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Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments

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Abstract. Antioxidants could improve sperm media, extending the viability of spermatozoa and protecting their DNA. The protective ability of lipoic acid, melatonin, Trolox and crocin was tested on red deer spermatozoa incubated at 37°C. Cryopreserved spermatozoa were thawed and incubated with 1 mM or 0.1 mM of each antioxidant, with or without oxidative stress (100 μM Fe^{2+}). Motility (CASA), viability, mitochondrial membrane potential and acrosomal status were assessed. Lipoperoxidation (malondialdehyde production), intracellular reactive oxygen species (ROS) and DNA status (TUNEL) were checked at 4 h. Incubation alone increased ROS and decreased motility. Oxidative stress intensified these effects, increasing lipoperoxidation and DNA damage. Lipoic acid had little protective effect, whereas 1 mM melatonin showed limited protection. Trolox lowered ROS and lipoperoxidation both in oxidised and non-oxidised samples. In oxidised samples, Trolox prevented DNA and acrosomal damage, and ameliorated motility. Crocin at 1 mM showed similar results to Trolox, but noticeably stimulated motility and had no effect on lipoperoxidation. In a second experiment, a broader range of crocin and melatonin concentrations were tested, confirming the effects of crocin (positive effects noticeable at 0.5–0.75 mM), but showing an increase in lipoperoxidation at 2 mM. Melatonin was increasingly effective at 2.5 and 5 mM (ROS, lipoperoxidation and DNA status). Crocin seems a promising new antioxidant, but its particular effects on sperm physiology must be further studied, especially the consequences of motility stimulation and confirming its effect on lipoperoxidation. Melatonin might be useful at relatively high concentrations, compared to Trolox.

Additional keywords: crocin, lipoic acid, melatonin, Trolox.

Introduction

Cryopreservation of epididymal spermatozoa is feasible and practical for the creation of germplasm banks for wild deer species (Garde *et al.* 2006; Martínez-Pastor *et al.* 2006*b*). Nevertheless, conditions must be optimised for the successful post-thawing application of such samples. The susceptibility of spermatozoa to oxidative damage arises as an important problem, since it might lead to loss of motility, membrane integrity, fertilising capability and other physiological changes in spermatozoa (Storey 1997; Aitken and Baker 2004; Cassani *et al.* 2005). This damage may be higher in thawed and epididymal spermatozoa, because of the lack of seminal plasma, which is a recognised source of antioxidants (Chen *et al.* 2003).

Antioxidants have an important role in maintaining the motility and the genetic integrity of sperm cells against oxidative damage (Hughes *et al.* 1998). Extenders can be supplemented with antioxidants before freezing (Peña *et al.* 2003, 2004; Gadea *et al.* 2005*a*; Roca *et al.* 2005; Fernández-Santos *et al.* 2007), or just after thawing (Berlinguer *et al.* 2003; Gadea *et al.* 2005*b*; Fernández-Santos *et al.* 2009*a*), which block the production of reactive oxygen species or counteract oxygen toxicity. We have demonstrated that some antioxidants improve the cryopreservation of red deer epididymal spermatozoa (Fernández-Santos *et al.* 2007), and, using ascorbic acid, we could mimic the protective effect of the epididymal environment (Fernández-Santos *et al.* 2009*b*). Furthermore, we have shown that seminal plasma

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improves the cryopreservation of Iberian red deer epididymal spermatozoa, which could be partly due to its antioxidant properties (Martínez-Pastor *et al.* 2006*a*).

In the present study, we evaluated four antioxidants with different chemical properties: lipoic acid, melatonin, Trolox and crocin. Our interest in testing these antioxidants is based on the fact that their mechanisms of action, radical preference and organelle accumulation varies greatly. We aimed at testing their effects in cryopreserved spermatozoa, in the context of post-thawing sperm work. Thawed spermatozoa might be submitted to stressful conditions in IVF settings or when used for sexing. In these applications, adding antioxidants to the medium would be advantageous. Indeed, even if spermatozoa were frozen in the presence of added antioxidants, simple routine techniques could remove this additional protection, for instance, by removing freezing extender (centrifugation, gradient centrifugation, swim-up, etc.). Previously, we found that washing increased the susceptibility of thawed spermatozoa to exogenous oxidative stress (Domínguez-Rebolledo *et al.* 2009).

Lipoic acid is an antioxidant belonging to the thiol-containing group (Navari-Izzo *et al.* 2002), which functions both in aqueous and membrane phases (Bast and Haenen 2003). It is found naturally in mitochondria as the coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Packer *et al.* 1995). Selvakumar *et al*. (2006) showed that oral administration of lipoic acid improved the semen quality and reduced the oxidative stress and DNA damage in rat, but it has not been tried directly on spermatozoa yet.

Melatonin, an endocrine product of the pineal gland belonging to the indole group, is an effective water-soluble and fat-soluble antioxidant (Acuña-Castroviejo *et al.* 2001), and it might be especially effective in protecting mitochondria (Leon *et al.* 2004). Its effects seem to be due to the signalling effects on the upregulation of antioxidant enzymes and downregulation of pro-oxidant enzymes and to direct radical scavenging. Moreover, metabolites resulting from the interaction of melatonin with several oxidising radicals are themselves potent antioxidants too (Leon *et al.* 2004; Hardeland 2005). Several studies have shown that melatonin protected spermatozoa against oxidative stress (Shang *et al.* 2004; Jang *et al.* 2009), although it seemed to be less efficient than other antioxidants such as Trolox (Gavella and Lipovac 2000).

Crocin, a glucosyl ester of crocetin, is one of several uncommon water-soluble carotenoids that represent the yellow pigments of saffron (*Crocus sativus*) (Carmona *et al.* 2005). The pharmacological effect of crocin has been studied recently, finding that crocin prevents apoptosis in several cell types (Ochiai *et al.* 2007) and ameliorates reperfusion-induced oxidative damage (Zheng *et al.* 2007). Moreover, Heidary *et al*. (2008) showed that oral administration of 50 mg of saffron improved sperm morphology and motility in fertile men, but it has not been tried directly on spermatozoa to date.

Trolox is a water-soluble vitamin E analogue with a high capacity for radical scavenging (Mickle and Weisel 1993) that is often used as a standard for reporting antioxidant capacity for other substances (Gavella and Lipovac 2000; Prior *et al.* 2005). The addition of Trolox to semen preservation media was shown to improve the longevity and quality of chilled and frozen–thawed spermatozoa (Peña *et al.* 2003, 2004), sperm motility and mitochondrial membrane integrity during post-thaw incubation (Peña *et al.* 2003), and effectively reduced induced lipid peroxidation (Gavella and Lipovac 2000). Furthermore, we have demonstrated that Trolox reduced intracellular reactive oxygen species (ROS) and lipid peroxidation, and preserved membrane integrity of red deer epididymal spermatozoa during post-thaw incubation, either with or without induced oxidative stress (Martínez-Pastor *et al.* 2008, 2009*a*). Recently, we reported that Trolox protected motility and viability and abolished DNA damage in samples submitted to oxidative stress after thawing and washing (Domínguez-Rebolledo *et al.* 2009).

Thus, we tested if the supplementation of incubation media with two concentrations of these antioxidants, in the millimolar to micromolar range (0.1 and 1 mM), would protect thawed red deer epididymal spermatozoa against oxidative stress (spontaneous and induced), considering the use of thawed samples for *in vitro* techniques (IVF, sorting, etc.). A second experimental series, designed to clarify the results obtained in the first trial, was focussed on studying broader ranges of concentrations for crocin and melatonin.

Materials and methods

Reagents and media

Fluorescence probes and the ApoTarget APO-BRDU Kit (TUNEL test) were purchased from Invitrogen (Barcelona, Spain). The spectrophotometric assay for malondialdehyde (BIOXYTECH MDA-586) was purchased from Oxis International (Beverly Hills, CA, USA). Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probes propidium iodide (PI) and peanut agglutinin (PNA-TRITC) were acquired from Sigma (Madrid, Spain). Stock solutions of the antioxidant solutions were prepared as 100 mM and 10 mM of antioxidant, in ethanol for lipoic acid, melatonin and Trolox (vitamin E analogue), and in water for crocin. Although lipoic acid, melatonin and Trolox are water-soluble, they have a limited solubility, and the concentrated stock solutions must be prepared in organic solvents. The oxidant stock was prepared as 10 mM $FeSO_4$ and 50 mM sodium ascorbate (Fe^{2+}–ascorbate) in water. Stock solutions of the fluorescence probes were: PI, 7.5 mM; PNA-TRITC, 0.2 mg mL^{-1}; YO-PRO-1, 50 μM; TO-PRO-1, 50 μM; Mitotracker Deep Red, 1 mM; CM-H_2DCFDA, 500 μM. All fluorescent stocks were prepared in DMSO, except for PI and PNA-TRITC, which were prepared in water, and kept at −20°C and in the dark until needed. The antioxidant and oxidant solutions were prepared on the same day. Bovine gamete medium (BGM-3) was composed of 87 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 μg mL^{-1} kanamicine, 10 μg mL^{-1} phenol red and 6 mg mL^{-1} BSA (pH 7.5).

Stags and testes collection

Epididymal samples were collected from mature stags (*Cervus elaphus hispanicus*, Helzheimer 1909) that were legally culled and hunted in their natural habitat during the rutting season

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(September and October). Hunting was in accordance with the harvest plan of game reserves, following Spanish Harvest Regulation (Law 2/93 of Castilla-La Mancha), which conforms to European Union Regulations. These operations were carried out as part of a project approved by the ethical committee of the University of Castilla-La Mancha, following the Guidelines for the Care and Use of Animals. Spermatozoa were collected from the cauda epididymis within 3 h post-mortem, and diluted at ambient temperature to 200×10^6 spermatozoa mL^{-1} in Triladyl (Minitüb, Tiefenbach, Germany) with 20% egg yolk. Extended spermatozoa were cooled down to 5°C (-2°C min^{-1}) and equilibrated for 2 h at the same temperature. Samples were loaded into 0.25-mL plastic straws (IMV, L'Aigle Cedex, France) and frozen in nitrogen vapour (4 cm above liquid nitrogen, -120°C) for 10 min. The straws remained for a minimum period of one year in liquid nitrogen.

Experimental design

Samples from four stags were used in this experiment. One straw per stag was thawed by dropping it into a water bath with saline solution at 37°C for 30 s. The straws were thoroughly wiped, and their contents poured into a tube, forming a pool. The pool was washed free of freezing extender by diluting with three volumes of BGM-3, centrifuging (600g, 5 min, room temperature) and removing the supernatant. The pellet was resuspended in BGM-3 up to 30×10^6 cells mL^{-1}.

Effect of antioxidants on the motility and physiology of thawed spermatozoa

The washed pool was split among nine tubes. One of the tubes was left as control and analysed immediately, and the other tubes were supplemented with a 1 : 100 dilution of either the 100 mM solution (1 mM final) or the 10 mM solution (0.1 mM final) of one of the antioxidants (melatonin, lipoic acid, crocin or Trolox). Since melatonin, lipoid acid and Trolox stocks were prepared in ethanol, control and crocin tubes received an equivalent volume of ethanol (absolute). The tubes were incubated at 37°C and analysed for motility, viability, mitochondrial status and acrosomal status after 2 h and 4 h. This experiment was replicated eight times.

