ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Marc Yeste, Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, 08193 Bellaterra, Cerdanyola del Vallès, Barcelona, Spain.
E-mail: marc.yeste@uab.cat

Keywords:
AI, boar sperm, cryopreservation, fertility, nucleoprotein structure, prolificacy, reduced glutathione

Received: 5-Jun-2013
Revised: 30-Aug-2013
Accepted: 16-Sep-2013

doi: 10.1111/j.2047-2927.2013.00144.x

Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen

[1]E. Estrada, [1]J. E. Rodríguez-Gil, [1]L. G. Rocha, [2]S. Balasch, [3]S. Bonet and [1]M. Yeste

[1]Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Barcelona, Spain, [2]Swine Genetic Services (Gepork, SL), Barcelona, Spain, and [3]Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, Girona, Spain

SUMMARY

The main aim of this work was to evaluate how supplementing freezing media with reduced glutathione (GSH) affected the 'in vivo' fertilizing ability of boar semen subjected to cryopreservation procedures. With this purpose, 12 ejaculates coming from 12 boars were cryopreserved in the presence or absence of 2 mM GSH, whereas the same number of extended ejaculates coming from the same boars was used as negative/farm controls. Eight different sperm parameters (levels of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels, and total and progressive sperm motility) were evaluated before freezing and after 30 and 240 min of thawing. In addition, a total of 180 multiparous sows were used in the field fertility trials, the females being randomly divided into three groups and inseminated with extended, frozen-thawed control or frozen-thawed semen supplemented with 2 mM GSH. The presence of GSH in the freezing media significantly ($p < 0.05$) increased farrowing rates and the number of total born piglets and alive born piglets, and partially counteracted the cryopreservation-induced damages inflicted on frozen-thawed spermatozoa. We can thus conclude that supplementing freezing media with 2 mM GSH greatly improves boar sperm cryopreservation technology, as it significantly improves the fertilizing ability of frozen-thawed spermatozoa.

INTRODUCTION

Artificial insemination (AI) with extended semen offers many benefits to the swine industry, through improving biosecurity and access to high-quality genetic material (Bailey *et al.*, 2008; Knox, 2011). However, AI with frozen-thawed semen only represents 2% of swine inseminations (Roca *et al.*, 2011), as it yields lower fertility rates and litter sizes than those achieved with extended semen (Almlid & Hofmo, 1995; Johnson *et al.*, 2000). New insights into the protocol of boar sperm cryopreservation have previously been made with the aim to increase fertility and prolificacy rates (Spencer *et al.*, 2010). In this regard, it is worth mentioning that the use of post-cervical artificial insemination (post-CAI) with frozen/thawed semen has represented a considerable step forward (Gil *et al.*, 2000; Rath, 2002; Casas *et al.*, 2010).

Previous studies reported that freeze-thawing of boar spermatozoa induces cell alterations that can affect sperm fertilizing ability. Some of these alterations are linked to damage in sperm plasmalemma (Meyers, 2005; Flores *et al.*, 2008; Casas *et al.*, 2009) and osmotic and oxidative stresses (Hernández *et al.*, 2007). Cryopreservation also induces destabilization of nucleoprotein structure, as the increase in the number of disrupted disulphide bonds between sperm nucleoproteins after freeze-thawing manifests (Flores *et al.*, 2011; Yeste *et al.*, 2012). Finally, boar sperm cryopreservation has also been reported to increase DNA fragmentation after 240 min of thawing (Yeste *et al.*, 2012, 2013), even though the extent of such damage is lower than in other mammalian species like horse and human (Baumber *et al.*, 2003; Silva & Gadella, 2006; Fraser *et al.*, 2011).

The antioxidant, reduced glutathione (GSH), is the most abundant thiol in cells and is considered of vital importance, among other functions, for the maintenance of the intracellular redox balance (Jacob *et al.*, 2003). Chatterjee *et al.* (2001) demonstrated that GSH maintains the normal distribution pattern of sulphydryl groups in bull sperm membrane proteins during

freeze-thawing, thereby avoiding the cryopreservation-induced changes in the quantity and distribution pattern of these sulphydryl groups. Furthermore, the addition of GSH to freeze-thawing media has been seen to increase sperm motility, slightly reduce intracellular peroxide levels and enhance the sperm-penetration-to-oocyte ability of boar spermatozoa (Gadea *et al.*, 2004, 2005; Yeste *et al.*, 2012). In this context, the addition of protective agents of disulphide bonds, such as GSH at a final concentration of 2 mM, seems to improve the ability of the nucleoprotein structure to resist cryoinjuries, as previous works of our group have shown (Yeste *et al.*, 2012). However, and despite the relevance of sperm chromatin integrity on fertilizing ability (Silva & Gadella, 2006), no previous study has been conducted to evaluate the actual impact of supplementing freezing extenders with GSH on pig 'in vivo' fertility and prolificacy.

On the basis of this background, this study aimed to determine the effects of adding 2 mM GSH to freeze extenders (LEY and LEYGO) on the field fertility and prolificacy of multiparous sows inseminated with extended, frozen-thawed control or frozen-thawed semen supplemented with 2 mM GSH. In addition, some additional sperm quality parameters (such as the amounts of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, ROS levels and sperm motility) were also assessed in this study and correlated with field fertility and prolificacy data to evaluate their relative importance in explaining boar sperm fertilizing ability 'in vivo' as well as their resistance to cryopreservation.

MATERIALS AND METHODS

The experimental protocol was designed following the guidelines established by the Animal Welfare Directive of the Regional Government of Catalonia (Spain), the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Spain), and according to Spanish welfare and protection standards in swine (R.D. 1392/2012).

Seminal samples

In total, 24 ejaculates coming from 12 healthy boars from the Pietrain breed, 2–3 years of age, were used in this study. These animals were housed in climate-controlled buildings (Swine Genetic Services, S.L., Roda de Ter, Barcelona, Spain), fed with an adjusted diet (2.3 kg/d) and provided with water ad libitum. In addition, all of them were of proven fertility according with the AI station records, and were found to present good ejaculate freezability ('good freezers').

From all of these 24 ejaculates, 12 were used for cryopreservation purposes, whereas the other 12 were used as farm controls (extended semen) for evaluating sperm parameters and performing in vivo fertility trials. In all cases, the sperm-rich fractions were collected manually twice per week using the hand-gloved method, diluted with a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain), and cooled down to 16–17 °C. Ejaculates used as farm controls were diluted 1 : 9 (v:v) with the above-mentioned commercial extender and split into seminal doses of 60 mL and 3×10^9 spermatozoa/dose each, suitable for post-CAI. Two of these seminal doses, stored at 17 °C, were sent to our laboratory to assess the following eight sperm parameters after incubation of the spermatozoa for both 30 and 240 min at 37 °C: levels of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels, and total and progressive sperm motility (PMOT). The other 12 ejaculates were diluted 1 : 2 (v:v) with the same commercial extender, packaged with plastic bags and also sent to our lab at 17 °C. An aliquot from each of these 12 ejaculates was also taken before starting the cryopreservation process to evaluate the eight previously mentioned parameters.

In all cases, the quality of all sperm samples was evaluated upon arrival to check that they satisfied the quality standards previously established as minimal conditions to proceed with the freeze-thawing procedure (i.e. total sperm motility >80%, PMOT >60%; morphologically normal spermatozoa >85%; sperm viability >85% (Yeste *et al.*, 2012).

