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Response of Thawed Epididymal Red Deer Spermatozoa to Increasing Concentrations of Hydrogen Peroxide, and Importance of Individual Male Variability

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Contents

Oxidative stress represents a challenge during sperm manipulation. We have tested the effect of increasing hydrogen peroxide (H_2O_2) levels on red deer spermatozoa after cryopreservation, and the role of male-to-male variation in that response. In a first experiment, eight thawed samples were submitted to 0, 25, 50, 100 and 200 μM H_2O_2 for 2 h at 37°C. Intracellular reactive oxygen species (H_2DCFDA-CM) increased with H_2O_2 concentration, but we only detected a decrease in sperm function (motility by CASA and chromatin damage by sperm chromatin structure assay) with 200 μM. Lipoperoxidation (TBARS) increased slightly with 50 μM H_2O_2 and above. In a second experiment, samples from seven males were submitted to 0 and 200 μM H_2O_2 for 2 h, triplicating the experiment within each male. Males differed at thawing and regarding their response to incubation and H_2O_2 presence. We found that the kinematic parameters reflected male-to-male variability, whereas the response of the different males was similar for lipid peroxidation and viability. A multiparametric analysis showed that males grouped differently if samples were assessed after thawing, after incubation without H_2O_2 or after incubation with H_2O_2. Red deer spermatozoa are relatively resilient to H_2O_2 after thawing, but it seems to be a great male-to-male variability regarding the response to oxidative stress. The acknowledgement of this individual variability might improve the development of optimized sperm work protocols.

Introduction

Oxidative stress is one of the major threats to sperm functionality, both *in vivo* and *in vitro*. Reactive oxygen species (ROS) have a fundamental role in sperm physiology, but in excess they can damage spermatozoa (Agarwal and Saleh 2002). Reactive oxygen species can be detrimental even within physiological levels, since they may trigger early capacitation and irreversible events, such as acrosome reaction (Hsu et al. 1999). Researchers generally use external sources of ROS to study oxidative stress on spermatozoa such as hydrogen peroxide (H_2O_2), a potent membrane-permeable oxidizing species (Oehninger et al. 1995). Armstrong et al. (1999) found that hydrogen peroxide was not only responsible for the loss of motility, but also it caused the loss of mitochondrial membrane potential. Moreover, ROS, including H_2O_2, have dual effects on mammalian sperm. Low concentrations of ROS exogenously added are believed to play a stimulatory role in sperm capacitation (Rivlin et al. 2004), hyperactivation (De Lamirade and Gagnon 1994), acrosome reaction (Griveau et al. 1995) and sperm-oocyte fusion (Aitken et al. 1995). However, excessive levels of ROS are linked to impaired sperm function and infertility (Sharma et al. 2004). Reactive oxygen species can be also detrimental to sperm DNA integrity (Baumber et al. 2003; Dominguez-Rebolledo et al. 2010a,b).

It has been previously reported by our own group that different ROS generators affected quality parameters differently in red deer, showing that hydrogen peroxide (H_2O_2) was more cytotoxic to red deer spermatozoa than Fe^{2+}/ascorbate (Martinez-Pastor et al. 2009a). Moreover, motility and mitochondrial membrane potential were quickly decreased by H_2O_2 (1 mM and 100 μM), and only H_2O_2 (1 mM) was able to reduce sperm viability. Thus, the present study was designed to deepen on our previous results, analysing a broader range of H_2O_2 concentrations.

Moreover, between-male variability represents a challenge for sperm cryopreservation, because that variability can affect spermatozoa cryosurvival (Soler et al. 2003) and fertility (Malo et al. 2005; Gomendio et al. 2006). That is probably because of differences regarding sperm biochemistry and metabolism (Loomis and Graham 2008), rooting in the genetic variability of individuals. The male-to-male variability could also affect the resistance of spermatozoa to oxidative stress, for instance through changes in the composition of sperm membranes (Waterhouse et al. 2006). In fact, high polyunsaturated fatty acids levels have been related to higher vulnerability to ROS (Ollero et al. 2001), and previous studies have shown that fatty acid profiles could be modified in deers exposed to heavy metals (Castellanos et al. 2010).

Thus, in the present study, we used thawed epididymal spermatozoa of Iberian red deer (*Cervus elaphus hispanicus*) to test the hypothesis that increasing concentrations of H_2O_2 affected differently to sperm characteristics, seeking for endpoints in which H_2O_2 could noticeably affect spermatozoa. It is well known that epididymal spermatozoa are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognized as the prime source of antioxidant protection (Chen et al. 2003). In this respect, it is needed a better understanding of the spermatozoa behaviour against oxidative damage, because this damage represents a serious challenge for these unprotected cells when they are outside the epididymal environment. Moreover, spermatozoa might be submitted to stressing

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situations during *in vitro* procedures (*in vitro* fertilization or sorting), which could increase ROS and other oxidative species. Therefore, this study could be useful to simulate the response of epididymal red deer spermatozoa to oxidative stress *in vitro*, allowing to explore procedures to alleviate it.

In the present work, it has been also tested if male-to-male variability reflects on the spermatozoa response to H_2O_2, expecting to observe this effect when submitting samples from different males to oxidative stress. Being a wild species, we have the advantage of working with samples coming from unselected populations, thus allowing us to better analyse that kind of variability (Garde et al. 2006).

Materials and Methods

Reagents and media

CM-H_2DCFDA, YO-PRO-1 and TO-PRO-3 were purchased from Invitrogen (Barcelona, Spain). Flow cytometry equipment, software and consumables (including the sheath fluid, BD FACSFlow) were purchased from BD Biosciences (San Jose, CA, USA). Acridine orange (chromatographically purified) was purchased from Polysciences Inc. (Warrington, PA, USA). Other fluorescence probes and chemicals (high grade) were obtained from Sigma Chemical Co. (Madrid, Spain). Stock solutions of the fluorescence probes were as follows: propidium iodide, 7.5 mM in water; CM-H_2DCFDA, 0.5 mM in DMSO; YO-PRO-1 and TO-PRO-3, 50 μM in DMSO. All solutions were stored at −20°C and in the dark until needed, except oxidant working solutions, which were prepared the same day. Preparation and staining of samples for flow cytometric analysis were performed by flow cytometer PBS (BD FACSFlow; BD Biosciences).

Animals, spermatozoa collection and cryopreservation

For this study, we used spermatozoa recovered from the epididymides of mature stags (age >4.5 years, weight >130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–October). Gamekeepers collected the complete male genitalia and provided the hour of the death. Hunting was in accordance with the harvest plan of the game reserve, which made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation.

Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at ambient temperature (approximately 22°C) within 2 h after being removed. The samples were processed as soon as they arrived at the laboratory. The elapsed time between animal death and sperm recovery ranged from 3 to 6 h, which is an adequate and reliable time interval for evaluating sperm parameters, as decreases in the quality of sperm traits begin to take place 12 h after the death of a male (Soler and Garde 2003). For the collection of epididymal spermatozoa, the testes and epididymides were removed from the scrotal sac. The cauda epididymides, which included 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes (Nunc, Roskilde, Denmark).

