± 0.5^{c}	47.2 ± 2.6^{b}	32.0 ± 2.0^{c}	28.6 ± 1.7^{i}	46.4 ± 2.5^{g}	2.9 ± 0.2^{c}	1.7 ± 0.2^{c}
G2P2	13.7 ± 0.7^{b}	9.5 ± 0.5^{c}	51.7 ± 2.8^{b}	36.6 ± 2.1^{ef}	24.5 ± 1.6^{b}	42.8 ± 2.3^{cg}	2.7 ± 0.2^{c}	1.7 ± 0.1^{c}

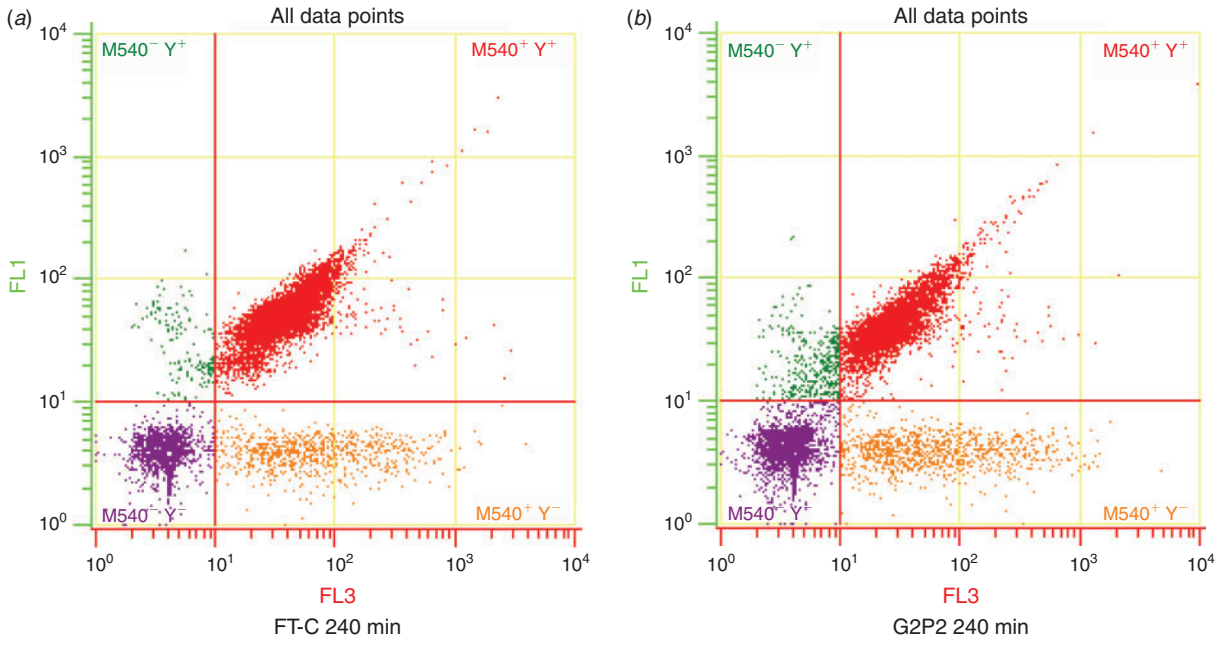

Fig. 4. Representative dot plots from M540/YO-PRO-1 staining after 240 min incubation at 37°C after thawing in the (*a*) frozen–thawed control (FT-C) and (*b*) 2 mM reduced glutathione + 2 mM procaine hydrochloride (G2P2)-treated groups. The upper left quadrants ($M540^-/YO\text{-}PRO\text{-}1^+$) contain non-viable spermatozoa with low levels of disordered membrane lipid organisation, the upper right quadrants ($M540^+/YO\text{-}PRO\text{-}1^+$) contain non-viable spermatozoa with high levels of disordered membrane lipid organisation, the lower left quadrants ($M540^-/YO\text{-}PRO^{®}\text{-}1^-$) contain viable spermatozoa with low levels of disordered membrane lipid organisation and the lower right quadrants ($M540^+/YO\text{-}PRO^{®}\text{-}1^-$) contain viable spermatozoa with high levels of disordered membrane lipid organisation.