Cryopreservation and thawing of sperm samples

When stated, semen samples were cryopreserved using the Westendorf method (Westendorf *et al.*, 1975) and adapted by Yeste *et al.* (2012). Briefly, all ejaculates were cryopreserved 24 h after extraction and storage at 17 °C. At that time, the ejaculates were centrifuged at 17 °C and 600***g*** for 5 min. Then, pellets were recovered and diluted at 1.5×10^9 spermatozoa/mL (using a Makler counting chamber; Sefi-Medical Instruments, Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Spermatozoa were next cooled down to 5 °C for 90 min, and subsequently diluted at 1×10^9 spermatozoa/mL in a second medium containing LEY glycerol and ES Paste (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA) (LEYGO), at final concentrations of 2 and 0.5% respectively. During the sperm cryopreservation process, each ejaculate was divided into two equal parts. The first one followed the conventional freezing process, whereas LEY and LEYGO cryopreservation extenders of the other part were supplemented with 2 mM reduced L-glutathione ($C_{10}H_{17}N_3O_6S$; GSH, Sigma-Aldrich®, St Louis, MO, USA). This GSH concentration was chosen because of our previously published results (Yeste *et al.*, 2012). Afterwards, sperm samples were packed in 0.5-mL plastic and labelled straws (Minitub Ibérica, SL) distinguishing between treatments [frozen-thawed control (FT C) vs. frozen-thawed supplemented with 2 mM GSH (FT GSH)], boars and ejaculates. The straws were then transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica, SL). The freezing programme (SY-LAB software; Minitub Ibérica, SL) consisted of 313 sec of cooling at the following rates: −6 °C/min from 5 to −5 °C (100 sec), −39.82 °C/min from −5 to −80 °C (113 sec), maintained for 30 sec at −80 °C, and finally cooled at −60 °C/min from −80 to −150 °C (70 sec). The straws were finally plunged into liquid N_2 (−196 °C) for further storage.

After at least 2 weeks of storage at −196 °C, four straws per ejaculate and treatment (FT C or FT GSH) were taken, thawed at 37 °C for 20 sec and immediately diluted with three volumes of warmed Androstar Plus® at 37 °C at a final dilution of 1/4 (Casas *et al.*, 2012). All the sperm parameters (amounts of free-cysteine residues in sperm nucleoproteins, DNA fragmentation, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels and sperm motility) were evaluated at 30 and 240 min after thawing at 37 °C.

Evaluation of free-cysteine residues in sperm nucleoproteins before and after freeze-thawing

The determination of free cysteine radicals in sperm nucleoproteins, as an indirect measure of disrupted disulphide bonds

within nucleoproteins, was carried out following the protocol adapted to boar spermatozoa and described by Flores *et al.* (2011). Briefly, samples were centrifuged at 600×***g*** and 17 °C for 20 min 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 leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator, Ika® Labortechnick; Staufen, Germany), and the homogenates centrifuged at 850***g*** and 4 °C for 20 min. Both the resultant supernatants and the upper layer of the pellet were discarded, and the pellets were subsequently resuspended in 500 μL of PBS. The purity of this separation was determined by observation under a phase-contrast microscope (Zeiss Primo Star, Karl Zeiss; Jena, Germany) at 400× magnifications (Zeiss Plan-Achromat 40×/0.65; Karl Zeiss). Samples purity was described as the percentage of loose heads in comparison with the presence of whole, non-fractioned spermatozoa and separated tails in the sample. In all cases, the mean purity percentage was higher than 95% of loose heads in comparison with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separating the heads from their respective mid-pieces.

The levels of free cysteine radicals in sperm nucleoproteins were determined in the samples obtained by using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma-Aldrich®) as described by Brocklehurst *et al.* (1979). Briefly, the 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. Ten-μL aliquots of cysteine standards from 0.1 to 5 mM (Sigma-Aldrich®) were also added to 990 μL of 0.4 mM 2,2′-dithiodipyridine for evaluation. In all the cases, mixtures were incubated at 37 °C for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalized through a parallel determination of the total protein content of samples by the Bradford (1976) method, using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad, Hercules, CA, USA). Three replicates per sample and treatment were evaluated, and the corresponding mean ± SEM (standard error of the mean) was subsequently calculated.

Evaluation of DNA fragmentation before and after freeze-thawing

DNA fragmentation was assessed using a sperm chromatin dispersion test (SCDt) specifically designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain) and following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this technique strongly correlated with those obtained with other tests, like the neutral comet assay (Enciso *et al.*, 2006).

Briefly, the lysing buffer included in the commercial kit was tempered to 22 °C and vials containing low-melting agarose were incubated at 100 °C for 5 min in a water bath. Vials were then left in another water bath at 37 °C for 5 min to equilibrate the agarose temperature. Twenty-five μL of each sperm sample (at a final concentration of 10^7 spermatozoa/mL) were added to a vial prior to mixing it thoroughly. One drop of 25 μL 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 air-bubble formation.

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 of lysis solution per slide were added. An incubation step at 22 °C for 5 min was performed, prior to washing for 5 min with miliQ® 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; Molecular Probes®, Eugene, OR, USA) and mounted in DABCO antifading medium (DABCO™ anti-fading medium; Sigma-Aldrich®). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 1000× magnification.

Three counts of 250 spermatozoa each using three different slides were carried out per sample, prior to calculating the corresponding mean ± SEM. 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

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) (Lee *et al.*, 2008). These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, PNA-FITC/PI, H_2DFCDA/PI, HE/YO-PRO®-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter, Fullerton, CA, USA; Serial Number AL300087, Technical specification at https://www.beckmancoulter.com/wsrportal/ajax/downloadDocument/721742AD.pdf?autonomyId=TP_DOC_32032&documentName=721742AD.pdf). This instrument, which had not been altered in the original configuration provided by the manufacturer, 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/volume was directly measured with the Cell Lab Quanta™ SC cytometer employing 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. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. In this system, the optical characteristics for these filters were as follows: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band pass filter: 525 nm, detection width 505–545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter:

575 nm, detection width: 560–590 nm); and FL3 (red fluorescence): long pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, YO-PRO®-1 and H_2DFCDA), whereas FL3 was used to detect red (HE and PI) fluorescence.

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 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 (particle diameter <7 μm) and cell aggregates (particle 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 below, compensation was used to minimize spill-over of green fluorescence into the red channel.

Information on the events was collected in List-mode Data files (LMD), and these generated files were then analysed using Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyse the cytometric histograms. In PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assessments, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina *et al.* (2010). Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± SEM.

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen, Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma).

Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner & Johnson (1995). Briefly, sperm samples were incubated at 38 °C for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 μM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, whereas PI fluorescence was detected through FL-3. After this assessment, three 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 for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Acrosome integrity (PNA-FITC/PI)

Acrosome integrity was assessed by costaining the spermatozoa with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocianate (FITC) and PI, according to the procedure described by Nagy *et al.* (2003). Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5 μg/mL) and PI (final concentration: 10 μM) and incubated at 38 °C for 10 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa were identified and placed in one of the four following populations: (i) viable spermatozoa with intact acrosomes (PNA-FITC$^-$/PI$^-$); (ii) viable spermatozoa with damaged (exocytosed) acrosomes (PNA-FITC$^+$/PI$^-$); (iii) non-viable cells with intact acrosomes (PNA-FITC$^-$/PI$^+$); and (iv) non-viable cells with damaged acrosomes (PNA-FITC$^+$/PI$^+$).

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC-spill over into the PI/FL-3 channel (2.45%).

Assessment of intracellular ROS levels

Intracellular peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) levels were determined using two different oxidation-sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed by costaining the spermatozoa either with PI or with YO-PRO®-1.

In the case of peroxides, spermatozoa were stained with H_2DCFDA at a final concentration of 200 μM and PI at a final concentration of 10 μM, and incubated at 25 °C for 60 min in the dark. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie & Welch, 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3. Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

In the case of superoxides, samples were stained with HE (final concentration: 4 μM) and YO-PRO®-1 (final concentration: 40 μM) and incubated at 25 °C for 40 min in the dark (Guthrie & Welch, 2006). Hydroethidine is freely permeable to cells and is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of ethidium (E$^+$) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means ± SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF$^+$ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E$^+$).

Correction of data: identification of non-DNA containing particles

The percentage of non-DNA-containing particles (alien particles) was determined since in some flow cytometry assessments, especially when working with cryopreserved spermatozoa, there may be an overestimation of sperm particles. Indeed, alien particles such as cytoplasmic droplets, cell debris or diluent components (such as egg yolk), will often show EV/FS and SS characteristics similar to those of spermatozoa and cannot thus be excluded via light scatter (Petrunkina *et al.*, 2010). For this reason, 5 μL of each sperm sample coming from cooling or post-thawing steps were diluted with 895 μL of milliQ®-distilled water. Samples were then stained with PI at a final concentration of 10 μM and incubated at 38 °C for 3 min, according to the procedure described by Petrunkina *et al.*, 2010. Percentages of alien particles (f) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and treatment after PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assays, according to the following formula:

$$q_1' = \frac{q_1 - f}{100 - f} \times 100$$

where q_1' is the percentage of non-stained spermatozoa after correction.