Spermatozoa were collected from the distal portion of the epididymis as described by Soler et al. (2003). Epididymal contents from both testicles of the same male were pooled for processing. Then, the sperm mass was diluted to a sperm concentration ~400×10^6 sperm/ml in fraction A of a Tris-Citrate-Fructose (TCF: Tris 27.0 g/l, citric acid 14.0 g/l, fructose 10.0 g/l and 20% clarified egg yolk) (Fernandez-Santos et al. 2006). Then, the diluted sperm was further diluted with the same volume of Fraction B of the extender (12%, v/v of glycerol), at ambient temperature (22°C). Samples were cooled down to 5°C and, after 2 h of equilibration, were loaded into 0.25 -ml plastic straws (IMV, L'Aigle Cedex, France) and frozen in liquid nitrogen vapour (4 cm above liquid nitrogen; −120°C) for 10 min. The straws remained for a minimum period of 1 year in liquid nitrogen (−196°C). Thawing was carried out by immersing straws in a water bath at 37°C for 30 s.

Experimental Design

Experiment 1. Effects of increasing doses of H_2O_2 on thawed epididymal spermatozoa from red deer

Experiment 1 was designed to explore the effect of several H_2O_2 concentrations on sperm parameters after thawing and to evaluate the relation of H_2O_2 with sperm parameters. Thawed semen was washed in TCF ($300 \times g$, 5 min) and diluted in the same medium to 30×10^6 spermatozoa/ml. The sperm solution was split among 5 aliquots in microtubes. One of them was left untreated as the control. The other aliquots were subjected to oxidative stress by adding H_2O_2 in four concentrations (25, 50, 100 and 200 μM). With this approach, we seeked to expand the study initiated previously (Martinez-Pastor et al. 2009a), exploring concentrations between 10 μM (which had no negative effects in that study) and 1 mM (which was patently cytotoxic). All treatments were split into two aliquots. One of them was incubated with 0.5 μM H_2DCFDA (for assessing ROS production), and the other was used to evaluated the rest of sperm parameters. The microtubes were incubated at 37°C and analysed 120 min after starting the incubation (the control was analysed at 0 and 120 min). This experiment was replicated 8 times with samples from eight different males (one straw per male).

Experiment 2. Individual male-to-male variation in the response to oxidative stress

This experiment evaluated the presence of male to male individual differences on the effect of oxidative stress. Thawed semen was washed in TCF ($300 \times g$, 5 min.) and diluted in the same medium to 30×10^6 spermatozoa/ml. The sperm solution was split among two aliquots in microtubes. One of them was left untreated as control, and the other was incubated with 200 μM H_2O_2 at 37°C, evaluating the samples after 120 min. The experiment was replicated with samples from seven

males, with triplicates within each male, using a different cryopreserved straw each time.

Sperm evaluation

Sperm motility

Sperm motility was assessed using a computer-assisted motility analyzer (SCA2002, CASA system; Microptic, Barcelona, Spain) coupled to an optical phase-contrast microscope (Nikon Eclipse 80i), equipped with negative phase-contrast objectives, a warming stage at 37°C and a Basler A302fs camera (Basler Vision Technologies, Ahrensburg, Germany). A pre-warmed Makler counting chamber (10 μm depth) was loaded with 5 μl of sample and analysed. The parameters used in this study were percentage of motile spermatozoa (total motility, TM, %), velocity according to the actual path (VCL, μm/s), linearity (LIN, %) and amplitude of the lateral displacement of the sperm head (ALH, μm). Sample acquisition rate was 25 images/s, and motile spermatozoa were defined as those with VCL > 10 μm/s. At least five fields per sample were recorded and analysed afterwards.

Sperm viability

Viability was assessed by the monomeric cyanine nucleic acid stain YO-PRO-1. Samples were diluted down to 10^6 spermatozoa/ml in flow cytometry PBS with 0.1 μM YO-PRO-1 and 10 μM PI. After 20 min in the dark, the samples were run through a flow cytometer. Labelling cells with the apoptotic marker YO-PRO-1 yielded three subpopulations: viable (unstained: YO-PRO-1-/PI−), apoptotic-like membrane changes (YO-PRO-1+/PI−) and non-viable (membrane damaged: PI+). Hoechst 33342 was included at 5.1 μM.

Detection of ROS

The derivative of fluorescein, CM-H_2DCFDA, was used for the detection of ROS. Oxidation of this probe is detected by monitoring the increase in fluorescence with a flow cytometer, using excitation sources and filters appropriate for fluorescein (green fluorescence). This fluorescence probe was combined with TO-PRO-1, a red-fluorescence analogue to YO-PRO-1. Stock solutions of the fluorescence probe were prepared as CM-H_2DCFDA 0.5 mM in DMSO, TO-PRO-3 50 μM in DMSO, to give a final concentration of 0.5 μM of CM-H_2DCFDA and 0.1 μM of TO-PRO-1. Hoechst 33342 was included at 5.1 μM.

Flow cytometry analyses

We used a Becton Dickinson LSR-I flow cytometer (BD Biosciences), furnished with a 325 nm He-Cd (excitation for Hoechst 33342), a 488 nm Ar-Ion laser (excitation for YO-PRO-1 and PI) and a 633 nm He-Ne laser (excitation for Mitotracker Deep Red). Hoechst 33342 fluorescence was read with the FL5 photodetector (424/44BP filter), YO-PRO-1 and CM-H_2DCFDA fluorescences were read with the FL1 photodetector (530/28BP filter), and PI and TO-PRO-1 fluorescences were read with the FL3 photodetector (670LP filter). FSC/SSC signals and Hoechst fluorescence were used to discriminate spermatozoa from debris. Fluorescence captures were controlled using the CELL QUEST PRO 3.1 software (BD Biosciences). All the parameters were read using logarithmic amplification. For each sample, 5000 spermatozoa were recorded at 200 events/s, saving the data in flow cytometry standard (FCS) v. 2 files. The analysis of the flow cytometry data was carried out using WEASEL v. 2.6 (WEHI, Melbourne, Australia). The YO-PRO-1/PI stain was analysed as previously described for red deer (Martinez-Pastor et al. 2008). From this stain, viability was defined as the percentage of membrane intact spermatozoa (PI−) and the 'apoptotic' ratio, as the relation among the YO-PRO-1+/PI− and PI− (YO-PRO-1-/PI− plus YO-PRO-1+/PI− spermatozoa) subpopulations, expressed as percentage. This ratio estimated the proportion of spermatozoa with apoptosis-like membrane changes within the PI–subpopulation.

