Title

Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels

Running header

Nuclear cryodamage in GFE and PFE

Authors

Marc Yeste[1,*], Efrén Estrada[1], Isabel Casas[2], Sergi Bonet[3], Joan Enric Rodríguez-Gil[1]

[1]Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra (Barcelona), Spain

[2]Andrology Lab, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center; 382 West Street Road, Kennett Square, PA 19348 USA

[3]Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, E-17071 Girona, Spain

***Corresponding author**

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.

Ph: +34 935 811 959, Fax: +34 935 812 006. E-mail address: marc.yeste@uab.cat

Abstract

The main aim of the present study was to determine whether differences in the amounts of free cysteine residues in sperm nucleoproteins, which are a direct marker of the integrity of the disulfide bonds between nucleoproteins, existed between good (GFE) and poor boar freezability ejaculates (PFE) during the different steps of the freeze-thawing process. The analyzed steps were: a) immediately before starting cryopreservation (17ºC), b) at the end of the cooling step (5ºC), and after c) 30, and d) 240 min post-thawing. In addition, the present study also sought to determine whether GFE and PFE differed in the amounts of peroxides and superoxides generated during freeze-thawing as an overall measure of the boar sperm reactive oxygen species (ROS) accumulation rate. According to our results, PFE present lower resistance than GFE to cryopreservation-induced alterations of disulfide bonds between nucleoproteins, since levels of cysteine free residues were higher in PFE than in GFE after 30 and 240 min post-thawing. On the other hand, no significant differences were observed between GFE and PFE in ROS levels during freeze-thawing. In conclusion, PFE are less resistant than GFE to cryopreservation not only in terms of sperm motility and membrane integrity, but also in the integrity of nucleoprotein structure. However, this difference between PFE and GFE in the resistance of the nucleoprotein structure to freeze-thawing is not linked with concomitant changes in ROS levels.

Keywords: Boar sperm, cryopreservation, freezability, sperm nucleus, ROS

1. Introduction

Sperm cryopreservation is the most efficient method for storing boar spermatozoa for a long period of time [1], even though their fertilizing ability is lower than that of fresh or refrigerated semen (see [2] for a review). In pigs, not all the ejaculates present the same ability to withstand freeze-thawing, but differences in sperm freezability (i.e. the ability of sperm to sustain cryopreservation procedures) have been reported to exist between breeds [3-4], between and within boars [5-7] and even between fractions coming from the same ejaculate [8]. For this reason, boars and their ejaculates have been respectively rated as 'good' or 'bad' freezers [9], and as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) [7].

Although the mechanisms underlying different ejaculate freezability remain to be elucidated, such freezability differences have been related to protein or lipid composition of the sperm membrane [4], to the composition of the seminal plasma and/or to the functionality of the accessory glands [5]. On the other hand, Thurston et al. [10] demonstrated that a consistent and genetically determined variation between boars exists in frozen-thawed sperm quality. Subsequently, Thurston et al. [11] even identified amplified fragment length polymorphism markers associated with 'good' and 'poor' boar freezers. Related to this, Safranski et al. [1] have suggested that direct selection for freezability might be successful since heritabilities for freezability are low to moderate but higher than for fresh semen traits.

One of the most studied changes induced by sperm cryopreservation in mammals concerns generation of reactive oxygen species (ROS) and chromatin damages. Regarding ROS, variations of these substances during boar sperm cryopreservation process are low [12-14], although boar sperm have been suggested to be susceptible to ROS-induced damage during cryopreservation process [15]. In addition, Gómez-

Fernández et al. [16], comparing peroxide levels in ‘good’ and ‘bad’ boar freezers, did not found significant differences between both groups either before starting cryopreservation or after thawing. Thus, the role of ROS levels in boar sperm cryodamaging is an unresolved question, which merits a more in-depth approximation.

Cryopreservation of boar spermatozoa also appears to alter sperm nucleus, since destabilizes nucleoprotein structure by disrupting disulfide bonds and, in a much lower extent, increases DNA fragmentation [14, 17]. Although Hernández et al. [6] studied the differences in terms of chromatin integrity in good and bad freezers immediately after thawing, and found low but significant differences between these two groups, no study has still conducted to evaluate whether GFE and PFE differ in the amounts of disulfide bonds disrupted during the cryopreservation process. In addition, it has been suggested that ROS generation during freeze-thawing can increase DNA fragmentation [6, 17], but no study has hitherto evaluated ROS generation and changes in nucleoprotein structure and chromatin integrity altogether during freeze-thawing, including the cooling step (5ºC).

Against this background, the present study sought to determine whether differences between GFE and PFE exist in terms of sperm chromatin packaging (assessed as levels of free cysteine radicals in sperm nucleoproteins, as a measure of the amounts of disrupted disulfide bonds, and DNA fragmentation) and ROS species, before starting cryopreservation procedure (extended semen at 17ºC), after the cooling step (i.e. when sperm has been diluted in LEY extender and cooled to 5ºC) and 30 and 240 min after thawing. In addition, other sperm functional parameters, such as sperm motility, and membrane and acrosome integrity have also been assessed.

2. Materials and Methods

2.1. Sperm samples

The experimental protocol was designed following 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, Cerdanyola del Vallès; Spain).

Thirty-five ejaculates coming from different healthy and adult boars (ages ranging between eighteen months and three years old) were used in this study. Each ejaculate came from a different boar. Animals were housed in climate-controlled buildings, fed with an adjusted diet (2.3 $Kg \cdot day^{-1}$) consisting of a basal diet and a 1% premix for boars (P174N; TecnoVit, Tarragona, Spain), and provided with water *ad libitum*. Ejaculates were collected twice per week by the gloved-hand technique in a local farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain) with an interval of at least three days between collections. After removing the gelatinous fraction by filtering through gauze, the total volume of the sperm-rich fraction was diluted 1:5 (v:v) in a long-term extender (Duragen®; Magapor S.L.; Zaragoza, Spain). These diluted sperm-rich fractions were transported within four hours after the extraction in an insulated container and stored in our laboratory at 17ºC for 20h. The quality of the sperm samples was then evaluated to check that they satisfied the quality standard (i.e. total sperm motility>80%, progressive sperm motility>60%; morphologically normal spermatozoa>85%; sperm viability>85%; see [14]). Since the quality of these thirty-five ejaculates involved in this study was over the set thresholds, they were frozen according to the experimental design described below.

2.2. Experimental design

In this study, seven parameters (sperm motility, sperm viability, acrosome integrity, levels of peroxides and superoxides, amounts of free cysteine residues in sperm nucleoproteins and sperm chromatin integrity) were examined for each ejaculate, and after the four following cryopreservation steps: 1) before starting the cryopreservation procedure (i.e. at 17ºC); 2) at the end of the cooling step (i.e. after sperm being cooled at 5ºC in LEY extender for 120 min); 3) after 30 min post-thawing (FT 30 min); and 4) after 240 min post-thawing (FT 240 min). For each ejaculate, an aliquot of 20 mL was taken to assess all the mentioned sperm parameters in the first step (before starting the cryopreservation protocol, i.e. at 17ºC). The remaining volume of each ejaculate was cooled to 5ºC for 120 min and an aliquot of 20 mL was then taken to assess the seven mentioned sperm parameters. Finally, the remaining sperm volume was cryopreserved and stored in liquid nitrogen at −196ºC for at least two months, for methodological purposes only. After thawing, samples were incubated 30 min or 240 min at 37ºC, prior to determining all the sperm parameters. These two time points (30 min and 240 min) corresponded to the third and fourth steps of our experimental design, and were chosen to assess the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses [18]. All this experimental design was replicated 35 times, using 35 different ejaculates, and each ejaculate came from a different boar.