Sperm motility

Sperm-motility analysis was performed by utilizing a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10× 0.30 PLAN objective lens, Olympus-Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 ms. Five to six separate fields were taken for each replicate, and three replicates were run per sample.

For each assessment, 15 μL of sperm sample was placed in a Makler counting chamber (Sefi-Medical Instruments), and total and progressive motility together with other kinetic parameters were recorded (Yeste *et al.*, 2008). Total motility was defined as the percentage of spermatozoa that showed a VAP > 10 μM/s, whereas progressive motility was defined as the percentage of spermatozoa that showed a VAP > 45 μm/s.

Sperm morphology

As stated, sperm morphology was assessed upon arrival of the seminal samples to verify that they satisfied the quality standard (i.e. morphologically normal spermatozoa >85%). For this purpose, 5 μL of each semen sample was placed on a slide and mounted with a cover slip. Slides were then incubated for 30 min in 100% humidity at 25 °C to immobilize the spermatozoa. Sperm morphology was assessed subjectively by making three counts of 100 spermatozoa each, prior to calculating the corresponding mean ± SEM and differentiating between morphologically normal spermatozoa, spermatozoa with cytoplasmic droplets and aberrant spermatozoa (coiled tails, tails folded at the connecting piece, at the intermediate piece or at Jensen's ring) (Yeste *et al.*, 2008). A phase-contrast microscope (Olympus BX41) was used, and the samples were observed at 200× magnification (Olympus 20× 0.40 PLAN objective lens, positive phase-contrast field).

Reproductive management and insemination of sows

In this study, a total of 180 multiparous sows from a breeding farm (Swine Genetic Services, S.L., Sant Sadurní d'Osormort, Barcelona, Spain) were used in the fertility trials. These sows, from Landrace and Large White breeds, were also housed in climate-controlled buildings, fed with an adjusted diet (2.2 kg/d) and provided with water ad libitum.

Sows were randomly divided into three groups of 15 animals each, and each group was inseminated with extended, frozen-thawed control (FT C) or frozen-thawed semen supplemented with 2 mM GSH (FT GSH). This experiment was repeated every 2 months and up to four times, according to an insemination programming system of all-in/all-out production followed by the breeding farm. Thus, a total of 60 sows per group (extended, FT C, FT GSH) were inseminated. Each ejaculate was used to inseminate five sows.

The insemination programme was carried out according to the management of sows at weaning. Detection of oestrus was monitored from Day 2 post-weaning by inspection of the vulva for reddening and swelling and response to a male teaser, and confirmed at Days 4 and 5 after pressing on the sow's back and determining for the presence/absence of the standing reflex. (Soede *et al.*, 2011). The time of onset of oestrus was defined as the first time at which a sow revealed a back-pressure response (Roberts & Bilkei, 2005).

Post-cervical insemination (Watson & Behan, 2002; Roberts & Bilkei, 2005; Casas *et al.*, 2010) through a Magaplus S® catheter (Magapor, Zaragoza, Spain) was used in all of the inseminations. This insemination device consists of a conventional foam-type disposable AI catheter that is inserted into the cervix of the female. After waiting for cervix relaxation and engagement, the catheter is inserted up into the uterine body where the semen is deposited. Double insemination was performed in all cases, with an interval of 12 h between both inseminations, thereby covering the insemination-to-ovulation interval recommended for extended and cryopreserved doses (Waberski *et al.*, 1994; Casas *et al.*, 2010).

Extended seminal doses used for AI were prepared as described in Section 2.1 (i.e. 3×10^9 sperm/dose in 60 mL/dose), whereas in the case of frozen-thawed semen, six frozen semen straws (stored at −196 °C) were taken per ejaculate and treatment (FT C or FT GSH), subsequently thawed by shaking for 20 sec into a water bath at 37 °C and diluted with three volumes of Androstar Plus®, also warmed at 37 °C (i.e. a final volume of 12 mL: 3 mL of FT sperm and 9 mL of Androstar Plus®). Finally, 48 mL of the same commercial extender were added to these 12 mL to reach a final volume of 60 mL (i.e. 3×10^9 spermatozoa/dose in doses of 60 mL, as in the case of extended semen).

Assessments of fertility rates and litter sizes

The rate of non-return to oestrus was assessed at 21-Day post-insemination (NRR_{21d}) with a male teaser, and pregnancy diagnosis (PR_{30d}) was verified at 30-Day by ultrasonography (Echoscan T-100; Import-vet SA, Barcelona, Spain). Farrowing rate (FR) was also recorded, together with the litter sizes, that is the total number of piglets born (TP), the number of live-born piglets (AP) and the number of stillborn piglets (SB).

Statistical analyses

Statistical analyses were performed using the IBM® SPSS® 20 (IBM Corp., Chicago, IL, USA) and the SYSTAT 12.0 for Windows statistical packages (SYSTAT Software Inc., Evanston, IL, USA). Data are presented as percentages and mean ± standard error of the mean (SEM). Each ejaculate was considered as an independent observation, and the minimal level of significance was set at $p < 0.05$ in all statistical analyses.

As a first step, fertility rates (i.e. non-return to oestrus rate at 21 days, pregnancy rate at 30 days, farrowing rate at parturition) were transformed using logistic (logit) transformation (e.g. $\text{logit} = \ln(NRR_{21d}/(1 - NRR_{21d}))$, the log-odds then being used for subsequent calculations. Then, both sperm parameters and fertility and prolificacy data were tested for normality and homocedasticity using the Kolmogorov–Smirnov and Levene tests. When needed, data on percentages were recalculated using the arcsine square root (x) transformation to match the parametric assumptions.

General linear models

In the case of sperm parameters (i.e. free cysteine radicals in sperm nucleoproteins, DNA fragmentation, sperm viability and acrosome integrity, intracellular peroxide and superoxide levels and sperm motility), a generalized linear mixed model for repeated measures was run where each sperm parameter was the independent variable, incubation time (30 or 240 min) was the intra-subject factor and the type of semen used (extended, FT C, FT GSH) and the boar were, respectively, the fixed-effect and random-effect factors. A post hoc Bonferroni's test was used for pair-wise comparisons.

The effects of semen type on fertility data, based on non-return to oestrus rates at 21 days, pregnancy rates at 30 days or farrowing rates, and litter sizes were determined through a linear mixed model with treatment as fixed-effects and boar as random-effects factors. A post hoc Bonferroni's test was also run for multiple comparisons.

Correlations and regression analyses

Correlations between the eight sperm parameters (free cysteine radicals in sperm nucleoproteins, DNA fragmentation, sperm viability and acrosome integrity, intracellular peroxide and superoxide levels, and total and PMOT) were calculated using the Pearson correlation. In addition, correlations of the eight mentioned sperm parameters with fertility and prolificacy data were also calculated using the Pearson correlation.

On the other hand, linear regression analyses (the Pearson correlation and multiple regression) were used to determine the ability of these sperm parameters to predict non-return rates at 21 days (NRR_{21d}), pregnancy rates at 30 days (PR_{30d}), farrowing rates (FR), TP and AP. The procedure used (the forward stepwise model) was the same one used in a previous article of our group (Yeste *et al.*, 2010) and consisted of optimizing the regression equation ($y = a + b_1x_1 + b_2x_2 + \ldots + b_kx_k;\ k \leq 8$) to increase the determination coefficient (R^2). The significance level for introducing each parameter in the multiple regression model was 0.10 and the significance level (α) for the model was 0.05.