pass through intact plasma membranes (Aitken 1995). In this way, the rate of destruction of $\cdot O_2^-$ would be very high, thus maintaining its intracellular values within a narrow range. In fact, conflicting reports exist in the literature regarding $\cdot O_2^-$ levels in cryopreserved boar spermatozoa. Specifically, although Awda *et al.* (2009) observed a decrease in the intracellular $\cdot O_2^-$ content of viable spermatozoa after freeze–thawing, Kim *et al.* (2011) observed a decrease in intracellular $\cdot O_2^-$ levels after the cooling step up to 5°C, but not after freeze–thawing.

Oxidative stress, which is a result of an imbalance between ROS generation and antioxidant scavenging activities (for a

Table 4. Percentage of spermatozoa and peroxide levels in spermatozoa in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. GMFI, geometric mean of fluorescence intensity of Optical Filter 1 (FL1); DCF, dichlorodihydrofluorecein-positive; PI, propidium iodide; DCF^+/PI^-, viable spermatozoa with a high H_2O_2 content; DCF^+, total spermatozoa; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

			GMFI (arbitrary units)			
	% DCF^+/PI^- spermatozoa		DCF^+/PI^-		DCF^+	
	30 min	240 min	30 min	240 min	30 min	240 min
Extended	2.4 ± 0.2^{a}	1.8 ± 0.2^{b}	86.4 ± 4.7^{a}	68.6 ± 3.8^{b}	82.0 ± 4.4^{a}	26.4 ± 1.6^{b}
FT-C	3.5 ± 0.3^{ce}	1.3 ± 0.1^{d}	106.0 ± 5.8^{c}	45.3 ± 2.5^{d}	100.3 ± 5.5^{c}	48.2 ± 2.6^{d}
G1	3.1 ± 0.3^{e}	1.4 ± 0.1^{d}	105.3 ± 5.8^{c}	35.7 ± 2.0^{e}	98.5 ± 5.4^{c}	38.7 ± 2.2^{e}
G2	2.4 ± 0.2^{a}	1.8 ± 0.2^{b}	83.1 ± 4.6^{a}	19.2 ± 1.1^{f}	79.8 ± 4.2^{a}	28.5 ± 1.6^{b}
P1	3.7 ± 0.3^{c}	1.2 ± 0.1^{d}	103.5 ± 5.5^{c}	47.1 ± 2.6^{d}	101.1 ± 5.6^{c}	49.8 ± 2.7^{d}
P2	3.7 ± 0.4^{ce}	1.3 ± 0.1^{d}	111.4 ± 5.9^{c}	49.2 ± 2.7^{d}	103.9 ± 5.5^{c}	52.8 ± 2.9^{d}
G1P1	3.2 ± 0.3^{ce}	1.4 ± 0.1^{d}	110.3 ± 6.0^{c}	37.0 ± 2.0^{e}	106.2 ± 5.7^{c}	39.7 ± 2.1^{e}
G1P2	3.2 ± 0.3^{ce}	1.3 ± 0.1^{d}	106.2 ± 5.6^{c}	41.6 ± 2.3^{de}	105.6 ± 5.8^{c}	41.7 ± 2.4^{e}
G2P1	2.6 ± 0.2^{a}	1.8 ± 0.2^{b}	86.0 ± 4.3^{a}	23.1 ± 1.2^{f}	83.5 ± 4.5^{a}	30.1 ± 1.7^{b}
G2P2	2.5 ± 0.2^{a}	1.7 ± 0.1^{b}	83.6 ± 4.5^{a}	21.1 ± 1.2^{f}	78.6 ± 4.3^{a}	29.1 ± 1.7^{b}