Classification of ejaculates as GFE or PFE was only performed when the seven parameters (i.e. sperm motility, sperm viability, acrosome integrity, levels of peroxides and superoxides, amounts of free cysteine radicals in sperm nucleoproteins and sperm chromatin integrity) had been evaluated in all the ejaculates and in all the cryopreservation steps. Then, and on the basis of sperm viability and motility post-thawing assessments, both groups were set and further comparisons were performed to examine whether differences between GFE and PFE existed in terms of sperm motility,

and membrane and acrosome integrity, levels of peroxides and superoxides, amounts of free cysteine radicals in sperm nucleoproteins, and in sperm chromatin integrity throughout the four cryopreservation steps (extended semen (17ºC), cooled semen (5ºC), 30 min after thawing, and 240 min after thawing).

2.3. Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method [19] adapted by Yeste et al. [14]. All the ejaculates were centrifuged at 17ºC and 400 ×*g* for 5 min. Pellets were recovered with 3-4 mL of supernatant and diluted at 1.5×10^9 spermatozoa·mL^{-1} (using a Makler counting chamber; Sefi-Medical Instruments; Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Spermatozoa were then cooled down to 5ºC for 120 min in a programmable freezer (Icecube14S-B; Minitüb Ibérica SL) with a cooling ramp of 0.1 °C·min^{-1}, and an aliquot was taken for the assessment of the seven mentioned sperm parameters. This aliquot corresponded to the second step (i.e. 5ºC as stated in Section 2.2). The remaining volume of LEY solution was subsequently diluted at 1×10^9 spermatozoa·mL^{-1} in a second medium (LEYGO) containing LEY with 6% glycerol and 1.5% Orvus ES Paste (OEP, Equex STM; Nova Chemical Sales Inc.; Scituate, MA, USA). Final concentrations of glycerol and OEP at LEYGO medium were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitüb Ibérica, SL; Tarragona, Spain) and transferred to a programmable freezer (Icecube14S-B; Minitüb Ibérica SL). The freezing program (SY-LAB software; Minitüb Ibérica SL) consisted of 313 sec of cooling at the following rates: −6ºC·min^{-1} from 5ºC to −5ºC (100 sec), −39.82ºC·min^{-1} from −5ºC to −80ºC (113 sec), maintained for 30 sec at −80ºC, and finally cooled at −60ºC·min^{-1} from

−80ºC to −150ºC (70 sec). The straws were then plunged into liquid N_2 (−196 ºC) for further storage.

After being at least two months in liquid N_2, four straws per ejaculate and treatment were thawed and diluted with three volumes of warmed BTS at 37ºC (at a final dilution of 1/4). Each straw was shaken for 20 sec under water bath at 37ºC. After 30 and 240 min of thawing, all the sperm parameters were again assessed, the results corresponding to the third and fourth steps as stated in Section 2.2.

2.4. Determination of sperm-head free-cysteine radicals

The determination of free cysteine radicals in sperm nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. [17]. Briefly, samples were centrifuged at 600 ×*g* and 17°C for 20 min and resuspended in an ice-cold 50mM Tris buffer (pH 7.4) containing 150mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycolate, 1mM benzamidine, 10µg·mL^{-1} leupeptin, 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 1mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator, Ika® Labortechnick; Staufen, Germany). Afterwards, homogenates were 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 40× 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 sperm 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 disulfide; Sigma; Saint Louis, USA) as described by Brocklehurst et al. [20]. Briefly, the 10-µL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 µL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. The mixture was incubated at 37°C for 60 min. Afterwards, levels of free cysteine radicals were 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 method [21], using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad; Hercules, California, USA). Three replicates per sample and cryopreservation step were evaluated, and the corresponding mean ± SEM (standard error of the mean) was subsequently calculated.

2.5. DNA fragmentation

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 [22].

Briefly, the lysing buffer included in the commercial kit was tempered to 22ºC and vials containing low-melting agarose were incubated at 100ºC for 5 min in a water bath.

Vials were then left in another water bath at 37ºC for 5 min to equilibrate the agarose temperature. Twenty-five µL of each sperm sample (at a final concentration of 10^7 spermatozoa·mL^{-1}) 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 4ºC for 5 min. The coverslip was then removed and 50 µL of lysis solution per slide were added. An incubation step at 22ºC 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^{-1}; Molecular Probes®, Oregon, USA) and mounted in DABCO antifading medium (DABCO™ anti-fading medium; Sigma-Aldrich®, St. Louis, MO, USA). 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.

2.6. Flow cytometric analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) [23]. These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), acrosome integrity and ROS levels in GFE and PFE and throughout the four mentioned steps In each case, the sperm concentration in each

treatment was adjusted to 1×10^6 spermatozoa·mL^{-1} 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, California, USA; Serial number AL300087, Technical specification at http://www.beckmancoulter.com). 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 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 has thus 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: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP 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): LP 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), while FL3 was used to detect red (HE and PI) fluorescence.

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 analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particles diameter<7 µm) and cell aggregates (particles diameter >12 µm). Therefore, the sperm-specific events, which usually appeared in a typically L-shape scatter profile, were positively gated on the basis of EV and SS distributions, while 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). These generated files were then analyzed using Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 *vs.* FL3) and to analyze the cytometric histograms. In all cases except for SYBR-14/PI assessment, data obtained from flow cytometry experiments at cooling and post-thawing steps were corrected according to the procedure described by Petrunkina et al. [24]. 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, Oregon, USA) and were diluted with dimethyl sulfoxide (DMSO; Sigma).

2.6.1. 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 and Johnson [25]. Briefly, sperm samples were incubated at 38ºC for 10 min with SYBR-14 at a final concentration of 100nM, and then with PI at a final concentration of 10µM for 5 min and at the same

295 temperature. FL-1 was used for measuring the SYBR-14 fluorescence, while PI
296 fluorescence was detected through FL-3. After this assessment, three sperm populations
297 were identified: i. viable green-stained spermatozoa (SYBR-14^{+}/PI^{-}); ii. non-viable red-
298 stained spermatozoa (SYBR-14^{-}/PI^{+}), and iii. non-viable spermatozoa that were stained
299 both green and red (SYBR-14^{+}/PI^{+}). Non-sperm particles (debris) were found in SYBR-
300 14^{-}/PI^{-} quadrant.
301 Single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and
302 FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI channel
303 (2.45%).
304
305 *2.6.2. Acrosome integrity (PNA-FITC/PI)*
306 Acrosome integrity was assessed by co-staining the spermatozoa with the lectin from
307 *Arachis hypogaea* (peanut agglutinin) conjugated with fluorescein isothiocianate (FITC)
308 and PI, according to the procedure described by Nagy et al. [26]. Briefly, spermatozoa
309 were stained with PNA-FITC (final concentration: 2.5µg·mL^{-1}) and PI (final
310 concentration: 10µM) and incubated at 38ºC for 5 min. PNA-FITC fluorescence was
311 collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa
312 were identified and placed in one of the four following populations: i. viable
313 spermatozoa with intact acrosome (PNA-FITC^{-}/PI^{-}); ii. viable spermatozoa with
314 damaged (exocytosed) acrosome (PNA-FITC^{+}/PI^{-}); iii. non-viable cells with intact
315 acrosome (PNA-FITC^{-}/PI^{+}), and iiii. non-viable cells with damaged acrosome (PNA-
316 FITC^{+}/PI^{+})
317 Unstained and single-stained samples were used for setting the electronic volume (EV)
318 gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC-spill over into
319 the PI channel (2.45%).