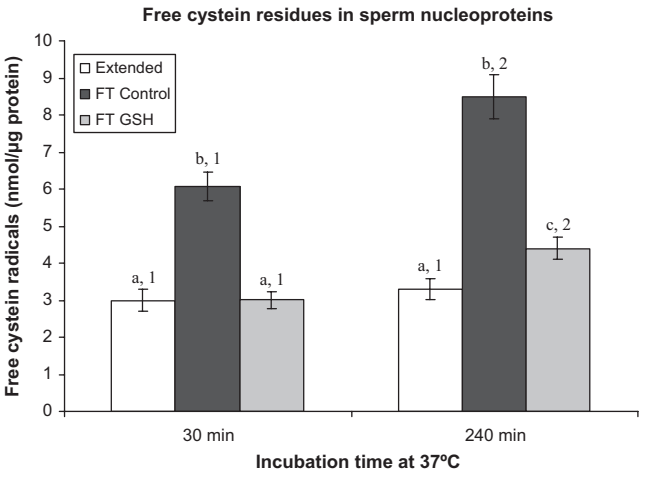
Finally, cross-validation using the Jackknife procedure was performed to estimate the bias of regression equation parameters: slope (b) and intercept (a) (Kott, 2001). In all cases, the values of the Pearson coefficient were transformed using Fisher Z-transform prior to jackknifing. The Jackknife estimation was performed on the resulting Z-transformed values, the final value of the r estimate being obtained by the inverse of the Fisher Z-transformation. The Jackknife estimate of the bias was calculated as the difference between the biased estimator ($\hat{\beta}$) and the unbiased Jackknife estimator ($\tilde{\beta}_J$), and the (1-α) confidence intervals (CI) were calculated as follows: $CI = \tilde{\beta}_J \pm t_{\upsilon,\alpha/2} \cdot \hat{\sigma}_{\tilde{\beta}_J}$, where $\bar{\beta}$ is the mean of the pseudo-values, $\hat{\sigma}_{\tilde{\beta}_J}$ is the standard error of the pseudo-values and $t_{\upsilon,\alpha/2}$ is the α-level critical value of a Student's t distribution with $\upsilon = n - 1$ degrees of freedom (Yeste *et al.*, 2010).

RESULTS

Effects of the addition of 2 mM GSH to cryopreservation extenders on free-cysteine residues of sperm nucleoproteins

Figure 1 shows the effects of treatment (extended, FT C, FT GSH) and post-thawing incubation time on the levels of free cysteine residues in sperm nucleoproteins. After 30 min of incubation at 37 °C, frozen-thawed control sperm presented significantly ($p < 0.05$) higher levels of free cysteine residues in sperm nucleoproteins than extended or frozen-thawed semen supplemented with 2 mM GSH. After 240 min of thawing, levels of free cysteine residues in frozen-thawed control were again significantly ($p < 0.05$) higher than were those observed in extended semen or frozen-thawed spermatozoa supplemented with GSH. However, at that time, frozen-thawed semen supplemented with 2 mM GSH presented significantly higher ($p < 0.05$) levels of free cysteine radicals than did extended semen (FT C: 8.5 ± 0.6 vs. FT GSH: 4.4 ± 0.3).

Figure 1 Free cysteine residues (as mean ± SEM) in sperm nucleoproteins in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.



Effects of the addition of 2 mM GSH to cryopreservation extenders on DNA fragmentation

DNA fragmentation levels were low in all treatments after 30 and 240 min of incubation at 37 °C. However, whereas extended semen and frozen-thawed spermatozoa supplemented with 2 mM GSH did not differ after incubation at 37 °C for either 30 or 240 min, frozen-thawed control semen presented significantly ($p < 0.05$) higher percentages of spermatozoa with fragmented DNA at 240 min post-thawing when compared with extended and frozen-thawed semen supplemented with 2 mM GSH (Fig. 2).

Effects of the addition of 2 mM GSH to cryopreservation extenders on sperm viability

Freeze-thawing of boar spermatozoa significantly ($p < 0.05$) reduced the percentages of viable spermatozoa both after 30 and 240 min post-thawing (Fig. 3). Nevertheless, the extent of this damage differed between frozen-thawed control and frozen-thawed semen supplemented with 2 mM GSH, as the former presented significantly lower percentages of viable spermatozoa than the latter, both after 30 and 240 min post-thawing (FT C: 33.5 ± 1.4 vs. FT GSH: 46.9 ± 1.9).

Figure 2 Percentages of spermatozoa with fragmented DNA (mean ± SEM) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.

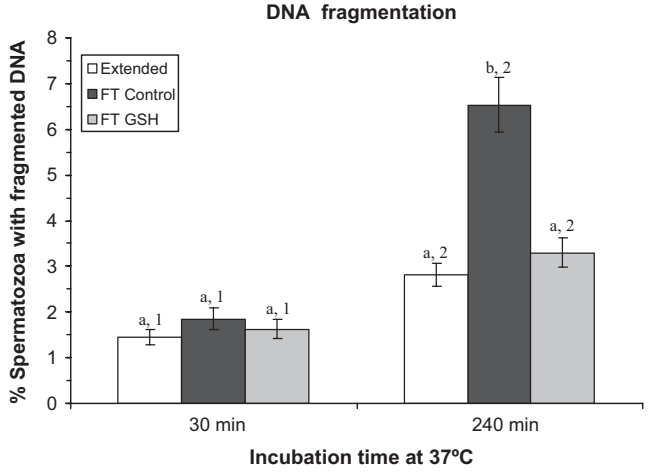
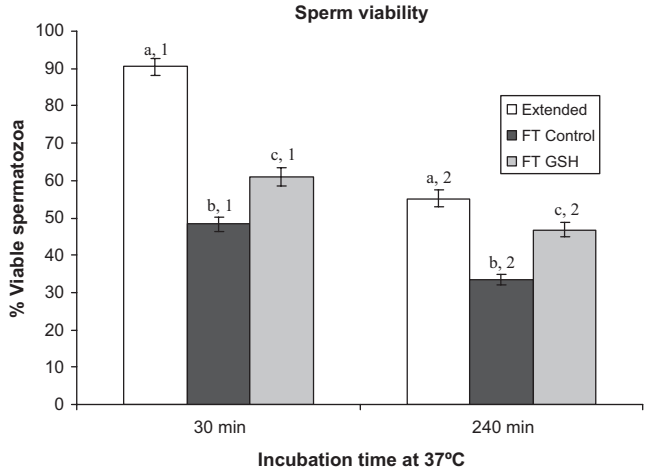


Figure 3 Percentages of viable spermatozoa (SYBR14$^+$/PI$^-$) (mean ± SEM) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min. Different letters (a–c) mean significant ($p < 0.05$) differences between treatments, whereas different numbers (1, 2) mean significant differences ($p < 0.05$) between time points within the same treatment.



Effects of the addition of 2 mM GSH to cryopreservation extenders on acrosome integrity

Table 1 shows (as mean ± SEM) the percentages of acrosome-intact spermatozoa in extended semen, frozen-thawed control and frozen-thawed supplemented with GSH after 30 and 240 min of incubation at 37 °C. Again, freeze-thawing of boar spermatozoa was seen to significantly ($p < 0.05$) decrease the percentage of acrosome-intact spermatozoa. Notwithstanding, this reduction was significantly higher ($p < 0.05$) in frozen-thawed control than in frozen-thawed semen supplemented with 2 mM GSH (e.g. at 240 min post-thawing, FT C: 20.9 ± 1.0 vs. FT GSH 38.8 ± 1.6).