Effect of antioxidants on the physiology of thawed spermatozoa submitted to oxidative stress

The nine tubes described above were replicated and supplemented with the oxidant solution, at a final concentration of 100 μM of Fe^{2+} and 500 μM of ascorbate. The tubes were incubated at 37°C and analysed for motility, viability, mitochondrial status and acrosomal status after 2 h and 4 h. This experiment was replicated eight times.

Effect of antioxidants on the level of lipid peroxidation, intracellular ROS and DNA damage of thawed spermatozoa submitted to oxidative stress

The same set of 18 tubes, supplemented with the respective antioxidants and with or without oxidant, was incubated for 4 h at 37°C and analysed for lipid peroxidation (malondialdehyde production), ROS production and DNA damage. The control sample was analysed before starting the incubation, as a reference value. This experiment was replicated eight times.

Study of an extended concentration range for crocin and melatonin

The previous experiments showed interesting results for melatonin and crocin, thus an additional experiment was carried out to study the effect of intermediate crocin concentrations and higher melatonin concentrations. Samples were processed as previously described, but supplemented with either 0.1, 0.25, 0.5, 0.75, 1 or 2 mM of crocin or 1, 2.5 or 5 mM of melatonin (leaving one tube without supplementation, as control). It is noteworthy to notice that 5 mM is close to the melatonin solubility limit in aqueous solutions, therefore it was not sensible to increase its concentration beyond this value. All tubes were split and the oxidant solution was added to one of the splits. After 4 h we analysed sperm motility, lipid peroxidation, intracellular ROS and DNA damage. This experiment was replicated six times.

CASA analysis

Sperm were diluted down to 10–20 $\times 10^6$ spermatozoa mL^{-1} and loaded into a Makler counting chamber (10-μm depth) at 37°C. The CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan), equipped with a warming stage at 37°C and a Basler A302fs 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 Sperm Class Analyzer (SCA2002) software (Microptic S.L., Barcelona, Spain). Sampling was carried out using a 10× negative phase-contrast objective (no intermediate magnification). Image sequences were saved and analysed afterwards. Software settings were adjusted to deer spermatozoa. The standard parameter settings were as follows: 25 frames s^{-1}; 20 to 90 μm^2 for head area; VCL $>10\ \mu m\ s^{-1}$ to classify a spermatozoon as motile. For each spermatozoon, the software rendered the percentage of motile spermatozoa, three velocity parameters (VCL, velocity according to the actual path; VSL, velocity according to the straight path; VAP, velocity according to the smoothed path), three track linearity parameters (LIN, linearity; STR, straightness: WOB, wobble), the amplitude of the lateral displacement of the sperm head (ALH), and the head beat-cross frequency (BCF). These parameters have been defined elsewhere (Mortimer *et al.* 1988).

Fluorescence probes for sperm analysis

Several physiological traits were assessed by using fluorescent probes and flow cytometry, which were described in previous studies (Peña *et al.* 2005; Martínez-Pastor *et al.* 2008, 2009*b*; Domínguez-Rebolledo *et al.* 2009). Samples were diluted down to 10^6 spermatozoa mL^{-1} in BGM-3, and stained using four fluorophore combinations. Sperm viability and 'apoptotic' status were assessed with 0.1 μM YO-PRO-1 and 10 μM PI. Since the term 'apoptotic' may be misleading, we will use the expression 'increased membrane permeability' instead. Mitochondrial activity and acrosomal status were assessed by combining 0.1 μM YO-PRO-1, 0.1 μM Mitotracker Deep Red

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and 4 μg mL^{-1} PNA-TRITC (peanut agglutinin). YO-PRO-1 allowed discrimination of viable spermatozoa, whilst active mitochondria were stained by Mitotracker Deep Red and damaged acrosomes were stained by PNA-TRITC. Spermatozoa stained in these two solutions were incubated 20 min in the dark before being run through a flow cytometer.

For assessing intracellular ROS, spermatozoa were diluted in BGM-3 with 0.5 μM CM-HDCFDA and 0.1 μM TO-PRO-1 (for assessing viability). The CM-H_2DCFDA is cleaved by cellular sterases being thus retained within the spermatozoa. Only after being oxidised, it acquires an intense fluorescence, indicating presence of intracellular ROS. Spermatozoa were incubated for 30 min in the dark at 37°C before being analysed.

DNA damage was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling assay (TUNEL) method, following the manufacturer's instructions (ApoTarget APO-BRDU Kit). Briefly, spermatozoa diluted in PBS (10^6 cells mL^{-1}) were fixed for 1 h in 2% paraformaldehyde. The cells were washed with PBS, pelleted and resuspended with ethanol at 0°C. The samples were left at −20°C overnight. Then, the cells were washed twice using the wash buffer provided with the Kit, adding the DNA-labelling mixture after removing the wash buffer. After 60 min at 37°C (with agitation), the cells were washed twice using the rinse buffer. Finally, the cells were resuspended in the antibody solution (FITC-anti-BrdUTP mAb) and incubated for 30 min at room temperature in the dark. Samples were resuspended in a PI–RNase A solution and analysed by flow cytometry within 2 h. Positive and negative controls (incubation of fixed cells with DNase A and substituting water for the DNA labelling mixture, respectively) were used to standardise the assay.

Flow cytometry analyses

Samples were analysed as shown previously (Domínguez-Rebolledo *et al.* 2009). Briefly, we used a Cytomics FC 500 flow cytometer, with a 488-nm Ar-Ion laser (excitation for YO-PRO-1, TRITC, PI, CM-H_2DCFDA and TUNEL), and a 633-nm He-Ne laser (excitation for Mitotracker Deep Red and TO-PRO-1). Fluorescence from YO-PRO-1, CM-H_2DCFDA and TUNEL (fluorescein isothiocyanate, FITC) were read using a 525/25BP filter, TRITC was read using a 575/20BP filter, PI was read using a 615DSP filter and Mitotracker Deep Red and TO-PRO-1 were read using a 675/40BP filter. FSC/SSC signals were used to discriminate spermatozoa from debris. Some authors have warned about the risk of misinterpreting spermatozoa for debris when using the FSC/SSC gating (Nagy *et al.* 2003; Petrunkina and Harrison 2009). Thus, in previous studies we used the fluorochrome Hoechst 33342 in a flow cytometer equipped with an UV laser to discriminate debris from spermatozoa (Domínguez-Rebolledo *et al.* 2009; García-Álvarez *et al.* 2009*a*, 2009*b*; Martínez-Pastor *et al.* 2009*a*), and we have confirmed that the amount of debris misinterpreted as spermatozoa when using the FSC/SSC discrimination was negligible if samples were previously washed. Fluorescence captures were controlled using the RXP software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 spermatozoa were recorded, 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, Victoria, Australia). The YO-PRO-1/PI and YO-PRO-1/PNA-TRITC/Mitotracker Deep Red staining were analysed as previously described for red deer (Martínez-Pastor *et al.* 2008). For analysing sperm viability, three subpopulations were identified: viable (YO-PRO-1−/PI−), increased membrane permeability (YO-PRO-1+/PI−) and membrane-damaged (PI+). Spermatozoa with active mitochondria were stained by Mitotracker, indicating high mitochondrial membrane potential ($\Delta\psi_m$). For assessing the acrosomal status, we considered the proportion of PNA$^+$ spermatozoa (damaged acrosome) within the viable (YO-PRO-1−) and the increased membrane permeability/necrotic (YO-PRO-1+) subpopulations. Example cytograms are shown in Fig. 1. Intracellular ROS were assessed only in viable (TO-PRO-1−) spermatozoa, showing results in arbitrary fluorescence units (mean fluorescence intensity, MFI). For TUNEL analysis, the negative control allowed the definition of the TUNEL− population, thus events with increased fluorescence were counted as TUNEL + cells.

Assessment of lipid peroxidation

The susceptibility of spermatozoa to peroxidation was assessed using the Bioxitech MDA-586 kit (Oxis International, Foster, CA, USA) to detect malonaldehyde (MDA) concentration. The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI) with MDA at 45°C. One molecule of MDA reacts with two molecules of NMPI to yield a stable carbocyanine dye with maximum absorption at 586 nm. Samples of 15 μL were diluted in 30 μL of PBS (10^7 cells mL^{-1}) and then incubated for 30 min at 37°C with 40 μM of Fe^{2+} and 200 μM ascorbate, in order to induce MDA release (Aitken *et al.* 1993). Then, samples were thoroughly mixed with the reagent provided in the kit. The mixture was heated at 45°C for 1 h. Then, the tubes were centrifuged at 10 000g for 10 min at roomtemperature to pellet the precipitate. The clear supernatant was collected and transferred to wells (200 μL well^{-1}) in a 96-well flat-bottom transparent plate (Nunc, Roskilde, Denmark). The plate was completed with a calibration curve prepared using the MDA standard provided in the kit. The plate absorbance at 586 nm was read on a multipurpose microplate reader (Synergy HT; BIO-TEK, Winooski, VT, USA). The MDA concentrations were calculated from the standard curve generated from known quantities of MDA (0, 1, 2, 4, 8 and 16 μM), and presented as nmol of MDA per 10^8 spermatozoa.

Statistical analysis

Data were analysed in the R statistical environment (R Development Core Team 2008). To analyse the effects of time, antioxidant supplement and oxidant effect on sperm parameters, we used linear mixed-effects models, with replicates as the random part of the models. Data are expressed as mean ± s.e.m. unless otherwise stated. Wherever results are expressed as effect size (mean ± s.e.m. of the coefficient), they must be interpreted as the variation (positive or negative) of the given parameter relative to the control treatment.

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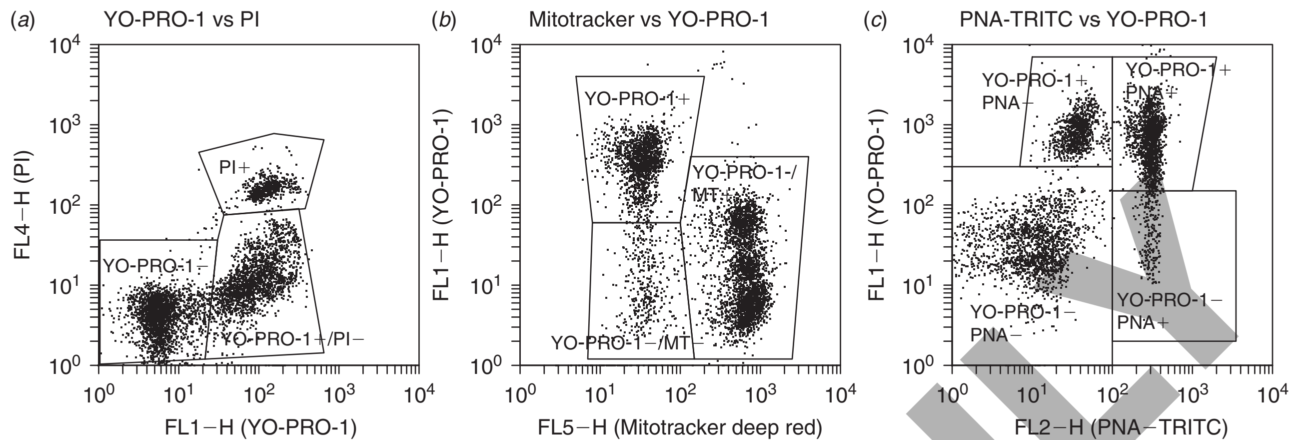

