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 \( \mu \)M 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. 2006b). 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. 2005a; Roca et al. 2005; Fernández-Santos et al. 2007), or just after thawing (Berlinger et al. 2003; Gadea et al. 2005b; Fernández-Santos et al. 2009a), 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. 2009b). Furthermore, we have shown that seminal plasma...
The addition of Trolox to semen preservation media was shown to be partly due to its antioxidant properties (Martínez-Pastor et al. 2006a).

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, 2009a). 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 FeSO₄ and 50 mM sodium ascorbate (Fe²⁺-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-H₂DCFDA, 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 CaCl₂, 0.4 mM MgCl₂, 0.3 mM NaH₂PO₄, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg mL⁻¹ kanamycin, 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.
Antioxidants on thawed red deer sperm

Production), ROS production and DNA damage. The control sample

Samples from four stags were used in this experiment. One straw

Experimental design

Effect of antioxidants on the motility and physiology of thawed spermatozoa

The washed pool was split among nine tubes. One of the tubes

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

The nine tubes described above were replicated and supple-

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

Studies of an extended concentration range for crocin and melatonin

The previous experiments showed interesting results for

CASA analysis

Sperm were diluted down to 10–20 × 10^6 spermatozoa mL^{-1}

Fluorescence probes for sperm analysis

Several physiological traits were assessed by using fluores-

(September and October). Hunting was in accordance with the

Harvest plan of game reserves, following Spanish Harvest Reg-

ulation (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 equi-

librated 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 two

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, lipoic 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 acroso-

mal 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 supple-

mented with the oxidant solution, at a final concentration of

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 ox-

idant 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.

CAS A analysis

Sperm were diluted down to 1–2 × 10^6 spermatozoa mL^{-1}

and loaded into a Makler counting chamber (10-µm depth

at 37°C). The CASA system consisted of a triscular 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× nega-

tive phase-contrast objective (no intermediate magnification).

Image sequences were saved and analysed afterwards. Software

settings were adjusted to deer spermatozoa. The standard param-

eter settings were as follows: 25 frames s^{-1}; 20 to 90 µm^2

for head area; VCL > 100 µm s^{-1} to classify a spermatozoon as

motile. For each spermatozoon, the software rendered the per-

centage 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, straight-

ness; 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 fluores-

cen t probes and flow cytometry, which were described in

previous studies (Peña et al. 2005; Martínez-Pastor et al.

2008, 2009b; 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 ‘apop-

totic’ 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
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-HDDCFDA and 0.1 μM TO-PRO-1 (for assessing viability). The CM-H²DCFDA is cleaved by cellular stereses 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 (10⁶ 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-H²DCFDA and TUNEL) and a 633-nm He-Ne laser (excitation for Mitotracker Deep Red and TO-PRO-1). Fluorescence from YO-PRO-1, CM-H²DCFDA and TUNEL (fluorescin isothioceyanate, 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; Petrunkin 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-Alvarez et al. 2009a, 2009b; Martínez-Pastor et al. 2009a), 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 (ΔΨm). 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 (10⁶ cells mL⁻¹) and then incubated for 30 min at 37°C with 40 μM of Fe²⁺ 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 10⁸ 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.
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 ± 0.7% at 0 h to 16.3 ± 0.8% at 2 h, $P = 0.028$, and 21.2 ± 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
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-Δψm 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-Δψm 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 −3.9 to 3.2% after 4 h (effect at 4 h: −3.1 ± 3.4 vs effect in non-oxidised samples: −18.2 ± 2.7) in samples not submitted to oxidative stress, and supplemented with each antioxidant-dose combination within each given time (P < 0.05; ***P < 0.01; **P < 0.001).

Table 1. Sperm motility (mean ± s.e.m.) in samples not submitted to oxidative stress, and supplemented with each antioxidant-dose combination