Table 1 Percentages of acrosome-intact spermatozoa (PNA-FITC$^-$/PI$^-$), viable spermatozoa with high intracellular peroxide levels (DCF$^+$/PI$^-$), viable spermatozoa with high superoxide levels (E$^+$/ YO-PRO-1$^-$), and total and progressive motile spermatozoa in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 or 240 min

	% Acrosome intact spermatozoa		% Spermatozoa DCF$^+$/PI$^-$		% Spermatozoa E$^+$/ YO-PRO-1$^-$		Total motility		Progressive motility	
	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min	30 min	240 min
Extended	85.3 ± 3.2^{a,1}	64.7 ± 2.6^{a,2}	2.2 ± 0.2^{a,1}	1.8 ± 0.2^{a,1}	3.3 ± 0.3^{a,1}	3.4 ± 0.4^{a,1}	90.2 ± 4.5^{a,1}	57.4 ± 2.7^{a,2}	64.8 ± 3.4^{a,1}	41.7 ± 2.2^{a,2}
FT C	47.4 ± 2.0^{b,1}	20.9 ± 1.0^{b,2}	3.3 ± 0.3^{b,1}	1.4 ± 0.1^{b,2}	3.4 ± 0.3^{a,1}	3.5 ± 0.4^{a,1}	57.6 ± 3.3^{b,1}	38.1 ± 1.9^{b,2}	34.0 ± 1.9^{b,1}	18.0 ± 1.1^{b,2}
FT GSH	62.6 ± 2.5^{c,1}	38.8 ± 1.6^{c,2}	2.6 ± 0.2^{a,1}	1.9 ± 0.1^{a,2}	3.2 ± 0.3^{a,1}	3.5 ± 0.3^{a,1}	72.9 ± 3.8^{c,1}	49.5 ± 2.4^{c,2}	52.5 ± 2.8^{c,1}	37.2 ± 2.0^{c,2}

Different letters (a–c) mean significant ($p < 0.05$) differences within a column and different numbers (1, 2) mean significant differences within a row for a given sperm parameter (i.e. % acrosome-intact spermatozoa, % spermatozoa DCF$^+$/PI$^-$, % spermatozoa E$^+$/ YO-PRO-1$^-$, % total motile spermatozoa, or % progressive motile spermatozoa).

Effects of the addition of 2 mM GSH to cryopreservation extenders on ROS levels

After 30 min of incubation at 37 °C, the percentage of viable spermatozoa with high peroxide levels (DCF^+/PI^-) was found to be slightly but significantly higher ($p < 0.05$) in frozen-thawed control than in extended and frozen-thawed semen supplemented with 2 mM GSH (Table 1). In contrast, the percentage of spermatozoa with high intracellular superoxide levels (E^+/YO-PRO-1^-) was not found to be affected by sperm cryopreservation or GSH supplementation.

Effects of the addition of 2 mM GSH to cryopreservation extenders on sperm motility

Table 1 also shows data on total and PMOT in extended semen, frozen-thawed control and frozen-thawed spermatozoa supplemented with 2 mM GSH. As expected, boar sperm cryopreservation significantly decreased total (TMOT) and PMOT. However, in both parameters, the reduction observed in frozen-thawed control was significantly ($p < 0.05$) higher than that observed in frozen-thawed spermatozoa supplemented with 2 mM GSH, both at 30 and 240 min post-thawing (e.g. PMOT at 240 min post-thawing, FT C: 18.0 ± 1.1 vs. FT GSH 37.2 ± 2.0).

Correlations between sperm quality parameters

Table 2a shows the correlation coefficients among all the eight sperm parameters evaluated at 30 min. Apart from the percentages of spermatozoa with fragmented DNA and of viable spermatozoa with high levels of superoxides, the other six sperm parameters were found to be significantly correlated each other. At 240 min post-thawing (Table 2b), all of the sperm parameters except those that evaluated ROS levels, that is percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-) levels, were found to be significantly correlated with each other.

Effects of the addition of 2 mM GSH to cryopreservation extenders on field fertility and prolificacy

The insemination with frozen-thawed control samples yielded significantly ($p < 0.05$) lower fertility results when measured as the non-oestrus return rate after 21 days (NRR_{21d}) of insemination, pregnancy rate after 30 days (PR_{30d}) of insemination and farrowing rate (FR) at parturition than those observed in sows inseminated with extended semen (e.g. FR, extended: 91.4 ± 5.1 vs. FT C: 67.2 ± 3.4, Table 3). Strikingly, the presence of 2 mM GSH counteracted the deleterious effect of freeze-thawing observed in frozen-thawed control samples (e.g. FR, FT C: 67.2 ± 3.4 vs. FT GSH: 92.7 ± 5.5). In this manner, fertility rates of boar semen frozen in the presence of 2 mM GSH were not significantly different to those observed in sows inseminated with extended semen (Table 3).

As far as litter sizes are concerned, both TP and AP per sow were significantly ($p < 0.05$) higher in females inseminated with extended semen or with frozen-thawed spermatozoa supplemented with 2 mM GSH than in sows inseminated with frozen-thawed control (e.g. AP, extended: 13.6 ± 0.5 and FT GSH: 12.4 ± 0.9 vs. FT C: 6.9 ± 2.3; Table 3). Again, no significant differences were observed between extended and frozen-thawed semen supplemented with 2 mM GSH. The number of stillborn piglets did not differ between treatments (Table 3).

Table 2 Correlations (Pearson coefficient) between the eight evaluated sperm parameters (levels of free cysteine residues (FCR) in sperm nucleoproteins, % spermatozoa with fragmented DNA, % viable spermatozoa ($SYBR14^+/PI^-$), % acrosome-intact spermatozoa (PNA-$FITC^-/PI^-$), % viable spermatozoa with high intracellular peroxide levels (DCF^+/PI^-), % viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-), and % total and % progressive motile spermatozoa) in extended, frozen-thawed control (FT C) and frozen-thawed spermatozoa supplemented with 2 mM GSH (FT GSH) after incubation at 37 °C for 30 min (a) or 240 min (b)

	FCR residues in sperm nucleoproteins	% Spermatozoa with fragmented DNA	% Viable spermatozoa	% Acrosome intact spermatozoa	% Spermatozoa DCF^+/PI^-	% Spermatozoa E^+/YO-PRO-1^-	% Total motile spermatozoa	% Progressive motile spermatozoa
(a)								
FCR in sperm nucleoproteins	1	0.131	−0.658**	−0.497*	0.401*	0.107	−0.404*	−0.501**
% Spermatozoa with fragmented DNA	0.131	1	0.047	0.078	0.124	0.054	0.036	0.048
% Viable spermatozoa	−0.658**	0.047	1	0.787**	−0.404*	−0.184	0.788**	0.750**
% Acrosome intact spermatozoa	−0.497*	0.078	0.787**	1	−0.448*	0.043	0.802**	0.733**
% Spermatozoa DCF^+/PI^-	0.401*	0.124	−0.404*	−0.448*	1	0.104	−0.362	−0.395*
% Spermatozoa E^+/YO-PRO-1^-	0.107	0.054	−0.184	0.043	0.104	1	−0.092	−0.051
% Total motile spermatozoa	−0.404*	0.036	0.788**	0.802**	−0.362	−0.092	1	0.935***
% Progressive motile spermatozoa	−0.501**	0.048	0.750**	0.733**	−0.395*	−0.051	0.935***	1
(b)								
FCR in sperm nucleoproteins	1	0.649**	−0.785**	−0.589**	−0.130	0.153	−0.639**	−0.619**
% Spermatozoa with fragmented DNA	0.649**	1	−0.487**	−0.797**	−0.218	−0.008	−0.812**	−0.855***
% Viable spermatozoa	−0.785**	−0.487**	1	0.640**	0.069	−0.082	0.602**	0.499**
% Acrosome intact spermatozoa	−0.589**	−0.797**	0.640**	1	0.282	−0.019	0.896***	0.832**
% Spermatozoa DCF^+/PI^-	−0.130	−0.218	0.069	0.282	1	0.004	0.270	0.203
% Spermatozoa E^+/YO-PRO-1^-	0.153	−0.008	−0.082	−0.019	0.004	1	−0.121	−0.046
% Total motile spermatozoa	−0.639**	−0.812**	0.602**	0.896**	0.270	−0.121	1	0.877***
% Progressive motile spermatozoa	−0.619**	−0.855***	0.499**	0.832**	0.203	−0.046	0.877***	1

Significances of correlation coefficient (r) test are shown as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

Table 3 Fertility performances of sows inseminated with extended, frozen-thawed control (FT C) or frozen-thawed semen supplemented with 2 mM GSH (FT GSH)

	Extended	FT C	FT GSH
Non-return rate to estrus 21 days (NRR_{21d}) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Pregnancy rate at 30 days (PR_{30d}) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Farrowing rate (FR) (%)	91.4 ± 5.1[a]	67.2 ± 3.4[b]	92.7 ± 5.5[a]
Total piglets born (TP) (*n*)	14.3 ± 0.4[a]	7.5 ± 2.4[b]	13.0 ± 1.0[a]
Alive born piglets (AP) (*n*)	13.6 ± 0.5[a]	6.9 ± 2.3[b]	12.4 ± 0.9[a]
Stillborn piglets (SP) (*n*)	0.8 ± 0.1[a]	0.6 ± 0.2[a]	0.6 ± 0.3[a]

Different superscripts within a row (a, b) mean significant differences between treatments (i.e. extended, FT C and FT GSH).