Fig. 1. Cytograms corresponding to (*a*) the YO-PRO-1 (YP)/PI stain and to (*b*, *c*) the YO-PRO-1/PNA-TRITC/Mitotracker deep red (MT) stain. (*a*) Plotting YO-PRO-1 vs PI rendered three subpopulations: YO-PRO-1− (viable), YO-PRO-1+/PI− (increased membrane permeability) and PI+ (membrane damaged, non-viable). (*b*) Plotting Mitotracker deep red vs YO-PRO-1 rendered three subpopulations: Mitotracker+ (viable spermatozoa with high $\Delta\psi_m$), YO-PRO-1−/Mitotracker− (viable spermatozoa with low $\Delta\psi_m$) and YO-PRO-1+ (increased membrane permeability and non-viable spermatozoa). (*c*) Plotting PNA-TRITC vs YO-PRO-1 rendered four subpopulations: YO-PRO-1−/PNA− (viable spermatozoa with intact acrosomes), YO-PRO-1−/PNA+ (viable spermatozoa with damaged acrosome), YO-PRO-1+/PNA− (increased membrane permeability and non-viable spermatozoa with intact acrosomes) and YO-PRO-1+/PNA+ (increased membrane permeability and non-viable spermatozoa with damaged acrosomes). The MT+ [YP−], PNA− [YP−] and PNA− [YP+] rates shown in Tables 2 and 4 were obtained as percentage rates of these subpopulations. MT+ [YP−] is the rate of YO-PRO-1−/MT− per YO-PRO-1− (sum of YO-PRO-1−/MT− and YO-PRO-1−/MT+) from (*b*). PNA− [YP−] is the rate of PNA−/YO-PRO-1− per YO-PRO-1−, and PNA− [YP+] is the ratio of PNA−/YO-PRO-1+ per YO-PRO-1+ from (*c*).

Results

Effect of antioxidants on the motility and physiology of thawed spermatozoa

Sperm physiology was clearly affected by several of the antioxidant treatments tested, even in the absence of exogenous oxidative stress. Motility results without induced oxidative stress are summarised in Table 1. Incubation time decreased the percentage of motile spermatozoa and lowered the values of kinematic parameters. Antioxidants did not affect this trend except for crocin, which stimulated motility. Crocin at 1 mM maintained the percentage of motile spermatozoa, VCL and ALH at 2 h ($P > 0.05$, compared with 0 h), and significantly increased motility, VCL and LIN at 4 h. Conversely, 0.1 mM crocin depressed motility, both at 2 h and 4 h, compared with the respective controls. The other antioxidants had some decreasing effects on motility parameters (generally, reducing LIN). Although significant, these effects were small.

The fluorescent probes did not show large changes in the samples incubated without oxidant (Table 2). There were not significant changes in YO-PRO-1− (viable) and YO-PRO-1+/PI− (increased membrane permeability) proportions, neither along time nor among treatments. Nevertheless, we could observe a slight decrease in both of them with time, which reflected a significant increase of membrane-damaged spermatozoa (PI+, not shown in the table) at 2 and 4 h (from $11.7 \pm 0.7\%$ at 0 h to $16.3 \pm 0.8\%$ at 2 h, $P = 0.028$, and $21.2 \pm 2.1\%$ at 4 h, $P < 0.001$). The percentage of spermatozoa with active mitochondria (high-$\Delta\psi_m$) and the rate of spermatozoa with intact acrosomes within the viable population (PNA− [YP−] in Table 2) underwent little changes along time and between treatments. However, the ratio of spermatozoa with intact acrosomes within the YO-PRO-1+ population (PNA− [YP+]) dropped at 2 h.

Effect of antioxidants on the physiology of thawed spermatozoa submitted to oxidative stress

The application of oxidative stress depressed sperm motility (Table 3), halving the percentage of motile sperm at 2 h and almost reducing it to the fifth part at 4 h (compared with the control samples of non-oxidised samples). Oxidative stress not only decreased the numbers of motile spermatozoa, but also their kinematic parameters. However, 1 mM crocin cushioned that decrease at 2 h (not significant differences among non-oxidised and oxidised samples for total motility, and improved results for VCL and ALH). Moreover, at 2 and 4 h, results were higher than the respective controls. Trolox also partially counteracted the effect of oxidative stress on motility (0.1 mM at 2 h, and 0.1 and 1 mM at 4 h). Samples with 1 mM Trolox at 2 h and melatonin tended to a higher total motility, but results were not significant ($P < 0.1$).

The fluorescent probes showed that oxidative stress modified sperm physiology (Table 4). YO-PRO-1− decreased at 4 h (effect size respect to control at 0 h: -9.4 ± 3.6, $P = 0.014$), whereas antioxidant supplementation could not improve that effect. YO-PRO-1+/PI− dropped as early as 2 h (effect size respect to control: -17.0 ± 3.4 at 2 h, $P < 0.001$; -10.5 ± 3.3 at 4 h, $P = 0.003$). Consequently, PI+ spermatozoa increased with time (effect size respect to control at 0 h: 18.8 ± 2.1 at

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Table 1. Sperm motility (mean ± s.e.m.) in samples not submitted to oxidative stress, and supplemented with each antioxidant-dose combination
Letters indicate significant differences ($P < 0.05$) between times for the control treatment. Asterisks indicate significant differences between the treatment and its control within each given time ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

Time	Antioxidant	Conc. (mM)	Motile (%)	VAP (μm s^{-1})	LIN (%)	ALH (μm)
0 h	Control		90 ± 6.6^{a}	88.4 ± 16^{a}	55.2 ± 3.5^{a}	3.9 ± 0.6^{a}
2 h	Control		63.2 ± 13.1^{b}	58.5 ± 5.8^{b}	52.8 ± 5.7^{a}	3.1 ± 0.3^{b}
	Lipoic acid	0.1	57.6 ± 16	53.2 ± 9.5	47.8 ± 3.4*	3 ± 0.2
		1.0	55.1 ± 13.6	45.6 ± 9.5***	48.7 ± 7.7*	2.7 ± 0.5
	Crocin	0.1	49.7 ± 10.8*	51.1 ± 3	44.6 ± 3.3***	3.3 ± 0.3
		1.0	86.9 ± 7.8***	84.1 ± 10.8***	55 ± 3.5	3.7 ± 0.6***
	Melatonin	0.1	60.2 ± 13.3	59.6 ± 8.6	53.9 ± 6.1	3 ± 0.2
		1.0	61.7 ± 8	54.2 ± 4.5	50.5 ± 4.5	3.1 ± 0.3
	Trolox	0.1	65.3 ± 14.4	59 ± 8.8	48.5 ± 4.4**	3.4 ± 0.2*
		1.0	63.1 ± 15.4	56.4 ± 7.7	48.2 ± 4.7**	3.3 ± 0.2
4 h	Control		52.6 ± 5.8^{c}	45.6 ± 1.9^{c}	44.1 ± 1.7^{b}	2.9 ± 0.1^{b}
	Lipoic acid	0.1	49.8 ± 10.8	43.6 ± 8.1	42.7 ± 2.9	2.9 ± 0.4
		1.0	49.8 ± 12.8	39.5 ± 3.9*	40.9 ± 2.5**	2.8 ± 0.2
	Crocin	0.1	40.6 ± 6.7*	41.7 ± 2.7	42.1 ± 2.7	2.9 ± 0.2
		1.0	74.9 ± 12.2***	70 ± 8.1***	54.6 ± 2***	3.1 ± 0.2
	Melatonin	0.1	50.1 ± 9.9	44.4 ± 7.4	42.3 ± 4.4	3 ± 0.2
		1.0	49.2 ± 9.4	42.8 ± 4	42 ± 2.8*	2.9 ± 0.1
	Trolox	0.1	49.6 ± 11.4	45.3 ± 5.9	43.3 ± 3.2	3 ± 0.3
		1.0	51.7 ± 12.1	43.3 ± 5.4	41.2 ± 3**	3 ± 0.2

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2 h, $P < 0.001$; 21.0 ± 2.0 at 4 h, $P < 0.001$). However, 1 mM crocin and both concentrations of Trolox maintained YO-PRO-1+/PI− proportions both at 2 and 4 h, therefore keeping the PI+ subpopulation low.

The absolute percentage of high-$\Delta\psi_m$ spermatozoa (58.2% ± 4.4 at 0 h, not shown in Table 4) decreased slowly with time (effect size respect to control at 4 h: −6.3 ± 2.7, $P = 0.028$). Crocin and lipoic acid at 1 mM and Trolox decreased slightly the proportion of high-$\Delta\psi_m$ spermatozoa within the viable population (MT+ [YP−] in Table 4). In fact, we found that 1 mM crocin was the only antioxidant that caused a real (although small) decrease in the absolute proportion of high-$\Delta\psi_m$ spermatozoa in the non-oxidised samples (effect size respect to control: −3.9 ± 1.5 at 2 h, $P = 0.056$; −3.8 ± 1.4 at 4 h, $P = 0.009$). In the case of Trolox and lipoic acid, the decrease of MT+ [YP−] was caused by a marginal, non-significant, increase of YO-PRO-1− spermatozoa, while maintaining the absolute proportion of high-$\Delta\psi_m$ spermatozoa.

Acrosomal status was considerably affected by oxidative stress. The percentage of spermatozoa with intact acrosomes dropped from 92.9 ± 1.9% at 0 h to 65.5 ± 2.7% at 2 h and 61.2 ± 3.2% after 4 h (effect at 4 h: −31.7 ± 3.4 vs effect in non-oxidised samples: −18.2 ± 3.9). Similarly to the non-oxidised samples, most of this decrease occurred in the YO-PRO-1+ subpopulation, which presented a decrease of acrosome-intact spermatozoa from 81.9 ± 4.5% at 0 h to 18.1 ± 1.0% at 2 h (Table 4, PNA− [YP+]), while the YO-PRO-1− subpopulation showed a high proportion of acrosome-intact spermatozoa. Interestingly, 1 mM crocin and Trolox decreased slightly the percentage of acrosome-intact spermatozoa in the YO-PRO-1− subpopulation, while considerably increasing that percentage in the YO-PRO-1+ subpopulation. Trolox at 1 mM achieved the higher effect at 4 h on protecting the acrosomes of YO-PRO-1+ spermatozoa.

Effect of antioxidants on the level of lipid peroxidation, intracellular ROS and DNA damage of thawed spermatozoa submitted to oxidative stress

Fig. 2 displays the results for intracellular ROS, lipoperoxidation (MDA production) and TUNEL assays. In general, MDA levels and the percentage of TUNEL+ spermatozoa mimicked ROS levels, with some interesting exceptions. In the absence of oxidative stress, intracellular ROS spontaneously increased from 0 h to 4 h ($P = 0.035$), and all antioxidants significantly reduced the CM-H_2DCFDA signal below control levels, except 1 mM crocin. When submitted to oxidative stress, the CM-H_2DCFDA signal increased five-fold. In these conditions, only 1 mM crocin, 0.1 mM Trolox and 1 mM Trolox significantly reduced the CM-H_2DCFDA signal ($P < 0.001$), remaining close to the non-oxidised values.