<table>
<thead>
<tr>
<th>Time</th>
<th>Antioxidant</th>
<th>Conc. (mM)</th>
<th>Motile (%)</th>
<th>VAP (µm s⁻¹)</th>
<th>LIN (%)</th>
<th>ALH (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>Control</td>
<td>90 ± 6.6</td>
<td>88.4 ± 16</td>
<td>55.2 ± 3.5</td>
<td>39 ± 0.6</td>
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<tr>
<td>2 h</td>
<td>Control</td>
<td>63.2 ± 13.1</td>
<td>58.5 ± 5.8</td>
<td>52.8 ± 5.7</td>
<td>3.1 ± 0.3</td>
<td></td>
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<tr>
<td></td>
<td>Lipoic acid</td>
<td>0.1</td>
<td>57.6 ± 16</td>
<td>53.2 ± 9.5</td>
<td>47.8 ± 3.4</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>55.1 ± 13.6</td>
<td>45.6 ± 9.5</td>
<td>48.7 ± 7.7</td>
<td>2.7 ± 0.5</td>
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<td></td>
<td>Crocin</td>
<td>0.1</td>
<td>49.7 ± 10.8</td>
<td>51.1 ± 3</td>
<td>44.6 ± 3.3</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>86.9 ± 7.8</td>
<td>84.1 ± 10.8</td>
<td>55 ± 3.5</td>
<td>3.7 ± 0.0</td>
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<td></td>
<td>Melatonin</td>
<td>0.1</td>
<td>60.2 ± 13.3</td>
<td>59.6 ± 8.6</td>
<td>53.9 ± 6.1</td>
<td>3.2 ± 0.2</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>61.7 ± 8</td>
<td>54.2 ± 4.5</td>
<td>50.5 ± 4.5</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>4 h</td>
<td>Control</td>
<td>52.6 ± 5.8</td>
<td>45.6 ± 1.9</td>
<td>41.1 ± 1.7</td>
<td>3.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipoic acid</td>
<td>0.1</td>
<td>49.8 ± 10.8</td>
<td>43.6 ± 8.1</td>
<td>42.7 ± 2.9</td>
<td>2.9 ± 0.4</td>
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<td></td>
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<td>43.3 ± 5.4</td>
<td>41.2 ± 3**</td>
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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-H₂DCFDA signal below control levels, except 1 mM crocin. When submitted to oxidative stress, the CM-H₂DCFDA signal increased five-fold. In these conditions, only 1 mM crocin, 0.1 mM Trolox and 1 mM Trolox significantly reduced the CM-H₂DCFDA 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 10⁶ 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 10⁶ spermatozoa, P < 0.001). In oxidised samples, MDA levels increased considerably (22.4 ± 2.5 nmol of MDA per 10⁶ 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-Δψm (active mitochondria) spermatozoa 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)

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<th>Time</th>
<th>Antioxidant</th>
<th>Conc. (mM)</th>
<th>YP−</th>
<th>YP+/PI−</th>
<th>MT+[YP−]</th>
<th>PNA−[YP−]</th>
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### 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)

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<th>ALH (μm)</th>
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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

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<tr>
<th>Time</th>
<th>Antioxidant</th>
<th>Conc. (mM)</th>
<th>YP−</th>
<th>YP+/PI−</th>
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<th>PNA− [YP−]</th>
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<td>35.8 ± 5.3a</td>
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<td>Control</td>
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<td>96.8 ± 1.7</td>
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<td>96.7 ± 1.6</td>
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Trolox concentrations lowered MDA yield to values close to control at 0 h (pooled concentrations: 13.9 ± 1.3 nmol of MDA per 10⁷ 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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+ TUNEL on dialdehyde (MDA) production; middle) and DNA damage (proportion of according to CM-HDCFDA stain; top), lipid peroxidation (LPO, as mal-

Intracellular ROS levels in viable (TO-PRO-1 Fig. 2. shown as 1 s.e.m. Significant differences between controls (0 h vs 4 h) are

mean values for samples submitted to oxidative stress, with vertical lines shows mean values for non-oxidised samples, whereas the dashed line shows

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

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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 $^**$ ($P < 0.01$) 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 $\mu$M Trolox, although being able to reduce intracellular ROS, could not prevent the effects of oxidative stress (Martínez-Pastor et al. 2009a). 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 $\mu$M 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-$\mu$M concentrations, considering the reduction from control levels, whereas for the 1-$\mu$M concentrations this ratio was ~2.5. Effectively, at 2.5 $\mu$M the reduction of MDA production was roughly equivalent to that achieved by 1 $\mu$M Trolox. ROS scavenging and TUNEL 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.

**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 $^**$ ($P < 0.01$) for the oxidised samples.
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\textsubscript{2}O\textsubscript{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\textsuperscript{2+}, 1 mM H\textsubscript{2}O\textsubscript{2} or 100 mM 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. 2009a). 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.
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We confirmed this effect in the second trial, which suggested using more accurate techniques (e.g. mass spectrometry). To the solutions, but we could not find such an effect. Whether even at low concentrations, yields a patent yellow-orange colour of the antioxidant with the analytical techniques (crocin, oxidised samples. Therefore, we checked if it was due to an inter-

ference of the antioxidant (de Lamirande and O’Flaherty 2008). In the present study, 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 spectrometry).

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-ΔΨm 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 mitochondrial toxicity, 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 deserve further comment. The oxidative stress caused a slight decrease of the YO-PRO-1−/− subpopulation and a noticeable drop of the YO-PRO-1+/−/− 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+/−/− 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+/−/− 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
increased membrane damage rate (a similar effect was observed using Fe\textsuperscript{2+} as oxidising agent; Martínez-Pastor et al. 2009a). 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 pre-freezing 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


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