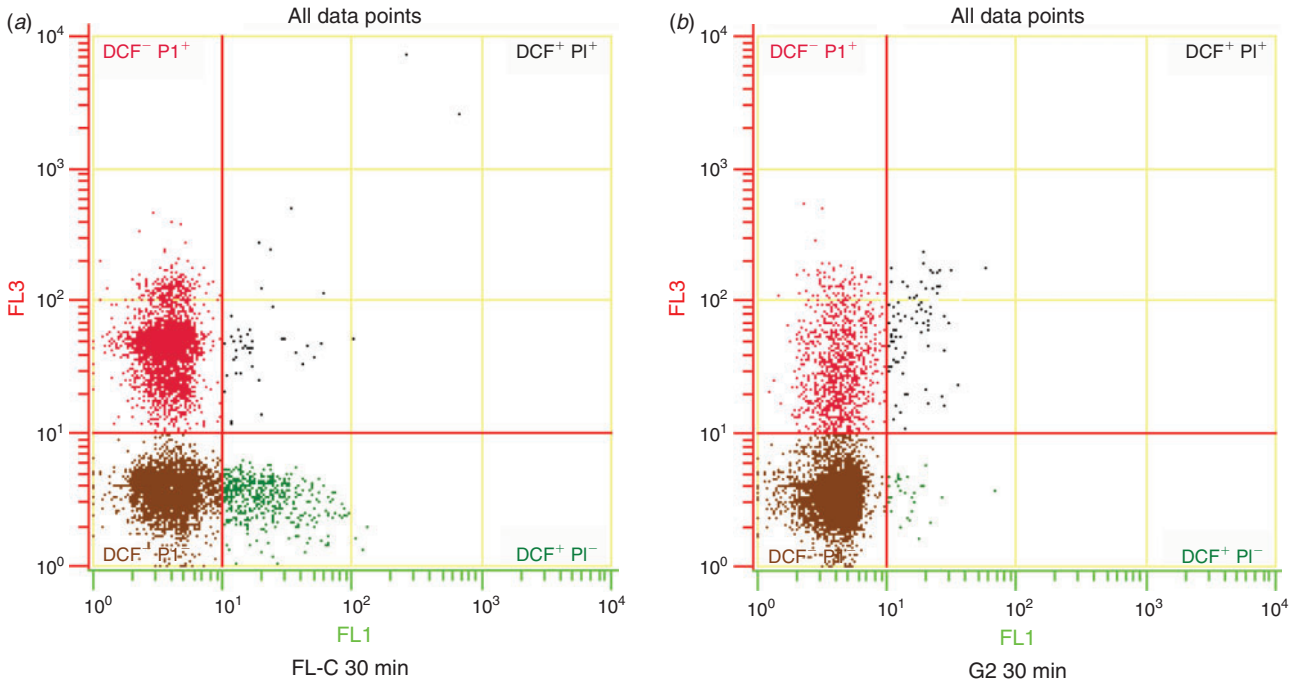

Fig. 5. Representative dot plots from 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA)/propidium iodide (PI) staining (peroxide levels) after 30 min incubation at 37°C after thawing in the (*a*) frozen–thawed control (FT-C) and (*b*) 2 mM reduced glutathione (G2)-treated groups. The upper left quadrants (DCF^-/PI^+) contain non-viable spermatozoa with low levels of intracellular H_2O_2, the upper right quadrants (DCF^+/PI^+) contain non-viable spermatozoa with high levels of intracellular H_2O_2, the lower left quadrants (DCF^-/PI^-) contain viable spermatozoa with low levels of intracellular H_2O_2 and the lower right quadrants (DCF^+/PI^-) contain viable spermatozoa with high levels of intracellular H_2O_2.