MDA levels followed ROS values in the non-oxidised samples. Control at 0 h yielded 10.8 ± 1.2 nmol of MDA per 10^8 spermatozoa, which increased non significantly after 4 h (14.6 ± 2.5, $P = 0.145$). Antioxidants significantly reduced MDA levels in non-oxidised samples, except 1 mM lipoic acid and 0.1 mM melatonin, whilst 1 mM crocin increased MDA (19.3 ± 1.5 nmol of MDA per 10^8 spermatozoa, $P < 0.001$). In oxidised samples, MDA levels increased considerably (22.4 ± 2.5 nmol of MDA per 10^8 spermatozoa in control at 4 h, $P < 0.001$). Crocin at 1 mM, despite abolishing intracellular ROS, did not show lower MDA levels. Conversely, both

Table 2. Sperm physiology as assessed by flow cytometry (mean ± s.e.m., units are %) in samples not submitted to oxidative stress, and supplemented with each antioxidant-dose combination

YP−, % of YO-PRO-1− spermatozoa (viable); YP+/PI−, % of YO-PRO-1+/PI− spermatozoa (increased membrane permeability); MT+ [YP−], % of high-$\Delta\psi_m$ (active mitochondria) spermatozoa within the YO-PRO-1− population; PNA− [YP−], % of PNA-TRITC− spermatozoa (acrosome intact) within the YO-PRO-1− population; PNA− [YP+], % of PNA-TRITC− spermatozoa within the YO-PRO-1+ population. Letters indicate significant differences ($P < 0.05$) between times for the control treatment. Asterisks indicate significant differences between the treatment and its control within each given time (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Time	Antioxidant	Conc. (mM)	YP−	YP+/PI−	MT+ [YP−]	PNA− [YP−]	PNA− [YP+]
0 h	Control		51.1 ± 5.9	35.8 ± 5.3	93.7 ± 2.4[a]	99.2 ± 0.7	81.9 ± 11.9[a]
2 h	Control		51.7 ± 6.7	31.5 ± 5.3	91.1 ± 3.3[b]	93.1 ± 4.5	45.5 ± 11.9[b]
	Lipoic acid	0.1	52.7 ± 6.6	30 ± 6	89.8 ± 4.4*	92 ± 5.2	45.5 ± 12.4
		1.0	50.4 ± 6.1	30.3 ± 4.2	91.4 ± 2.1	92.2 ± 4.6	44.5 ± 9.9
	Crocin	0.1	53.8 ± 7	28.8 ± 7.5	90 ± 3.4	89.3 ± 12.8	45.5 ± 11.5
		1.0	49.5 ± 7.3	29.2 ± 7.7	89.8 ± 2.4	88.4 ± 14.9	43.7 ± 10.5
	Melatonin	0.1	52.2 ± 6.3	31.2 ± 4.3	90.9 ± 2.8	89.5 ± 13.4	46.8 ± 9.8
		1.0	52.9 ± 4.6	30.1 ± 3.7	89.7 ± 2.4	89.5 ± 11.5	44.6 ± 9.5
	Trolox	0.1	53.9 ± 7.8	27.9 ± 8.2	91.1 ± 2.5	89.6 ± 13.5	48.5 ± 12
		1.0	55.4 ± 4.8	27.3 ± 6.6	91 ± 2.3	90 ± 13.1	46.3 ± 11.9
4 h	Control		45.7 ± 8.7	32.2 ± 4.5	91.6 ± 2.9[ab]	86 ± 18.1	56.3 ± 4.9[c]
	Lipoic acid	0.1	48.7 ± 4.9	32.4 ± 4.1	88.7 ± 5*	85.4 ± 18.3	54 ± 8.1
		1.0	49 ± 5.7	30.6 ± 3.2	89.9 ± 3	85.8 ± 17.2	51.8 ± 11.7*
	Crocin	0.1	48.8 ± 8.1	31.9 ± 8.1	90.8 ± 2.5	85.1 ± 18.8	53.7 ± 11.8
		1.0	44.8 ± 10.9	31.7 ± 9.3	89.8 ± 2.7	85.1 ± 18.4	49.4 ± 8.9**
	Melatonin	0.1	44.9 ± 8.3	33.2 ± 4.1	91.5 ± 2.7	85.7 ± 17.4	54.2 ± 7
		1.0	45.4 ± 7.3	33.2 ± 7.3	91 ± 2.3	85.3 ± 17.4	54.1 ± 6.4
	Trolox	0.1	46.9 ± 5.5	31.1 ± 4	89.8 ± 3	84.7 ± 18.8*	55.9 ± 8.8
		1.0	48.8 ± 5.4	30.7 ± 5	89.4 ± 2.5*	85.8 ± 17.1	55.6 ± 11.1

Table 3. Sperm motility (mean ± s.e.m.) in samples submitted to oxidative stress (control at 0 h excepted), and supplemented with each antioxidant-dose combination

Letters indicate significant differences ($P < 0.05$) between times for the control treatment. Asterisks indicate significant differences between the treatment and its control within each given time (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Time	Antioxidant	Conc. (mM)	Motile (%)	VAP ($\mu m\,s^{-1}$)	LIN (%)	ALH (μm)
0 h	Control		90 ± 6.6[a]	88.4 ± 16[a]	55.2 ± 3.5[a]	3.9 ± 0.6[a]
2 h	Control		33.1 ± 15.8[b]	38.1 ± 12.9[b]	56.9 ± 7[a]	1.9 ± 0.2[b]
	Lipoic acid	0.1	34.2 ± 29.5	38.2 ± 21.1	56.2 ± 11	1.8 ± 0.4
		1.0	25 ± 20	31.5 ± 19.9	51.6 ± 15.7	1.6 ± 0.4
	Crocin	0.1	34.3 ± 13.3	34.1 ± 7.9	52.9 ± 5**	1.9 ± 0.2
		1.0	78.2 ± 8.8***	65.7 ± 9.5***	65.9 ± 3.7	2.2 ± 0.1**
	Melatonin	0.1	45 ± 25.3	43.4 ± 17	59.7 ± 9.8	1.9 ± 0.3
		1.0	42.6 ± 18.5	40.5 ± 11.8	59.5 ± 8.3	1.9 ± 0.2
	Trolox	0.1	53.7 ± 25.3**	44.8 ± 18.6	55.8 ± 11.5	2.1 ± 0.3*
		1.0	44.5 ± 16	40.3 ± 10.9	53.7 ± 7.3	2.1 ± 0.3*
4 h	Control		12.9 ± 7.9[c]	18.5 ± 7.3[c]	39.4 ± 9[b]	1.5 ± 0.2[b]
	Lipoic acid	0.1	18.4 ± 15.9	20.6 ± 10.7	45.8 ± 12	1.5 ± 0.2
		1.0	16.1 ± 13.7	18.2 ± 10.9*	42.1 ± 10.6	1.6 ± 0.7
	Crocin	0.1	13.6 ± 4.9	16.7 ± 7.3	37 ± 7.8	1.6 ± 0.4
		1.0	35.5 ± 9***	28.5 ± 10.4***	51.1 ± 9.6**	1.6 ± 0.2
	Melatonin	0.1	22.3 ± 14.6	18.7 ± 11.9	41.5 ± 10.1	1.5 ± 0.3
		1.0	20.6 ± 11	18.2 ± 8.3	40.4 ± 6.9	1.6 ± 0.3
	Trolox	0.1	24.2 ± 11.6*	23.5 ± 12.8	40.8 ± 9.8	1.8 ± 0.4
		1.0	23.9 ± 9.3*	21 ± 8.9	38.9 ± 7.6	1.8 ± 0.3

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Table 4. Sperm physiology as assessed by flow cytometry (mean ± s.e.m., units are %) in samples submitted to oxidative stress (control at 0 h excepted), and supplemented with each antioxidant-dose combination

YP−, % of YO-PRO-1− spermatozoa (viable); YP+/PI−, % of YO-PRO-1+/PI− spermatozoa (increased membrane permeability); MT+ [YP−], % of high-$\Delta\psi_m$ (active mitochondria) spermatozoa within the YO-PRO-1− population; PNA− [YP−], % of PNA-TRITC− spermatozoa (acrosome intact) within the YO-PRO-1− population; PNA− [YP+], % of PNA-TRITC− spermatozoa within the YO-PRO-1+ population. Letters indicate significant differences ($P < 0.05$) between times for the control treatment. Asterisks indicate significant differences between the treatment and its control within each given time ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

Time	Antioxidant	Conc. (mM)	YP−	YP+/PI−	MT+ [YP−]	PNA− [YP−]	PNA− [YP+]
0 h	Control		51.1 ± 5.9^{a}	35.8 ± 5.3^{a}	93.7 ± 2.4	99.2 ± 0.7	81.9 ± 11.9^{a}
2 h	Control		51.3 ± 6.7^{ab}	16.8 ± 7.1^{b}	93 ± 2.9	96.8 ± 1.7	18.1 ± 2.4^{b}
	Lipoic acid	0.1	50.2 ± 6	18 ± 6.8	92.1 ± 2.9	96.7 ± 1.6	21.8 ± 6.5
		1.0	48.2 ± 8.1	21.2 ± 5.7	91.3 ± 2.9	96.4 ± 1.5*	24.9 ± 7.8**
	Crocin	0.1	51.9 ± 7.2	18.2 ± 7.5	92.9 ± 2.1	96.3 ± 1.7	22.2 ± 8
		1.0	48 ± 5.5*	29.9 ± 6.5***	88 ± 3.3***	92.1 ± 3.8***	39.5 ± 5.1***
	Melatonin	0.1	52.5 ± 6.5	17.9 ± 6.6	92.6 ± 2.4	96.5 ± 2.2	20.1 ± 4.4
		1.0	52.5 ± 6	19.8 ± 7.5	92.7 ± 2.6	96.2 ± 2	22 ± 4.1
	Trolox	0.1	52 ± 6.3	28.5 ± 7.9***	90.2 ± 2.8**	93.6 ± 2.7***	42.1 ± 3.4***
		1.0	52.7 ± 6.9	27.4 ± 7.4***	90.3 ± 3.2**	94 ± 2.7***	41.8 ± 3.5***
4 h	Control		41.8 ± 11^{b}	25.5 ± 8.4^{b}	91.3 ± 3.8	90.9 ± 12.7	22.3 ± 4.4^{b}
	Lipoic acid	0.1	43.4 ± 7.8	22.5 ± 4	91.1 ± 2.5	90 ± 13.3	21.9 ± 3.4
		1.0	43.4 ± 8.2	22.4 ± 4.4	88 ± 3.7**	95.2 ± 3.5	28.5 ± 7.5*
	Crocin	0.1	42.9 ± 8	23.3 ± 1.9	91.1 ± 2.5	90 ± 14.6	26.7 ± 6.5
		1.0	40.4 ± 7.2	34.7 ± 8.9***	84.8 ± 4.3***	84.3 ± 15.7***	37.9 ± 3.3***
	Melatonin	0.1	43.6 ± 6.8	22.3 ± 2.8	89.1 ± 5.6	90 ± 14	26.7 ± 10.1
		1.0	44.1 ± 8.7	23 ± 3.7	89.9 ± 4.3	89 ± 13.3	26.9 ± 5.3
	Trolox	0.1	44.4 ± 7	32.9 ± 7.4**	87 ± 4.4***	86 ± 15.9***	43.9 ± 10.2***
		1.0	45.5 ± 6.5	31.9 ± 7.4**	86.9 ± 3.4***	86.9 ± 13.6***	50.7 ± 9.2***

Trolox concentrations lowered MDA yield to values close to control at 0 h (pooled concentrations: 13.9 ± 1.3 mmol of MDA per 10^6 spermatozoa). Although less effective, 1 mM melatonin significantly lowered MDA levels (effect size: −3.5 ± 1.8, $P = 0.049$).

