The effect of oxidative stress on thawed bulk sorted red deer sperm.

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Abstract

The aims of this study were to assess the effects of the sex sorting process on post-thaw sperm quality as well as on induced oxidative stress damage (H_2O_2 0 mM = H000; H_2O_2 50 mM = H050; H_2O_2 100 mM = H100) and the protective action of reduced glutathione (GSH) and Trolox, when comparing sorted (BSS) and non-sorted (NS) red deer spermatozoa incubated at 37 ºC. Sperm samples from 3 stags were collected by electroejaculation and frozen. Immediately after thawing, sperm motility was higher ($p < 0.05$) for NS (59%±3.3) than BSS (36.9%±5.8) sperm. Moreover, the percentage of apoptotic sperm was higher ($p < 0.05$) for BSS (21.6%±5.0) than NS sperm (14.6%±1.2). The presence of H_2O_2 increased DNA damage in NS (H000=4.1%±0.9; H050=9.3%±0.7; and H100=10.9%±2.3), but not BSS sperm. However, in the presence of oxidant, GSH addition improved ($p<0.05$) sperm motility in both groups of sperm samples as compared to their controls (NS: 44.5±4.8 *vs.* 21.1±3.9 and BSS 33.3±8.1 *vs.* 8.9±1.8). These results demonstrate that the sperm sorting process induces sublethal effects, albeit selecting a sperm population with a chromatin more resistant to oxidative stress than that in non-sorted sperm. Moreover, addition of GSH at 1 mM may be a good choice for maintaining the quality of stressed sperm samples, unlike Trolox, which inhibited sperm motility.

Keywords: Red deer, sex-sorting, cryopreservation, oxidative stress, antioxidant, reducted glutathione, trolox.

1. Introduction

Pre-selection of sperm based on the relative difference in DNA content between X- and Y- chromosomes has become one of the most important reproductive technologies to improve livestock production (Evans et al. 2004; Garner 2006). Sex sorting by flow cytometry, is an established method that has been introduced into commercial cattle production (Garner and Seidel Jr. 2008). The use of this technology can help farmers obtain an optimal offspring sex ratio in the animal production system of their interest with the advantages that this entails. This becomes especially interesting in the production of red deer for hunting given that only males are considered a trophy with the corresponding economic value. However, studies carried out to date to assess the effect of the sex sorting process in sperm from this species are limited. Similarly, few studies have addressed the potential beneficial effects of antioxidant use upon sorted sperm sample quality.

Previous studies (Gosálvez et al. 2011) demonstrated a decrease in the proportion of DNA-damaged sperm following sex selection, likely stemming from the removal of non-viable and non-oriented sperm during the sorting process. In addition, mammalian sperm with flattened, oval heads tended to be more readily oriented in a sperm sorter using hydrodynamics than those possessing more rounded or angular shaped heads (Garner 2006). Similarly, Dean et al. (1978) discussed that morphologically abnormal sperm could not align properly in the flow stream, whereas Sun et al. (1998) showed that poor-quality sperm samples displayed an increased level of DNA fragmentation.

In contrast, during sex sorting sperm are also exposed to some stressors such as incubation with the fluorescent dye Hoechst 33342 at 36ºC, high dilution ratio, mechanical processing, and subsequent passage through the electric field during sorting.

All of these stressors may cause oxidative stress in the sperm samples. In addition, sex-sorted sperm are often cryopreserved for logistic reasons. Freeze-thaw damage has been reported to increase the sperm susceptibility to reactive oxygen species ROS in other species such as stallion (Ball et al. 2001) or bull (Chatterjee and Gagnon 2001). The most common ROS generated by sperm are superoxide anion (O-2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-), with H_2O_2 being the most toxic ROS for sperm given its ability to penetrate biological membranes (Aitken 1995). Oxidative stress in sperm results in a loss of motility, membrane integrity and/or fertilizing ability (Aitken 1995; Aitken and Baker 2004; Domínguez-Rebolledo et al. 2011). In this context, antioxidants may help prevent oxidative stress-driven damage. Antioxidants play an important role in maintaining the motility and the DNA integrity of sperm against oxidative stress and damage (Hughes et al. 1998). Extenders can be supplemented with antioxidants, before freezing (Peña et al. 2004; Roca et al. 2005; Fernández-Santos et al. 2007; Anel-López et al. 2012) or immediately after thawing (Fernández-Santos et al. 2009; Domínguez-Rebolledo et al. 2010), which scavenge the excess of ROS.

One of the antioxidants widely used has been reduced glutathione (GSH). GSH is a tripeptid ubiquitously distributed in living cells. It plays an important role in protecting cells from the noxious effects of oxidative stress, directly and as a cofactor of glutathione peroxidase (Atmaca 2004). This enzyme uses GSH to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The addition of GSH to cryopreservation extenders has yielded variable results in several species (Câmara et al. 2011; Anel-López et al. 2012). Supplementing GSH to epididymal red deer sperm before freezing (Anel-López et al. 2012) or to electroejaculated red deer sperm after thawing (Anel-López et al. 2015) increased the sperm quality in this species, proving to be a high value additive.