review, see Sikka 2001), has been observed in human (Gadea *et al.* 2011), horse (Ball *et al.* 2001), bull (Bilodeau *et al.* 2000) and dog (Kim *et al.* 2010) spermatozoa in response to cryopreservation. However, in the boar, the link between ROS production and freeze–thawing remains unclear. In the boar, H_2O_2 has been identified as being the primary source of ROS damage in viable spermatozoa not only during cryopreservation (Kim *et al.* 2011), but also when using ROS-generating systems

Table 5. Percentage of motile spermatozoa and spermatozoa exhibiting progressive motility in the different treatment groups after 30 or 240 min incubation at 37°C after thawing

Data are the mean ± s.e.m. Different superscripts indicate significant differences ($P < 0.05$) between a given treatment at a given time point and the rest of the treatments and time points within the same category of spermatozoa. TMOT, total motile spermatozoa; PMOT, spermatozoa exhibiting progressive motility; Extended, extended control (semen refrigerated at 17°C); FT-C, frozen–thawed control; G1, 1 mM reduced glutathione (GSH); G2, 2 mM GSH; P1, 1 mM procaine hydrochloride (ProHCl); P2, 2 mM ProHCl

	TMOT (%)		PMOT (%)	
	30 min	240 min	30 min	240 min
Extended	88.0 ± 4.9^{a}	58.0 ± 2.9^{b}	64.3 ± 3.2^{a}	39.1 ± 2.1^{bf}
FT-C	60.5 ± 3.4^{bg}	38.7 ± 2.3^{c}	35.1 ± 2.0^{bg}	17.6 ± 1.0^{c}
G1	70.2 ± 3.8^{d}	46.1 ± 2.5^{ef}	51.7 ± 2.9^{d}	28.7 ± 1.7^{ei}
G2	74.0 ± 3.8^{d}	50.4 ± 2.9^{f}	53.6 ± 3.0^{d}	36.5 ± 2.0^{b}
P1	59.0 ± 2.9^{b}	40.2 ± 2.3^{c}	40.0 ± 2.2^{bf}	30.6 ± 1.8^{e}
P2	64.9 ± 3.8^{g}	42.9 ± 2.3^{ce}	43.6 ± 2.2^{f}	31.8 ± 1.9^{e}
G1P1	62.4 ± 3.3^{g}	40.5 ± 2.4^{c}	52.6 ± 2.9^{d}	32.5 ± 1.7^{eg}
G1P2	61.0 ± 3.2^{bg}	41.2 ± 2.1^{c}	48.1 ± 2.6^{d}	31.4 ± 1.6^{e}
G2P1	70.3 ± 3.8^{d}	47.8 ± 2.7^{ef}	42.4 ± 2.5^{f}	24.3 ± 1.4^{i}
G2P2	73.0 ± 3.9^{d}	55.1 ± 2.8^{b}	53.1 ± 2.9^{d}	35.8 ± 1.6^{bg}

(Guthrie and Welch 2006). In the present study, a lack of catalase and exhaustion of the other antioxidants may explain why the H_2O_2 levels were slightly higher after the freeze–thawing procedures, in agreement with the findings reported by Kim *et al.* (2011). In this regard, the positive effect of the addition of 2 mM GSH to the reduction of H_2O_2 levels could be related to a mechanism linked to the deactivation of the oxidising agents by reduction (Stenesh 1998), thereby compensating for the lack of other antioxidants and/or catalase.