Sperm chromatin assessment

Chromatin stability was assessed following the sperm chromatin structure assay (SCSA), 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. 1980). Acridine orange (AO) fluorescence shifts from green (dsDNA; double strand) to red (ssDNA; single strand) depending on the degree of DNA denaturation. Samples were diluted in TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final sperm concentration of 2×10^6 cells/ml in cryotubes. Samples were frozen in liquid nitrogen and stored in an ultracold freezer at −80°C until needed. For analysis, the samples were thawed on crushed ice. Acid-induced denaturation of DNA *in situ* was achieved by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) to 200 μl of sample. After 30 s, the cells were stained by adding 1.2 ml of an acridine orange solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 6 μg/ml acridine orange pH 6.0). The stained samples were analysed by flow cytometry exactly at 3 min after adding the acridine orange solution.

Samples were run through the LSR-I flow cytometer described earlier. Green fluorescence was detected using the FL-1 photodetector and red fluorescence with the FL-3 photodetector. Data were collected from 10 000 events at 200 events/s for further analysis with CELL-QUEST software (Becton Dickinson). A tube with 0.4 ml of detergent-acid solution and 1.2 ml of acridine orange solution was run through the system before running any samples and between samples. At the beginning of each session, a standard semen 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 to total intensity of the fluorescence (red/[red + green] × 100), called DFI (DNA fragmentation index; formerly called αt) for each

spermatozoa, representing the shift from green to red fluorescence. High values of DFI indicate chromatin abnormalities. Flow cytometry data were processed to obtain % DFI (% of spermatozoa with DFI > 25) and high DNA stainability (HDS: % of spermatozoa with green fluorescence higher than channel 600, of 1024 channels).

TBARS assay for quantification of lipid peroxidation (LPO)

The susceptibility of the spermatozoa to lipoperoxidation (LPO) was estimated by the thiobarbituric acid reactive substance (TBARS) method according to Ohkawa et al. (1979). Samples of 100 µl were thoroughly mixed with 200 µl of a stock solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 M HCl. This mixture was heated at 90°C for 15 min, and then the reaction was stopped by placing the tubes in ice-cold water for 5 min. The tubes were centrifuged at $1500 \times g$ for 15 min to pellet the precipitate, and the clear supernatant was collected and transferred to wells (200 µl/well) in a 96-well flat bottom transparent plate (Nunc). The plate was completed with a calibration curve prepared from a malondialdehyde (MDA) stock (1,1,3,3-tetramethoxypropane). Sample absorbance at 532 nm was read on a multipurpose microplate reader (Synergy HT, BIO-TEK, Winooski, VT, USA). Malondialdehyde concentration was calculated from a standard curve. The lipid peroxidation index was calculated as nmol of MDA per 10^8 sperm. This assay was duplicated for each sample.

Statistical analysis

Statistical analyses were carried out using the R statistical package (http://www.r-project.org). For the analysis of H_2O_2 (Experiment 1), data were analysed using linear mixed-effects models, treating the male effect as the random part of the model, and time or H_2O_2 concentration as the fixed part of the model. For the analysis of the male-to-male variability (Experiment 2), results were arc sine (proportions) or log-transformed (other variables), and male, treatment (values at 0, 2 and 2 h with 200 µM of H_2O_2) and their interaction were analysed by ANOVA. For the graphical analysis of the data, we used interaction plots and principal component analysis (with TM, VCL, LIN, ALH, LPO and viability). Unless otherwise stated, results are presented as mean ± SEM, and statistical significance was accepted for $p < 0.05$.

Results

Experiment 1. Effects of increasing doses of H_2O_2 on thawed epididymal spermatozoa from red deer

We evaluated how increasing H_2O_2 concentration affected sperm quality parameters, looking after H_2O_2 concentrations that might induce critical changes on sperm quality during incubation. Intracellular ROS (Fig. 1) spontaneously increased from 0 to 2 h (210 ± 12 at 0 h and 309 ± 9 at 2 h, in mean fluorescence units; $p < 0.001$). When H_2O_2 was added to the samples, ROS concentration increased with H_2O_2, comparing with incubation without H_2O_2 (25 µM: 379 ± 13, $p = 0.011$; 50 µM: 413 ± 27, $p < 0.001$; 100 µM: 428 ± 22, $p < 0.001$; 200 µM: 521 ± 37, $p < 0.001$).

The effect of H_2O_2 on incubated spermatozoa is shown in Fig. 2 as effect sizes respect to the control at 2 h (0 µM H_2O_2). In general, only the highest H_2O_2 concentration (200 µM) showed an effect in this experiment. Total motility decreased with the incubation (from 36.6 ± 5.7% to 28.6 ± 8.4% at 2 h; $p = 0.031$). It did not decreased further with H_2O_2 (effect size not significant; Fig. 2a), except for 200 µM, which decreased motility down to 21.4 ± 7.5% ($p = 0.037$). While linearity changed neither with incubation nor with H_2O_2 treatments (Fig. 2c), velocity and ALH decreased after 2 h of incubation (101.2 ± 6.2–88.5 ± 6.1 µm/s, $p < 0.031$; 4.1 ± 0.2–3.6 ± 0.2 µm, $p < 0.020$). Their values when they were incubated with 200 µM H_2O_2 were 72.7 ± 9.1 µm/s and 3.0 ± 0.2 µm ($p = 0.020$

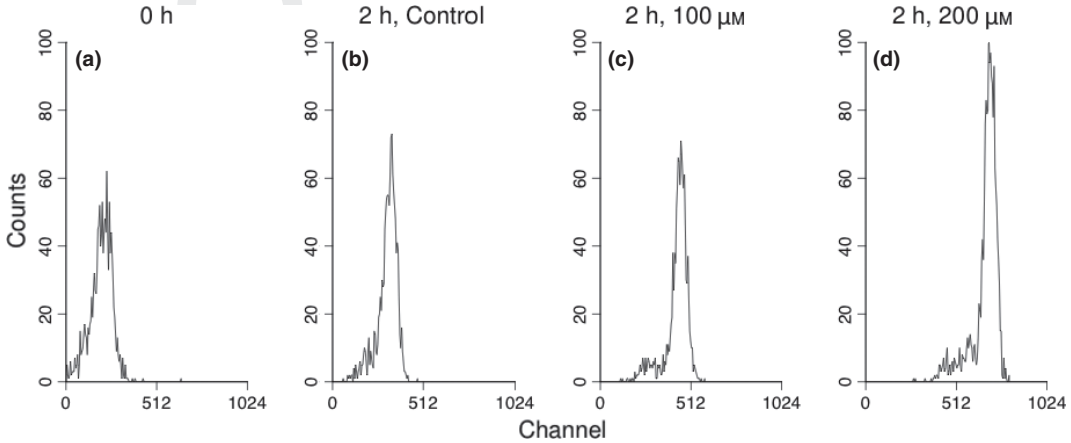

Fig. 1. Representative histograms from samples stained with CM-H_2DCFDA/TO-PRO-3, showing fluorescence intensity for CM-H_2DCFDA in the TO-PRO-1– subpopulation (viable spermatozoa). A higher fluorescence (given as fluorescence channel number, 1–1024) indicates higher intracellular reactive oxygen species. The mean fluorescence increased from baseline values at 0 h (a) to 2 h (b), and within 2 h, with increasing H_2O_2 concentrations [100 µM (c) and 200 µM (d) are shown here]

