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Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37 °C in presence of several antioxidants

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Abstract

Antioxidants may be useful for supplementing sperm extenders. We have tested dehydroascorbic acid (DHA), TEMPOL, N-acetyl-cysteine (NAC) and rutin on epididymal spermatozoa from red deer, during incubation at 37 °C. Cryopreserved spermatozoa were thawed, washed and incubated with 1 mM or 0.1 mM of each antioxidant, including oxidative stress (Fe^{2+}/ascorbate). Motility (CASA and clustering of subpopulations), viability, mitochondrial membrane potential, and acrosomal status were assessed at two and four h. Lipoperoxidation, intracellular reactive oxygen species (ROS) and DNA damage (DNA) status (TUNEL) were checked at 4 h. Oxidative stress increased ROS, lipoperoxidation and DNA damage. Overall, antioxidants negatively affected motility and physiological parameters. Only DHA 1 mM protected motility, increasing the fast and progressive subpopulation. However, it had a detrimental effect on acrosomal and DNA status, in absence of oxidative stress. Tempol and rutin efficiently reduced lipoperoxidation, ROS, and DNA damage in presence of oxidative stress. NAC was not as efficient as TEMPOL or rutin reducing lipoperoxidation or protecting DNA, and did not reduce ROS, but its negative effects were lower than the other antioxidants when used at 1 mM, increasing the subpopulation of hyperactivated-like spermatozoa at 2 h. Our results show that these antioxidants have mixed effects when spermatozoa are incubated at physiological temperatures. DHA may not be suitable because of prooxidant effects, but TEMPOL, NAC and rutin may be considered for cryopreservation trials. In general, exposure of red deer spermatozoa to these antioxidants should be limited to low temperatures, when only protective effects may develop.

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Keywords: Red deer; Spermatozoa; Antioxidant; Oxidative stress; DNA damage

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1. Introduction

Antioxidants have an important role maintaining the motility and the genetic integrity of spermatozoa [1]. Not only pathologic situations put spermatozoa under oxidative stress, but also assisted reproductive techniques may elicit the generation of free radicals [2]. Artificial reproductive techniques (ART), such as cryopreservation, washing and incubation, produce deleterious effects, which can be linked to decreasing fertility. One of the strategies to prevent these deleterious

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effects is to remove free radicals from the sperm medium, adding antioxidants [3–8].

There is a great variety of antioxidant substances, including vitamins, enzymes and other free radical scavengers. The mechanism of action, toxicity and effectiveness vary enormously. Moreover, the effect of antioxidants may change depending on species, medium and protocols (e.g., refrigerated storage vs. cryopreservation vs. IVF).

This study aims at testing a series of antioxidants for their use on the manipulation and conservation of red deer spermatozoa. The protocol follows a previous design, incubating thawed spermatozoa in a buffered medium supplemented with two typical concentrations (1 and 0.1 mM) of several antioxidants [9]. The purpose of such a protocol is to define the general effects of the tested antioxidants on red deer spermatozoa and their possible toxicity, if any. Thus, future studies may use the results of this preliminary test for refining specific applications of these antioxidants (for instance, making up new cryopreservation extenders and testing a particular concentration range). The particularity of the antioxidants chosen in this study–dehydroascorbic acid, TEMPOL, rutin and N-acetyl-cysteine-is that they belong to antioxidant families with different mechanisms of action. In this manner, the results of this study might be useful for researchers testing antioxidants of similar chemical structure and, thus, similar solubility, toxicity and similar effects on spermatozoa.

Dehydroascorbic acid (DHA) is the oxidized form of vitamin C (ascorbic acid). Cells can readily import DHA into the cytoplasm and organelles (rather than ascorbic acid [10]), and reduce it to ascorbic acid, which contributes to the antioxidant system of the cell [11]. It has been confirmed that spermatozoa of several mammals express glucose transporters (GLUT family), and that spermatozoa can import DHA through these transporters [12]. Therefore, we hypothesized that DHA could be more efficient than ascorbic acid –typically used in previous studies with spermatozoa [5,13–17]-as an intracellular antioxidant and for supplementing sperm media.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) is a membrane-permeable nitroxide with SOD-like activity [18]. Due to its ability to permeate membranes, TEMPOL could be a good candidate to provide intracellular protection against free radicals produced in the mitochondria, especially the superoxide radical. Tempol has been tested in several ruminant species, preserving sperm motility during refrigerated storage of goat and ram spermatozoa [19,20]. However, TEMPOL did improve neither motility in cryopreserved bull spermatozoa [21] nor the kidding rate after refrigerated storage of goat semen [20].