Concerning DNA damage, the percentage of TUNEL+ spermatozoa was low at 0 h (4.0 ± 1.0%), and it did not significantly vary after 4 h, with or without antioxidant supplement. Oxidative stress caused an increase of TUNEL+ spermatozoa (23.0 ± 5.0%, $P < 0.001$). Crocin at 1 mM and the two Trolox concentrations prevented the increase of TUNEL+ spermatozoa ($P < 0.001$), yielding a proportion of TUNEL+ spermatozoa close to that at 0 h.

Study of an extended concentration range for crocin and melatonin

Figs 3 and 4 summarise the results of the study on extended concentration ranges for crocin and melatonin. Intermediate crocin concentrations in the 0.1–2 mM range yielded results similar to those previously reported, presenting a turning point around 0.5 mM. As previously observed, crocin stimulated motility above control results, both in the presence and absence of oxidative stress. In oxidised and non-oxidised samples total motility increased with crocin concentration, possibly starting by 0.5 mM ($P < 0.1$), and being significant at 0.75 mM and higher. In the case of oxidised samples, 0.75 mM and above practically abolished the effect of the oxidant treatment. VAP and LIN similarly increased with crocin concentration, and ALH rose slightly at 1 and 2 mM only in the oxidised samples.

When confronted with exogenous oxidative stress, crocin at 0.25 mM and above significantly decreased intracellular ROS. However, in the absence of oxidative stress, crocin tended to increase fluorescence values, which were significantly higher than control in the 0.75–2 mM range. Lipid peroxidation was not improved by any crocin concentration, and MDA values were significantly higher when crocin was added at 2 mM (a linear increase with increasing crocin concentration was detected for the non-oxidised samples, $P < 0.001$). Nevertheless, crocin was very effective at abolishing the effect of the oxidative stress on DNA, with 0.5 mM being enough to maintain samples close to the non-oxidised control.

Increasing melatonin concentration was positive overall for sperm quality. For non-oxidised samples, any concentration reduced intracellular ROS slightly ($P < 0.05$), but in oxidised samples, 2.5 mM reached the lowest results, with no apparent improvement with 5 mM. Nevertheless, only 5 mM increased total motility in oxidised samples (although neither VAP, LIN or ALH improved significantly), although it decreased LIN in non-oxidised samples (that effect was observed at 1 mM in the first trial, Table 1). Melatonin at 2.5 and 5 mM reduced MDA in oxidised samples to the levels of the non-oxidised control. The 5-mM concentration had the added benefit of significantly decreasing MDA in the non-oxidised samples. DNA damage in oxidised samples linearly decreased with melatonin concentration, reaching the non-oxidised control levels at 2.5 mM ($P < 0.05$).

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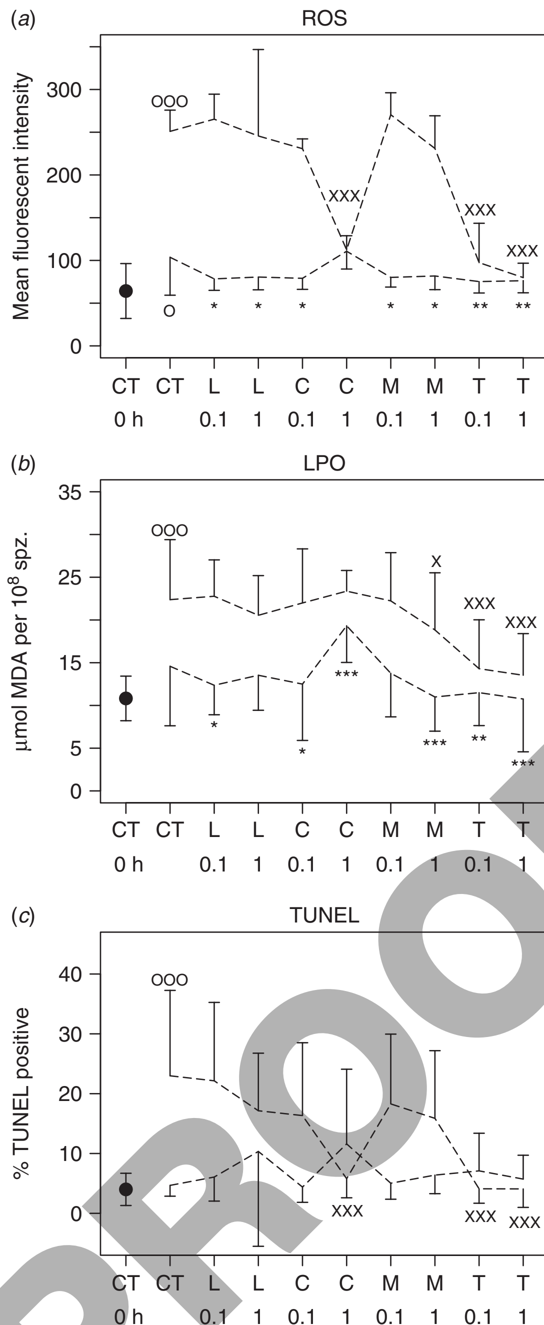

Fig. 2. Intracellular ROS levels in viable (TO-PRO-1−) spermatozoa (according to CM-HDCFDA stain; top), lipid peroxidation (LPO, as malondialdehyde (MDA) production; middle) and DNA damage (proportion of TUNEL+ spermatozoa; bottom). Axis labels correspond to the controls at 0 h (CT 0 h) and at 4 h (CT), and to the antioxidant treatments at 4 h (L, lipoic acid; C, crocin; M, melatonin; T, Trolox, at 0.1 and 1 mM). The solid line shows mean values for non-oxidised samples, whereas the dashed line shows mean values for samples submitted to oxidative stress, with vertical lines showing 1 s.e.m. Significant differences between controls (0 h vs 4 h) are shown as ∘ ($P < 0.05$) and ∘∘∘ ($P < 0.001$). Significant differences between the control at 4 h and treatments are shown as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) for the non-oxidised samples, and as × ($P < 0.05$) and ××× ($P < 0.001$) for the oxidised samples.

Discussion

The antioxidant concentrations used in the present study were chosen after bibliographical research and our own experience using Trolox. Concentrations in the order of magnitude of 0.1 mM seem to render good results (Gavella and Lipovac 2000; Peña *et al.* 2003, 2004). Lower concentrations were not considered, since we found that 10 µM Trolox, although being able to reduce intracellular ROS, could not prevent the effects of oxidative stress (Martínez-Pastor *et al.* 2009*a*). We obtained only a slight improvement in a previous study using 0.1 mM of Trolox (Martínez-Pastor *et al.* 2008), and other antioxidants might be less effective than Trolox (Gavella and Lipovac 2000). Thus, as a first approach, we chose 1 mM as a secondary concentration, which would yield more pronounced effects in all antioxidants, as shown previously (Domínguez-Rebolledo *et al.* 2009). The results showed a great variability among antioxidants. Whereas Trolox and 1 mM crocin yielded very promising results, melatonin effects were quite limited, and lipoic acid was mostly ineffective.

The results of lipoic acid (LA) were disappointing, considering previous studies in other cell types (Navari-Izzo *et al.* 2002; Bast and Haenen 2003). LA can be reduced to the more potent dihydrolipoic acid (DHLA) (Navari-Izzo *et al.* 2002), which regenerates the stock of reduced antioxidants. However, it is known that low concentrations of DHLA can recycle Fe^{2+} (Scott *et al.* 1994), which could explain the lack of effect in the samples submitted to oxidative stress. Due to its multi-functional nature, it is possible that the benefits of LA could be expressed in more concrete experimental settings beyond the purpose of the present study, or in more complex media formulations, such as those used for IVF. Direct addition of DHLA could be a suitable option for future experiments, since that strategy would not rely on the ability of sperm cells to convert LA to DHLA, but media formulation must take into account that metal cations (e.g. iron or copper) must be absent. Supplementing the medium with several antioxidants (such as reduced glutathione), in order to achieve a synergistic effect, could be a sensible approach.

Similarly, melatonin at 0.1 or 1 mM did not improve sperm status. However, 1 mM melatonin caused a discrete reduction of lipid peroxidation, and, when we used higher concentrations, melatonin showed a high ROS-scavenging activity, reflected in reduced lipoperoxidation and DNA damage results. These results agree with Gavella and Lipovac (2000), who, using human spermatozoa, observed a reduction in the lipid peroxidation induced using a ferrous–ascorbate model after adding 2–6 mM of melatonin. These authors estimated that melatonin was 40-fold more efficient than Trolox in achieving the same reduction of lipid peroxidation. In the present study, we found a similar ratio (above 50-fold) for the 0.1-mM concentrations, considering the reduction from control levels, whereas for the 1-mM concentrations this ratio was ∼2.5. Effectively, at 2.5 mM the reduction of MDA production was roughly equivalent to that achieved by 1 mM Trolox. ROS scavenging and TUNEL results at 2.5–5 mM support this trend, showing that melatonin can be an effective antioxidant. Therefore, we suggest using melatonin concentrations in the millimolar range, and approaching the saturating concentration in water. Nevertheless, other authors have