As per other antioxidant agents, Trolox is an analogue of vitamin E with high capacity to capture free radicals (Mickle and Weisel 1993), and is usually used as the standard to check the antioxidant capacity of others molecules (Lipovac 2000; Ronald et al. 2005). Extender supplementation with Trolox was shown to improve sperm motility and mitochondrial membrane integrity during post-thaw incubation in ejaculated boar sperm (Peña et al. 2003). Furthermore, we previously demonstrated that Trolox reduced intracellular reactive oxygen species and lipid peroxidation, and preserved membrane integrity during post-thaw incubation of red deer epididymal sperm, whether exposed or not to induced oxidative stress (Martínez-Pastor et al. 2008; Martínez-Pastor et al. 2009). Similarly, Trolox protected motility and viability and abolished DNA damage in red deer sperm samples submitted to oxidative stress following thawing and washing (Domínguez-Rebolledo et al. 2009). In contrast, when Trolox was added in the millimolar range concentration to post-thaw electroejaculated red deer sperm samples it exerted a negative effect on motility (Anel-López et al. 2015).

The aims of this study were to assess the effect of sex sorting process on post-thaw sperm quality, as well as the effect of induced oxidative stress on sorted vs. non-sorted sperm. We also aimed to determine if the antioxidants GSH and Trolox may provide protection against damage induced by the most common reactive oxygen species. The results of this study may provide new avenues for improving biotechnology of reproduction in red deer.

2. Materials & Methods

2.1. Reagents and media

The fluorescence probe YO-PRO-1 and Hoeschst 33342 were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) was acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Stock solutions of the fluorescence probes were: PI: 1.5 mM; YO-PRO-1: 50 μM. YO-PRO-1 stock was prepared in DMSO. PI and Hoeschst 33342 were prepared in water. PI and YO-PRO-1 were stored at -20 ºC and Hoeschst 33342 was stored at 5 ºC, all of them in the dark. The stock solution of acridine orange was prepared in water at 1 mg/mL and kept in the dark at 5ºC. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA) or Becton Dickinson (San Jose, CA, USA). Stock solutions of the antioxidants (Sigma-Aldrich®) were prepared at 100 mM in DMSO (Trolox) or in water (reduced glutathione, GSH) and stored at -20 ºC.

The collecting medium during sorting was a Tris-Citrate-Glucose (TCG) (pH: 7.3 and pOsm: 380 mOsm/kg) containing: glucose (250 mM), sodium citrate (12 mM), EDTA (1.6 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk (EY) at 5% (V/V) penicillin (0.7 mM), and streptomycin (1.14 mM). The washing medium of ejaculates consists in the same extender for transport with EY at 2.5% (V/V). The transport medium was a Tris-Citrate-Fructose (TCF) (pH: 7.3 and pOsm: 330 mOsm/kg), containing: Tris (213 mM), citric acid monohydrate (71.83 mM), fructose (55.51 mM), EY at 20% (V/V), penicillin (0.7 mM), and streptomycin (1.14 mM). The working medium for cytometry assessment was the bovine gamete medium (BGM-3), composed of: NaCl (87 mM), KCl (3.1 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.4 mM), NaH_2PO_4 (0.3

146 mM), HEPES (40mM), sodium lactate (21.6 mM), sodium pyruvate (1 mM),
147 kanamycin (0.017 mM), phenol red (28.22 mM) and 6mg/mL BSA (Bovine serum
148 albumin) (pH 7.5). Solutions for SCSA® (Sperm Chromatin Structure Assay) were
149 prepared following Evenson and Jost (2000): TNE buffer Tris-HCl (0.01 M), NaCl
150 (0.15 M), EDTA (1 mM), pH 7.4), acid-detergent solution (Triton X-100 (0.17%), NaCl
151 (0.15 M), HCl (0.08 N), pH 1.4) and acridine orange solution (citric acid (0.1 M),
152 Na2HPO4 (0.2 M), EDTA (1 mM), NaCl (0.15 M), pH 6.0; acridine orange was added
153 from the stock up to 6 μg/mL). These solutions were kept at 5 ºC in the dark.
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155 **2.2. Stags, ejaculate collection and sperm sample preparation**
156 One ejaculate was obtained from each of 3 mature stags during the breeding season
157 (mid-September). Animals were housed in a semi-free ranging regime at Las Lomas
158 Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were
159 performed in accordance with Spanish Animal Protection Regulation RD53/2013 which
160 conforms to European Union Regulation 2010/63/UE. Electroejaculation procedure was
161 carried out as described Martínez et al. (2008). Semen was collected by fractions in
162 graduated glass tubes. Sperm concentration was assessed using a hemocytometer
163 (Bürker chamber; Brand Gmbh, Germany), after diluting the sample in a glutaraldehyde
164 solution (5 mL of sample in 500 mL of 2% glutaraldehyde solution—29 g/L glucose
165 monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate).
166 We discarded the fractions with urine contamination, as assessed by positive Urea Test
167 Strips (Diagnostic Systems GmbH, Holzheim, Germany). Samples with total motility
168 below 80 % were discarded.
169 Semen was diluted 1:3 in TCG 2.5% and then centrifuged at 600 xg 5 min. The
170 supernatant was removed and then the concentration of the pellet was calculated. Once

concentration was determined sperm aliquots were individually diluted to a concentration of 800 x 10^6 sperm/mL in TCF medium supplemented with 20% (v/v) and transported to the sorting facilities (about 8h at 17ºC). Upon arrival to the laboratory sperm samples were split into two aliquots. One of these aliquots was used to perform the Control (NS) and the other one was used for sperm sorting (bulk sorted samples; BSS). Sperm samples for sorting were re-diluted to 100 x 10^6 sperm/mL with TCG (0% EY) medium and stained with 2.6 µL of Hoeschst33342 (H-42) (Stock solution: 25 mg/mL) during 50 min at 34ºC as previously described by Parrilla et al. (2012).

2.3. Flow cytometric sperm sex sorting

Just prior to flow sorting, stained sperm samples were filtered through a 30 µm nylon mesh filter and 1µL of food color dye (0.002% w/v; FD&C #40, Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to each sample to quench the fluorescence of H-42 (food dye) in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Then, X and Y-chromosome-bearing sperm were separated (bulk sorting) according to the Beltsville Sperm Sorting Technology method (Johnson and Welch 1999) using a high-speed cell sorter (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) modified for sperm sorting. The cell sorter was operated at 40 psi and was equipped with a UV-laser set at an output of 175 mW (Spectra Physics 1330, Terra Bella Avenue, Mountain View, California). Samples were sorted in the presence of HEPES-buffer based sheath fluid (Buss, 2005) supplemented with 0.1% of EDTA (w/v) and collected in 50-mL tubes prefilled with 2.5 mL of TCG medium containing 5% (v/v) of EY. A total of 20 x 10^6 bulk-sorted sperm were collected per tube in an approximate volume of 25 mL.

196 **2.4 Sperm cryopreservation**

197 Sorted sperm were centrifuged at 3000 xg for 4 min at 21ºC. The supernatant was
198 discarded, and the pellets were re-extended to 20 x 10^6 sperm/mL using Triladyl®
199 (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of EY. Then sperm
200 samples were immersed in a programmable temperature-controlled water bath
201 (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21
202 to 5ºC over 90 min, and left for an equilibration time of 2 h. Following, sperm were
203 packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen
204 vapor (4 cm above liquid nitrogen) for 10 min, and then plunged into liquid nitrogen
205 prior to storage. After 1 year of storage, two straws from each group were thawed in a
206 circulating water bath at 37ºC for 30 s.

207 A control group consisting of non-sorted (NS) sperm frozen under the same
208 conditions as the sorted sperm was included in this experiment. Therefore, control
209 sperm aliquots were gradually diluted to 1 x 10^6 sperm/mL using HEPES-buffer based
210 sheath fluid in the presence of collection medium, mimicking the conditions at which
211 sorted sperm are exposed. After dilution, sperm samples were stored at flow cytometer
212 room temperature (21–22°C) for approximately 4 h before being processed for freezing
213 together with the sorted samples. Storage and thawing was performed as described for
214 sorted samples.

215

216 **2.5. Experimental design**

217 Three post-thaw replicates from the same ejaculate per stag were used for these
218 experiments. Seven straws per stag, replicate and sperm type (NS or BSS) were thawed
219 by dropping them into a water bath with saline solution at 37ºC for 30 s. Then, straws of
220 each sperm type (NS or BSS) and replicate where pooled and evaluated. Experiment 1

consisted of the assessment of the effect of sex sorting on sperm quality characteristics immediately after thawing (0h) and following 2 h of incubation at 37ºC (2h). Experiment 2 consisted on the assessment of the susceptibility of NS and BSS sperm to oxidative stress. Immediately after thawing, each group (NS and BSS) was divided in three aliquots and then supplemented with H_2O_2 to a final concentration of 50 µM and 100 µM (H050 and H100); a sample was left without H_2O_2 as a control (H000). Then, the samples were incubated 2 h at 37 ºC and evaluated. Experiment 3 consisted on the determination of any benefits from GSH or Trolox addition to a final concentration of 1 and 2 mM each, in both post-thaw BSS and NS sperm samples challenged with oxidative stress. After thawing, each H_2O_2 group (H000, H050 and H100) was divided into 5 aliquots and then they were supplemented with GSH or Trolox, respectively, each to a final concentration of 1 or 2 mM. An aliquot with no antioxidant addition was used as a control. Then, the samples were submitted to an incubation of 2 h at 37 ºC and evaluated.

2.6. Motility analysis by CASA

Motility characteristics were objectively evaluated in all sperm samples after thawing and following 2 h of incubation at 37ºC using a CASA system. Samples were loaded into a Makler counting chamber (10 µm depth) at 37ºC. The CASA system consisted of a triocular optical phase contrast microscope (Eclipse E400; Nikon, Tokyo, Japan), equipped with a warming stage at 37ºC and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analysed using the ISAS software v. 1.2 (Proiser, Valencia, Spain). Sampling was carried out using a 10X negative phase contrast objective (no intermediate magnification). Image sequences were saved and

analysed afterwards. The standard parameter settings were: 50 frames/s; 20 to 90 μm^2 for head area; and, VCL > 10 μm/s, in order to classify a spermatozoon as motile.

2.7. Flow cytometry analyses: evaluation of sperm viability and apoptotic markers

Several physiological traits were assessed using fluorescent probes and flow cytometry, as previously described for red deer (Anel-López et al. 2012). Samples were diluted to 10^6 sperm/mL in BGM-3, and stained using the fluorophore combinations PI/YO-PRO-1 to assess membrane permeability and viability (PI at 6 μM, YO-PRO-1 at 0.1 μM). In all cases, Hoechst 33342 was added at 5 mM, in order to discriminate debris. Sperm stained in these two solutions were incubated for 10 min in the dark before being analysed by flow cytometry. The sperm populations described in this study were: PI negative (viable) and PI negative\YO-Pro-1 positive (apoptotic).

Flow cytometry analyses were carried out with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA), with semiconductor lasers emitting at 405 nm (violet; Hoechst 33342), 488 nm (blue; YO-PRO-1 and PI). Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342, 530/40 (green) for YO-PRO-1 and 613/20 (red) for PI. The system and event analyses were controlled using the Summit software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 sperm were recorded, saving the data in flow cytometry standard (FCS) v. 3 files. The analysis of the flow cytometry data was carried out using WEASEL v. 3 (WEHI, Melbourne, Australia). The YO-PRO-1/PI/ Hoeschst33342 combination was analysed as previously described for red deer (Anel-López et al. 2012).

2.8. Sperm chromatin structure assay

Chromatin stability was assessed following the SCSA® (Sperm Chromatin Structure Assay), based on the susceptibility of sperm DNA to acid-induced denaturation in situ and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson et al. 2002).

For the analysis of SCSA, we used a FACScalibur flow cytometer (Becton Dickinson) and the acquisition software CellQuest v. 3. At the beginning of each session, a standard sperm sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence versus total intensity of the fluorescence (red/ [red+green] ×100), called DFI (DNA fragmentation index; formerly called αt) for each sperm, representing the shift from green to red fluorescence. High values of DFI indicate chromatin abnormalities. Flow cytometry data was processed to obtain %DFI (% of sperm with DFI>25).

2.9. Statistical analysis

Data were analysed using the SAS™ V.9.1. package (SAS Institute Inc., Cary, NC, USA). Results are shown as mean ± standard error of the mean. Data analysis was carried out using linear mixed-effects models (MIXED procedure, ML method), including sample (non-sorted vs. sorted), incubation time after thawing (0 vs. 2 h), H_2O_2 concentration (0, 50 and 100 µM) and type and concentration of antioxidant (GSH and Trolox at 1 and 2 mM) as fixed factors, and the replicate (pool of sperm samples) as the random effect. Significant fixed effects were further analysed using pairwise comparisons of means with the Tukey test. A significance level of $p < 0.05$ was used.

3. Results

Experiment 1: effect of sex sorting process on red deer sperm immediately after thawing and after 2 hours of incubation at 37ºC.

Immediately after thawing (0h), the motility of sperm samples differed ($p < 0.05$) with higher values for NS sperm (59%±3.3) than BSS sperm (36.9%±5.8) (Table 1). In addition, there was a higher percentage of apoptotic sperm ($p < 0.05$) for BSS (21.6%±5) than NS (14.6%±1.2) samples (Table 1). However, viability and %DFI did not differ at this time point (0h) (Table 1). Following incubation (2h), there was no decrease in motility for either NS or for BSS sperm. Conversely, viability decreased ($p < 0.05$) and the %DFI increased ($p < 0.05$) over time for both sperm groups. However, viability and %DFI did not differ between both sperm sample types following incubation (2h) (Table 1).

Experiment 2: Susceptibility of non-sorted and sorted sperm to oxidative stress.

In the presence of H_2O_2, motility was clearly affected, showing a significant decrease for both sperm sample groups (NS and BSS) (Figure 1a). In contrast, 100 µM H_2O_2 exerted a positive effect ($p < 0.05$) on NS sperm viability when compared to sperm incubated in the absence of H_2O_2 (41.7%±2.4 vs. 31.6%±1.5). In the presence of H_2O_2, viability was higher ($p < 0.05$) for NS sperm than BSS sperm (Fig. 1b) and the percentage of apoptotic cells was higher in BSS sperm than NS sperm (Fig. 1c). However, while the %DFI was not affected by the presence of oxidant for BSS sperm, the values for this parameter were increased ($p < 0.05$) with the concentration of H_2O_2 for NS sperm (H050=9.3±0.7 and H100=10.9%±2.3 Vs. H000=4.1±0.9) (Fig 1d).

Experiment 3: The effect of the addition of antioxidants GSH and Trolox at different concentrations against oxidative stress

The addition of GSH at concentrations of 1 and 2 mM (GSH1 and GSH2) in the presence of oxidant had a beneficial effect on sperm motility in both types of sperm samples (Table 2). At both H050 and H100, supplementation with GSH1 (44.5%±4.8) or GSH2 (47.7%±6.6) maintained the motility higher ($p < 0.05$) than that from control (21.1%±3.9) NS sperm samples (Table 2). However, in the presence of 50 or 100 µM H_2O_2, Trolox (TRX1 and TRX2) supplementation, regardless of concentration (1 or 2 mM, respectively) had either no beneficial effect on sperm motility or decreased this parameter in comparison to control NS sperm samples (Table 2). Antioxidants had no effect whatsoever on sperm viability, regardless of concentration or sperm sample type (Table 2). The addition of GSH at 2 mM increased the percentage of apoptotic sperm, while Trolox at 1 mM decreased this value in relation to Control for BSS sperm and both concentrations of oxidant (Table 2). In regards to the %DFI, values were lower ($p < 0.05$) in the presence of antioxidants in NS samples challenged with oxidants (Table 2). However, no positive effect of antioxidant addition was observed on %DFI for BSS challenged with oxidants (Table 2).

4. Discussion

The aim of this work was to assess the effect of the sex-sorting process on post-thaw quality of red deer sperm. To achieve this goal, sperm samples were sorted and then cryopreserved. Immediately after thawing, viability and %DFI did not differ between non-sorted (NS) and bulk sorted (BSS) sperm samples. However, the percentage of apoptotic sperm was higher for BSS than NS sperm, whereas the percentage of motile sperm was significantly lower for BSS than NS sperm. The increase in apoptotic sperm

following the sex sorting process is in agreement with that observed by Balao da Silva et al. (2013), who showed that sex sorting increased the permeability of the membrane in stallion sperm. This may partially explain the decreased motility and increased apoptosis rates observed in sorted when compared to NS sperm. Similarly, following incubation, viability was higher and the percentage of apoptotic cells was lower for NS sperm.

Regarding oxidative stress, both sperm sample groups (NS and BSS) were susceptible as evidenced by the decrease in total motility in the presence of an oxidant. In agreement, we previously reported that post-thaw incubation of red deer sperm in100 µM and 1 mM but not 10 µM H_2O_2 for 1 hour depressed motility (Martínez-Pastor et al. 2009). The exact mechanism by which H_2O_2 inhibits motility is unclear, but it is known that it inhibits enzymes such as glucose-6-phosphate dehydrogenase (Maneesh and Jayalekshmi 2006).

Viability did not decrease in the presence of H_2O_2. In fact, NS sperm exposed to H100 showed a significant increase in viability. It is known that ROS are essential factors in many metabolic pathways, so it is plausible that the ROS produced by the addition of H_2O_2 exerted a positive effect. Similarly, Leahy et al. (2010) was first to report a beneficial effect of ROS on thawed ram sperm viability following a 3-hour incubation at 37ºC in the presence of 45 µM H_2O_2.

Conversely, DNA damage was strongly increased by the presence of H_2O_2 in NS but not in BSS sperm samples. Thus, sorted sperm were more resistant to oxidative stress than NS sperm, as evidenced by an increase in the %DFI in the presence of oxidant after 2 hours of incubation at 37ºC. This effect may be due to a step during the sorting process that is performed to remove the dead sperm subpopulation. Boe-Hansen et al. (2005) showed significant differences in the degree of DNA damage between

371 unsorted (higher) and sorted (lower) bull sperm samples when DNA integrity was
372 measured using both the sperm chromatin structure assay (SCSA®) and the neutral
373 comet assay. The authors suggested that this effect could be linked to the exclusion of
374 non-viable sperm by the sorting process. This was later confirmed by Gosálvez et al.
375 (2011), who demonstrated that sperm with DNA damage were separated into the dead
376 sperm subpopulation at sorting. In our study, there were no differences in DNA damage
377 between NS and BSS immediately after thawing or even following incubation.
378 However, differences were striking when sperm samples were incubated in presence of
379 H_2O_2. We speculate that the sorting process, which includes a step that removes
380 nonviable and non-flow oriented sperm, indirectly selected for a subpopulation with a
381 higher resistance of chromatin to oxidative stress than that in non-sorted sperm samples.
382 In addition, a recent study showed that capacitation and apoptosis are linked processes
383 joined by their common dependence on the continued generation of ROS (Aitken et al.
384 2015). Because of the higher number of sperm positive for apoptotic markers within
385 BSS samples, we hypothesize that the sorting process induced some capacitation-like
386 process as it has been previously described in ram or boar (Catt et al. 1997; Maxwell
387 and Johnson 1999; Maxwell et al. 1998) .