2.6.3. Assessment of oxidative stress: peroxides (H_2DCFDA/PI) and superoxides (HE/YO-PRO®-1)

ROS levels were determined through two different oxidation-sensitive fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE), used to analyze the intracellular content of peroxides (H_2O_2) and superoxide anions ($O_2^{-\bullet}$), respectively. Following a procedure modified from Guthrie and Welch [12], a simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa either with PI or with YO-PRO®-1.

In the first case, 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 25ºC 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 [12]. This DCF fluorescence was collected through FL-1, while PI fluorescence was detected through FL-3. Measurements were expressed as the geometric mean of green intensity fluorescence units (GMFI, geometric mean in FL-1) and this was used as the index of ROS generation. Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and data was not compensated.

In the second probe, samples were stained with HE (final concentration: 4µM) and with YO-PRO®-1 (final concentration: 40µM) and incubated at 25ºC for 40 min in the dark [12]. Hydroethidine is freely permeable to cells and it is oxidised 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 expressed as the percentage of viable sperm with high $O_2^{-\bullet}$) (high ethidium fluorescence; E^+) and the geometric

mean of red-intensity fluorescence (geometric mean channel in the FL-3). Data was not compensated.

2.6.4. 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 (as egg yolk), will often show EV/FS and SS characteristics similar to those of spermatozoa and can not thus be excluded via light scatter [24]. 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 10µM and incubated at 38ºC for 3 min, according to the procedure described by Petrunkina et al. [24]. Percentages of alien particles (*f*) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and dual-staining analysis, except in SYBR-14/PI assay (i.e. PNA-FITC/PI, H_2DFCA/PI and HE/YO-PRO®-1), 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.

2.7. Sperm motility

Sperm-motility analysis was performed by utilizing a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system was 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 10x 0.30 PLAN objective lens). 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 msec. 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 were placed in a Makler counting chamber (Sefi-Medical Instruments), and total and progressive motility together with other kinetic parameters were recorded [14]. Total motility was defined as the percentage of spermatozoa that showed a VAP>10 $\mu m \cdot s^{-1}$, whereas progressive motility was defined as the percentage of spermatozoa that showed a VAP>45 $\mu m \cdot s^{-1}$. In the case of extended (17ºC) and cooled (5ºC) sperm, samples were incubated at 37ºC for 15 min before evaluating sperm motility.

2.8. Sperm morphology

As stated, sperm morphology was assessed upon arrival of 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 were placed on a slide and mounted with a cover slip. Slides were then incubated for 30 min in 100% humidity at 25 ºC 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 the Jensen's ring). A phase contrast microscope (Olympus BX41) was used, and the samples observed at 200x magnification (Olympus 20x 0.40 PLAN objective, positive phase-contrast field).

2.9. Statistical analyses

Statistical analyses were performed using IBM SPSS 19.0 for Windows (SPSS Inc.; Chicago, Illinois) and data are presented as mean ± SEM. The data obtained from the analysis of all sperm parameters were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests. The present study was developed with 35 ejaculates from 35 different boars. In all statistical analyses, the minimal level of significance was set at $P<0.05$.

2.9.1. Obtaining GFE and PFE groups

After evaluating the seven mentioned parameters in all the 35 ejaculates and throughout the four cryopreservation steps, GFE and PFE groups were set by running a hierarchical cluster analysis for dissimilarities that used the values obtained for sperm progressive motility (Table 1) and for sperm viability after 30 and 240 min post-thawing, following the procedure described by Casas et al. [7]. This procedure consisted of calculating the chi-squared frequencies from the sperm progressive motility and the sperm viability after 30 and 240 min post-thawing, and constructed a dissimilarity dendrogram as a result.

2.9.2. Comparisons between GFE and PFE during freeze-thawing

After separating the ejaculates in these two groups, the sperm parameters of GFE and PFE were compared with independent sample t-tests for repeated measures, where the inter-subject factor was the freezability ejaculate group (i.e. GFE vs. PFE) and the intra-subject factor was the cryopreservation step (i.e. before starting cooling, at cooling to 5ºC, FT after 30 min and FT after 240 min).

3. Results

3.1. Differences between GFE and PFE in terms of free cysteine radicals in sperm nucleoproteins during freeze-thawing

After evaluating post-thaw sperm motility and viability of the 35 ejaculates, 19 were classified as GFE, while the others 16 belonged to the PFE group. The classification of ejaculates obtained after 30 min post-thawing agreed with the obtained after 240 min post-thawing (data not shown).

Regarding free cysteine radicals in sperm nucleoproteins during cryopreservation, freeze-thawing increased the levels of free cysteine residues in sperm nucleoproteins, which went from 3.02 ± 0.22 nmol·μg^{-1} protein (mean ± SEM) in GFE before freezing to 7.05 ± 0.45 nmol·μg^{-1} protein in GFE after 240 min post-thawing (Fig. 1). This increase was not observed at the end of the cooling step, but only after 30 and 240 min post-thawing. When comparing GFE with PFE, no significant differences were found either before starting the cryopreservation procedure (i.e. extended semen at 17ºC) or at the end of the cooling step (5ºC). In contrast, the levels of free cysteine residues in sperm nucleoproteins were significantly (P<0.001) higher in PFE than in GFE, both after 30 and 240 min post-thawing (Fig. 1).

3.2. Differences in chromatin integrity between GFE and PFE during freeze-thawing

Freeze-thawing increased the percentage of spermatozoa with fragmented DNA after thawing, but GFE and PFE differed in the time point when this increase was observed (Fig. 2). Thus, whereas the percentage of spermatozoa with fragmented DNA in PFE was significantly higher (P<0.05) than that of extended semen (17ºC) after 30 min and 240 post-thawing, sperm DNA fragmentation in GFE group only increased significantly after 240 min post-thawing but not before. In addition, the levels of sperm DNA

fragmentation between GFE and PFE did not significantly differ either at 17ºC or after being cooled to 5ºC for 120 min (Fig. 2). Nevertheless, the percentages of spermatozoa with fragmented DNA in PFE were significantly higher than those observed in GFE, both after 30 (P<0.05) and 240 min (P<0.001) post-thawing.

3.3. Differences in sperm viability between GFE and PFE during freeze-thawing

Sperm viability (% spermatozoa SYBR14^{+}/PI^{-}) dramatically decreased (P<0.01) after thawing (i.e. at FT 30 and FT 240 min) in both GFE and PFE, as Fig. 3 shows (mean ± SEM). Moreover, significant differences (P<0.01) between GFE and PFE were only observed after thawing, but neither before starting cryopreservation (i.e. extended semen at 17ºC) nor at the cooling step (i.e. at 5ºC).

3.4. Differences in sperm motility between GFE and PFE during freeze-thawing

Total sperm motility underwent a significant (P<0.01) decrease after 30 min and 240 min post-thawing in both GFE and PFE (Table 1). Furthermore, PFE presented a significant (P<0.05) lower percentage of total motile spermatozoa than GFE both after 30 min and 240 min post-thawing.