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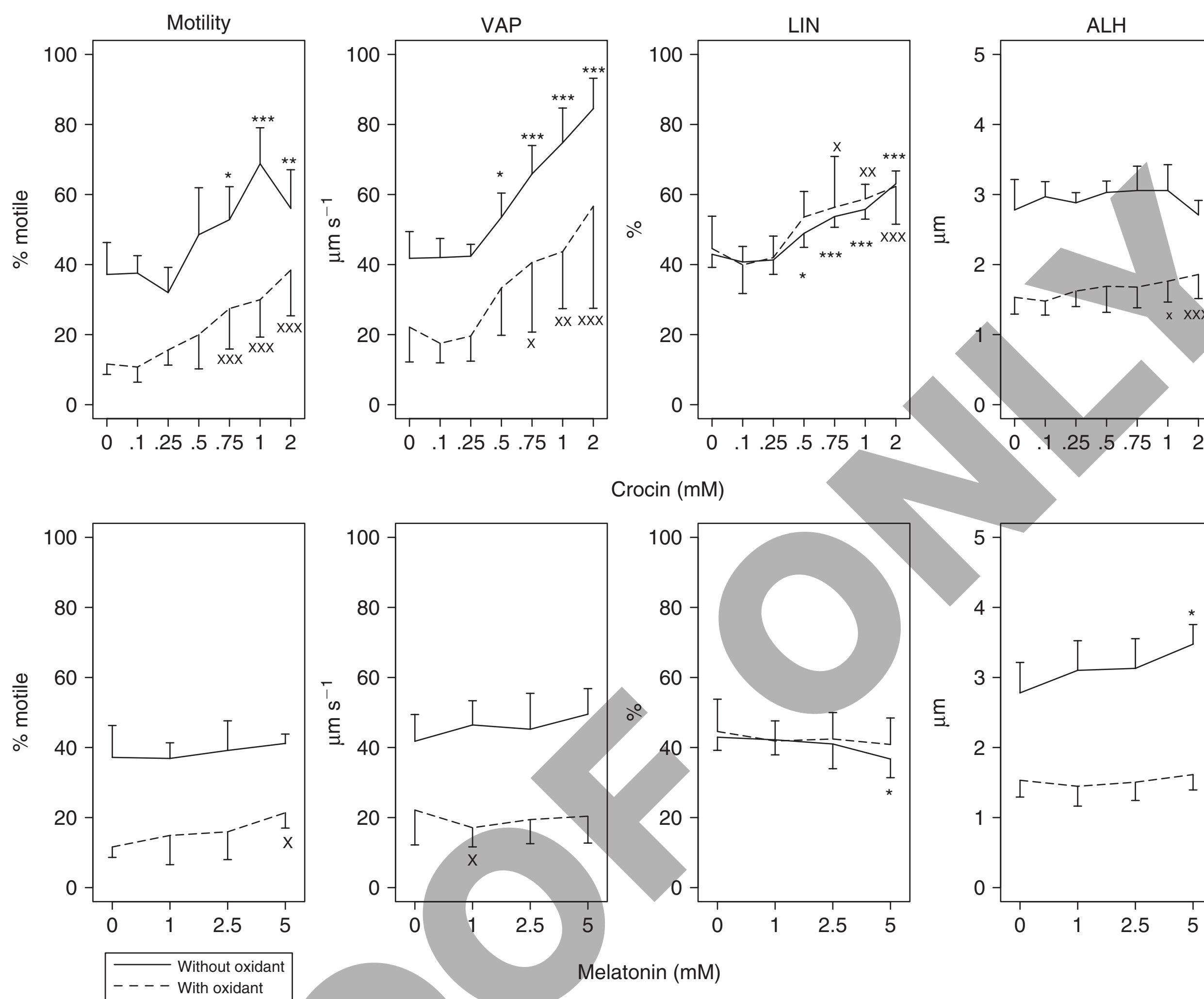

Fig. 3. Effect of extended concentration ranges of crocin (top) and melatonin (bottom) on motility. The solid lines show mean results for non-oxidised samples, and the dashed lines show mean results for oxidised samples. Vertical lines show 1 s.e.m. Significant differences between the control at 4 h (0) and antioxidant concentrations are shown as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) for the non-oxidised samples, and as × ($P < 0.05$), ×× ($P < 0.01$) and ××× ($P < 0.001$) for the oxidised samples.

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noted positive effects of melatonin at lower doses. For instance, Rao and Gangadharan (2008) found that 0.1 mM melatonin was enough to protect rat spermatozoa from stress induced by mercury. Recently, Jang *et al*. (2009) showed that the treatment of boar spermatozoa with that same concentration of melatonin protected them from added H_2O_2, and improved the developmental ability of IVM–IVF embryos. These results indicate that melatonin effects might greatly depend on the experimental conditions and the type of sample.

Trolox was the most effective antioxidant in removing ROS and preventing lipoperoxidation and DNA damage. Previous results showed that 10 μM Trolox was effective in reducing intracellular ROS levels and lipid peroxidation of red deer spermatozoa submitted to 100 μM Fe^{2+}, 1 mM H_2O_2 or 100 mU mL^{-1} xanthine oxidase (with 2 mM hypoxanthine), although this reduction was only partial, and it could not protect sperm motility or viability (Martínez-Pastor *et al.* 2009*a*). Therefore, it seems that the concentration at which Trolox reached an optimal degree of protection for our samples was 0.1 mM, achieving little advantage when increased 10-fold. The results of this experiment seem to contradict previous trials using Trolox as an antioxidant for freezing epididymal deer spermatozoa (Fernández-Santos *et al.* 2007). In that experiment, 6.4 mM Trolox yielded lower-quality results after thawing, and 3.2 mM showed a decrease after 2 h of incubation at 37°C. However, the action of Trolox during freezing–thawing might be different from that during post-thawing incubation, and Trolox concentrations were much higher in that experiment. Cao and Cutler (1993) indicated that Trolox could have a dual effect, acting as a pro-oxidant at high concentrations and given some conditions.

Donnelly *et al*. (1999*b*) showed that a combination of ascorbic acid and α-tocopherol could induce DNA damage in human spermatozoa. The same authors (Donnelly *et al.* 1999*a*), working in the 20–60 μM range, showed that increasing α-tocopherol concentrations depressed motility. Nevertheless, in these same studies the authors showed that α-tocopherol alone both reduced ROS production and DNA damage. Other studies suggested that α-tocopherol had little effect on ram (Upreti *et al.* 1997) or human (Askari *et al.* 1994) spermatozoa (although globally they found positive effects). However, these studies focussed on sperm motility, a parameter that does not reflect the protective action of Trolox except in stringent conditions, as shown in the present study. Other studies have supported the effectiveness of α-tocopherol in improving the lipoperoxidation status and the general quality of spermatozoa (Peña *et al.* 2003, 2004).

Crocin, a carotenoid tested for the first time on spermatozoa, showed a high efficacy at the 1-mM concentration, reducing intracellular ROS and protecting sperm DNA in a manner comparable to 0.1 mM Trolox. Nevertheless, several details merit further analysis. First of all, and despite its demonstrated ability as a radical scavenger, crocin could not prevent the rise of MDA levels when sperm samples were submitted to oxidant stress. Contrarily, in the absence of that stress, 1 mM crocin not only did not decrease ROS below 4-h control levels (which was attained by the rest of the antioxidant treatments), but also increased MDA levels. This effect was also noticed in the second experiment, when 2 mM yielded a significantly higher result both for oxidised and non-oxidised samples. Although the MDA increase was accompanied by an increment of the ROS readings in the non-oxidised samples, we noticed a gradual ROS decrease in the oxidised samples. Therefore, we checked if it was due to an interference of the antioxidant with the analytical techniques (crocin, even at low concentrations, yields a patent yellow-orange colour to the solutions), but we could not find such an effect. Whether crocin is causing an increase in lipoperoxidation or if the results are due to artefacts should be taken into account in future studies, using more accurate techniques (e.g. mass spectometry).

A surprising result from crocin in the first trial was the notable stimulation of motility at 1 mM, both in the absence and presence of oxidative stress, whereas 0.1 mM seemed to cause a transient decrease of motility at 2 h in non-oxidised samples. We confirmed this effect in the second trial, which suggested that this stimulatory effect could take place at around 0.5 mM. It seems that crocin would stimulate sperm metabolism, and the ROS results seem to support this explanation. In fact, we observed a linear ROS increase in non-oxidised samples with increasing crocin concentration. In fact, despite the fact that crocin decreased ROS in oxidised samples, it was not as efficient as Trolox or melatonin at the same concentrations, and we hypothesise that this could be due to *de novo* production of ROS induced by crocin. Other authors have found that sperm motility is regulated by kinase phosphorylation (Vijayaraghavan *et al.* 1996; González-Fernández *et al.* 2009), and that these pathways, in which endogenously generated ROS act as second messengers, are involved in sperm capacitation and hyperactivation (de Lamirande and O'Flaherty 2008). In the present study, we did not find that any treatment caused sperm hyperactivation. Rather, crocin kept linearity high and it did not caused an important increase in ALH (a sharp decrease of linearity and rise of ALH would have suggested hyperactivation; Mortimer and Maxwell 1999). Other authors have pointed out that some free radicals, such as nitric oxide, are related to high motility and kinematics in spermatozoa (Ortega Ferrusola *et al.* 2009). Crocin could act through the modulation of the redox balance, resulting in higher motility and possibly in other effects. In fact, the good level of DNA protection of crocin at 0.5 mM and above suggests that the excess of intracellular ROS might not be reaching dangerous levels. The action of crocin must be thoroughly tested, since our results suggest that crocin, and maybe other carotenoids, might affect sperm physiology beyond their protective effect as radical scavengers. Molecular studies, including detection of differential protein phosphorylation and production of specific free radicals, must be carried out, and crocin should be tested in different experimental sets before deciding on its suitability for sperm media.

Crocin at 1 mM tended to decrease, although slightly, the absolute percentage of high-$\Delta\psi_m$ spermatozoa. Other carotenoids have a recognised protective effect on mitochondria (Wolf *et al.* 2009), and crocin itself has been cited as a mitochondrial protector (Venkatraman *et al.* 2008). Nevertheless, it has been shown that carotenoid breakdown products may exert mitochondriotoxicity, inhibiting mitochondrial respiration, impairing cell redox state and increasing malondialdehyde (Siems *et al.* 2009), and other studies have shown that the carotenoid derivatives retinol and retinoic acid had differential effects in the proliferative and redox status of different cell types (Zanotto-Filho *et al.* 2008). It is possible that similar processes were taking place in the crocin-treated samples in the present study (explaining high MDA levels and mitochondrial inactivation), and it must be tested in future studies. In fact, Siems *et al*. (2009) suggested that the negative effect of carotenoid derivatives could be prevented by using additional antioxidants. Nevertheless, it is doubtful that an extensive detrimental effect could be taking place in the present experiment, given the long incubation time (4 h) and the good motility results. A possibility is that a sperm subpopulation could be certainly undergoing negative effects from the crocin supplementation, but another, much larger subpopulation, could be being stimulated.

Finally, two other effects of 1 mM crocin and Trolox in the oxidised samples deserver further comment. The oxidative stress caused a slight decrease of the YO-PRO-1− subpopulation and a noticeable drop of the YO-PRO-1+/PI− subpopulation (whilst increasing PI+ spermatozoa), and it caused a considerable increase in acrosomal damage (in the YO-PRO-1+ spermatozoa, though). Crocin at 1 mM and Trolox prevented both effects. YO-PRO-1+/PI− spermatozoa are cells that maintain plasmalemma integrity, but which present increased YO-PRO-1 permeability. YO-PRO-1 staining has been associated to apoptosis in somatic cells (Idziorek *et al.* 1995), and, while the concept in spermatozoa is still controversial (Grunewald *et al.* 2009), it seems that YO-PRO-1 staining in spermatozoa is related to changes in membrane permeability similar to those associated with apoptosis (Peña *et al.* 2005; Martínez-Pastor *et al.* 2008; Kumaresan *et al.* 2009). In fact, YO-PRO-1+/PI− spermatozoa are able to sustain motility for some time (Martínez-Pastor *et al.* 2008), but that population showed a great sensitivity to oxidative stress, with an