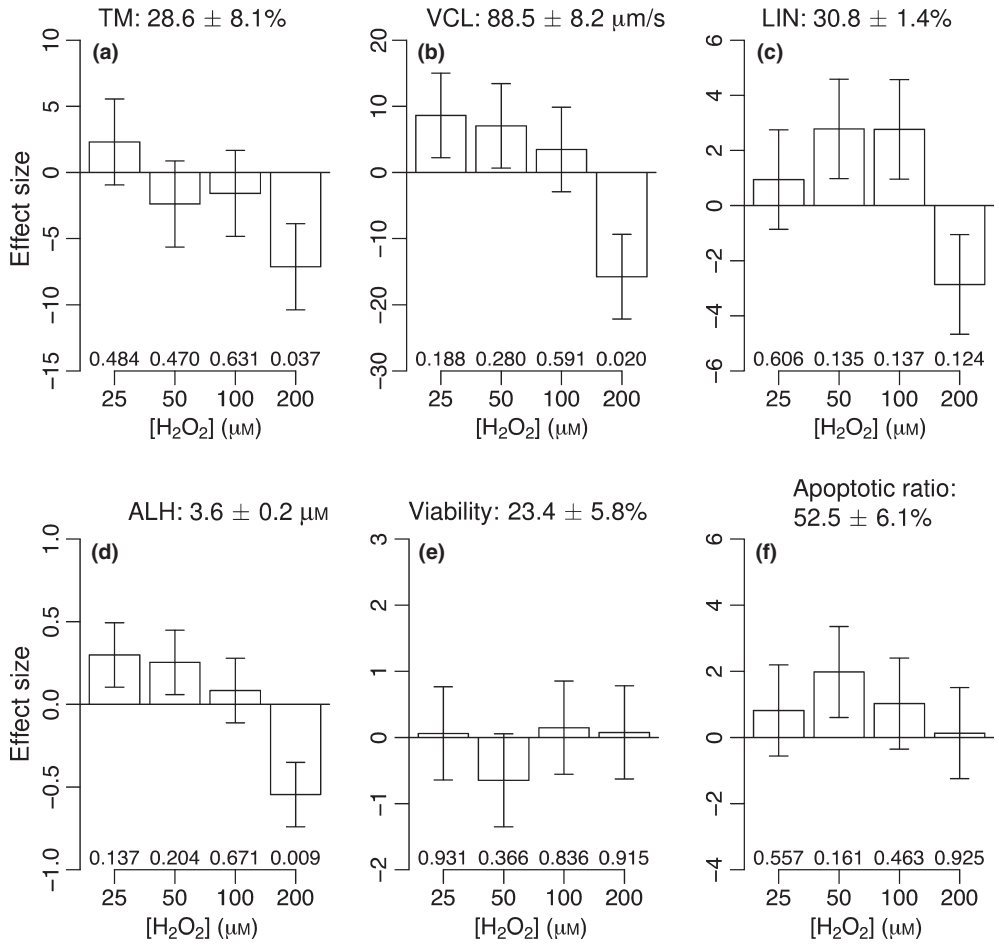

Fig. 2. Effect sizes of the H_2O_2 treatments, for the CASA analysis and YO-PRO-1/PI stain (viability and apoptotic ratio). In each case, the control value at 2 h (0 μM H_2O_2) was used as the intercept of the model (mean ± SEM showed), effect sizes being the relative variation of the parameter from the control value. For each H_2O_2 treatment, p values are given above the *x*-axis (H_0: effect not different from 0). Total motility (TM; a), curvilinear velocity (VCL; b) and the mean amplitude of the lateral movement of the head (ALH; d) were significantly reduced after 200 μM H_2O_2 treatment, whereas linearity (LIN; c), viability (e) and the apoptotic ratio (f) were not significantly affected by H_2O_2 addition

and p = 0.009, respectively), not changing significantly with other H_2O_2 treatments (Fig. 2b, d).

Incubation decreased the proportion of viable spermatozoa (55.2 ± 4.4–46.1 ± 5.1%; p = 0.018), tended to increase the apoptotic ratio (47.9 ± 5.3–52.5 ± 5.9%; p = 0.060) and slightly increased the lipid peroxidation of the samples, as estimated by the LPO by-product MDA, but not significantly (3.8 ± 0.7 nmol MDA/10^8 spermatozoa to 3.9 ± 0.6 nmol MDA/10^8 spermatozoa; p = 0.051). Addition of H_2O_2 did not modify the proportion of viable spermatozoa or the apoptotic ratio, comparing with 2 -h incubation without H_2O_2 (Fig. 2e, f). LPO levels did not increase in any H_2O_2 treatment comparing with the incubation without H_2O_2 (Fig. 3a); nevertheless, when comparing with the results at 0 h, 50 μM H_2O_2 and above significantly increased MDA concentration (effect sizes of +0.4 ± 0.1 for 50 μM, +0.5 ± 0.1 for 100 μM and +0.4 ± 0.1 for 200 μM, indicating increases above the 0 h levels, p < 0.05; the effect size of 2 -h incubation without H_2O_2 was +0.3 ± 0.1, p = 0.051).

The SCSA test revealed that incubation alone did not cause significant changes to chromatin stability (% DFI: 4.9 ± 1.5% at 0 h and 3.2 ± 0.6% at 2 h, p = 0.353; HDS: 2.7 ± 0.8% at 0 h and 4.1 ± 1.3% at 2 h, p = 0.198). Only 200 μM H_2O_2 increased % DFI significantly, up to 10.3 ± 2.9% (Fig. 3b), not having effect on HDS (Fig. 3c).

Experiment 2. Individual male-to-male variation in response to oxidative stress

In this experiment, we tested the effect of male-to-male variation during the incubation, including the response to oxidative stress caused by the higher dose of H_2O_2. In general, males differed at thawing (p < 0.01 for TM, VCL, ALH and viability). Three groups were differentiated (see group at 0 h in Fig. 4): males 1, 2 and 6 were characterized by higher motility (TM: 40.9 ± 3.0%; VCL: 104.7 ± 5.3 μm/s; ALH: 4.1 ± 0.2 μm) and viability (64.3 ± 2.2%); males 3 and 4 were characterized by low motility (TM: 18.9 ± 2.2%; VCL: 66.7 ± 6.5 μm/s; ALH: 3.0 ± 0.2 μm) while maintaining a relatively high viability (54.1 ± 2.4%); and males 5 and 7 were characterized by low motility (TM: 16.8 ± 1.4%), while maintaining high kinematic parameters (VCL: 94.9 ± 2.0 μm/s; ALH: 4.0 ± 0.1 μm) and lower viability (40.7 ± 2.8%).