On the other hand, sperm progressive motility (PMOT) gradually decreased after starting the cryopreservation process, as the percentages of progressive motile spermatozoa at the cooling step were significantly (P<0.05) lower than those observed before starting the cryopreservation process, and those observed after 30 and 240 min post-thawing significantly lower (P<0.05) than those found at the cooling step (Table 1). In addition, the percentages of progressive motile spermatozoa were not only significantly (P<0.05) higher in GFE than in PFE after 30 and 240 post-thawing but also at the cooling step.

3.5. Differences in acrosome integrity of spermatozoa between GFE and PFE during freeze-thawing

Similarly to the observed in sperm viability assessment, a significant (P<0.01) decrease in the percentage of acrosome-intact spermatozoa was only observed after thawing but not before (Table 1). On the other hand, percentages of spermatozoa with an intact acrosome in extended (17ºC) and cooled (5ºC) semen did not differ between GFE and PFE. In contrast, percentages of acrosome-intact spermatozoa in GFE were significantly (P<0.05) higher than those observed in PFE after 30 and 240 min post-thawing.

3.6. Differences in peroxide and superoxide levels of spermatozoa between GFE and PFE during freeze-thawing

Table 2 shows the percentage of viable spermatozoa with high peroxide levels (DCF^+/PI^-) and geometric means of fluorescence intensity (GMFI) in FL1 channel in viable (DCF^+/PI^-) and total (DCF^+) spermatozoa. No significant differences (P>0.05) were observed between GFE and PFE in any of the cryopreservation steps.

Table 3 shows the percentage of viable spermatozoa with high superoxide levels (E^+/YO-PRO®-1^-) and geometric means of fluorescence intensity in FL3 channel in viable (E^+/YO-PRO®-1^-) and total (E^+) spermatozoa. The percentage of viable spermatozoa with high superoxide levels (E^+/YO-PRO®-1^-) did not differ among the four cryopreservation steps. In contrast, the geometric means of fluorescence intensity (FL3) in viable spermatozoa with high superoxide levels (E^+/YO-PRO®-1^-) progressively decreased from cooling step (5ºC) to 240 min after thawing. The geometric mean of E^+-fluorescence intensity in total spermatozoa was significantly (P<0.05) higher after 240 min post-thawing than at the first two steps (i.e. extended

semen at 17ºC and cooling step at 5ºC). Despite these changes, no significant differences were found in any of these three parameters between GFE and PFE during the entire cryopreservation procedure (Table 3).

4. Discussion

The results shown in this work clearly indicate that boar sperm cryotolerance is concomitant with the resistance of these cells to sustain alterations in their nucleoprotein structure during freeze-thawing. This is evident when comparing the levels of free cysteine radicals in sperm nucleoproteins between GFE and PFE after thawing. On the contrary, boar sperm resistance to cryopreservation does not appear to be concomitant with differences in the sperm's ability to modulate their ROS intracellular levels, since changes in ROS during freeze-thawing are essentially the same when comparing GFE and PFE. These results could seem a paradox, because previous works have suggested that ROS generation during boar sperm cryopreservation can induce damages in sperm chromatin [6, 17], in a similar fashion to what occurs in other species [27-29]. However, levels of DNA fragmentation in boar spermatozoa after freeze-thawing, as a method to evaluate nuclear cryodamage, are very low [6, 14, 17]. These very low values make difficult to establish a clear relationship between chromatin damage and ROS levels. On the other hand, the disruption of disulfide bonds between nucleoproteins induced by boar sperm cryopreservation is much more intense than the damage inflicted on DNA integrity [14]. For this reason, the disruption of disulfide bonds between boar sperm nucleoproteins can be regarded as a more sensitive parameter to determine nuclear cryodamage rather than DNA fragmentation. In this regard, we must bear in mind that disulfide bonds play a main role in stabilizing the protein conformation and in the union between protamines [30]. When osmotic conditions are greatly modified, as it is the case of boar sperm cryopreservation, disulfide bonds can be weakened and disrupted, thereby causing an irreversible damage to nucleoprotein structure [31].

Our results strongly suggest that ejaculate freezability is related with the integrity of nucleoprotein structure, since the disruption of disulfide bonds between boar sperm nucleoproteins caused by freeze-thawing is better counteracted in GFE than in PFE. Although the mechanism that explains the higher resistance of such disulfide bonds in GFE than in PFE still remains to be elucidated, we suggest that these differences could be explained by a defective spermatogenesis and/or epididymal maturation in the spermatozoa of PFE, since at these steps histones are replaced by protamines and disulfide bonds between and within protamines are formed [32]. On the other hand, another possible explanation would be related with the higher amount of HSP90AA1 and other proteins [33] in GFE than in PFE, which might protect sperm cells from cold and osmotic shocks.

Another cryopreservation-induced damage that affects sperm nucleus is DNA fragmentation. However, as indicated above, the extent of this damage differs among species. Thus, whereas cryopreservation clearly increases sperm DNA fragmentation in some species, like bulls [34] and stallions [29], in others, such as rams [35] and boars [14, 17], this increase is more moderate and does not appear immediately after thawing but only after 2-4 h of post-thawing incubation at 37°C. Interestingly, in the present work a significant cryopreservation-induced increase in sperm DNA fragmentation was observed before in PFE (after 30 min post-thawing) than in GFE (after 240 min post-thawing). These results, together with other studies [14], allow us to hypothesize that the destabilization of nucleoprotein structure, due to the disruption of disulfide bonds observed after freeze-thawing, seems to underlie the subsequent increase of DNA fragmentation in boar spermatozoa. Indeed, the fragility of nucleoprotein structure in the case of PFE, clearly detected in the levels of free cysteine residues after 30 min post-thawing, seems to underlie the chromatin fragmentation observed at the same time

548 point. In contrast, GFE present lower levels of free cysteine radicals than PFE after 30
549 min post-thawing, and the increase in sperm DNA fragmentation in GFE is only
550 observed after 240 min post-thawing. In fact, less inter- and intra-protamine interactions
551 make DNA more susceptible to damage [36], because reduced chromatin packaging
552 leads to lower resistance against strong acids, proteases, DNases and/or detergents [37].
553 In addition, after 240 min post-thawing, there are higher levels of leakage products from
554 very significant and increasing numbers of dead and dying spermatozoa, including
555 nucleases released from spermatozoa with damaged plasma membranes, that can
556 fragment DNA [38]. Moreover, there can also be traces of components of the
557 cryopreservation medium, including glycerol and yolk particles, which can be
558 detrimental for spermatozoa. Thus, we suggest that all these detrimental products may
559 directly damage sperm chromatin and/or accelerate DNA fragmentation upon
560 destabilization of the nucleoprotein structure, especially in the case of PFE that clearly
561 present a lower resistance to freeze-thawing. Finally, it is worth noting that the observed
562 alterations in the nucleoprotein structure of the boar sperm-head might affect the
563 sperm's fertilizing ability, because protamines play a critical role for proper sperm
564 chromatin packaging [30] and protamine-deficient sperm adopt a less-stable chromatin
565 structure [36]. Related to this, ejaculates classified as GFE have been reported to present
566 higher rates of oocyte penetration, cleavage and of blastocyst formation than those
567 classified as PFE [39]. This could also be related with the lower amounts of disrupted
568 disulfide bonds between and within sperm nucleoproteins, and with the lesser degree of
569 chromatin fragmentation in GFE than in PFE observed in our study.
570 Some previous reports have suggested that ROS could be one of the contributors to
571 cryopreservation-induced DNA fragmentation in boar spermatozoa [6, 40]. In fact, in
572 other species like humans, there is a significant positive correlation between DNA

fragmentation and ROS [41]. In the present study, however, we have found significant differences in the integrity of nucleoprotein structure and DNA fragmentation between GFE and PFE, but not in ROS levels. Although the main cause of DNA damage in frozen-thawed spermatozoa is still open for discussion [40], our results do not support the hypothesis that ROS is the main cause of cryopreservation-induced DNA fragmentation in boar sperm. Notwithstanding, basal ROS formation and membrane lipid peroxidation in the absence of ROS generators are quite low in fresh and frozen-thawed boar spermatozoa, in contrast to what happens in other species, like human, cattle, and poultry [42]. Our data also match with another previous study from our group [14], where we observed that even though both ProHCl and GSH protected the nucleoprotein structure of boar sperm during freeze-thawing with a similar extent, only the latter reduced peroxide generation. This indicates that the beneficial action of ProHCl on sperm nucleus is independent from modulating ROS generation, and backs our current observations in GFE and PFE, because both groups differ in the extent of damages in sperm nucleus but not in the ROS levels.