Correlation analyses among sperm parameters, fertility and litter size data

From all of the sperm parameters evaluated after 30 min of incubation at 37 °C, the levels of free cysteine residues in sperm nucleoproteins were found to be significantly ($p < 0.05$) and negatively correlated with non-return at 21 days, pregnancy rates at 30 days and farrowing rates (Table 4). In contrast, the other sperm parameters were not found to be significantly correlated with pregnancy or fertility rates. On the other hand, the levels of free cysteine residues in sperm nucleoproteins and the percentages of viable and acrosome-intact spermatozoa were found to be significantly correlated with both TP and AP (Table 4). Sperm motility data, percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-), and percentages of spermatozoa with fragmented DNA were not significantly correlated with either fertility rates or litter sizes at 30 min post-thawing (Table 4).

In the case of sperm parameters evaluated after 240 min of incubation at 37 °C, levels of free cysteine residues in sperm nucleoproteins, percentages of spermatozoa with fragmented DNA and percentages of viable and acrosome-intact spermatozoa were found to be correlated with non-return at 21 days, pregnancy rate at 30 days and farrowing rate, and with both TP and AP (Table 4). In contrast, percentages of progressive motile spermatozoa were found to be positively and significantly correlated with both TP and AP, but not with fertility rates (i.e. non-return at 21 days, pregnancy rate at 30 days and farrowing rate). Again, percentages of viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide (E^+/YO-PRO-1^-) levels were not found to be correlated with fertility rates or litter sizes (Table 4).

Regression analyses among sperm parameters, fertility and litter size data

Following the stepwise forward model, linear regression equations between sperm parameters evaluated at 30 or 240 min post-thawing and fertility rates and litter sizes were worked out and are shown in Table 5.

In the case of fertility rates, determination coefficients (R^2) were lower than in the case of litter size variables. In addition, sperm parameters that were included in the model were only two, namely levels of free cysteine residues in sperm nucleoproteins determined both after 30 and 240 min after thawing, and the percentage of spermatozoa with fragmented DNA at 240 min post-thawing.

As far as the regression equations for litter sizes (TL and AP), two parameters evaluated 30 min after incubation at 37 °C were included in the model (levels of free cysteine residues in sperm nucleoproteins and percentage of acrosome-intact spermatozoa). In the case of 240 min post-thawing, three sperm parameters (levels of free cysteine residues in sperm nucleoproteins, percentage of spermatozoa with fragmented DNA and percentage of viable spermatozoa) rather than two were included in the model.

Cross-validation through the Jackknife test in the 10 regression equations depicted in Table 5 showed that they correctly predicted all of the dependent variables (Logit(NRR_{21d}), Logit(PR_{30d}), Logit(FR), TP and AP), both the β and $\widetilde{\beta}$ falling into 95% confidence intervals calculated with the mean of the pseudo-values and their standard errors (data not shown).

DISCUSSION

Our results strongly indicate that supplementing the freezing media with 2 mM GSH has a very strong improving effect on the 'in vivo' fertilizing ability of boar sperm subjected to freeze-thawing. This is a very important asset, especially if one takes

Table 4 Correlation analyses (Pearson's coefficients) of sperm parameters evaluated either at 30 or 240 min post-thawing with fertility rates [non-return rate at 21 days (NRR_{21d}), pregnancy rate at 30 days (PR_{30d}), farrowing rate (FR)] and litter sizes [total number of piglets born (TP), piglets born alive (AP) and stillborn piglets (SP)]

Incubation time	Sperm parameter	Logit(NRR_{21d})	Logit(PR_{30d})	Logit(FR)	TP	AP	SP
30 min	Free cystein residues in sperm nucleoproteins	−0.339*	−0.339*	−0.339*	−0.471**	−0.474**	0.168
	% Spermatozoa with fragmented DNA	−0.163	−0.163	−0.163	0.054	0.027	−0.139
	% Viable spermatozoa	0.274	0.274	0.274	0.373*	0.375*	−0.052
	Acrosome intact spermatozoa	0.289	0.289	0.289	0.459**	0.459**	−0.199
	% Spermatozoa $DCF^+/PI-$	−0.140	−0.140	−0.140	−0.262	−0.270	0.044
	% Spermatozoa E^+/ YO-PRO-1^-	−0.065	−0.065	−0.065	−0.076	−0.057	0.184
	% Total motile spermatozoa	0.256	0.256	0.256	0.297	0.292	−0.146
	% Progressive motile spermatozoa	0.177	0.177	0.177	0.306	0.307	−0.098
240 min	Free cystein residues in sperm nucleoproteins	−0.427**	−0.427**	−0.427**	−0.580**	−0.590**	0.158
	% Spermatozoa with fragmented DNA	−0.387*	−0.387*	−0.387*	−0.492**	−0.513**	0.029
	% Viable spermatozoa	0.321*	0.321*	0.321*	0.526**	0.529**	−0.188
	Acrosome intact spermatozoa	0.309*	0.309*	0.309*	0.441**	0.445**	−0.151
	% Spermatozoa DCF^+/PI^-	0.118	0.118	0.118	0.045	0.031	−0.126
	% Spermatozoa E^+/ YO-PRO-1^-	−0.079	−0.079	−0.079	−0.052	−0.061	0.091
	% Total motile spermatozoa	0.153	0.153	0.153	0.298	0.296	−0.037
	% Progressive motile spermatozoa	0.262	0.262	0.262	0.330*	0.337*	−0.118

Significances of correlation coefficient (*r*) test are shown as follows: *$p < 0.05$, **$p < 0.01$.

Table 5 Multiple regression analyses of fertility rates (NRR_{21d}, PR_{30d} and FR) or litter sizes (TP, AP and SP) with sperm parameters evaluated either at 30 or 240 min post-thawing. Regression equations, together with determination coefficients (R^2) and *F* and *p* values of the model are depicted

	Regression equation	R^2	*R*	*F*	*p* value model
NRR_{21d}					
30 min	Logit (NRR_{21d}) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (NRR_{21d}) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
PR_{30d}					
30 min	Logit (PR_{30d}) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (PR_{30d}) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
FR					
30 min	Logit (FR) = $-0.52(FCR_{30}) + 5.90$	0.11	0.34	5.20	<0.05
240 min	Logit (FR) = $-0.37(FCR_{240}) - 0.40(SDF_{240}) + 7.30$	0.20	0.45	4.97	<0.05
TP					
30 min	TP = $-0.61(FCR_{30}) + 0.11(ACR_{30}) + 6.79$	0.30	0.55	8.42	0.001
240 min	TP = $-0.46(FCR_{240}) - 0.56(SDF_{240}) + 0.07(VB_{240}) + 12.67$	0.37	0.61	7.58	<0.001
AP					
30 min	AP = $-0.59(FCR_{30}) + 0.10(ACR_{30}) + 6.43$	0.30	0.55	8.49	0.001
240 min	AP = $-0.44(FCR_{240}) - 0.60(SDF_{240}) + 0.06(VB_{240}) + 12.36$	0.39	0.62	8.11	<0.001

NRR_{21d}, non-return rate after 21 days of insemination; PR_{30d}, pregnancy rate after 30 days of insemination; FR, farrowing rate; TP, total piglets born; AP, live-born piglets; FCR, free cysteine radicals in sperm nucleoproteins; SDF, % spermatozoa with fragmented DNA; VB, % viable spermatozoa, ACR, % acrosome-intact spermatozoa.