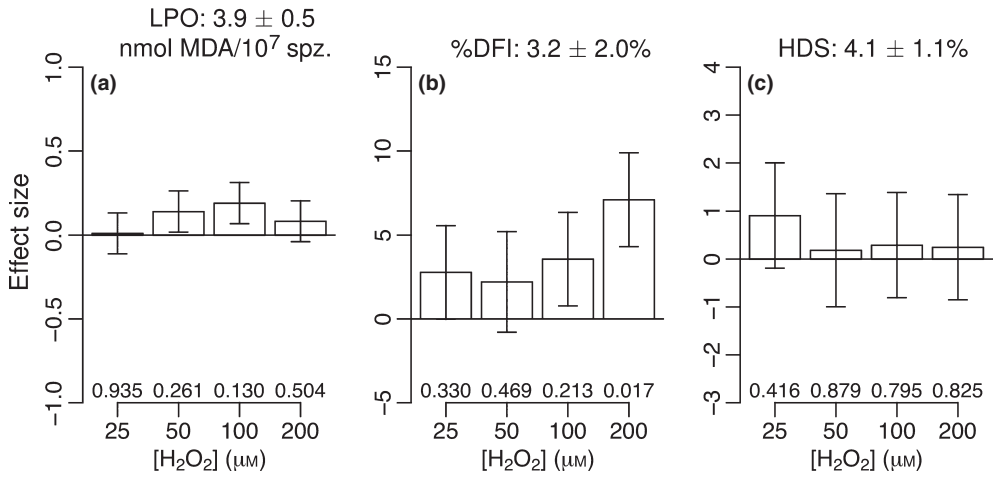

Fig. 3. Model effect sizes of the H_2O_2 treatments showed for the lipoperoxidation analysis (LPO) and sperm chromatin structure assay (DNA damage). In each case, the control value at 2 h (0 μM H_2O_2) was used as the intercept of the model (mean ± SEM showed), and the effect sizes are the relative variation of the parameter from the control value. For each H_2O_2 treatment, p values are given above the *x*-axis (H_0: effect not different from 0). Neither LPO (a) nor high DNA stainability (HDS; c) was significantly affected by the tested H_2O_2 concentrations, but the percentage of spermatozoa with high DNA fragmentation index (% DFI; b) significantly increased after incubation with 200 μM H_2O_2

Considering the whole experiment, male-to-male variability did not disappear after incubation with or without H_2O_2, but it was a significant factor for all studied parameters ($p < 0.001$ for TM, VCL, ALH and viability; $p < 0.01$ for apoptotic ratio; $p < 0.05$ for LIN and LPO). What is more important that variability affected how samples from different males responded to the incubation and oxidative stress (male ± treatment interaction). We found that interaction significant for VCL ($F_{12,27} = 3.036$, $p = 0.007$), LIN ($F_{12,27} = 3.107$, $p = 0.007$) and ALH ($F_{12,27} = 2.662$, $p = 0.017$). These differences throughout treatments can be appreciated in the interaction plots showed in the Fig. 4. It is clear that the behaviour of the samples was similar in the case of LPO and viability (Fig. 4e, f, change during incubation and little difference among 2 and 2 h plus H_2O_2), whereas most of the variability was shown in motility parameters. Total motility (Fig. 4a) suggested some degree of male-to-male variability on the response to treatments, but not reaching significance ($F_{12,27} = 1.838$, $p = 0.092$).

Therefore, most of the variability concerning treatment response was expressed on the kinematic parameters. For VCL (Fig. 4b), males 1, 3 and 5 underwent little change after incubation, but dropped if H_2O_2 was included in the medium, whereas males 2, 6 and 7 were affected by incubation without H_2O_2 (and in a higher degree, specially for 6, in presence of H_2O_2), and male 4 was little affected by the treatments. A similar pattern was detected for ALH (Fig. 4d). For LIN (Fig. 4c), a different grouping developed. In a first group (males 2, 4 and 5), LIN was little affected by incubation or oxidative stress. Contrarily, LIN dropped during incubation in the samples of males 6 and 7, whereas it did not decrease during incubation in the samples of 1 and 3 (in fact, increased for 3), but decreased (considerably for 1) in presence of H_2O_2.

A principal component analysis of averaged results for each male and treatment allowed to show these results in the bidimensional space defined by the first two principal components extracted (Fig. 5a). The male-to-male variability (initial characteristics, after incubation characteristics—either in presence or in absence of H_2O_2—and the response to the treatments) is displayed in the Fig. 5b. In that plot it is made clear that samples from different males behaved differently, as showed by the different directions and lengths of the vectors joining the points for each sample. According to the direction of change after incubation without H_2O_2, males could be grouped in three groups: one grouping males 1 and 3, other with males 2 and 7 and a third one with males 4, 5 and 6. When H_2O_2 was included, male 2 was just affected by the effect size, while maintaining the same direction. Others showed a different response (males 4, 5 and 7), but only in one or two parameters and not too large; male 6 could be included in this group, although the differences were much larger for that male. Finally, males 1, 3 showed a dramatically different response if incubated in absence or presence of H_2O_2.

Discussion

Oxidative stress has an important role in sperm physiology. In this study, we have studied this topic on cryopreserved epididymal spermatozoa; therefore, we must to point out that results might be different in fresh or ejaculated doses. Cryopreservation not only reduces sperm quality, but also induces oxidative stress and decreases the antioxidants in semen (Aisen et al. 2005; Peris et al. 2007), and epididymal spermatozoa have not contacted with seminal plasma, which contributes to the antioxidant defence of semen. Furthermore, male-to-male variability also affects to the resistance to cryopreservation-derived damage (Esteso et al. 2006; Loomis and Graham 2008), possibly enhancing post-thawing differences among males. These facts were considered when planning this study, and therefore our analysis and conclusions are within the context of cryopreserved epididymal spermatozoa.

In a previous study on oxidative agents of our own group (Martinez-Pastor et al. 2009a), it was found that 10 μM H_2O_2 did not affect thawed spermatozoa, but