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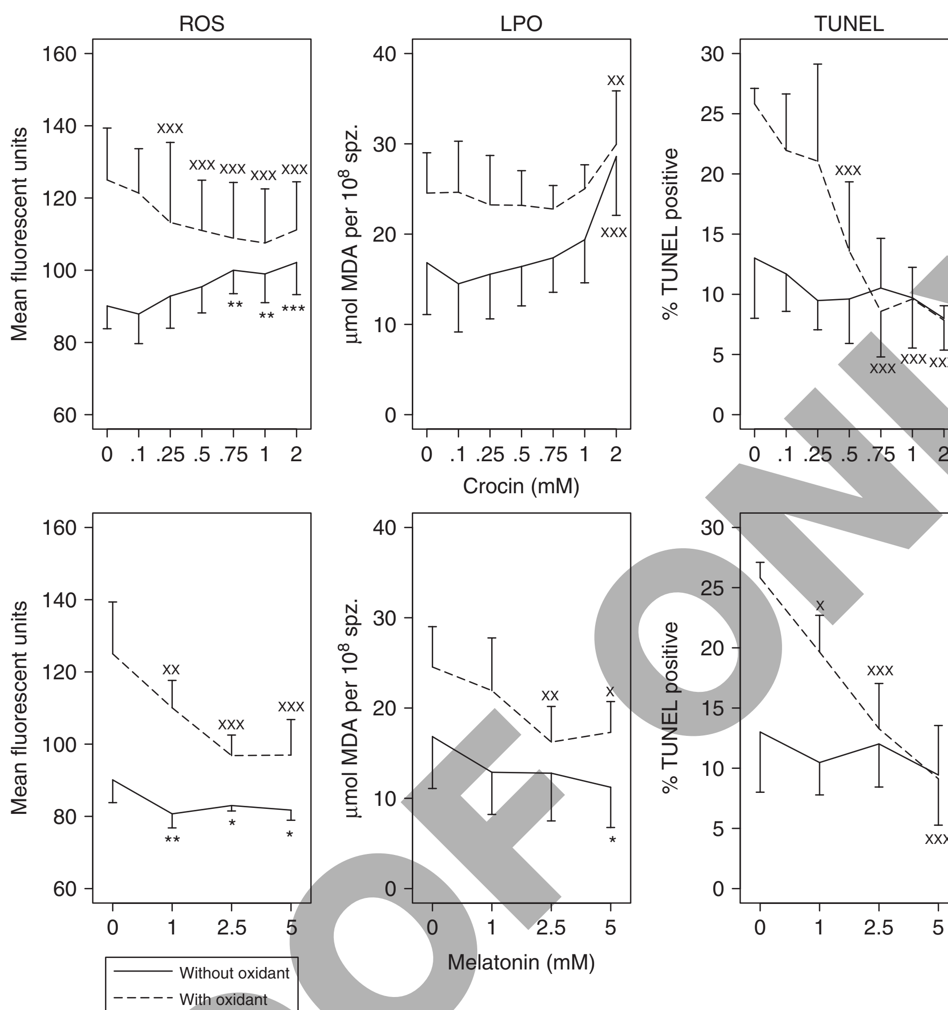

Fig. 4. Effect of extended concentration ranges of crocin (top) and melatonin (bottom) on intracellular ROS levels in viable (TO-PRO-1−) spermatozoa (according to CM-HDCFDA stain; left), lipid peroxidation (LPO, as malondialdehyde (MDA) production; centre) and DNA damage (proportion of TUNEL+ spermatozoa; right). The solid lines show mean results for non-oxidised samples, and the dashed lines show mean results for oxidised samples. Vertical lines show 1 s.e.m. Significant differences between the control at 4 h (0) and antioxidant concentrations are shown as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) for the non-oxidised samples, and as × ($P < 0.05$), ×× ($P < 0.01$) and ××× ($P < 0.001$) for the oxidised samples.

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increased membrane damage rate (a similar effect was observed using Fe^{2+} as oxidising agent; Martínez-Pastor *et al.* 2009*a*). It is possible that this event was related to the widespread acrosomal damage observed in the YO-PRO-1+ subpopulation. High intracellular ROS levels might be involved in these changes. Whereas YO-PRO-1− spermatozoa might cope with redox unbalancing, the YO-PRO-1+ spermatozoa, with an altered physiology, might not be able to control pathways leading to membrane damage or acrosome reaction. We have shown that the mitochondria of YO-PRO-1+ spermatozoa are always inactive (Martínez-Pastor *et al.* 2008), which possibly leads to ATP depletion for housekeeping processes (Silva and Gadella 2006), rendering these cells unable to cope with stressing events. Nevertheless, we must point out that 1 mM crocin and Trolox not only prevented acrosomal damage in the YO-PRO-1+ spermatozoa, but they induced a slight increase of reacted acrosomes in the YO-PRO-1− spermatozoa with oxidative stress. Although we have not estimated the capacitation status of these spermatozoa, it is possible that these differences were due to part of the YO-PRO-1− subpopulation undergoing capacitation and a small number undergoing spontaneous acrosome reaction. If that hypothesis were right, it would suggest that these antioxidants were able to sustain normal physiological pathways in spermatozoa, since the conditions we used were expected to be capacitating.

In conclusion, among the tested antioxidants, only Trolox and 1 mM crocin achieved a high degree of radical scavenging. Melatonin was effective at higher concentrations than Trolox. Lipoic acid might be useful in different conditions, but results

were not promising. Whereas Trolox demonstrated a high protective capacity, crocin was able to considerably enhance sperm motility. Both antioxidants deserve further research in order to fully understand other effects noted in this study, especially regarding their interactions with pathways involving mitochondrial and acrosomal fate. These or similar antioxidants could be candidates for improving sperm manipulation media, either for IVF and similar techniques or for other kind of techniques, such as sex sorting. Of course, the enrichment of post-thawing manipulation media with antioxidants does not preclude that antioxidants could be used to improve collection media and freezing extenders, in order to prevent oxidative damage prefreezing and during the freezing–thawing process. Antioxidants used post-thawing could be useful to prevent further damage, but it is unlikely that they alone could revert any damage caused beforehand.

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References

Acuña-Castroviejo, D., Martín, M., Macías, M., Escames, G., León, J., Khaldy, H., and Reiter, R. J. (2001). Melatonin, mitochondria and cellular bioenergetics. *J. Pineal Res.* **30**, 65–74. doi:10.1034/J.1600-079X.2001.300201.X

Aitken, R. J., and Baker, M. A. (2004). Oxidative stress and male reproductive biology. *Reprod. Fertil. Dev.* **16**, 581–588. doi:10.1071/RD03089

Aitken, R. J., Harkiss, D., Buckingham, D. W. (1993). Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol. Reprod. Dev.* **35**, 302–315. doi:10.1002/MRD.1080350313

Askari, H. A., Check, J. H., Peymer, N., and Bollendorf, A. (1994). Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze–thaw process. *Arch. Androl.* **33**, 11–15. doi:10.3109/01485019408987797

Bast, A., and Haenen, G. R. M. M. (2003). Lipoic acid: a multifunctional antioxidant. *Biofactors* **17**, 207–213. doi:10.1002/BIOF.5520170120

Berlinguer, F., Ledda, S., Rosati, I., Bogliolo, L., Leoni, G., and Naitana, S. (2003). Superoxide dismutase affects the viability of thawed European mouflon (*Ovis g. musimon*) semen and the heterologous fertilization using both IVF and intracytoplasmatic sperm injection. *Reprod. Fertil. Dev.* **15**, 19–25. doi:10.1071/RD02048

Cao, G., and Cutler, R. G. (1993). High concentrations of antioxidants may not improve defence against oxidative stress. *Arch. Gerontol. Geriatr.* **17**, 189–201. doi:10.1016/0167-4943(93)90050-R

Carmona, M., Zalacain, A., Pardo, J. E., López, E., Alvarruiz, A., and Alonso, G. L. (2005). Influence of different drying and ageing conditions on saffron constituents. *J. Agric. Food Chem.* **53**, 3974–3979. doi:10.1021/JF0404748

Cassani, P., Beconi, M. T., and O'Flaherty, C. (2005). Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. *Anim. Reprod. Sci.* **86**, 163–173. doi:10.1016/J.ANIREPROSCI.2004.06.006

Chen, H., Chow, P. H., Cheng, S. K., Cheung, A. L. M., Cheng, L. Y. L., and O, W. S. (2003). Male genital tract antioxidant enzymes: their source, function in the female, and ability to preserve sperm DNA integrity in the golden hamster. *J. Androl.* **24**, 704–711.

de Lamirande, E., and O'Flaherty, C. (2008). Sperm activation: role of reactive oxygen species and kinases. *Biochim. Biophys. Acta* **1784**, 106–115.

Domínguez-Rebolledo, A. E., Fernández-Santos, M. R., García-Alvarez, O., Maroto-Morales, A., Garde, J. J., and Martínez-Pastor, F. (2009). Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. *Theriogenology* **72**, 1073–1084. doi:10.1016/J.THERIOGENOLOGY.2009.06.027

Donnelly, E. T., McClure, N., and Lewis, S. E. (1999*a*). Antioxidant supplementation *in vitro* does not improve human sperm motility. *Fertil. Steril.* **72**, 484–495. doi:10.1016/S0015-0282(99)00267-8

Donnelly, E. T., McClure, N., and Lewis, S. E. (1999*b*). The effect of ascorbate and alpha-tocopherol supplementation *in vitro* on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* **14**, 505–512. doi:10.1093/MUTAGE/14.5.505

Fernández-Santos, M. R., Martínez-Pastor, F., García-Macías, V., Esteso, M. C., Soler, A. J., Paz, P., Anel, L., and Garde, J. J. (2007). Sperm characteristics and DNA integrity of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. *J. Androl.* **28**, 294–305. doi:10.2164/JANDROL.106.000935

Fernández-Santos, M. R., Domínguez-Rebolledo, A. E., Esteso, M. C., Garde, J. J., and Martínez-Pastor, F. (2009*a*). Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity. *Int. J. Androl.* **32**, 353–359. doi:10.1111/J.1365-2605.2008.00871.X

Fernández-Santos, M., Domínguez-Rebolledo, A., Esteso, M., Garde, J., and Martínez-Pastor, F. (2009*b*). Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin C supplementation. *Reprod. Domest. Anim.* **44**, 212–220. doi:10.1111/J.1439-0531.2007.01032.X

Gadea, J., Garcia-Vazquez, F., Matas, C., Gardon, J. C., Canovas, S., and Gumbao, D. (2005*a*). Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. *J. Androl.* **26**, 396–404. doi:10.2164/JANDROL.04155

Gadea, J., Gumbao, D., Matas, C., and Romar, R. (2005*b*). Supplementation of the thawing media with reduced glutathione improves function and the *in vitro*-fertilizing ability of boar spermatozoa after cryopreservation. *J. Androl.* **26**, 749–756. doi:10.2164/JANDROL.05057

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Fernández-Santos, M. R., Esteso, M. C., Pérez-Guzmán, M. D., and Soler, A. J. (2009*a*). Heterologous *in vitro* fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. *Theriogenology* **71**, 643–650. doi:10.1016/J.THERIOGENOLOGY.2008.09.036

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J. J., Ramón, M., Fernández-Santos, M. R., Esteso, M. C., Pérez-Guzmán, M. D., and Soler, A. J. (2009*b*). Sperm characteristics and *in vitro*-fertilization ability of thawed spermatozoa from Black Manchega ram: electroejaculation and postmortem collection. *Theriogenology* **72**, 160–168. doi:10.1016/J.THERIOGENOLOGY.2009.02.002

Garde, J. J., Martinez-Pastor, F., Gomendio, M., Malo, A. F., Soler, A. J., Fernandez-Santos, M. R., Esteso, M. C., Garcia, A. J., Anel, L., and Roldan, E. R. S. (2006). The application of reproductive technologies to natural populations of red deer. *Reprod. Domest. Anim.* **41**(Suppl 2), 93–102. doi:10.1111/J.1439-0531.2006.00773.X

Gavella, M., and Lipovac, V. (2000). Antioxidative effect of melatonin on human spermatozoa. *Arch. Androl.* **44**, 23–27. doi:10.1080/014850100262371

González-Fernández, L., Ortega-Ferrusola, C., Macias-Garcia, B., Salido, G. M., Peña, F. J., and Tapia, J. A. (2009). Identification of

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protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. *Biol. Reprod.* **80**, 1239–1252. doi:10.1095/BIOLREPROD.108.073486

Grunewald, S., Sharma, R., Paasch, U., Glander, H., and Agarwal, A. (2009). Impact of caspase activation in human spermatozoa. *Microsc. Res. Tech.* **72**, 878–888. doi:10.1002/JEMT.20732

Hardeland, R. (2005). Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. *Endocrine* **27**, 119–130. doi:10.1385/ENDO:27:2:119

Heidary, M., Vahhabi, S., Reza Nejadi, J., Delfan, B., Birjandi, M., Kaviani, H., and Givrad, S. (2008). Effect of saffron on semen parameters of infertile men. *Urol. J.* **5**, 255–259.