In somatic cells, ProHCl shows a greater ability to bind purines than do pyrimidines, which is why it appears to bind DNA cytosine–phosphorous–guanine (CpG) islands (Ping *et al.* 2006), and has an antioxidant effect on ROS-induced endothelial damage in the rabbit aorta (Lee *et al.* 2010), potentially via H_2O_2 scavenging. However, although GSH slightly reduced the H_2O_2 content in boar spermatozoa in the present study, ProHCl did not exhibit an H_2O_2-scavenging effect. This leads us to suggest that these two agents have different mechanisms of action. Nonetheless, it is difficult to determine exactly what the extent of ROS impairment is, because $\cdot O_2^-$ levels were not modified by the addition of GSH even though a significant decrease was seen in H_2O_2, although to a lesser extent (from ~105 to 80 a.u. on GMFI). The low production of H_2O_2 in boar spermatozoa due to freeze–thawing procedures observed in the present and other studies (Guthrie and Welch 2006; Kim *et al.* 2011), along with the different effects of GSH and ProHCl on H_2O_2 levels and the accuracy of the detection mechanisms used in the present and other studies, opens reasonable doubts as to the real role of ROS on the function and survival of boar spermatozoa.

The integrity of sperm chromatin is usually assessed through DNA fragmentation tests. However, the results of previous studies in boar investigating DNA fragmentation after freeze–thawing procedures are not clear. Thus, Fraser and Strzeżek (2005), using the neutral comet assay that detects double-strand DNA breaks, observed an increase in DNA fragmentation in boar spermatozoa after freeze–thawing procedures, but Hernández *et al.* (2006), using a sperm chromatin dispersion assay (SCSA), and Flores *et al.* (2008, 2011), using the SCDt, found similar sperm DNA fragmentation indices in extended and frozen–thawed boar spermatozoa. It is worth noting that the SCSA consists of *in situ* DNA denaturation and further staining with acridine orange (Evenson *et al.* 1999), whereas the SCDt assesses the level of DNA damage through chromatin dispersion after controlled DNA denaturation and protein depletion (Enciso *et al.* 2006).

In the present study, differences in sperm DNA fragmentation were not found after 30 min incubation after thawing. These findings are in agreement with other previous reports (Hernández *et al.* 2006; Flores *et al.* 2008, 2011), as well as the results reported by Rybar *et al.* (2004) and Boe-Hansen *et al.* (2005), who found only 5%–10% changes in chromatin structure after direct freezing in liquid nitrogen. Thus, sperm DNA fragmentation appears to be not increased by freeze–thawing procedures if we take into account levels of sperm DNA fragmentation after 30 min incubation after thawing. However, in the FT-C group, the percentage of spermatozoa with fragmented DNA increased from $1.8 \pm 0.3\%$ after 30 min incubation after thawing to $6.2 \pm 0.8\%$ after 240 min incubation after thawing. Despite this being a marginal increase, significantly higher levels of sperm DNA fragmentation were seen in the FT-C group after 240 min incubation after thawing than in the G2, P2, G2P1 and G2P2 groups. In addition, the levels of sperm DNA fragmentation in these four treatment groups were similar to those in the extended control group after 240 min incubation after thawing.

Together, these results allow us to hypothesise that destabilisation of the nucleoprotein structure, due to disruption of disulfide bonds, after 30 min incubation after thawing of boar spermatozoa leads to chromatin decondensation (Flores *et al.* 2008, 2011) and seems to underlie the subsequent increase in DNA fragmentation. In fact, less inter- and intraprotamine interactions make DNA more susceptible to damage (Fuentes-Mascorro *et al.* 2000; Nasr-Esfahani *et al.* 2004), because DNA strands are tightly wrapped around the protamine molecules and reduced chromatin packaging leads to lower resistance against strong acids, proteases, DNases and/or detergents (Chapman and Michael 2003). Thus, after 240 min incubation after thawing, there are higher levels of leakage products from very significant and increasing numbers of dead and dying spermatozoa. These products are likely to include acrosomal lytic enzymes, as well as sperm nuclear, cytoplasmic and mitochondrial components, including nucleases released from spermatozoa with damaged plasma membranes. Endogenous nucleases, found in hamster, mouse and human spermatozoa, can fragment DNA by cleaving it into loop-sized fragments (Ward and Ward 2004) and, in the mouse, the nucleases can be activated during freeze–thawing procedures (Sotolongo *et al.* 2005). In addition, there can be traces of components of the cryopreservation medium, including glycerol and yolk particles, that may be detrimental for spermatozoa. Thus, we suggest that all these