into account that sperm cryopreservation has been regarded as being the most efficient method for storing boar sperm samples for a long period of time (Holt, 2000). However, a greater utilization of this technique has been hampered so far because of its lower fertility rates and litter sizes when compared with extended semen, despite all of the efforts being made in this regard (Almlid & Hofmo, 1995; Johnson *et al.*, 2000; Casas & Flores, 2013). In this way, our results may be relevant to further develop boar sperm freeze-thawing protocols that could yield 'in vivo' fertility results not far below to those obtained with extended semen. This is clearly stated by our data, in which the addition of 2 mM GSH to the freezing media significantly increased non-return rates at 21 days, pregnancy rates at 30 days and farrowing rates from 67.2 ± 3.4% (FT control) to 92.7 ± 5.5% (FT GSH). In a similar way, adding 2 mM GSH to the freezing media also significantly increased the total number of piglets and the number of piglets born alive. Supporting our results, another study conducted 'in vitro' has demonstrated that the addition of GSH to the thawing extender is beneficial for boar frozen-thawed spermatozoa, as it increases the sperm-penetration-to-oocyte ability (Gadea *et al.*, 2004). Taking all of these results together, and despite this being only a preliminary study, the finding that 2 mM GSH can greatly improve the 'in vivo' fertilizing ability of frozen boar spermatozoa is a very valuable result.

One issue that still remains undetermined is whether the fertility increase mediated by the GSH addition to frozen-thawed boar spermatozoa is specifically based on the improvement of a single, or more than one, sperm parameter. This is one of the aspects that has led us to evaluate a reasonably wide array of separate boar semen quality parameters. Such sperm parameters were evaluated before and after 30 and 240 min of freeze-thawing, the latter post-thawing time being set both to ensure the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses (Waberski *et al.*, 1994; Casas *et al.*, 2010) and as a sperm resistance probe. When considering the results of all of these sperm parameters, cryopreservation, as expected, causes a general impairment of boar sperm function and survival. This impairment includes damage in the nucleoprotein structure, reduction in sperm viability, motility and acrosome integrity, and a slight increase in intracellular peroxide levels. Most of this damage is visible after 30 min of thawing at 37 °C, but the case of DNA fragmentation is particularly noteworthy, as damage is only observed at 240 min post-thawing. Of course, these data are not new and match other previous reports (Casas *et al.*, 2009; Yeste *et al.*, 2012, 2013). In fact, it is widely known that sperm cryopreservation impairs sperm quality not only in porcine but also in other mammalian species (Baumber *et al.*, 2003).

Another question concerns the mechanism/s by which GSH is able to counteract, at least partially, boar sperm cryodamage. On this point, it is well-known that GSH has a strong counteracting effect on the cryopreservation-induced alterations of the sperm nucleoprotein structure and DNA integrity (Tuncer *et al.*, 2010; Yeste *et al.*, 2012). The relationship between the overall sperm nucleoprotein structure and DNA integrity is highlighted by our results. Thus, percentages of spermatozoa with fragmented DNA at 240 min post-thawing were correlated with the levels of free cysteine residues in sperm nucleoproteins. In addition, percentages of spermatozoa with fragmented DNA at 240 min post-thawing were also included in all of the regression equations that were set from the sperm parameters evaluated at this time-point.

From our results, however, it is not possible to know exactly how GSH improves boar sperm fertilizing ability. This is because of the fact that GSH does not only maintain nucleus integrity but also keeps membrane integrity (plasmalemma and acrosome membrane) and sperm motility, even though with a lesser intensity. Despite this, the joint study of data from fertility trials and sperm quality through correlation and regression analyses suggests that the beneficial effects of GSH are mainly related to the stabilization of the sperm nucleoprotein structure. Indeed, Pearson correlation coefficients and their significance tests are the highest when fertility rates (NRR_{21d}, PR_{30d} and FR) or both TP and AP are correlated with the levels of free cysteine residues in boar sperm nucleoproteins, as an indirect measurement of the integrity of the sperm nucleoprotein structure. Furthermore, when linear regression equations are worked out through a stepwise model, the statistical weight of the integrity of the

nucleoprotein structure is the highest. As such, all 10 regression equations included this parameter as an independent variable. This finding emphasizes the relevance of the integrity of the nucleoprotein structure (e.g. free cysteine residues in sperm nucleoproteins at 30 min post-thawing, FT C: 6.1 ± 0.4 vs. FT GSH: 3.0 ± 0.2) when linked to the fertilizing ability of a given ejaculate and to the increase in field fertility and prolificacy (FR, FT C: 67.2 ± 3.4 vs. FT GSH: 92.7 ± 5.5; AP, FT C: 6.9 ± 2.3 vs. FT GSH: 12.4 ± 0.9). On the contrary, the GSH-mediated increases in sperm viability, motility and acrosome-integrity (e.g. Sperm viability at 30 min post-thawing, FT C: 48.3 ± 1.9 vs. FT GSH: 60.9 ± 2.5) are linked to a much lesser extent to the analysed 'in vivo' fertilizing ability parameters. These results suggest that the effects of GSH on the maintenance of the boar sperm nucleoprotein structure after freeze-thawing are, among all of the analysed effects, those most related to the improving action of GSH on boar sperm fertilizing ability. Therefore, we can reasonably hypothesize that one of the most important mechanisms by which the addition of GSH increases fertility and prolificacy of frozen-thawed spermatozoa is the protection of the nucleoprotein structure and subsequent DNA integrity. In this regard, it is worth noting that previous reports have emphasized the relevance of chromatin integrity on sperm fertilizing ability (Silva & Gadella, 2006), and that the oviductal selection mechanisms in the establishment of sperm reservoir, so important in eutherian mammals (Yeste, 2013), have been reported to be endowed with stable and non-fragmented chromatin structure (Ardón *et al.*, 2008). All of these data appear to highlight the importance of a correct nucleoprotein structure to explain boar sperm fertilizing ability.

Nonetheless, this finding does not preclude that other sperm parameters, such as sperm viability or acrosome integrity, were included in regression equations predicting litter sizes (TP and AP), despite not being in those predicting fertility rates. On the other hand, sperm progressive motility after 240 min of incubation was found to be correlated with litter sizes, but not included in any of the regression equations set, and the parameters evaluating intracellular levels of peroxides and superoxides were not correlated with either fertility rates or litter sizes. All of these data also emphasize the relevance of sperm viability and acrosome integrity in sperm fertilizing ability, and at the same time suggest that GSH acts primarily on nucleoprotein structure and, to a lesser extent, on membrane integrity of frozen-thawed boar sperm rather than on ROS levels. The low production of H_2O_2 and $O_2^{-\bullet}$ in boar spermatozoa owing to freeze-thawing procedures observed in this and other studies (Guthrie & Welch, 2006; Kim *et al.*, 2011; Yeste *et al.*, 2012), along with the effects of 2 mM GSH on intracellular ROS levels, raise, in fact, reasonable doubts about the real role of these ROS in the fertilizing ability of boar frozen-thawed spermatozoa.

CONCLUSION

In conclusion, supplementing freezing media with 2 mM GSH improves the fertilizing ability of frozen-thawed boar semen, and it is suggested to be used to improve 'in vivo' fertility performance when utilizing cryopreserved boar spermatozoa. This improvement appears to be related to the protection effect of GSH on the integrity of the nucleoprotein structure and DNA fragmentation and, to a lesser extent, on the general function of the sperm cell.

ACKNOWLEDGEMENTS

This work has been supported by grant AGL-2008-01792/GAN (Ministerio de Economía y Competitividad, Spain). The authors thank Mr Chuck Simmons, from the Language facility at the Autonomous University of Barcelona, for help with the English of this manuscript.

AUTHORS' CONTRIBUTIONS

E.E. conducted the experimental analyses in the laboratory and participated in AI. J.E. R.-G. also participated in lab analyses and in AI, and revised the manuscript. L.G.R. and S.B. took part in AI trials. As senior author, M.Y. conducted the experimental analyses in the laboratory, was also involved in AI trials, performed the statistical analyses, and wrote the MS.