Previous studies have demonstrated that ROS generation and lipid peroxidation occur during cryopreservation in human [43], horse [28], bull [44] and dog [45] spermatozoa. In boar sperm, however, ROS production linked to freeze-thawing procedures is quite low [12-14, 46], and small differences are only observed post-thawing. In our study, freeze-thawing slightly increased the percentage of viable spermatozoa with high levels of H_2O_2, but no significant differences were found when comparing GFE with PFE in any of the freeze-thawing steps, matching Gómez-Fernández et al. [16].

With regard to superoxides, there was a significant reduction in the GMFI in viable spermatozoa (E^+/ YO-PRO-1^-) after cooling (5^o) and after thawing, in agreement with Awda et al. [46] and Kim et al. [13]. However, we did not find significant differences

between GFE and PFE. These data may back Casas et al. [7] who did not find significant differences in Cu/Zn superoxide dismutase content between GFE and PFE. In fact, previous reports have suggested that the endogenous ROS defense system in boar sperm is either very efficient or essentially unchallenged during cryopreservation [47] and that boar spermatozoa has a substantial amount of intracellular superoxide dismutase (SOD) for scavenging $O_2^{-\bullet}$ and for rapidly dismutating $O_2^{-\bullet}$ to H_2O_2 [13]. Another possible explanation for our results is that levels of $O_2^{-\bullet}$ would not be affected by cryopreservation, because $O_2^{-\bullet}$ presents a very short life and is too polar to penetrate intact plasma membranes [27].

As expected, GFE and PFE differed in plasma membrane (sperm viability) and acrosome integrity after freeze-thawing but not before starting cryopreservation (i.e. extended semen at 17ºC) and at the cooling step. Thus, sperm viability and acrosome integrity decreased in both groups after 30 and 240 post-thawing, but the impairment in PFE was higher than in GFE. In terms of sperm motility, significant differences between GFE and PFE were observed in the percentage of progressive motile spermatozoa at the cooling and post-thawing steps. This matches other previous studies that have reported differences in post-thaw sperm motility between breeds [3] and ejaculates [7].

Finally, and as previously mentioned, one of the difficulties of boar sperm preservation is to predict the ejaculate freezability before starting cryopreservation process. Indeed, several times, the variations in semen freezability cannot be detected by a standard spermiogram of the ejaculate before freezing [7, 33]. In this regard, previous reports have found that some motility parameters (such as LIN, STR and PMOT) differ between GFE and PFE at the cooling step (5ºC) and after 240 min post-thawing [7], and that the HSP90AA1-content in sperm can be used as freezability marker before starting

623 cryopreservation [33]. However, and in the light of our results, levels of chromatin
624 fragmentation and number of cysteine free residues in sperm nucleoproteins are not
625 good predictors of ejaculate freezability, since GFE and PFE do not differ in these two
626 parameters neither before starting cryopreservation (i.e. extended semen at 17ºC) nor at
627 the cooling step (5ºC).
628 In conclusion, freeze-thawing of boar spermatozoa impaired sperm motility and
629 membrane and acrosome integrity, destabilizes nucleoprotein structure by disrupting
630 disulfide bonds and increases the levels of DNA fragmentation, although the extent of
631 this damage is higher in PFE than in GFE after thawing, but not at the cooling step. In
632 addition, our results indicate that variations in the sperm nuclear damages in GFE and
633 PFE are not directly related with the generation peroxides and superoxides during
634 cryopreservation.
635

Acknowledgements

This research has been supported by project AGL-2008-01792GAN of the Spanish Ministry of Science and Innovation and by Postdoctoral JdC fellowship (JCI-2010-08620) granted to Marc Yeste.

References

[1] Safranski TJ, Ford JJ, Rohrer GA, Guthrie HD. Plenary contribution to International Conference on Boar Semen Preservation 2011. Genetic selection for freezability and its controversy with selection for performance. Reprod Domest Anim 2011; 46 Suppl 2: 31-34.

[2] Rath D, Bathgate R, Rodríguez-Martínez H, Roca J, Strzezek J, Waberski D. Recent advances in boar semen cryopreservation. Soc Reprod Fertil Suppl 2009; 66: 51-66.

[3] Park CS, Yi YJ. Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. Anim Reprod Sci 2002; 73: 53-61.

[4] Waterhouse KE, Hofmo PO, Tverdal A, Miller Jr RR. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction 2006; 131: 887–894.

[5] Holt WV, Medrano A, Thurston LM, Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 2005; 63: 370-382.

[6] Hernández M, Roca J, Ballester J, Vázquez JM, Martínez EA, Johannisson A, Saravia F, Rodríguez-Martínez H. Differences in SCSA outcome among boars with different sperm freezability. Int J Androl 2006; 29: 583-591.

660 [7] Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, Bonet S. Freezability
661 prediction of boar ejaculates assessed by functional sperm parameters and sperm
662 proteins. Theriogenology 2009; 72: 930-948.
663 [8] Peña FJ, Saravia F, Núñez-Martínez I, Johannisson A, Wallgren M, Rodríguez
664 Martínez H. Do different portions of the boar ejaculate vary in their ability to sustain
665 cryopreservation? Anim Reprod Sci 2006; 93: 101-113.
666 [9] Watson PF. Recent developments and concepts in the cryopreservation of
667 spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev
668 1995; 7: 871-891.
669 [10] Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm
670 subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with
671 variation in boar semen quality following cryopreservation. J Androl 2001; 22: 382-394.
672 [11] Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of
673 amplified restriction fragment length polymorphism markers linked to genes controlling
674 boar sperm viability following cryopreservation. Biol Reprod 2002; 66: 545-554.
675 [12] Guthrie HD, Welch GR. Determination of intracellular reactive oxygen species and
676 high mitochondrial membrane potential in Percoll-treated viable boar sperm using
677 fluorescence-activated flow cytometry. J Anim Sci 2006; 84: 2089-2100.
678 [13] Kim SH, Lee YJ, Kim YJ. Changes in sperm membrane and ROS following
679 cryopreservation of liquid boar semen stored at 15 °C. Anim Reprod Sci 2011; 124:
680 118-124.
681 [14] Yeste M, Flores E, Estrada E, Bonet S, Rigau T, Rodríguez-Gil JE. Reduced
682 glutathione and procaine hydrochloride protect the nucleoprotein structure of boar
683 spermatozoa during freeze-thawing by stabilising disulfide bonds. Reprod Fertil Dev
684 2012; doi: 10.1071/RD12230.

685 [15] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim
686 Reprod Sci 2000; 61: 481-492.
687 [16] Gómez-Fernández J, Gómez-Izquierdo E, Tomás C, Mocé E, de Mercado E Is
688 Sperm Freezability Related to the Post-Thaw Lipid Peroxidation and the Formation of
689 Reactive Oxygen Species in Boars? Reprod Domest Anim 2012; doi: 10.1111/j.1439-
690 0531.2012.02126.x
691 [17] Flores E, Ramió-Lluch L, Bucci D, Fernández-Novell JM, Peña A, Rodríguez-Gil
692 JE. Freezing-thawing induces alterations in histone H1-DNA binding and the breaking
693 of protein-DNA disulfide bonds in boar sperm. Theriogenology 2011; 76: 1450-1464.
694 [18] Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, Bonet S. Fertility after
695 post-cervical artificial insemination with cryopreserved sperm from boar ejaculates of
696 good and poor freezability. Anim Reprod Sci 2010a; 118: 69-76.
697 [19] Westendorf P, Richter L, Treu H. Zur Tiefgefrierung von Ebersperma. Labor-und
698 Besamungsergebnisse mit dem Hülsenberger Pailetten-Verfahren. Dtsch. Tierärztl.
699 Wschr. 1975; 82: 261-267.
700 [20] Brocklehurst K, Stuchbury T, Malthouse JPG. Reactivities of neutral and cationic
701 forms of 2,2=-dipyridyl disulphide towards thiolate anions: detection of differences
702 between the active centres of actinidin, papain and ficin by a three-protonic-state
703 reactivity probe. Biochem J 1979; 183: 233-238.
704 [21] Bradford MM. A rapid and sensitive method for the quantification of microgram
705 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;
706 72: 248-254.
707 [22] Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez AI, Gosálvez J.
708 A new method to analyze boar sperm DNA fragmentation under bright-field or
709 fluoresecence microscopy. Theriogenology 2006; 65: 308-316.

710 [23] Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto
711 M, Goldberg M, Goralczyk EM, Hyun B, Jansen K, Kollmann T, Kong M, Leif R,
712 McWeeney S, Moloshok TD, Moore W, Nolan G, Nolan J, Nikolich-Zugich J, Parrish
713 D, Purcell B, Qian Y, Selvaraj B, Smith C, Tchuvatkina O, Wertheimer A, Wilkinson P,
714 Wilson C, Wood J, Zigon R, Scheuermann RH, Brinkman RR. MIFlowCyt: The
715 minimum information about a flow cytometry experiment. Cytometry A 2008; 73: 926–
716 930.
717 [24] Petrunkina AM, Waberski D, Bollwein H, Sieme H. Identifying non-sperm
718 particles during flow cytometric physiological assessment: a simple approach.
719 Theriogenology 2010; 73: 995-1000.
720 [25] Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-
721 14 and propidium iodide. Biol Reprod 1995; 53: 276-284.
722 [26] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method
723 to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm
724 immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003; 68:
725 1828-1835.
726 [27] Aitken RJ. Free radicals, lipid peroxidation and sperm function. Reprod Fertil Dev
727 1995; 7: 659-668.
728 [28] Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine
729 spermatozoa. Am J Vet Res 2001; 62. 508–515.
730 [29] Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and
731 cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 2003;
732 24: 621-628.
733 [30] Balhorn R. The protamine family of sperm nuclear proteins. Genome Biol 2007;
734 8:227-238.

735 [31] Oganesyan N, Ankoudinova I, Kim SH, Kimb R. Effect of osmotic stress and heat
736 shock in recombinant protein overexpression and crystallization. Prot Express Purif
737 2007; 52: 280-285.
738 [32] Calvin HI, Bedford JM. Formation of disulphide bonds in the nucleus and
739 accessory structures of mammalian spermatozoa during maturation in the epididymis. J
740 Reprod Fertil Suppl 1971; 13: 65-75.
741 [33] Casas I, Sancho S, Ballester J, Briz M, Pinart E, Bussalleu E, Yeste M, Fàbrega A,
742 Rodríguez-Gil JE, Bonet S. The HSP90AA1 sperm content and the prediction of the
743 boar ejaculate freezability. Theriogenology 2010b; 74: 940-950.
744 [34] Hallap T, Nagy S, Håård M, Jaakma U, Johannisson A, Rodríguez-Martínez H.
745 Sperm chromatin stability in frozen-thawed semen is maintained over age in AI bulls.
746 Theriogenology 2005; 63: 1752-1763.
747 [35] López-Fernández C, Johnston SD, Fernández JL, Wilson RJ, Gosálvez J.
748 Fragmentation dynamics of frozen-thawed ram sperm DNA is modulated by sperm
749 concentration. Theriogenology 2010; 74; 1362-1370.
750 [36] Nasr-Esfahani MH, Razavi S, Mozdarani H, Mardani M, Azvagi H. Relationship
751 between protamine deficiency with fertilization rate and incidence of sperm premature
752 chromosomal condensation post-ICSI. Andrologia 2004; 36: 95-100.
753 [37] Chapman JC, Michael SD. Proposed mechanism for sperm chromatin
754 condensation/decondensation in the male rat. Reprod Biol Endocrinol 2003; 1: 20.
755 [38] Ward MA, Ward WS. A model for the function of sperm DNA degradation.
756 Reprod Fertil Dev 2004; 16: 547-554.
757 [39] Gil MA, Roca J, Cremades T, Hernández M, Vázquez JM, Rodríguez-Martínez H,
758 Martínez EA. Does multivariate analysis of post-thaw sperm characteristics accurately

759 estimate in vitro fertility of boar individual ejaculates? Theriogenology 2005; 64 : 305-
760 316
761 [40] Fraser L, Parda A, Filipowicz K, Strzeżek J. Comparison of post-thaw DNA
762 integrity of boar spermatozoa assessed with the neutral comet assay and Sperm-Sus
763 Halomax test kit. Reprod. Domest. Anim. 2010; 45: 155-160.
764 [41] Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential
765 cause for DNA fragmentation in human spermatozoa. Hum Reprod 1998; 13: 896-900.
766 [42] Guthrie HD, Welch GR. Effects of reactive oxygen species on sperm function.
767 Theriogenology 2012; 78: 1700-1708.
768 [43] Álvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss
769 of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm
770 during cryopreservation. J Androl 1992; 13: 232-241.
771 [44] Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses
772 are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod
773 Dev 2000; 55: 282–288.
774 [45] Kim SH, Yu DH, Kim YJ. Effects of cryopreservation on phosphatidylserine
775 translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm.
776 Theriogenology 2010; 73: 282–292.
777 [46] Awda BJ, Mackenzie-Bell M, Buhr MM. Reactive oxygen species and boar sperm
778 function. Biol Reprod 2009; 81, 553-561.
779 [47] Guthrie HD, Welch GR, Long JA. Mitochondrial function and reactive oxygen
780 species action in relation to boar motility. Theriogenology 2008; 70: 1209-1215.
781

782 FIGURE LEGENDS

783 **Figure 1** Free cysteine radicals in sperm head proteins (as an indicator of disrupted
784 disulfide bonds) in GFE and PFE during freeze-thawing. Different superscripts (*a*-*e*)
785 mean significant differences ($P<0.05$) between ejaculate groups (GFE *vs.* PFE) and
786 cryopreservation steps (i.e. extended at 17ºC, cooled at 5ºC, FT 30 min and FT 240
787 min).

788

789 **Figure 2** Percentage of spermatozoa with fragmented DNA in GFE and PFE during
790 freeze-thawing. Different superscripts (*a*-*d*) mean significant differences ($P<0.05$)
791 between ejaculate groups (GFE *vs.* PFE) and cryopreservation steps (i.e. extended at
792 17ºC, cooled at 5ºC, FT 30 min and FT 240 min).

793

794 **Figure 3** Percentage of viable spermatozoa ($SYBR14^+/PI^-$) in GFE and PFE during
795 freeze-thawing. Different superscripts (*a*-*d*) mean significant differences ($P<0.05$)
796 between ejaculate groups (GFE *vs.* PFE) and cryopreservation steps (i.e. extended at
797 17ºC, cooled at 5ºC, FT 30 min and FT 240 min).

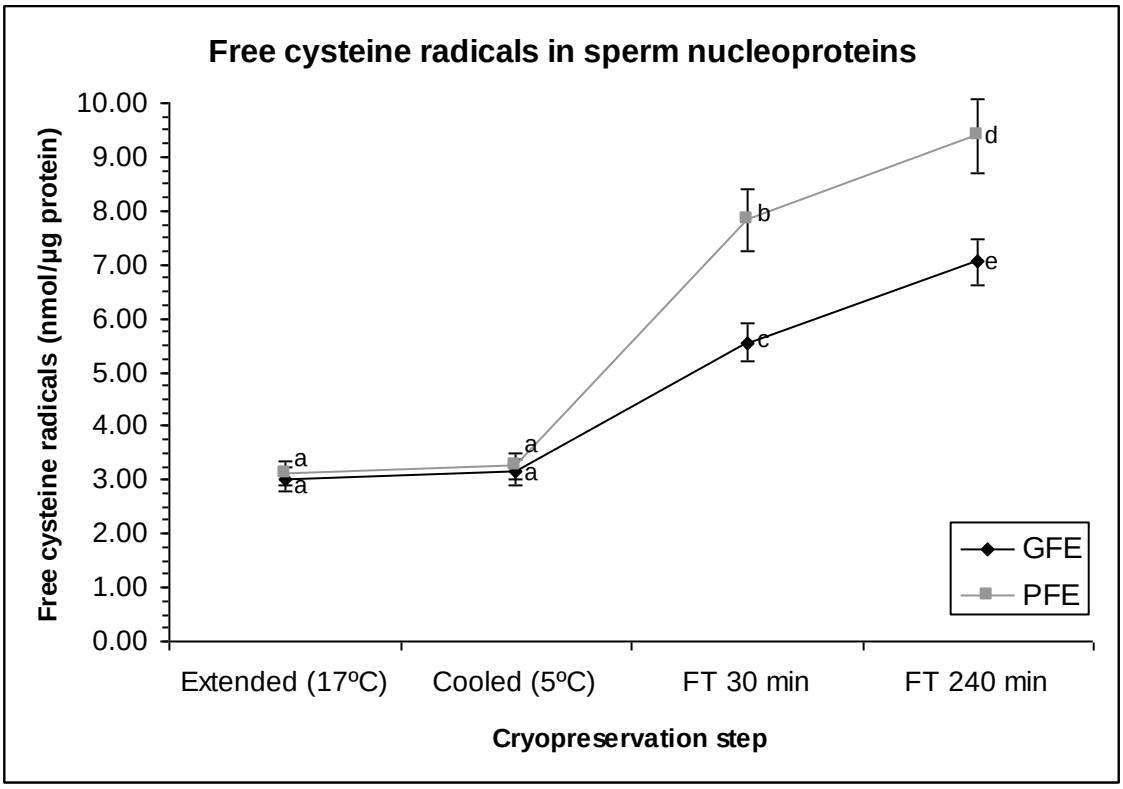
798

TABLE LEGENDS

Table 1 Percentages of acrosome-intact spermatozoa (PNA^-/PI^-) and total (TMOT) and progressive motile (PMOT) spermatozoa during freeze-thawing. Different superscripts (*a-f*) mean significant differences ($P<0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e. extended at 17ºC, cooled at 5ºC, FT 30 min and FT 240 min) within the same parameter (% Spermatozoa PNA^-/PI^-, % TMOT, % PMOT).

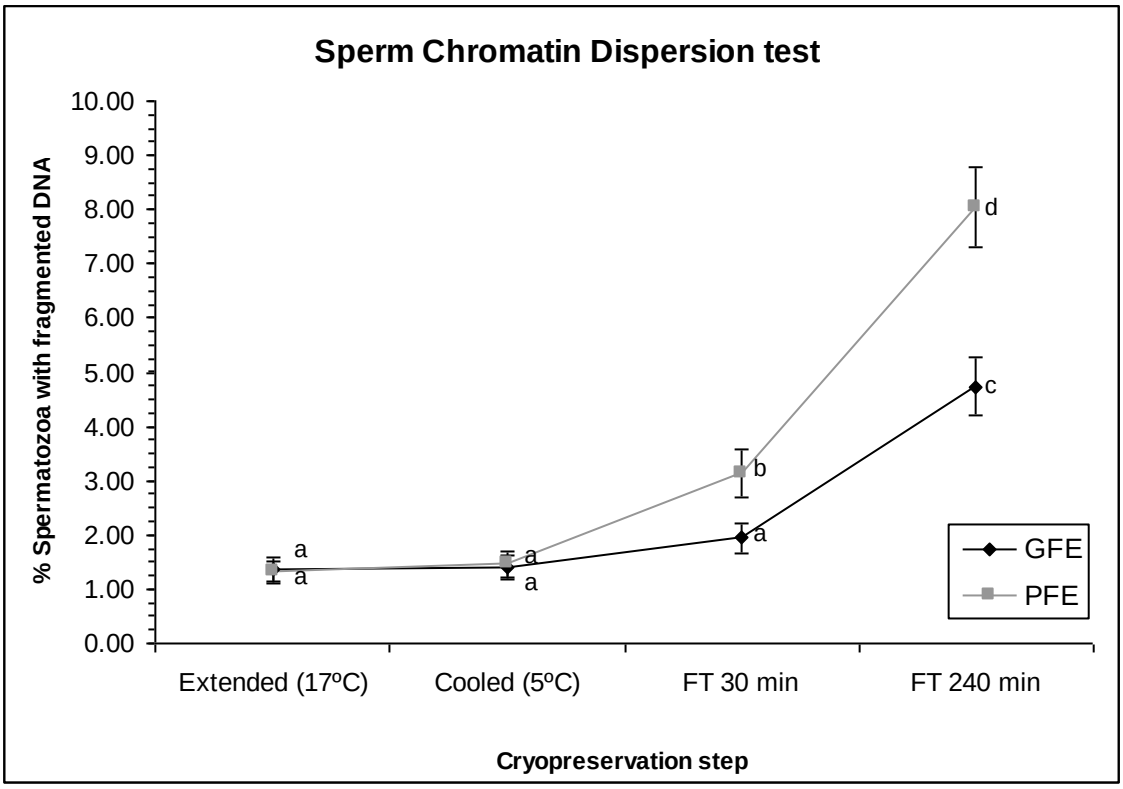
Table 2 Peroxide (H_2O_2) levels in boar spermatozoa during freeze-thawing. Different superscripts (*a, b* and *c*) mean significant differences ($P<0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e. extended at 17ºC, cooled at 5ºC, FT 30 min and FT 240 min) within the same parameter, i.e. % Spermatozoa DCF^+/PI^-, GMFI (FL1) DCF^+/PI^- (Viable spermatozoa with high H_2O_2, and GMFI (FL1) DCF^+ (Total spermatozoa). GMFI: Geometric mean of fluorescence intensity (arbitrary units).