LOW RESOLUTION FIG

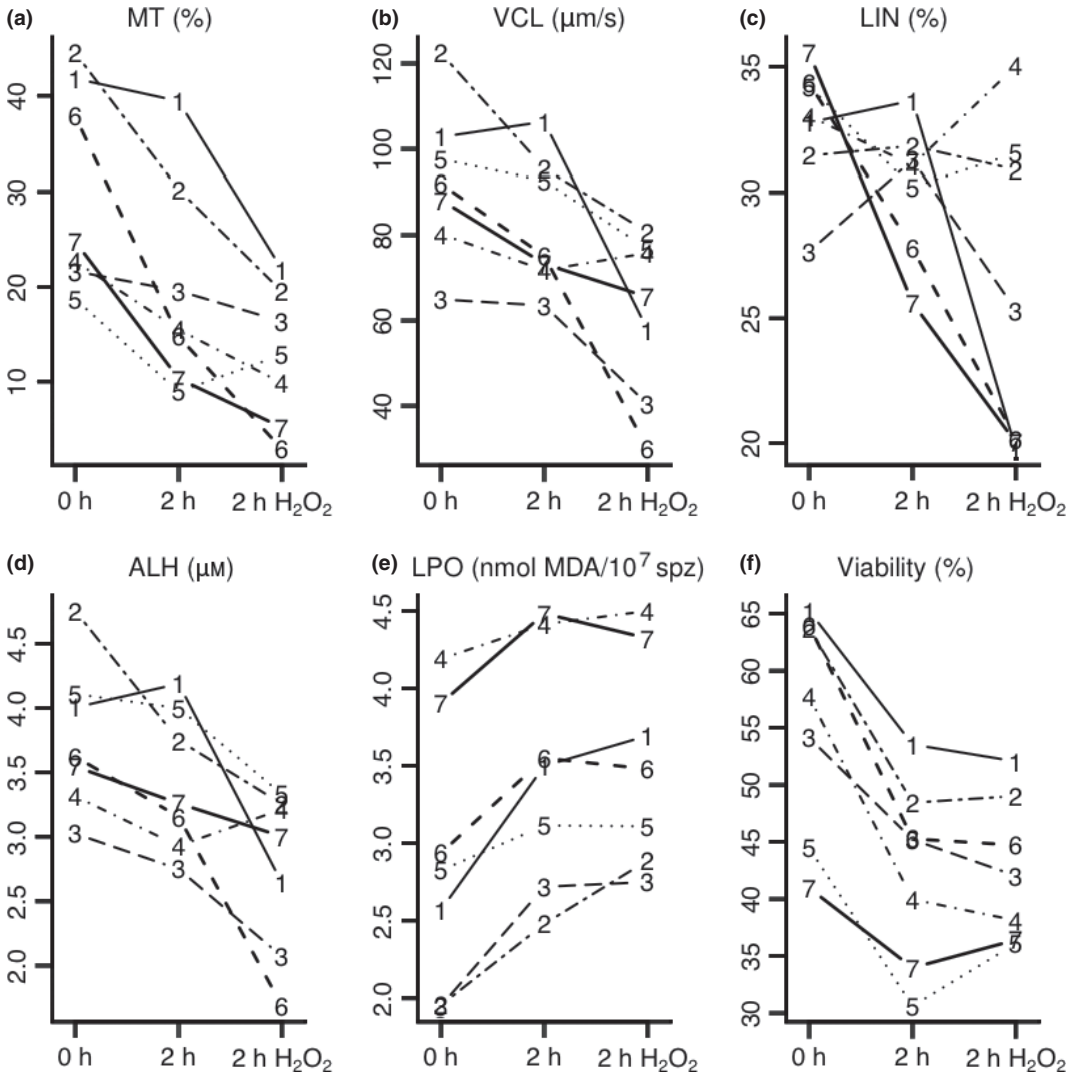

Fig. 4. These interaction plots show the effect of the individual males (1–7) and treatments (0, 2 h incubation and 2 h incubation with 200 μM H_2O_2) on sperm parameters (mean values displayed). Lines do not imply a continuity among 2 h incubation and 2 h incubation plus 200 μM H_2O_2, but they are used to highlight the different values and changes, among the treatments, of samples from different males. Differences among males are evident for motility parameters, while male-to-male differences (regarding different behaviour among treatments) were minimal for LPO and viability

100 μM and 1 mM depressed motility within 1 h of incubation (in fact, 1 mM abolished sperm motility almost immediately after adding it to the sample). We determined that a similar effect of xanthine oxidase/hypoxanthine was in fact caused by H_2O_2 generation. In our study, the only significant effects of H_2O_2 were caused by 200 μM, not by 100 μM. Apart from some differences on the experimental design (Dominguez-Rebolledo et al. 2009), it is possible that the limit above which H_2O_2 causes a detectable effect (regarding our experimental tests) would lay in the order of magnitude of 10^{-4} M. Individual males might present a different sensitivity to H_2O_2 within that order of magnitude, as suggested by the male-to-male variability experiment. In our previous study, we used samples from other set of males, which could be the source of the observed differences.

Although addition of H_2O_2 increased intracellular ROS, no other effects were observed below 200 μM. We found that even 10 μM H_2O_2 could increase intracellular ROS above control (Martinez-Pastor et al. 2009a), but this increase did not result in a noticeable change of motility or sperm physiology. However, Peris et al. (2007), working with fresh ram sperm, found that 50 μM H_2O_2 decreased motility in only 1 h of incubation. Nevertheless, these authors did not observe capacitation-related changes (chlortetracycline stain) among different H_2O_2 concentrations (0, 50 and 150 μM), except for 300 μM, which caused a significant increase in acrosome-reacted spermatozoa at 1 h of incubation (but not after 4 or 24 h). In the present study, H_2O_2 did not induce changes in the apoptotic ratio of the samples, a parameter depending on YO-PRO-1 stain, putatively related to membrane condition and possibly connected to the physiological status of the sperm cell (Peña et al. 2007; Martinez-Pastor et al. 2008). Previous studies have highlighted the role of ROS on the modulation of sperm physiology and their role activating capacitation (O'Flaherty et al. 1999; Baumber et al. 2003; Awda et al. 2009). For instance, Roy and Atreja

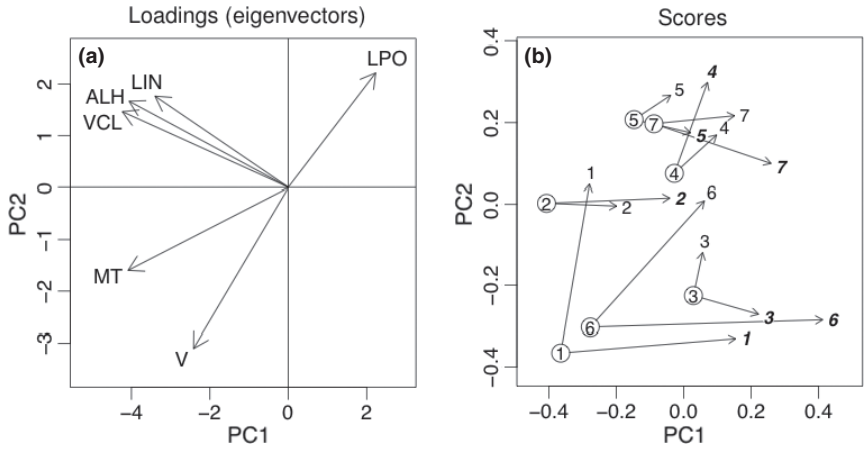

Fig. 5. Representation of the multivariate data showed in Fig. 4 in the bidimensional space resulting from performing a principal component analysis (PCA) with total motility, VCL, LIN, ALH, viability (V) and LPO (the first two principal components, PC1 and PC2, were selected). Subfigure (a) shows the variable loadings (linear relationships among the principal components and the variables), represented by the six eigenvectors, to help to interpret subfigure (b): for instance, in subfigure (b), samples 'moving' towards the lower-right quadrant would indicate samples with decreasing kinematic parameters, while those 'moving' towards the upper-right quadrant would have decreasing motility and viability, while increasing LPO. Subfigure (b) presents the changes underwent by samples from different males (1–7) as translations throughout the PC coordinates. Samples at 0 h are represented by circled numbers, which are the starting point for vectors representing the change underwent by these samples after 2 h of incubation (plain numbers) or 2 h of incubation with 200 μM of H_2O_2 (italic-bold numbers). The male-to-male differences showed in Fig. 4, regarding treatment effects, are evident in this plot

(2008) induced capacitation and associated tyrosine phosphorylation in buffalo spermatozoa by incubating with 50 μM H_2O_2. More detailed studies, such as analysis of tyrosine phosphorylation of specific proteins, should be performed in small ruminants, to determine whether H_2O_2 induces physiological changes beyond those reported by Peris et al. (2007) and us. The detection of these changes is of capital importance, since they might be unnoticed, affecting sperm functionality farther in sperm work protocols.