REFERENCES

Almlid T & Hofmo PO. (1995) A brief review of frozen semen application under Norwegian AI service conditions. *Reprod Dom Anim* 31, 169–173.

Ardón F, Helms D, Sahin E, Bollwein H, Töpfer-Petersen E & Waberski D. (2008) Chromatin-unstable boar spermatozoa have little chance of reaching oocytes in vivo. *Reproduction* 135, 461–470.

Bailey J, Lessar CH, Jacques J, Breque CH, Dobrisnski I, Zeng W *et al.* (2008) Cryopreservation of boar semen and its future importance to the industry. *Theriogenology* 70, 1251–1259.

Baumber J, Ball BA, Linfor JJ & Meyers SA. (2003) Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *J Androl* 24, 621–628.

Bradford MM. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Brocklehurst K, Stuchbury T & Malthouse JPG. (1979) Reactivities of neutral and cationic forms of 2,2-dipyridyl disulphide towards thiolate anions: detection of differences between the active centres of actinidin, papain and ficin by a three-protonic-state reactivity probe. *Biochem J* 183, 233–238.

Casas I & Flores E. (2013) Gene banking, the freezing strategy. In: *Boar Reproduction: Fundamentals and New Biotechnological Trends* (eds S Bonet, I Casas, WV Holt & M Yeste), pp. 551–588. Springer: Berlin, Germany. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8. doi:10.1007/978-3-642-35049-8.

Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M *et al.* (2009) Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology* 72, 930–948.

Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M *et al.* (2010) Fertility after post-cervical artificial insemination with cryopreserved sperm from boar ejaculates of good and poor freezability. *Anim Reprod Sci* 118, 69–76.

Casas I, Torner E, Yeste M & Bonet S. (2012) Boar sperm thawing practices: the number of straws does matter. *Theriogenology* 77, 1487–1494.

Chatterjee S, De Lamirande E & Gagnon C. (2001) Cryopreservation alters membrane sulphydryl status of bull spermatozoa: protection by oxidized glutathione. *Mol Reprod Develop* 60, 498–506.

Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez AI & Gosálvez J. (2006) A new method to analyze boar sperm DNA fragmentation under bright-field or fluoresecence microscopy. *Theriogenology* 65, 308–316.

Flores E, Cifuentes D, Fernández-Novell JM, Medrano A, Bonet S & Briz MD. (2008) Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm. *Theriogenology* 69, 1083–1094.

Flores E, Ramió-Lluch L, Bucci D, Fernández-Novell JM, Peña A & Rodríguez-Gil JE. (2011) Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulphide bonds in boar sperm. *Theriogenology* 76, 1450–1464.

Fraser L, Strzeżek J & Kordan W. (2011) Effect of freezing on sperm nuclear DNA. *Reprod Domest Anim* 46, 14–17.
Gadea J, Sellés E, Marco MA, Coy P, Matás C, Romar R *et al.* (2004) Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology* 62, 690–701.
Gadea J, García-Vázquez FA, Matás C, Gardón JC, Cánovas S & Gumbao D. (2005) Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. *J Androl* 26, 396–404.
Garner DL & Johnson LA. (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 53, 276–284.
Gil J, Tortades JM & Alevia A. (2000) Post cervical insemination. Proceedings of the 16th International Pig Veterinary Society Congress, Melbourne, 399.
Guthrie HD & Welch GR. (2006) Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci* 84, 2089–2100.
Hernández M, Vázquez JM, Martinez EA & Roca J. (2007) Oxidative stress during the cryopreservation of boar spermatozoa. *Reprod Fertil Dev* 19, 177.
Holt WV. (2000) Basics aspects of frozen storage of semen. *Anim Reprod Sci* 62, 2–32.
Jacob C, Giles GI, Giles NM & Sies H. (2003) Sulfur and selenium: the role of oxidation state in protein structure and function. *Angew Chem* 42, 4742–4758.
Johnson LA, Weitze KF, Fiser P & Maxwell WMC. (2000) Storage of boar semen. *Anim Reprod Sci* 62, 143–172.
Kim S, Lee YJ & Kim YJ. (2011) Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15°C. *Anim Reprod Sci* 124, 118–124.
Knox RV. (2011) The current value of frozen–thawed boar semen for commercial companies. *Reprod Dom Anim* 46, 4–6.
Kott PS. (2001) The delete-a-group Jackknife. *J Off Stat* 17, 521–526.
Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M *et al.* (2008) MIFlowCyt: the minimum information about a flow cytometry experiment. *Cytometry A* 73A, 926–930. doi:10.1002/CYTO.A.20623.
Meyers SA. (2005) Spermatozoa response to osmotic stress. *Anim Reprod Sci* 89, 57–64.
Nagy S, Jansen J, Topper EK & Gadella BM. (2003) A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol Reprod* 68, 1828–1835.
Petrunkina AM, Waberski D, Bollwein H & Sieme H. (2010) Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. *Theriogenology* 73, 995–1000.
Rath D. (2002) Low dose insemination in the sow – a review. *Reprod Dom Anim* 37, 201–205.
Roberts PK & Bilkei G. (2005) Field experiences on post-cervical artificial insemination in the sow. *Reprod Dom Anim* 40, 489–491.
Roca J, Parrilla I, Rodriguez-Martinez H, Gil MA, Cuello C, Vázquez JM *et al.* (2011) Approaches towards efficient use of boar semen in the pig industry. *Reprod Dom Anim* 46, 79–83.
Silva PFN & Gadella BM. (2006) Detection of damage in mammalian sperm cells. *Theriogenology* 65, 958–978.
Soede NM, Langendijk P & Kemp B. (2011) Reproductive cycles in pigs. *Anim Reprod Sci* 124, 251–258.
Spencer KW, Purdy PH, Blackburn HD, Spiller SF, Stewart TS, Knox RV *et al.* (2010) Effect of number of motile, frozen-thawed boar sperm and number of fixed-time inseminations on fertility in estrous-synchronized gilts. *Anim Reprod Sci* 121, 259–266.
Tuncer PB, Bucak MN, Büyükleblebici S, Sarıözkan S, Yeni D, Eken A *et al.* (2010) The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology* 61, 303–307.
Waberski D, Weitze KF, Gleumes T, Schwarz M, Willmen T & Petzoldt R. (1994) Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* 42, 831–840.
Watson PF & Behan JR. (2002) Intrauterine insemination of sows with reduced sperm numbers: results of a commercially based field trial. *Theriogenology* 57, 1683–1693.
Westendorf P, Richter L & Treu H. (1975) Zur Tiefgefrierung von Ebersperma Labor-und Besamungsergebnisse mit dem Hülsenberger Pailetten-Verfahren [Deep freezing of boar sperm. Laboratory and insemination results using the Hülsenberger paillete method]. *Dtsch Tierärztl Wschr* 82, 261–267.
Yeste M. (2013) Boar Spermatozoa within the Oviductal Environment (I). In: *Boar reproduction: fundamentals and new biotechnological trends* (eds S Bonet, I Casas, WV Holt & M Yeste), pp. 169–280. Springer: Berlin, Germany. ISBN: 978-3-642-35048-1. ISBN (ebook): 978-3-642-35049-8. doi:10.1007/978-3-642-35049-8.
Yeste M, Briz M, Pinart E, Sancho N, García-Gil N, Badia E *et al.* (2008) Boar spermatozoa and prostaglandin F2alfa. Quality of boar sperm after addition of prostaglandin F2alfa to the short-term extender over cooling time. *Anim Reprod Sci* 108, 180–195.
Yeste M, Briz M, Pinart E, Sancho S, Bussalleu E & Bonet S. (2010) The osmotic tolerance of boar spermatozoa and its usefulness as sperm quality parameter. *Anim Reprod Sci* 119, 265–274.
Yeste M, Flores E, Estrada E, Bonet S, Rigau T & Rodríguez-Gil JE. (2012) Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-thawing by stabilizing disulphide bonds. *Reprod Fertil Dev* 25, 1036–1050. doi:10.1071/RD12230.
Yeste M, Estrada E, Casas I, Bonet S & Rodríguez-Gil JE. (2013) Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. *Theriogenology* 79, 929–939.