Table 3 Superoxide ($O_2^{-\bullet}$) levels in boar spermatozoa during freeze-thawing. Different superscripts (*a, b* and *c*) mean significant differences ($P<0.05$) between ejaculate groups (GFE vs. PFE) and cryopreservation steps (i.e. extended at 17ºC, cooled at 5ºC, FT 30 min and FT 240 min) within the same parameter, i.e. % Spermatozoa E^+/YO-PRO-1^-; GMFI (FL3) E^+/YO-PRO-1^- (Viable spermatozoa with high $O_2^{-\bullet}$; GMFI (FL3) E^+ (total spermatozoa). GMFI: Geometric mean of fluorescence intensity (arbitrary units).



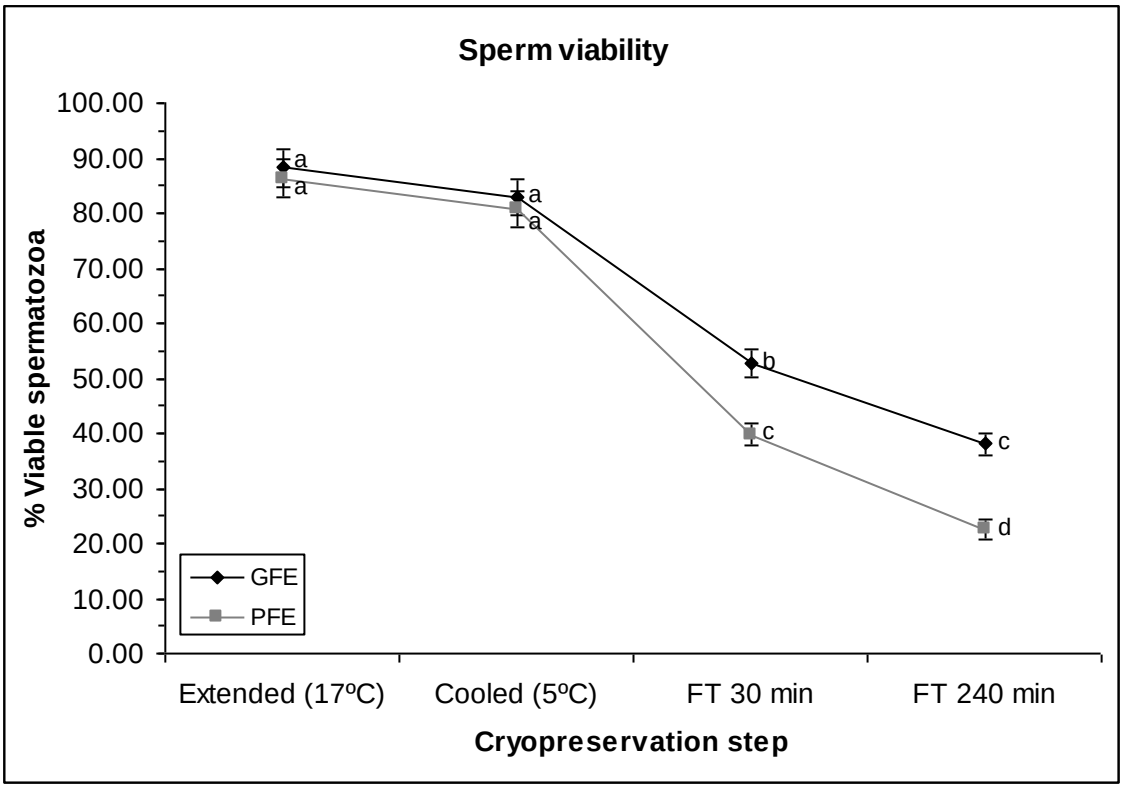
820 **Figure 1**

821



822 **Figure 2**

823



824 **Figure 3**

825

		Extended (17ºC)	Cooled (5ºC)	FT 30 min	FT 240 min
% Spermatozoa PNA^{-}/PI^{-}	GFE	87.5 ± 3.4^{a}	81.8 ± 3.2^{a}	50.9 ± 2.2^{b}	35.1 ± 1.6^{c}
	PFE	85.4 ± 3.5^{a}	80.0 ± 3.1^{a}	36.2 ± 1.8^{c}	18.7 ± 0.9^{d}
% Total sperm motility	GFE	89.3 ± 4.1^{a}	84.1 ± 3.8^{a}	62.5 ± 2.9^{b}	42.8 ± 2.0^{c}
	PFE	87.5 ± 3.9^{a}	81.7 ± 3.8^{a}	45.2 ± 2.1^{c}	27.6 ± 1.3^{d}
% PMOT	GFE	68.5 ± 3.1^{a}	59.7 ± 2.9^{b}	38.2 ± 1.7^{d}	25.6 ± 1.2^{e}
	PFE	67.9 ± 3.0^{a}	50.6 ± 2.4^{c}	27.8 ± 1.3^{e}	12.1 ± 0.6^{f}

Table 1

826
827

828

		Extended (17ºC)	Cooled (5ºC)	FT 30 min	FT 240 min
% Spermatozoa	GFE	2.5 ± 0.2[a]	2.3 ± 0.2[a]	3.9 ± 0.3[b]	1.5 ± 0.1[c]
DCF⁺/PI⁻	PFE	2.6 ± 0.2[a]	2.3 ± 0.2[a]	3.4 ± 0.3[b]	1.4 ± 0.1[c]
GMFI (FL1)	GFE	89.4 ± 4.7[a]	92.7 ± 5.1[a]	110.3 ± 5.9[b]	49.2 ± 2.8[c]
DCF⁺/PI⁻	PFE	90.5 ± 4.9[a]	94.8 ± 5.3[a]	108.4 ± 5.6[b]	51.8 ± 3.0[c]
GMFI (FL1)	GFE	85.1 ± 4.4[a]	86.8 ± 4.9[a]	102.2 ± 5.4[b]	52.9 ± 3.1[c]
DCF⁺	PFE	83.7 ± 4.3[a]	89.4 ± 4.8[a]	97.5 ± 5.2[b]	47.1 ± 2.5[c]

Table 2

829
830

831

		Extended (17ºC)	Cooled (5ºC)	FT 30 min	FT 240 min
% Spermatozoa	GFE	3.4 ± 0.3^{a}	2.9 ± 0.2^{a}	3.3 ± 0.3^{a}	3.5 ± 0.3^{a}
E^{+}/YO-PRO-1^{-}	PFE	3.5 ± 0.3^{a}	3.1 ± 0.2^{a}	3.5 ± 0.3^{a}	3.7 ± 0.3^{a}
GMFI (FL3) E^{+}/	GFE	106.9 ± 6.0^{a}	87.8 ± 4.9^{b}	69.4 ± 3.8^{c}	71.5 ± 4.2^{c}
YO-PRO-1^{-}	PFE	110.7 ± 6.3^{a}	90.1 ± 5.1^{b}	73.2 ± 4.5^{c}	76.4 ± 4.3^{c}
GMFI (FL3) E^{+}	GFE	125.6 ± 7.3^{ab}	121.5 ± 7.1^{a}	134.1 ± 7.7^{ab}	136.2 ± 7.5^{b}
	PFE	130.4 ± 7.4^{ab}	124.8 ± 7.3^{a}	140.7 ± 7.9^{b}	143.9 ± 8.0^{b}

Table 3

832
833