Agreeing with previous studies (Aitken et al. 1993; Armstrong et al. 1999; Peris et al. 2007; Martinez-Pastor et al. 2009a), sperm motility was the most sensitive parameter to H_2O_2. Motility loss by H_2O_2 has been primarily attributed to the inactivation of glycolytic enzymes, leading to energetic draining in the flagellum (Armstrong et al. 1999; Baumber et al. 2000). However, the sensitivity of spermatozoa to H_2O_2 varies dramatically among studies. Ramos and Wetzels (2001) found an almost total loss of motility after incubating 5 min human spermatozoa with 25 μM H_2O_2. Bilodeau et al. (2002), testing a wide range of H_2O_2 concentrations on bovine semen, found that 75 μM of H_2O_2 immediately decreased sperm motility and that just 12.5 μM H_2O_2 decreased motility after 1 h of incubation. This might imply that small ruminant spermatozoa might be more resilient to this effect, as we suggested in a previous study (Martinez-Pastor et al. 2009a).

A non-significant increase in MDA with time was observed, which seemed to be accelerated by H_2O_2 presence. This increase in LPO was unrelated to motility changes. These observations suggest that red deer sperm might be little prone to H_2O_2-induced lipoperoxidation. Peris et al. (2007) did not found increasing LPO levels when submitting the samples to H_2O_2 levels up to 300 μM, but after incubating their samples for 24 h. However, these authors found correlations among MDA concentration and other sperm parameters, which were not noticed in our study. It seems that there are between-species differences regarding susceptibility and consequences of lipid peroxidation. For instance, Alvarez and Storey (1989) could increase LPO and loss of motility in human and mouse sperm by adding H_2O_2 (1 and 5 mM), whereas the same concentrations of H_2O_2 were insufficient to induce LPO in rabbit sperm. Similarly, we could detect an increase in LPO using the TBARS technique in deer spermatozoa after incubating with 1 mM H_2O_2, but we could not detect a significant increase when applying 100 μM H_2O_2 (Dominguez-Rebolledo et al. 2010a,b).

Subjecting thawed spermatozoa to oxidative stress can affect chromatin integrity, and the SCSA test can be used to detect it (Fernandez-Santos et al. 2009; Martinez-Pastor et al. 2009b). Sperm chromatin integrity was affected by 200 μM H_2O_2. Previously (Martinez-Pastor et al. 2009a), we could not identify such chromatin insult, possibly because of the different set of males used. Again, it is possible that individual sample quality (different stocks of semen doses) could have a role, although we cannot discard variations in the experimental protocol (lack of sperm washing in our previous study). In fact, we found that washed samples were more vulnerable to oxidative stress than unwashed ones (Dominguez-Rebolledo et al. 2009). Other studies have shown apparently lower chromatin damage susceptibility in similar species. For instance, Peris et al. (2007) reported that SCSA showed that 150 and 300 μM H_2O_2 increased the % DFI on ram spermatozoa, but only after 24 -h incubation. In human spermatozoa, Ramos and Wetzels (2001) did not detect DNA damage when sperm from normospermic men were incubated for 1 h in the presence of 25 μM of H_2O_2, but damage was observed after 24 h (using TUNEL), alerting that low levels of ROS can be damaging given long incubation

times. Another study (Hughes et al. 1996), using the COMET assay, showed that applying 100 and 200 μM H_2O_2 for only one hour caused an important increase in DNA damage and that only 40 μM H_2O_2 was required to cause a small increase in DNA damage in asthenozoospermic samples (although baseline levels were similar to those of normozoospermic samples). This study highlights the importance of previous susceptibility to oxidative stress and the importance of between sample heterogeneity, regarding ROS resistance.

In the second part of our study, we aimed at studying the male-to-male variability on the response to H_2O_2. Although we worked with a limited number of males, it was evident that male-to-male variability had an effect, not only regarding the resilience to oxidative stress, but also to incubation without oxidants. In other studies, we have reported that sperm male-to-male variability seems to be high in red deer, possibly because of the unselected nature of the populations from which we obtained our samples (Garde et al. 2006). In fact, working with those wild populations allows us to easily detect and study male-to-male variability, which would be harder to detect working with animals submitted to strong human selection. We have previously showed that red deer present evident male-to-male differences in sperm characteristics and fertility (Malo et al. 2005) and in sperm sensitivity to cryopreservation (Soler et al. 2003). Moreover, we have proposed that variability could even reflect in biased sex ratios, depending on the fertility of different males (Gomendio et al. 2006).

In the present study, motility parameters were affected by incubation in some males, whereas in others motility was maintained almost unaltered for the duration of incubation, being only affected if oxidative stress was present. Contrarily, although individual variability was evident considering initial MDA concentration and sperm viability, it did not affect the changes on these variables after incubation with or without oxidative stress. It is known that many factors can affect membrane composition, among them individual variability, and that its composition influences its resistance and susceptibility to oxidative stress (Lenzi et al. 2002). It is possible that the small increase in LPO observed after incubation and H_2O_2 might have prevented us from detecting the interaction among males and treatments. Another reason could be that the high dilution and the freezing-thawing of the cryopreserved samples would have dimmed membrane-related differences, a hypothesis that could be tested in another study using fresh spermatozoa. It is important to consider that *in vivo* studies (Reglero et al. 2009) have shown no differences on LPO between deer living in areas contaminated with heavy metals and other living in uncontaminated areas, but the same study found differences among deer living in different estates. These findings suggest that some oxidative stress markers, such as LPO, could indeed depend more on the male than on environmental stressors.

Motility can be affected by multiple factors; and therefore, it is a good candidate to detect variability among males or samples (Malo et al. 2005). The resistance of sperm samples to incubation was apparently not dependent on their initial quality. Thus, male 1 and 6 had similar initial motility, but whereas male 1 maintained the same motility after incubation, and it was halved when H_2O_2 was present, it dropped dramatically for male 6, and it was abolished by H_2O_2. This example not only shows the impact of between-male differences, but also that the initial quality of a sample (just after thawing, in this case) might be not informative of its real potentiality. Therefore, sperm 'freezability' (comparison of the pre-freezing and post-thawing quality) might not suffice when characterizing samples from a male in the laboratory, being necessary to test the real resistance of spermatozoa by challenging them in physiological and non-physiological conditions (Roth et al. 1999; Soler et al. 2008). Furthermore, molecular techniques may be used to predict the performance of spermatozoa beyond cryopreservation (Thurston et al. 2002; Grunewald et al. 2008).