388 In the last experiment of this study we assessed the effect of the addition of 2
389 antioxidants in order to prevent the damaged induced by the addition of H_2O_2. Viability
390 was not affected by GSH, but apoptotic cells were increased by 2mM GSH in the
391 presence of H050 and H100. In contrast, GSH improved total motility for both groups
392 of sperm (NS and BSS) and kept low values of %DFI for NS sperm, in the presence of
393 H_2O_2. The lack of a beneficial effect of GSH on BSS sperm may have been due to low
394 starting values of DNA damage. Regarding the effects on motility, these results agree
395 with a previous report by our group showing that GSH also exerted a beneficial effect

on motility in thawed epididymal red deer sperm following a 6-hour incubation at 37ºC (Anel-López et al. 2012). Similarly, a recent study carried out in red deer sperm obtained by electroejaculation the GSH supplementation also had a protective effect on the quality of electroejaculated post-thaw red deer sperm after an incubation of 2 h at 39ºC (Anel-López et al. 2015). However, results with GSH in sperm from other ruminant species have not been so encouraging. In bull sperm, Foote et al. (2002) reported that 0.5 mM GSH exerted a marginal improvement in motility only after 12 h of incubation with superoxide dismutase and Tuncer et al. (2010) reported low values of DNA fragmentation when sperm were incubated in the presence of GSH. Similarly, studies on ram sperm have yielded few positive results when using GSH (Bucak et al. 2008; Câmara et al. 2011).

Conversely, Trolox appeared as an attractive option to maintain the quality of deer sperm. Previous studies reported that Trolox at 1 mM greatly decreased the susceptibility of post-thaw washed epididymal deer sperm to oxidative stress (Domínguez-Rebolledo et al. 2009). In addition, Trolox showed a high free radical scavenging activity in red deer sperm at only 10 µM (Martínez-Pastor et al. 2009). In contrast, the present study revealed that Trolox at the low millimolar range (1 and 2 mM) was not a good option to maintain the quality of NS and BSS sperm after incubation both in the absence or presence of oxidants. Its main effect was to inhibit the motility of samples. These results were according with some previous studies of our group in red deer epididymal sperm (Fernandez-Santos et al. 2007) and in sperm samples obtained by electroejaculation (Anel-López et al. 2015), in which Trolox also exerted a detrimental effect on motility. Conversely, Trolox at 1 mM had a protective effect on sperm membranes (lower apoptotic cells) of BSS sperm in the presence of both concentrations of H_2O_2 tested. In agreement, Peña et al. (2004) found positive

results when cryopreserving boar sperm with Trolox at 100 and 200 µM, having a protective effect on sperm membranes dependent upon the semen fraction frozen.

GSH at 1 mM yielded the best results in the study. In the presence of an oxidative agent, it kept high values of motility and low values of DNA damage for both sperm groups. In addition, unlike 2 mM GSH, this concentration did not induce an increase in the percent of apoptotic cells in BSS sperm. In contrast, Trolox at 1 and 2 mM did not yield positive results. While it protected NS sperm against DNA damage in the presence of an oxidant, total motility was inhibited by Trolox in the absence of oxidative stress and did not overcome the effects of oxidants for either group of sperm samples.

In conclusion, the sex sorting process prior to freeze-thawing sperm samples induced a sublethal effect by increasing the percentage of apoptotic cells although while concomitantly selecting a sperm population with a chromatin more resistant to oxidative stress injury. Therefore, GSH at 1 mM may be a good option to maintain sperm quality in sex-sorted samples, unlike Trolox (1 and 2 mM), which showed a high ability to inhibit sperm motility.

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Table 1. Effect of the sex sorting process on non-sorted (NS) and bulk sorted (BSS) thawed red deer sperm, immediately after thawing (0h) and following incubation (2h, 37 ºC).

SPERM	TIME AT	TM (%)	Viability (%)	Apoptotic (%)	%DFI
NS	**0 h**	59±3,3^{A}	56,5±1,5^{A}	14,6±1,2^{A}	2,1±0,1^{A}
	2 h	57,5±4,1^{A}	31,6±1,5^{B}	3±0,3^{B}	4,1±0,9^{B}
BSS	**0 h**	36,9±5,8^{B}	56,4±4,8^{A}	21,6±5^{C}	1,7±0,5^{A}
	2 h	36,2±3,8^{B}	22,5±1^{C}	6,4±0,4^{B}	3,2±0,2^{B}

Three replicates from one ejaculate from each of 3 males were assessed. Data are shown as the model-derived mean ± s.e.m. Data shown: Total motility (%TM), Viability, percentage of apoptotic sperm (Apoptotic) and DNA fragmentation index (%DFI). Different letters within column denote significant differences ($p < 0.05$) among treatments.

Table 2. Effect of reduced glutathione (GSH) and Trolox (TRX) against induced oxidative stress in non-sorted (NS) and sorted (BSS) sperm after 2 hours of incubation at 37ºC in medium containing H_2O_2 (H050, 50 µM; H100, 100 µM).

SPERM	H_2O_2	ANTIOX	(%)TM	(%)Viability	(%)Apoptotic	%DFI
NS	**H050**	**Control**	21,1±3,9	35,6±1,6	2,8±0,3	9,3±0,7
		GSH1	44,5±4,8 *	34,1±2,5	2,7±0,4	3,4±0,6 *
		GSH2	47,7±6,6 *	33,6±4,2	2,9±0,4	2,3±0,1 *
		TRX1	12,1±1,5 *	35,1±1,1	2,4±0,2	6,2±0,6 *
		TRX2	13,2±1,8 *	33,8±1,3	2,7±0,1	7,2±0,1 *
	H100	**Control**	11,3±2,2	41,7±2,4	3±0,2	10,9±2,3
		GSH1	28,4±2,2 *	39,8±1,2	3±0,1	5,9±1,9 *
		GSH2	38,7±3,4 *	36,9±1,8	2,6±0,2	5,1±1 *
		TRX1	5,7±1,2	35,2±2,5	3,2±0,4	5,8±1 *
		TRX2	4,5±1,7	35,9±3,4	3,3±0,3	5,5±0,7 *
BSS	**H050**	**Control**	8,9±1,8	25,8±2,5	5,8±0,9	3,3±0,2
		GSH1	33,3±8,1 *	23,8±0,9	6,7±0,5	2,4±0,6
		GSH2	31,4±2,9 *	25,2±0,8	7,3±1,3 *	1,9±0,4
		TRX1	7,3±2,2	25±1,5	4,5±0,5 *	2±0,3
		TRX2	9±1,7	25,9±1,6	4,4±0,4 *	1,8±0,2
	H100	**Control**	5±0,8	27,6±5,8	5,4±1,3	2,5±0,5
		GSH1	23±2,3 *	28,2±1	6,5±1,1	2±0,2
		GSH2	28,3±0,5 *	25,7±0,4	6,9±0,2 *	1,3±0,2
		TRX1	2,9±0,7	26,3±2,2	3,8±0,3 *	1,8±0,2
		TRX2	3,6±0,3	29,3±3	4,4±0,8	1,8±0,6

Three replicates from one ejaculate from each of 3 males were assessed. Data are shown as the model-derived mean ± s.e.m. Data shown: Total motility (%TM), Viability, percentage of apoptotic sperm (% Apopt) and DNA fragmentation index (%DFI). For each treatment (SPERM + H_2O_2 + ANTIOX) an asterisk (*) denotes a significant difference ($p < 0.05$) between the sample and its corresponding Control.

Figure Legends

Figure 1 Effect of 2 hours of incubation at 37ºC in medium containing H_2O_2 (H050, 50 µM; H100, 100 µM; vs. H000 ,0 µM) on non-sorted (NS) and sorted (BSS) red deer thawed

Three replicates from one ejaculate from each of 3 males were assessed. Data shown: Total motility (%TM) Fig. 1a; Viability (%Viab) Fig. 1b; % of apoptotic cells (%Apoptotic) Fig. 1c; DNA fragmentation index (%DFI) Fig. 1d. Different textures denote significant differences ($p < 0.05$) among concentrations of H_2O_2 within sperm group .(NS or BSS). Different capital letters denote differences ($p < 0.05$) between sperm groups (NS and BSS) at equal concentration of H_2O_2.

Figure 1

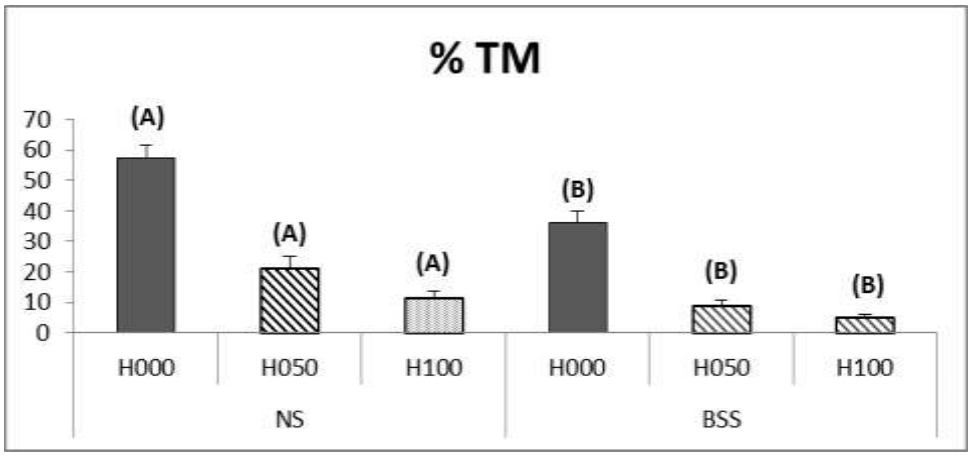


Figure 1a

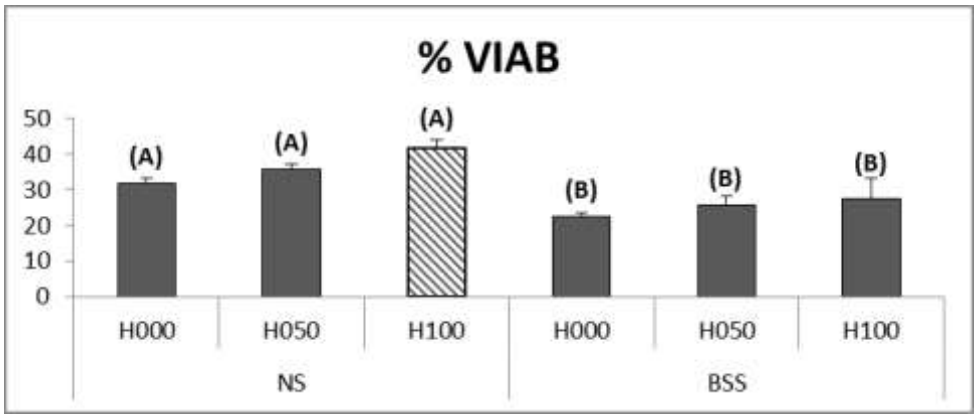
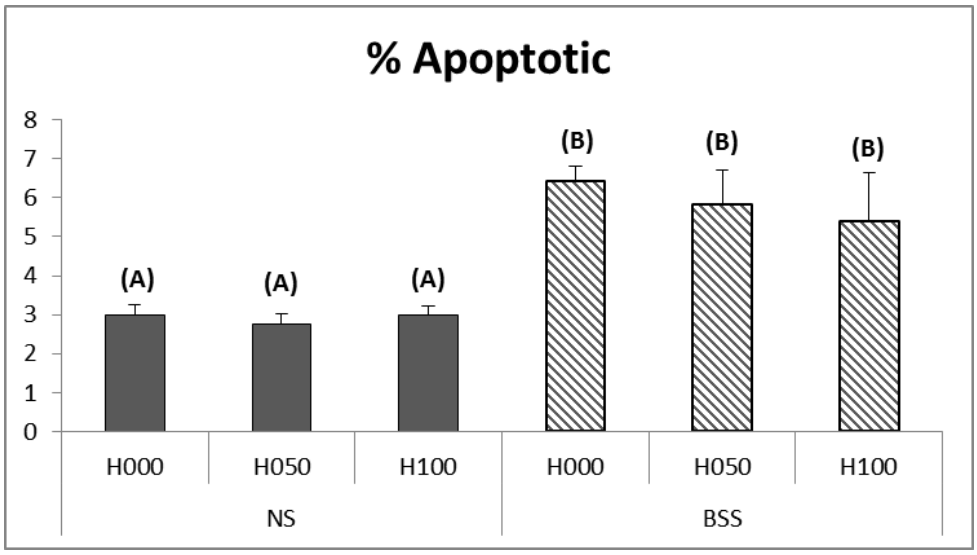


Figure 1b



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623 **Figure 1c**

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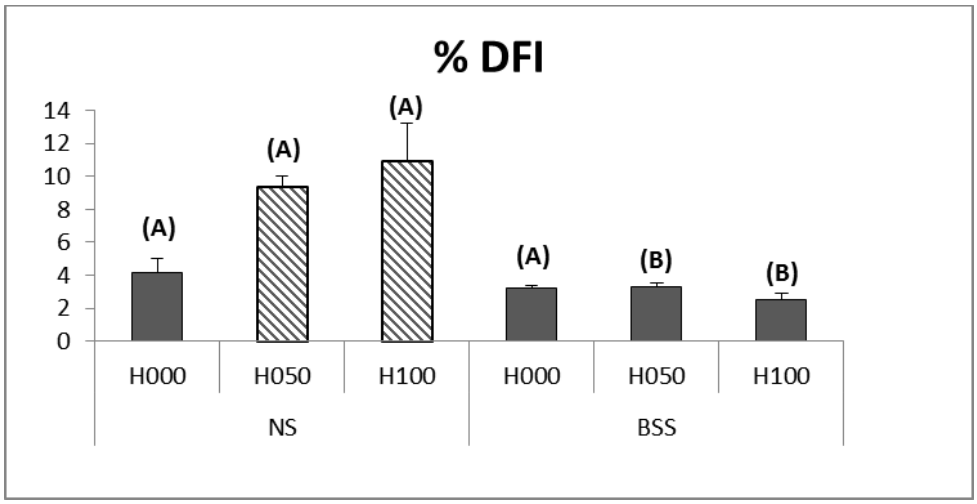

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626 **Figure 1d**

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