detrimental products may directly damage sperm chromatin and/or accelerate DNA fragmentation upon destabilisation of the nucleoprotein structure already observed after 30 min incubation after thawing. According to our hypothesis, cryopreservation of boar spermatozoa would alter the nucleoprotein structure without the necessity to arrive at the end-point that DNA fragmentation represents, as observed immediately after thawing. However, after 240 min incubation after thawing, this and other factors (e.g. ROS species and particles from dying spermatozoa) concomitantly act to increase levels DNA fragmentation. Our hypothesis also explains the contradictory results reported in the literature thus far. Indeed, it could explain why previous reports (Hernández *et al.* 2006; Flores *et al.* 2011) did not observe increases in DNA fragmentation immediately after thawing. In a similar fashion, DNA fragmentation does not appear in the ovine immediately after thawing, only after a 2–6 h incubation period.

In this context, GSH and ProHCl seem to counteract the increase in sperm DNA fragmentation, having beneficial effects on the spermatozoa not only during the freezing procedures, but also during incubation after thawing. This counteracting effect may be related to their protection of disulfide bonds and to their antioxidant activity, as well as to the lower levels of dying and/or dead spermatozoa observed in the GSH and ProHCl treatment groups. Furthermore, Lopes (1997) have reported that GSH protects DNA against ROS-induced fragmentation in humans.

Finally, the positive effects of 2 mM GSH (G2, G2P1 and G2P2 treatment groups) in the freezing medium were observed on sperm viability (SYBR-14/PI), acrosome integrity (PNA-FITC/PI) and changes in membrane permeability (YO-PRO-1/PI assay), because the results for these parameters were better in these three treatment groups (G2, G2P1 and G2P2) than in the FT-C group after both 30 and 240 min incubation after thawing. Although freeze–thawing increased the percentage of non-viable spermatozoa with high levels of disordered membrane lipid organisation (M540/YO-PRO-1), the addition of 2 mM GSH (G2, G2P1 and G2P2) to the freezing extenders resulted in a lower percentage of non-viable spermatozoa with high levels of disordered membrane lipid organisation. Conversely, despite ProHCl inducing sperm capacitation and hyperactivation in several species (bull, stallion and guinea-pig) without triggering the acrosome reaction (Mújica *et al.* 1994; Márquez and Suárez 2004; McPartlin *et al.* 2009), this effect was not observed in the present study.

Positive effects of GSH were seen on several parameters of sperm motility (TMOT, VSL, VCL, VAP and LIN) after 30 and 240 min incubation after thawing, in agreement with previous studies (Gadea *et al.* 2005). In the present study, we could hypothesise that GSH may compensate for ROS production linked to freeze–thawing procedures, because ROS impairs motility via ATP depletion mediated by the inhibition of oxidative phosphorylation and/or glycolysis by H_2O_2 (de Lamirande and Gagnon 1992).

Together, these findings indicate that the freeze–thawing of boar spermatozoa impairs sperm motility and membrane integrity, destabilises the nucleoprotein structure by disrupting disulfide bonds and increases levels of both DNA fragmentation and intracellular H_2O_2 in viable spermatozoa, although this occurs with a low incidence. During freeze–thawing, both ProHCl and GSH protect disulfide bonds between the cysteines residues of nucleoproteins and sperm chromatin, especially after 240 min incubation after thawing. This provides the rationale for the addition of GSH and ProHCl to the freezing medium, even though more research is needed to determine the exact mechanisms of action of these two agents and how they may affect fertilisation and subsequent embryo development. In addition, our data warrant further investigation to determine whether GSH and/or ProHCl are able to stabilise sperm nucleoprotein structure during freeze–thawing in other mammalian species.

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