In summary, we conclude that oxidative stress caused by H_2O_2 clearly affected kinematic parameters of cryopreserved red deer spermatozoa, but only at relatively high concentrations (considering previous studies, at a magnitude of 10^{-4} M). It did not seem to influence sperm viability or apoptotic markers (as defined with YO-PRO-1). This may be a consequence of membrane resilience to oxidative stress, but also to the effect of cryopreservation, which might have already removed susceptible spermatozoa from the samples. We have to point out that we utilized only epididymal spermatozoa and that results might vary when using ejaculated samples. In a previous study on red deer (Martínez-Pastor et al. 2006), we found that cryopreservation conditions of epididymal and ejaculated samples might vary. These differences could also affect the response of oxidative stress of ejaculated samples.

In conclusion, cryopreservation of gametes and embryos and the development of Genetic Resource Banks allow us to have a gene resource for an indefinite time (Watson and Holt 2001). These assisted reproductive technologies (ART) are potentially capable of improving the propagation and conservation of wild and endangered species (Wildt et al. 1997). Of the genetic material in cryobanks, the collection, storage and subsequent use of spermatozoa has found the most widespread application (Watson and Holt 2001). According to this, cryopreservation of spermatozoa combined with artificial insemination (AI) has been the method of ART that has been most extensively applied to deer species (Asher et al. 2000; Garde et al. 2006). In the present work, male-to-male variability was evident in the response to incubation both with and without H_2O_2. This male-to-male variability is important, because it reflects on fertility and in the outcome of other artificial reproductive techniques (AI and IVF success). Thus, we must discriminate among samples from different males not only according to their 'freezability', but also according to their performance after thawing and in stressing situations. We must take into account these differences as much to improve freezing protocols as in the post-thawing protocols, considering protective agents such as antioxidants and adjusting them to the characteristics of different kinds of samples. This is especially important when dealing with valuable specimens of endangered animals, which is

usual working with wild species. Actually, there is a remarkable interest in the use of ART for the management of Iberian deer (*C. elaphus hispanicus*) populations. Specifically, ART may play an important role for the purpose of ensuring genetic preservation and/or genetic progress. Moreover, the results of the present work can contribute to the development of adequate protocols for red deer as a farming species and also for other small ruminants.

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Conflict of interest

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Author contributions

All authors have contributed to the work, from experimental design to writing the manuscript.

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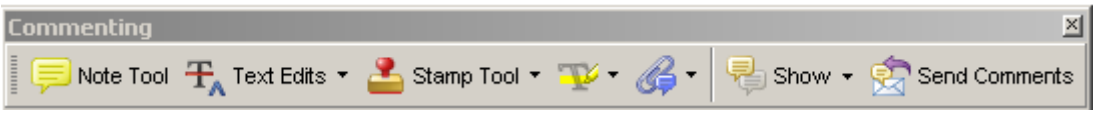

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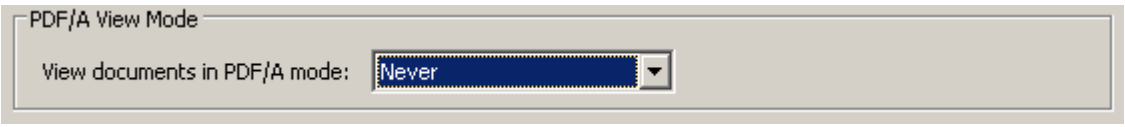
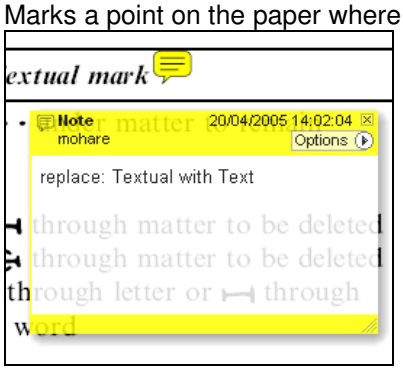


Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.

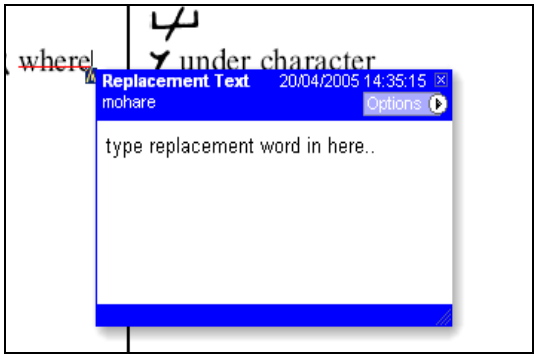


How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.

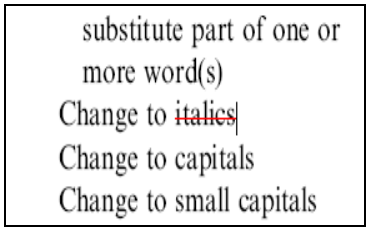


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

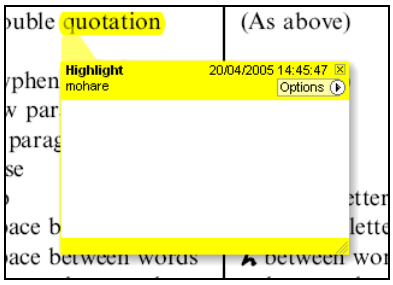
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

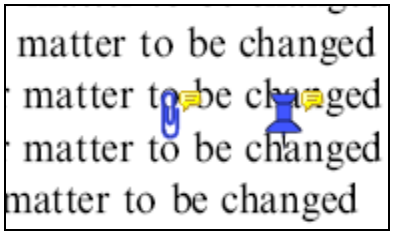


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

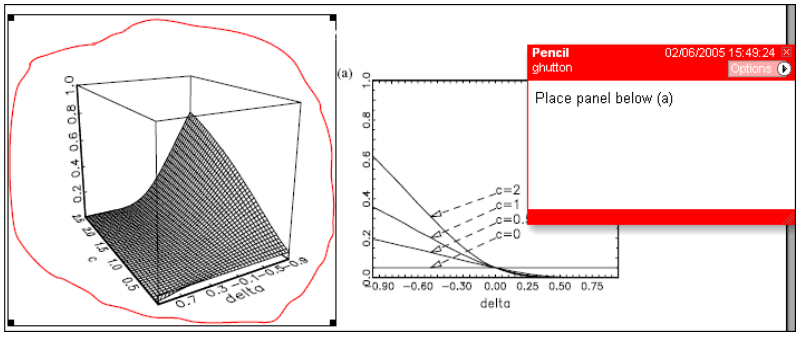


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: