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Effect of Several Antioxidants on Thawed Ram Spermatozoa Submitted to 37°C up to Four Hours

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Contents

Thawed ram spermatozoa were incubated at 37°C in the presence of dehydroascorbic acid (DHA), TEMPOL (TPL), *N*-acetyl-cysteine (NAC) and rutin (RUT), at 0.1 and 1 mM, in order to test their effects on sperm physiology. Cryopreserved spermatozoa from four rams were thawed, pooled, washed and incubated in TALP-Hepes with 1 mM or 0.1 mM of each antioxidant, performing a replicate with induced oxidative stress (Fe^{2+}/ascorbate). Motility (CASA), viability and mitochondrial membrane potential (flow cytometry) were analysed at 2 and 4 h. Lipoperoxidation (MDA production), intracellular reactive oxygen species (ROS) and DNA status (TUNEL) were analysed at 4 h. Antioxidants, except DHA 0.1 mM, decreased motility and kinematic parameters, but had little effect on viability or mitochondrial activity. Except 1 mM DHA, the antioxidants reduced ROS at 4 h. Moreover, NAC 1 mM, rutin and TEMPOL reduced ROS and DNA damage in the presence of oxidative stress. *N*-acetyl-cysteine, rutin 1 mM and TEMPOL reduced lipoperoxidation in the presence of oxidative stress. However, DHA did not affect lipoperoxidation. At 1 mM, DHA increased DNA damage in the absence of oxidative stress. Dehydroascorbic acid effects could arise from spermatozoa having a low capacity for reducing it to ascorbic acid, and it may be tested in the presence of other antioxidants or reducing power. Future research should focus in testing whether the inhibition of motility observed for NAC, rutin and TEMPOL is reversible. These antioxidants might be useful at lower temperatures (refrigerated storage or cryopreservation) when their protective effects could be advantageous.

Introduction

Artificial insemination (AI) with frozen-thawed semen has been proposed as a valuable tool for genetic improvement programmes for sheep (Anel et al. 2006). This technique has still to gain a widespread use, because of very variable and frequently low fertility with cervical AI, forcing the use of short-term refrigerated semen or laparoscopic insemination. The main problem with the cervical AI consists in the difficulty in performing a deep insemination, because of the sheep anatomy and to the convoluted shape of the cervical channel, forcing to deposit the semen in the vagina or to perform shallow intracervical inseminations (Kaabi et al. 2006; Druart et al. 2009). Moreover, cryopreservation impairs sperm quality (Salamon and Maxwell 2000), and possibly its ability to migrate to the oviduct, which explains the requirement of laparoscopic AI to achieve acceptable results when using cryopreserved semen in sheep.

Antioxidants have been proposed for improving results of artificial reproductive techniques. Many attempts have been tried in different species (Bilodeau et al. 2001; Gadea et al. 2005; Roca et al. 2005; Fernandez-Santos et al. 2007; Domìnguez-Rebolledo et al. 2009; Fernández-Santos et al. 2009), although field trials have not been as successful as *in vitro* experiments (Foote et al. 2002; Mara et al. 2007). In this study, we have tested the effect of several antioxidants on ram sperm quality, following an *in vitro* design that we used previously in red deer (Domìnguez-Rebolledo et al. 2010). Such a test was designed as a preliminary step to study the physiological changes of spermatozoa upon being submitted to the antioxidants at 37°C and to uncover toxic effects.

The antioxidants TEMPOL, *N*-acetyl-cysteine (NAC), rutin and deydroascorbic acid (DHA) were used in our experiment. TEMPOL has been tried for the refrigerated storage of ram spermatozoa (Mara et al. 2005), apparently improving the conservation of sperm quality and the *in vitro* fertility. However, the base extenders used for TEMPOL and non-TEMPOL treatments were different, preventing a proper comparison on the effect of this antioxidant. Furthermore, TEMPOL did not affect the motility of cryopreserved bull spermatozoa (Foote et al. 2002), and it could not improve the kidding rate of goats inseminated with refrigerated semen (Mara et al. 2007). However, this antioxidant seems promising, because it has been defined as having a SOD-like activity (Mitchell et al. 1990).

The other antioxidants have not been tested in ram spermatozoa before, although *N*-acetyl-cysteine (NAC) has yielded good results in other species. *N*-acetyl-cysteine is a thiol antioxidant, which are regarded as excellent radical scavengers and blockers of lipid peroxidation (Deneke 2000). Thus, Oeda et al. (1997) showed that NAC decreased ROS in human semen. Several studies in cryopreserved and refrigerated dog semen have reported positive effects of NAC supplementation of semen extenders (Michael et al. 2007, 2009, 2010), although no significant ROS reduction was observed. *N*-acetyl-cysteine has been also tested in bull (Bilodeau et al. 2001), with good results against induced oxidative stress.

Rutin (a flavonol) and DHA (the oxidized form of vitamin C) have not been tested in the context of artificial reproductive techniques previously. Rutin has been chosen because of the interesting results of flavonols in genotoxicity assays. Flavonols have a double-edge behaviour, because they can act as pro-oxidants or antioxidants, depending on concentration

and experimental conditions (Liu and Zheng 2002; Liu et al. 2010). Moreover, several studies have shown that quercetin, another flavonol, can modulate the capacitation of bull spermatozoa, while lowering the concentration of ROS (Córdoba et al. 2006, 2007, 2008). Moreover, this flavonol prevented DNA damage in spermatozoa in genotoxicity studies (Anderson et al. 1998). Regarding DHA, our interest in this molecule arises from previous studies showing that it can enter the spermatozoon through glucose transporters (GLUT family) (Angulo et al. 1998). Once in the mitochondria, DHA is reduced to ascorbic acid, increasing the antioxidant pool of the cell (KC et al. 2005). These studies suggest that the addition of DHA to cell media could increase intracellular ascorbic acid more efficiently than the addition of ascorbic acid itself, owing to the preference of GLUT transporters for the oxidized form.

In this study, we tested these four antioxidants during a 4-h incubation at 37°C and at 0.1 and 1 mM. The main objective of the study is to identify the physiological changes that the antioxidants produce at that temperature, trying to characterize their effects and possible toxicity. We aimed at providing basic information about the use of these antioxidants on ram spermatozoa, providing information prior to testing their usefulness on the refrigerated storage or cryopreservation of ram spermatozoa.

Materials and Methods

Reagents and media

Common reagents (Reagent grade or higher) and antioxidants were acquired from Sigma (Madrid, Spain). 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). Spermatozoa were incubated in a TALP-Hepes medium, 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 kanamicine, 10 μg/ml phenol red and 6 mg/ml BSA (pH 7.5). The antioxidants were prepared as stock solutions of 100 mM and 10 mM in double-distilled water, except rutin, which was diluted in 1 M NaOH in water. An oxidant solution was prepared with 10 mM $FeSO_4$ and 50 mM sodium ascorbate (Fe^{2+}/ascorbate) in water. Stocks of fluorescence probes were prepared in DMSO and kept at −20°C in the dark: YO-PRO-1: 50 μM; Mitotracker Deep Red: 1 mM; CM-H_2DCFDA: 500 μM. Antioxidant stocks and the oxidant solution were prepared fresh just before starting each experimental session.

Animals and semen processing

We used four adult males (2–9 years old) of the Churra breed, of proven fertility and trained for semen collection by artificial vagina. Semen collection was performed during the breeding season (Autumn). Ejaculates were collected by artificial vagina (40°C), and the tubes were maintained at 35°C during the initial evaluation of semen quality. The volume was estimated by using the graduation marks of the collection tube. Mass motility was assessed by microscopy (warming stage at 37°C, ×40; score: 0–5), and the sperm concentration was assessed by the photocolorimetric method (540 nm), on a specifically calibrated scale. Only ejaculates of good quality were used and frozen (volume: ≥0.5 ml; mass motility: ≥4; sperm concentration: $\geq 3000 \times 10^6$ ml).

Semen was diluted with the same volume of freezing extender. The freezing extender (Anel et al. 2003) consisted of a TTF medium (TES-Tris-fructose, 320 mOsm/kg, pH 7.2) supplemented with 10% egg yolk and 4% glycerol. The sample was then refrigerated to 5°C for 2 h. Samples were packed into 0.25-ml plastic straws and equilibrated for 1 h at 5°C. Then, the straws were frozen using a programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) using a rate of −20°C/min down to −100°C. The straws were kept in liquid nitrogen containers. For each experimental session, one straw per male was thawed in a water bath at 37°C for 30 s. The contents of the four straws were pooled and diluted with three volumes of TALP-Hepes. After centrifugation ($600 \times g$ for 5 min), the supernatant was discarded and the pellet was slowly resuspended in TALP-Hepes up to 30×10^6 cells/ml. The washed pool was assessed (motility, membrane and mitochondrial status, DNA, lipoperoxidation and ROS) 10 min after washing.

Experimental design

The experiments followed a factorial design. In all experiments, the washed pool was split among nine tubes. Eight of them were supplemented with either 1/100 of the 100 mM solution (1 mM final) or the 10 mM solution (0.1 mM final) of each antioxidant: TEMPOL, *N*-acetyl-cysteine (NAC), rutin or dehydroascorbic acid (DHA). The ninth tube was used as control (no antioxidant). All the experiments were replicated seven times. Half of the volume of each tube was passed to another series of tubes, which were submitted to oxidative stress by adding 1/100 of the oxidant solution (100 μM of $FeSO_4$ and 500 μM of sodium ascorbate). The tubes were incubated at 37°C and analysed at 2 h and 4 h.

Sperm motility assessment

The motility of the tubes without oxidative stress was assessed at 2 and 4 h. Sperm were diluted down to $10–20 \times 10^6$ spermatozoa/ml 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. The standard parameter settings were as follows: 25 frames/s, 20–90 μm for head area and VCL > 10 μm/s (curvilinear velocity) to classify a spermatozoon as motile. We used four motility parameters in this study: total motility, progressive motility (VCL > 25 and STR – straightness – > 80%), VCL and ALH (amplitude of the lateral displacement of the sperm head).

Fluorescence probes and flow cytometry analysis

The membrane and mitochondrial status of the tubes without oxidative stress were assessed at 2 and 4 h. Samples were diluted down to 10^6 spermatozoa/ml in TALP-Hepes containing 0.1 μM YO-PRO-1 and 0.1 μM Mitotracker Deep Red. YO-PRO-1 stains spermatozoa with increased membrane permeability, while spermatozoa with high mitochondrial membrane potential ($\Delta\psi_m$) were stained by Mitotracker Deep Red. Spermatozoa were incubated 20 min in the dark before being run through a flow cytometer.

The DNA status, lipoperoxidation and ROS production of all the tubes were assessed at 4 h. For assessing intracellular ROS, spermatozoa were diluted in TALP-Hepes with 0.5 μM CM-H_2DCFDA and incubated for 30 min in the dark at 37°C before being analysed by flow cytometry. CM-H_2DCFDA is retained within cells after being cleaved by cellular sterases. When it is oxidized, it fluoresces green, indicating the presence of intracellular ROS.

DNA damage was assessed by TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling assay], as carried out previously (Domìnguez-Rebolledo et al. 2009). Briefly, samples were diluted in PBS at 10^6 cells/ml and fixed for 1 h with 2% paraformaldehyde. The cells were washed and stored at −20°C in 70% ethanol. Cells were washed and labelled for 60 min at 37°C. The cells were washed, incubated 30 min in the antibody solution (FITC-Anti-BrdUTP mAb) at room temperature and resuspended in a PI/RNase A solution before being analysed by flow cytometry. Positive and negative controls (incubation with DNase A and substituting water for the DNA labelling solution, respectively) were used to standardize the assay.

Lipoperoxidation was assessed by measuring malondialdehyde concentration. We used the Bioxitech® MDA-586 kit (Oxis International, Foster, CA, USA) to detect malondialdehyde (MDA) in the samples, as described by Dominguez-Rebolledo et al. (2010). Samples were diluted with PBS to 10×10^6 ml and incubated for 30 min at 37°C with 40 μM of Fe^{2+} and 200 μM ascorbate, to release MDA (Aitken et al. 1993). Samples were mixed with the reactive provided in the kit and incubated at 45C for 1 h. The tubes were centrifuged, and the supernatant was transferred to wells in a 96-well flat-bottom transparent plate (Nunc, Roskilde, Denmark). 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 a standard curve generated from know quantities of MDA and presented as nmol of MDA per 10^8 spermatozoa.

Flow cytometry analyses were carried out with a Cytomics™ FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA), with a 488 nm Ar-Ion laser [excitation for YO-PRO-1, CM-H_2DCFDA and FITC (TUNEL)] and a 633 nm He-Ne laser (excitation for Mitotracker Deep Red). Fluorescence from YO-PRO-1, CM-H_2DCFDA and FITC were read using a 525/25BP filter, and Mitotracker Deep Red (MT) was read using a 675/40BP filter. FSC/SSC signals were used to discriminate spermatozoa from debris. 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, Vic., Australia). The YO-PRO-1/MT stain was analysed as previously described for red deer (Martinez-Pastor et al. 2008). We obtained three populations: YO-PRO-1+ spermatozoa (increased membrane permeability or damaged membranes), YO-PRO-1−/MT− (viable spermatozoa with inactive mitochondria) and YO-PRO-1−/MT+ (viable spermatozoa with active mitochondria). For ROS assessment, we recorded the median fluorescence intensity (MFI) corresponding to CM-H_2DCFDA. For TUNEL analysis, the negative control allowed to define the TUNEL– population; thus, events with increased fluorescence were considered as TUNEL+ cells. Only events with high PI fluorescence (single nucleus) were taken into account for TUNEL analysis. PI– (debris) or events with very high PI fluorescence (cell aggregates) were discarded.

Statistical Analysis

Data were analysed in the R statistical environment (R Development Core Team, 2011). To analyse the effects of time, antioxidant supplement and oxidant effect on sperm parameters, we used linear mixed-effects models, with incubation time, antioxidant type and antioxidant concentration as fixed effects. Replicate was the grouping factor in the random part of the models. Results are presented as means and 95% confidence intervals (within parentheses). $p < 0.05$ was considered significant.

Results

Results are showed in Figs 1–4 as means and 95% confidence intervals. Considering the effect of the incubation on the control samples (without antioxidants), we noted a slight decrease in motility variables at 2 h (Fig. 1), which was not significant for total, progressive motility nor mean VCL. Nevertheless, the mean ALH of the samples decreased significantly, from an initial value 3.20 (0.30) μm to 2.57 (0.08) μm at 2 h. After 4 h, the total motility decreased from 80.8% (3.3) to 63.13% (3.8) and VCL from 125.9 (11.4) μm/s to 94.4 (6.0) μm/s ($p < 0.05$). ALH did not show

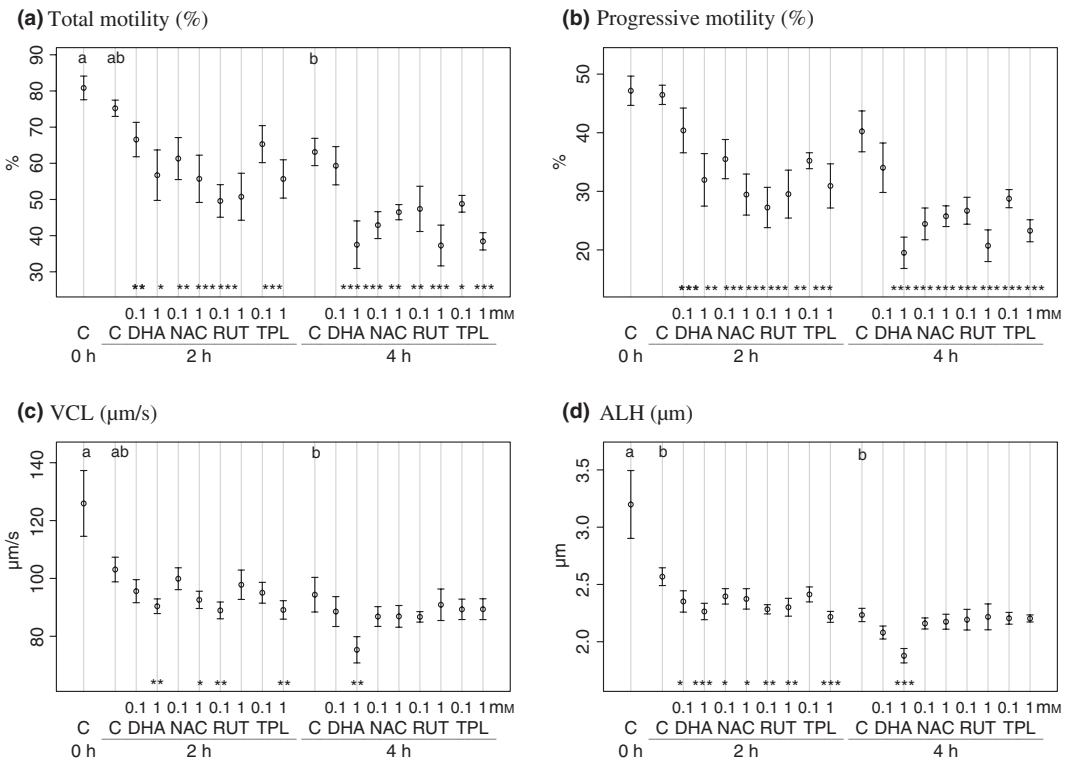

Fig. 1. Effects of antioxidant treatments on motility parameters at 2 h and 4 h of incubation (VCL: curvilinear velocity; ALH: amplitude of the lateral movement of the head). Mean and 95% CI are showed for each treatment: C: Control; DHA: Dehydroascorbic acid; NAC: *N*-acetyl-cisteine; RUT: rutin; TPL: TEMPOL. Letters on the top show significant differences among the three incubation times for the control samples (different letters indicate $p < 0.05$). Asterisks indicate significant differences among the antioxidant treatments and the control within each sampling time (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

significant changes respect to 2 h, with 2.23 (0.06) μm at 4 h. Viability and mitochondrial status (Fig. 2), good indicators of the overall integrity of spermatozoa, did not vary at 2 h [0 h: 42.4% (4.6) and 40.9% (4.5), respectively; 4 h: 43.5% (1.9) and 41.0% (2.0)]. At 4 h, the mean values decreased not significantly to 33.2% (2.84) and 31.23% (2.68), respectively. The variables related with oxidative stress, ROS and malondialdehyde production (Fig. 3), and DNA damage (TUNEL, Fig. 4) did not vary with incubation [0 h: 11.3 (1.5) MFI, 18.3 (2.6) nmol MDA/10 cells and 10.0% (5.1), respectively; 4 h: 13.1 (1.9) MFI, 15.6 (3.5) nmol MDA/10 cells and 1.8% (0.42)]. Induced oxidative stress during the incubation caused an increase in these parameters, which became significantly higher: 22.9 (2.7) MFI for ROS, 29.2 (3.6) nmol MDA/10 cells and 34.7% (4.7) TUNEL+ spermatozoa.

The incubation of spermatozoa with antioxidants caused important changes in motility. Except for DHA 0.1 mM and TEMPOL 0.1 mM, all treatments

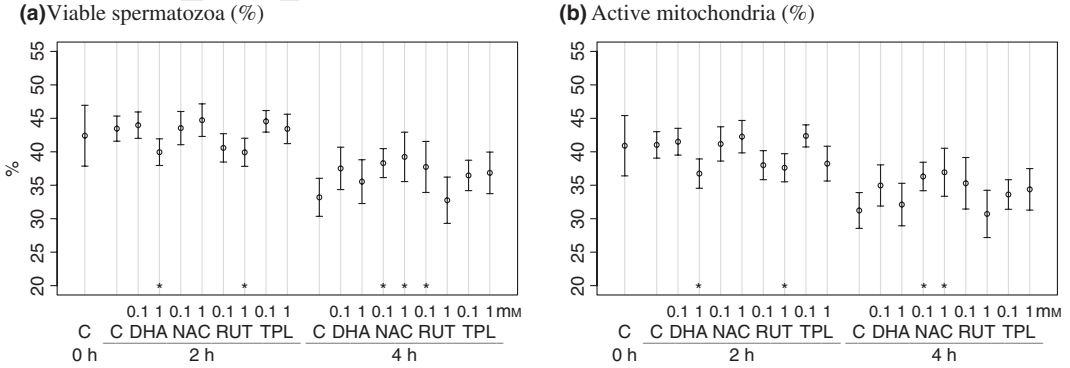

Fig. 2. Effects of antioxidant treatments on the results of the YO-PRO-1/Mitotracker Deep Red stain, at 2 h and 4 h of incubation. Viable spermatozoa are the proportion of YO-PRO-1− events, and active mitochondria is the proportion of YO-PRO-1−/Mitotracker Deep Red+ events. Mean and 95% CI are showed for each treatment: C: Control; DHA: Dehydroascorbic acid; NAC: *N*-acetyl-cisteine; RUT: rutin; TPL: TEMPOL. Letters on the top show significant differences among the three incubation times for the control samples (different letters indicate $p < 0.05$). Asterisks indicate significant differences among the antioxidant treatments and the control within each sampling time (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

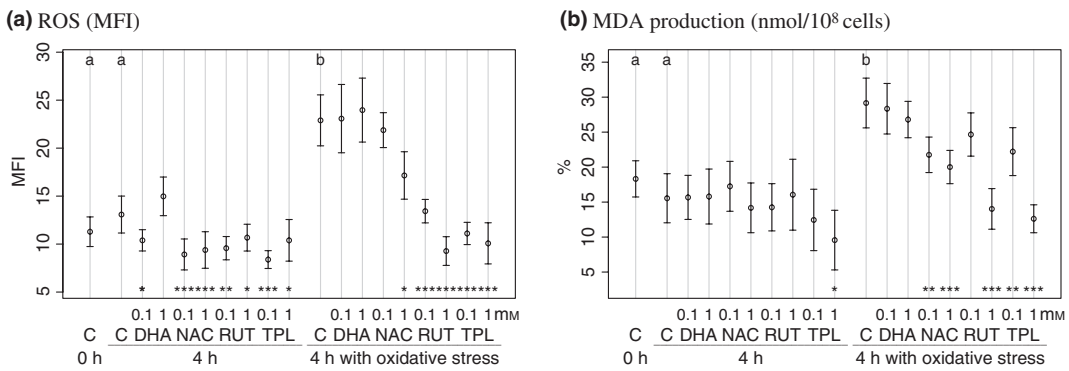

Fig. 3. Effects of antioxidant treatments on reactive oxygen species (ROS) and malondialdehyde (MDA) production, after 4 h of incubation without or with oxidative stress. ROS were assessed as median fluorescence intensity (MFI) of oxidized CM-H_2DCFDA. Mean and 95% CI are showed for each treatment: C: control; DHA: dehydroascorbic acid; NAC: *N*-acetyl-cisteine; RUT: rutin; TPL: TEMPOL. Letters on the top show significant differences among the three incubation times for the control samples (different letters indicate $p < 0.05$). Asterisks indicate significant differences among the antioxidant treatments and the control within each sampling time (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

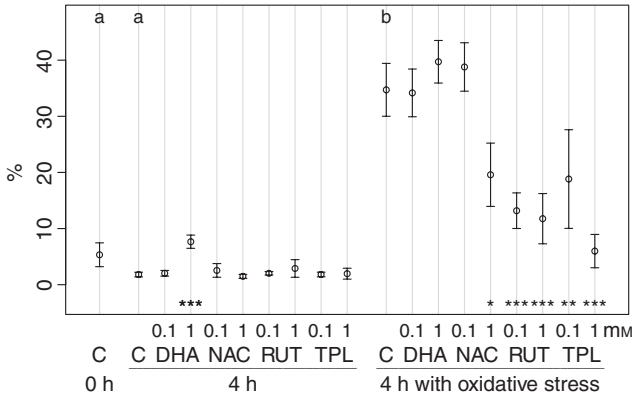

Fig. 4. Effects of antioxidant treatments on TUNEL results (DNA damage), after 4 h of incubation without or with oxidative stress. Mean and 95% CI are showed for each treatment: C: control; DHA: dehydroascorbic acid; NAC: *N*-acetyl-cisteine; RUT: rutin; TPL: TEMPOL. Letters on the top show significant differences among the three incubation times for the control samples (different letters indicate $p < 0.05$). Asterisks indicate significant differences among the antioxidant treatments and the control within each sampling time (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

significantly affected total motility at 2 h, causing an overall decrease of approximately 23 points (Fig. 1a). At 4 h, total motility further decreased in all treatments, except in DHA 0.1 mM (no significantly different to the control). This decrease in motility was larger in DHA 1 mM, RUT 1 mM and TEMPOL 1 mM, with an average decrease of 25 points respect to the control at 4 h. Progressive motility (Fig. 1b) showed a similar trend at both times, whereas VCL changes were less evident (Fig. 1c). Dehydroascorbic acid 1 mM, NAC 1 mM, Rutin 0.1 mM and TEMPOL 1 mM induced a small decrease of mean VCL at 2 h (13 μm/s less on average), while at 4 h, only DHA 1 mM had a significant effect, decreasing mean VCL to 75.3 (4.5) μm/s. ALH (Fig. 1 d) was slightly affected by all the antioxidants at 2 h (average decrease of 0.29 μm, $p < 0.05$), except for TEMPOL 0.1 mM. At 4 h, only DHA 1 mM (1.88 (0.06) μm) significantly decreased ALH respect to the control.

Contrarily, only a few antioxidants affected sperm viability and mitochondrial activity (Fig. 2). After 2 h of incubation, DHA and rutin at 1 mM decreased significantly these parameters, but at 4 h, NAC at 0.1 and 1 mM increased significantly both parameters above the control, and rutin 0.1 mM increased the proportion of viable spermatozoa. These changes were small, four points less for 2 h and five points more for 4 h, on average.

The production of ROS (Fig. 3a) dropped on many antioxidant treatments, both with and without induced oxidative stress. All antioxidants reduced intracellular ROS significantly after 4 h of incubation, except for DHA 1 mM, with an average drop of 3.4 in the MFI of the samples. *N*-acetyl-cysteine 1 mM, rutin and TEMPOL were able to significantly reduce the MFI values even in the presence of exogenous oxidative stress. In fact, MFI values of rutin and TEMPOL (0.1 and 1 mM) were not significantly different from those of the control at 0 h. Malondialdehyde production in the absence of oxidative stress was similar to the control (Fig. 3b), with only TEMPOL 1 mM significantly decreasing it (9.57 (4.26) nmol/10^8 cells). With induced oxidative stress, NAC, rutin 1 mM and TEMPOL caused a significant decline of MDA concentrations (average decrease of 8 nmol/10^8 cells for NAC and TEMPOL 0.1 mM, and of 16 nmol/10^6 cells for rutin and TEMPOL at 1 mM).

DNA damage (Fig. 4) was very low, even after 4 h of incubation, with no significant differences with the control, except DHA 1 mM, which increased TUNEL+ spermatozoa to 7.7% (1.2). *N*-acetyl-cysteine 1 mM [19.6% (5.6)], rutin [0.1 mM: 13.2% (3.2); 1 mM: 11.8% (4.5)] and TEMPOL [0.1 mM: 18.8% (8.8); 1 mM: 6.0% (3.0)] decreased the high proportion of TUNEL+ spermatozoa induced by oxidative stress. The values achieved by rutin and TEMPOL at 1 mM were not significantly different from the control at 0 h.

Discussion

Many studies have tested the effects of antioxidants on spermatozoa, with variable results. While there is a

general agreement that spermatozoa are highly vulnerable to the oxidative stress (Donnelly et al. 1999; Aitken and Sawyer 2003) and that the use of antioxidants could improve the results of artificial reproductive techniques (Donnelly et al. 2000; Foote et al. 2002), some authors have reported a lack of benefits or even detrimental effects of antioxidant supplementation in sperm media (Donnelly et al. 1999, 2000; Foote et al. 2002; Fernandez-Santos et al. 2007). In our study, we have found that most antioxidant treatments exerted an inhibitory effect on sperm motility, although most of them were efficient removing free radicals and protecting DNA and membranes from oxidation. Moreover, whereas NAC, rutin and TEMPOL behaved similarly, dehydroascorbic acid behaved differently regarding antioxidant activity and DNA protection.

Rutin and TEMPOL were especially efficient removing intracellular ROS, even in the presence of induced oxidative stress, and this efficiency was further demonstrated by indirect measures of oxidative stress such as MDA production and DNA fragmentation. *N*-acetylcysteine was not so effective in the presence of oxidative stress, but it also decreased ROS during the incubation and had an effect on lipoperoxidation and DNA protection. The loss of motility could be related to this efficient removal of free radicals from the spermatozoon cytoplasm. Free radicals take part in the physiological regulation of spermatozoa (Aitken and Curry 2010), and several studies have shown that the application of oxidative stress promotes capacitation and tyrosine phosphorylation, whereas the application of radical scavengers inhibits these processes (O'Flaherty et al. 2006; de Lamirande and O'Flaherty 2008). Thus, motility can be affected by ROS concentration via transduction signals affecting the flagellar beat (Aitken 2000). Some studies have reported loss of motility upon addition of antioxidants to the sperm media. For instance, Aitken et al. (1995) reported an inhibitory effect of 1 mM dithiothreitol in human spermatozoa motility.

Our results support that, for NAC, rutin and TEMPOL, the inhibition of motility could be due to excessive ROS scavenging, rather than to a direct toxic effect. Toxicity would have expressed in the form of decreased viability and increased apoptotic features (loss of mitochondrial activity and DNA fragmentation). Contrarily, these antioxidants had little effect on sperm viability and mitochondrial status and, in fact, NAC had a significantly positive effect in these parameters after 4 h of incubation. Considering these results, and the fact that these antioxidants could block lipid peroxidation and protect the sperm DNA, future studies could explore the possibility that motility could be resumed given the adequate conditions, by washing or adding stimulating factors. Moreover, the application of these antioxidants using different conditions (media, temperature, cryopreservation) may prevent the inhibition of motility while preserving the antioxidant and DNA-protecting effects.

Moreover, these effects could depend on the experimental conditions. For instance, we obtained good results incubating thawed spermatozoa from red deer in the presence of Trolox (a soluble form of vitamin E) (Domìnguez-Rebolledo et al. 2009, 2010), but results were suboptimal when the cryopreservation extender was supplemented with this antioxidant (Fernandez-Santos et al. 2007). Foote et al. (2002) reported that TEMPOL had toxic effects in bull spermatozoa frozen in whole milk extender (with only 0.2 mM), while these effects were greatly decreased when using an egg yolk-Tris extender. Moreover, Mara et al. (2005) reported that 2 mM TEMPOL in sodium citrate buffer supported the refrigerated storage of ram spermatozoa. Similarly, Bilodeau et al. (2001) tested 0.5 and 1 mM NAC in thawed bull spermatozoa, finding a positive effect in sperm motility after 6 h. In this case, the authors incubated the spermatozoa in the freezing extender (a Tris-egg yolk medium), in which the antioxidants were added. Other authors have tested NAC in fresh human semen (Oeda et al. 1997) and refrigerated stallion spermatozoa (Pagl et al. 2006), obtaining no motility inhibition. Another possible confounding effect is the presence of pyruvate in the incubation media, which has antioxidant properties (Upreti et al. 1998). This reinforces the hypothesis that the effect of these antioxidants on motility could be modulated by the medium and incubation conditions.

The DNA protective ability of rutin deserves a comment, because some studies have shown genotoxic effects in lymphocytes (Liu and Zheng 2002). Flavonoids can act either as genotoxicants or antimutagens, owing to their ability to interact with DNA, although *in vivo* assays have not found genotoxic effects (Utesch et al. 2008). In previous studies, flavonoids showed a pro-oxidant activity at low concentrations (100 μM) and protective effects at higher concentrations of 500 μM (Cemeli et al. 2009; Liu et al. 2010). In studies with human lymphocytes and spermatozoa, rutin at low concentrations (50–250 mM) did not prevent – and, in some cases, exacerbated – the genotoxic effects of mutagens, but prevented the genotoxic effects of the mutagens if used at 0.5 mM (Anderson et al. 1997, 1998). Our results show that rutin prevented DNA breaks as assessed by TUNEL at both concentration levels (100 μM and 1 mM). We have not observed the effects reported for other flavonoid, quercetin, which has been used to induce bull sperm capacitation because of its effects as a calcium ATPase inhibitor (Córdoba et al. 2007).

The effect of DHA was different to the other antioxidants tested. At 0.1 mM, it reduced intracellular ROS in the absence of oxidative stress, while not affecting motility (except for a slight decrease of ALH at 2 h). Nevertheless, its ROS scavenging effect seemed weak, not having effect in the presence of oxidative stress, nor reducing lipoperoxidation and DNA damage. However, at 1 mM, DHA not only had a strong effect on motility, but also affected sperm functionality, did not reduce intracellular ROS nor lipoperoxidation, and induced a small increase in DNA damage in samples incubated without oxidative stress. These paradoxical results could be explained considering that DHA needs to be reduced to ascorbic acid upon entering the cell (KC et al. 2005) in order to contribute to the antioxidant pool. However, the mammal spermatozoon has a very reduced cytoplasm and seems to have a limited capacity to regenerate antioxidants to its reduced form (Bilodeau et al. 2001). In these conditions, DHA may

enter in the sperm cytoplasm efficiently, but its usefulness as an antioxidant would be very limited owing to its slow reduction to ascorbic acid. Therefore, it may be acting as a pro-oxidant if applied at high concentrations.

In conclusion, NAC (at 1 mM), TEMPOL and rutin showed a strong antioxidant activity, accompanied by a high capacity for protecting sperm DNA in the presence of oxidative stress. It is necessary to test whether the inhibition of motility observed in our experiment is transient or irreversible and how these antioxidants affect sperm physiology in other conditions (especially in refrigerated or frozen storage). We have to consider that we incubated the spermatozoa at 37°C, and thus the results might be different if spermatozoa are exposed to the antioxidants at lower temperatures, or if they are removed before or shortly after taking the sample to physiological temperatures. Moreover, the fertility of samples supplemented with these antioxidants might not reflect the sperm quality showed in this paper. Dehydroascorbic acid could still be used pairing it with another antioxidant or with a source of reducing power – improving the capacity of spermatozoa to reduce it to ascorbic acid – while retaining the advantage of its efficient internalization via GLUT transporters.

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Conflict of interest

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