Hughes, C. M., Lewis, S. E., McKelvey-Martin, V. J., and Thompson, W. (1998). The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum. Reprod.* **13**, 1240–1247. doi:10.1093/HUMREP/13.5.1240

Idziorek, T., Estaquier, J., De Bels, F., and Ameisen, J. C. (1995). YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immunol. Methods* **185**, 249–258. doi:10.1016/0022-1759(95)00172-7

Jang, H., Kim, Y., Kim, B., Park, I., Cheong, H., Kim, J., Park, C., Kong, H., Lee, H., and Yang, B. (2009). Ameliorative effects of melatonin against hydrogen peroxide-induced oxidative stress on boar sperm characteristics and subsequent *in vitro* embryo development. *Reprod. Domest. Anim.*, In press. doi:10.1111/J.1439-0531.2009.01466.X

Kumaresan, A., Kadirvel, G., Bujarbaruah, K. M., Bardoloi, R. K., Das, A., Kumar, S., and Naskar, S. (2009). Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis-like changes in spermatozoa. *Anim. Reprod. Sci.* **110**, 162–171. doi:10.1016/J.ANIREPROSCI.2008.01.006

Leon, J., Acuna-Castroviejo, D., Sainz, R. M., Mayo, J. C., Tan, D. X., and Reiter, R. J. (2004). Melatonin and mitochondrial function. *Life Sci.* **75**, 765–790. doi:10.1016/J.LFS.2004.03.003

Martínez-Pastor, F., Anel, L., Guerra, C., Álvarez, M., Soler, A. J., Garde, J. J., Chamorro, C., and de Paz, P. (2006*a*). Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. *Theriogenology* **66**, 1847–1856. doi:10.1016/J.THERIOGENOLOGY.2006.04.036

Martínez-Pastor, F., Martínez, F., García-Macias, V., Esteso, M., Anel, E., Fernández-Santos, M., Soler, A., de Paz, P., Garde, J., and Anel, L. (2006*b*). A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. *Theriogenology* **66**, 1165–1172. doi:10.1016/J.THERIOGENOLOGY.2006.03.027

Martínez-Pastor, F., Fernández-Santos, M. R., del Olmo, E., Domínguez-Rebolledo, A. E., Esteso, M. C., Montoro, V., and Garde, J. J. (2008). Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. *Reprod. Fertil. Dev.* **20**, 547–556. doi:10.1071/RD08002

Martínez-Pastor, F., Aisen, E., Fernández-Santos, M. R., Esteso, M. C., Maroto-Morales, A., Garcia-Álvarez, O., and Garde, J. J. (2009*a*). Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction* **137**, 225–235. doi:10.1530/REP-08-0357

Martínez-Pastor, F., Martínez, F., Álvarez, M., Maroto-Morales, A., Garcia-Alvarez, O., Soler, A. J., Garde, J. J., de Paz, P., and Anel, L. (2009*b*). Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa obtained by electroejaculation. *Theriogenology* **71**, 628–638. doi:10.1016/J.THERIOGENOLOGY.2008.09.033

Mickle, D. A., and Weisel, R. D. (1993). Future directions of vitamin E and its analogues in minimizing myocardial ischemia-reperfusion injury and its analogues in minimizing myocardial ischemia-reperfusion injury. *Can. J. Cardiol.* **9**, 89–93.

Mortimer, D., Serres, C., Mortimer, S. T., and Jouannet, P. (1988). Influence of image sampling frequency on the perceived movement characteristics of progressively motile human spermatozoa. *Gamete Res.* **20**, 313–327. doi:10.1002/MRD.1120200307

Mortimer, S. T., and Maxwell, W. M. C. (1999). Kinematic definition of ram sperm hyperactivation. *Reprod. Fertil. Dev.* **11**, 25–30. doi:10.1071/RD99019

Nagy, S., Jansen, J., Topper, E. K., and Gadella, B. M. (2003). A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol. Reprod.* **68**, 1828–1835. doi:10.1095/BIOLREPROD.102.011445

Navari-Izzo, F., Quartacci, M. F., and Sgherri, C. (2002). Lipoic acid: a unique antioxidant in the detoxification of activated oxygen species. *Plant Physiol. Biochem.* **40**, 463–470. doi:10.1016/S0981-9428(02)01407-9

Ochiai, T., Shimeno, H., Mishima, K. I., Iwasaki, K., Fujiwara, M., Tanaka, H., Shoyama, Y., Toda, A., Eyanagi, R., and Soeda, S. (2007). Protective effects of carotenoids from saffron on neuronal injury *in vitro* and *in vivo*. *Biochim. Biophys. Acta* **1770**, 578–584.

Ortega Ferrusola, C., González Fernández, L., Macías García, B., Salazar-Sandoval, C., Morillo Rodríuez, A., Rodríguez Martinez, H., Tapia, J. A., and Peña, F. J. (2009). Effect of cryopreservation on nitric oxide production by stallion spermatozoa. *Biol. Reprod.* **81**, 1106–1111. doi:10.1095/BIOLREPROD.109.078220

Packer, L., Witt, E. H., and Tritschler, H. J. (1995). α-lipoic acid as a biological antioxidant. *Free Radic. Biol. Med.* **19**, 227–250. doi:10.1016/0891-5849(95)00017-R

Peña, F. J., Johannisson, A., Wallgren, M., and Rodriguez Martinez, H. (2003). Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim. Reprod. Sci.* **78**, 85–98. doi:10.1016/S0378-4320(03)00049-6

Peña, F. J., Johannisson, A., Wallgren, M., and Martinez, H. R. (2004). Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. *Zygote* **12**, 117–124. doi:10.1017/S096719940400262X

Peña, F. J., Saravia, F., Johannisson, A., Walgren, M., and Rodríguez-Martínez, H. (2005). A new and simple method to evaluate early membrane changes in frozen–thawed boar spermatozoa. *Int. J. Androl.* **28**, 107–114. doi:10.1111/J.1365-2605.2005.00512.X

Petrunkina, A. M., and Harrison, R. A. P. (2009). Systematic mis-estimation of cell subpopulations by flow cytometry: a mathematical analysis. *Theriogenology*, in press. doi:10.1016/J.THERIOGENOLOGY.2009.09.007

Prior, R. L., Wu, X., and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **53**, 4290–4302. doi:10.1021/JF0502698

R Development Core Team (2008). 'R: A Language and Environment for Statistical Computing'. (R Foundation for Statistical Computing: Vienna.)

Rao, M. V., and Gangadharan, B. (2008). Antioxidative potential of melatonin against mercury-induced intoxication in spermatozoa *in vitro*. *Toxicol. In Vitro* **22**, 935–942. doi:10.1016/J.TIV.2008.01.014

Roca, J., Rodriguez, M. J., Gil, M. A., Carvajal, G., Garcia, E. M., Cuello, C., Vazquez, J. M., and Martinez, E. A. (2005). Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J. Androl.* **26**, 15–24.

Scott, B. C., Aruoma, O. I., Evans, P. J., O'Neill, C., Van der Vliet, A., Cross, C. E., Tritschler, H., and Halliwell, B. (1994). Lipoic and dihydrolipoic acids as antioxidants. A critical evaluation. *Free Radic. Res.* **20**, 119–133. doi:10.3109/10715769409147509

PROOF ONLY

Selvakumar, E., Prahalathan, C., Sudharsan, P. T., and Varalakshmi, P. (2006). Chemoprotective effect of lipoic acid against cyclophosphamide-induced changes in the rat sperm. *Toxicology* **217**, 71–78. doi:10.1016/J.TOX.2005.08.020

Shang, X., Huang, Y., Ye, Z., Yu, X., and Gu, W. (2004). Protection of melatonin against damage of sperm mitochondrial function induced by reactive oxygen species. *Zhonghua Nan Ke Xue* **10**, 604–607.

Siems, W., Salerno, C., Crifò, C., Sommerburg, O., and Wiswedel, I. (2009). Beta-carotene degradation products – formation, toxicity and prevention of toxicity. *Forum Nutr.* **61**, 75–86. doi:10.1159/000212740

Silva, P. F. N., and Gadella, B. M. (2006). Detection of damage in mammalian sperm cells. *Theriogenology* **65**, 958–978. doi:10.1016/J.THERIOGENOLOGY.2005.09.010

Storey, B. T. (1997). Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol. Hum. Reprod.* **3**, 203–213. doi:10.1093/MOLEHR/3.3.203

Upreti, G. C., Jensen, K., Oliver, J. E., Duganzich, D. M., Munday, R., and Smith, J. F. (1997). Motility of ram spermatozoa during storage in a chemically-defined diluent containing antioxidants. *Anim. Reprod. Sci.* **48**, 269–278. doi:10.1016/S0378-4320(97)00054-7

Venkatraman, M., Konga, D., Peramaiyan, R., Ganapathy, E., and Dhanapal, S. (2008). Reduction of mitochondrial oxidative damage and improved mitochondrial efficiency by administration of crocetin against benzo[a]pyrene-induced experimental animals. *Biol. Pharm. Bull.* **31**, 1639–1645. doi:10.1248/BPB.31.1639

Vijayaraghavan, S., Stephens, D., Trautman, K., Smith, G., Khatra, B., da Cruz e Silva, E. F., and Greengard, P. (1996). Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. *Biol. Reprod.* **54**, 709–718. doi:10.1095/BIOLREPROD54.3.709

Wolf, A. M., Asoh, S., Hiranuma, H., Ohsawa, I., Iio, K., Satou, A., Ishikura, M., Ohta, S. (2009). Astaxanthin protects mitochondrial redox state and functional integrity against oxidative stress. *J. Nutr. Biochem.*, in press. doi:10.1016/J.JNUTBIO.2009.01.011

Zanotto-Filho, A., Schröder, R., and Moreira, J. C. F. (2008). Differential effects of retinol and retinoic acid on cell proliferation: a role for reactive species and redox-dependent mechanisms in retinol supplementation. *Free Radic. Res.* **42**, 778–788. doi:10.1080/10715760802385702

Zheng, Y. Q., Liu, J. X., Wang, J. N., and Xu, L. (2007). Effects of crocin on reperfusion-induced oxidative/nitrative injury to cerebral microvessels after global cerebral ischemia. *Brain Res.* **1138**, 86–94. doi:10.1016/J.BRAINRES.2006.12.064

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