Rutin belongs to the chemical family of the flavonoids. These are plant secondary metabolites based on the backbone of 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone). Flavonoids possess a wide range of biological activities, and they are potent antioxidants with anti-genotoxic activity [22]. However, flavonoids can act as prooxidants and genotoxics at low concentrations (100 μM), while protective effects occurred at 500 μM [22,23]. Other flavonoids have been tested in spermatozoa [24], suggesting interesting effects of these compounds beyond their antioxidant effects. Thus, Córdoba, et al. [25] showed that quercetin lowered reactive oxygen species (ROS) levels while modulating capacitation in bull spermatozoa.

Finally, we have included N-acetyl-cysteine (NAC) as a representative of the thiol antioxidant family. Reduced thiols are not only capable to remove free radicals from the medium, but also to regenerate oxidized thiols, improving the redox balance of the cell. Bilodeau, et al. [26] preserved the motility of thawed bull spermatozoa in the presence or absence of oxidative stress, using reduced glutathione, cysteine, NAC of 2-mercaptoethanol at the mM range. Studies in other species have shown that NAC is an efficient ROS scavenger when used in sperm incubation media, improving sperm function [27,28]. However, Pagl, et al. [29] reported that NAC had no effect on the refrigerated storage of stallion spermatozoa.

In our previous report [9], we analyzed many physiological parameters of red deer spermatozoa while testing lipoic acid, melatonin, trolox and crocin. We showed that lipoic acid had no effect, but melatonin, trolox and crocin were efficient antioxidants and protected sperm DNA damage (DNA), whereas crocin had other effects, such as stimulating motility and lipid peroxidation. Our objective in this study is to obtain novel information for the proposed set of antioxidants, which may be useful in future studies and applications. These antioxidants have been never tested in red deer spermatozoa, and very scarcely in other ruminants. In the case of DHA, it has never been tested on spermatozoa.

2. Materials and methods

2.1. Reagents and media

Fluorescence probes YO–PRO-1 and Mitotracker Deep Red, and the ApoTarget APO-BRDU Kit

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(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 PNA-TRITC were acquired from Sigma (Madrid, Spain). Stock solutions of the antioxidant solutions (dehydroascorbic acid, TEMPOL and N-acetylcysteine) were prepared as 100 mM and 10 mM of antioxidant in double-distilled water, except rutin, which was diluted in 1 M NaOH. The oxidant stock was prepared as 10 mM FeSO4 and 50 mM sodium ascorbate (Fe^{2+}/ascorbate) in water. The function of ascorbate is to recycle Fe^{2+}, reducing Fe^{3+}. Therefore, DHA would not interfere with this reaction, since it is already oxidized.

Stock solutions of the fluorescence probes were PI: 7.5 mM; PNA-TRITC: 0.2 mg/mL; YO–PRO-1: 50 μM; TO-PRO-3: 50 μM; Mitotracker Deep Red: 1 mM; CM-H_{two}DCFDA: 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 the oxidant solutions were prepared the same day. BGM-3 (Bovine Gamete Medium) 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 kanamycine, 10 μg mL^{-1} phenol red and 6 mg/mL BSA (pH 7.5).

2.2. *Animals and spermatozoa collection and cryopreservation*

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 (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. Spermatozoa were collected from the cauda epididymis within 3 h post-mortem, and diluted at ambient temperature to 200 × 10^6 spermatozoa/mL in Triladyl (Minitüb, Tiefenbach, Germany) with 20% egg yolk. Extended spermatozoa were cooled down to 5 °C (−2 °C/min) 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 vapor (4 cm above liquid nitrogen, −120 °C) for 10 minutes. The straws remained for a minimum period of 1 year in liquid nitrogen. When needed, doses were thawed by dropping them into a water bath with saline solution at 37 °C for 30 s.

2.3. *Experimental design*

Straws from our cryobank corresponding to four males were thawed and mixed forming a pool. The pool was washed free of freezing extender by diluting with three volumes of BGM-3, centrifuging (600*g*, 5 min) and removing the supernatant. The pellet was slowly resuspended in BGM-3 up to 30 × 10^6 cells/mL.

The washed pool was split among nine tubes. Eight of them were supplemented with either one-hundredth of the 100-mM solution (1 mM final) or the 10-mM solution (0.1 mM final) of each antioxidant: dehydroascorbic acid (DHA), TEMPOL, rutin or NAC. The ninth tube was used as control. All the experiments were replicated seven times.

2.3.1. *Effect of antioxidants in the motility of thawed spermatozoa*

The tubes were incubated at 37°C. Motility was assessed after 2 h and 4 h.

2.3.2. *Effect of antioxidants in the physiology of thawed spermatozoa submitted to oxidative stress*

Half of the volume of each tube was passed to another series of tubes, which were submitted to oxidative stress by adding 100 μM of FeSO4 and 500 μM of sodium ascorbate. The tubes were incubated at 37 °C and analyzed for viability, mitochondrial status and acrosomal status after 2 h and 4 h.

2.3.3. *Effect of antioxidants in markers of oxidative stress 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 analyzed for lipid peroxidation (malondialdehyde production), ROS production and DNA damage. Control was analyzed before starting the incubation, as a reference value.

2.4. *CASA analysis*

Sperm were diluted down to 10 to 20 × 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, Ahrens-

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burg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed 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 analyzed afterward. The standard parameter settings were 25 frames/sec; 20 to 90 μm^2 for head area; VCL >10 μm/sec to classify a spermatozoon as motile. For each spermatozoa, 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 ALH (amplitude of the lateral displacement of the sperm head) and the BCF (head beat-cross frequency).

2.5. Fluorescence probes for sperm analysis

Flow cytometry analyses using fluorescence probes were carried out as described previously [9]. Briefly, samples were diluted down to 10^6 spermatozoa/mL in BGM-3, and stained using four fluorophore combinations. Sperm viability (intact plasmalemma, normal permeability) and increased membrane permeability (apoptotic-like) were assessed with 0.1 μM YO–PRO-1 and 10 μM PI. 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 PNA-TRITC (peanut agglutinin). Yo-PRO-1 allowed discriminating membrane-intact spermatozoa, while 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-H_{two}DCFDA and 0.1 μM TO-PRO-3. CM-H_{two}DCFDA is retained within cells after being cleaved by cellular esterases. When it is oxidized, it acquires an intense fluorescence, indicating presence of intracellular ROS. Spermatozoa were incubated for 30 min in the dark at 37 °C before being analyzed.

DNA damage was assessed by TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling assay], following the manufacturer's instructions (ApoTarget APO-BRDU Kit). Briefly, spermatozoa diluted in PBS (10^6 cells/mL) were fixed for 1 h in 2% paraformaldehyde. The cells were washed twice with PBS and resuspended in 70% 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 labeling 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 analyzed by flow cytometry within 2 h. Positive and negative controls (incubation of fixed cells with DNase A and substituting water for the DNA labeling mixture, respectively) were used to standardize the assay.

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, TRITC, PI, CM-H_2DCFDA and TUNEL), and a 638 nm LED (excitation for Mitotracker Deep Red and TO-PRO-3). Fluorescence from YO–PRO-1, CM-H_2DCFDA and TUNEL (fluorescein isothiocyanate—FITC) were read using a 525/25 BP filter, TRITC was read using a 575/20 BP filter, PI was read using a 620/20 BP filter, and Mitotracker Deep Red and TO-PRO-3 were read using a 675/40 BP filter. FSC/SSC signals were used to discriminate spermatozoa from debris (Fig. 1). Fluorescence captures were controlled using the MPX 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) vs. 2 files. The analysis of the flow cytometry data were carried out using WEASEL vs. 2.6 (WEHI, Melbourne, Australia). The YO–PRO-1/PI and YO–PRO-1/PNA-TRITC/Mitotracker Deep Red staining were analyzed as previously described for red deer [30].

2.6. Assessment of lipid peroxidation

The susceptibility of the spermatozoa to lipoperoxidation was assessed using the Bioxitech MDA-586 kit (Oxis International, Foster, CA, USA) to detect malondialdehyde (MDA) concentration, as described by Domínguez-Rebolledo, et al. [9]. Briefly, samples were diluted with PBS to 10^7 mL^{-1} and incubated for 30 min at 37 °C with 40 μM of Fe^{2+} and 200 μM ascorbate, to induce MDA release [31]. Samples were mixed with the reactive provided in the kit and incubated at 45 °C for 1 h. Then, the tubes were centrifuged and the supernatant was transferred to wells (200 μL/well) in a

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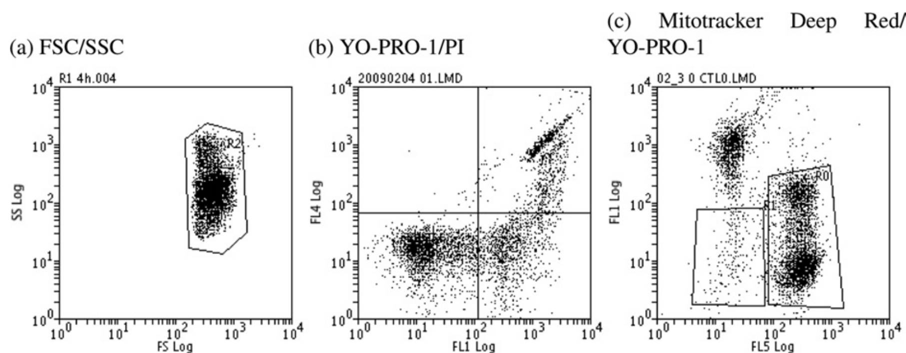

Fig. 1. Representative flow cytometry dot plots for the FSC/SSC signals, YO–PRO-1/PI and Mitotracker Deep Red/YO–PRO-1 stains. After washing, samples contained almost no debris, and any remaining debris was easily gated out using FSC/SSC signals (a) No debris can be detected in fluorescence dot plots (b). The quadrants in the YO–PRO-1/PI dot plot (b) separates the three types of stain (YO–PRO-1-/PI–, viable, intact plasmalemma, normal permeability; YO–PRO - 1+/PI–, apoptotic-like, increased membrane permeability; YO–PRO - 1+/PI+, damaged membrane). In Mitotracker/YO–PRO-1 dot plots (c), regions were used for enclosing MT+ spermatozoa (right; active mitochondria) and MT-/YO-PRO-1-spermatozoa (left; inactive mitochondria, normal membrane permeability). Flow cytometry analyses are described in detail in Martínez-Pastor, et al. [61].

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 known quantities of MDA, and presented as nmol of MDA per 10^8 spermatozoa.

2.7. Statistical analysis

Data were analyzed in the R statistical environment [32]. To analyze the effects of time, antioxidant supplement and oxidant effect on sperm parameters, we used linear mixed-effects models, with incubation time, antioxidant and antioxidant concentration in the fixed part of the models, and, including the replicates as the grouping factor in the random part of the models. Results are presented as mean ± SEM, or effect sizes (mean ± SEM of the respective coefficients) respect to the control (that is, the variation of the given parameter relative to the control).

To study sperm hyperactivation and changes in the motility patterns, we carried out a cluster analysis on CASA data [2]. Briefly, we first chose VAP, STR, WOB, ALH as the most informative parameters (minimizing correlations). Spermatozoa were initially grouped by using a non-hierarchical method (k-means; Clara algorithm), to produce 25 homogeneous clusters. These clusters were grouped using a model-based clustering (hierarchical method), using the Bayesian Information Criterion (BIC) to find the optimal model and final number of clusters. Then, the proportion of each cluster in each sampling was studied in the same manner than the rest of the parameters.

3. Results

Motility was affected by most treatments, being most noticeable at 4 h (Table 1). Total motility (TM), progressive motility (PM), VAP and ALH of the Control decreased steadily but significantly with time. At 2 h, some antioxidants produced some inhibition of motility. Rutin 1 mM depressed all the five motility parameters showed in Table 1, and TEMPOL 1 mM decreased PM, VAP and ALH. The lower concentrations of these antioxidants affected PM (rutin) and VAP (TEMPOL) in a lesser degree but still significantly. This negative effect was also displayed in NAC-treated samples, with lower PM (1 mM), VAP (0.1 mM) and LIN (1 and 0.1 mM). Contrarily, DHA enhanced several motility parameters above Control: PM and LIN at 1 and 0.1 mM, and VAP at 1 mM, while not showing a significant motility depression.

At 4 h, rutin 1 mM produced the largest inhibitory effect on motility, decreasing TM by −25.0 ± 3.1 respect to Control. As for 2 h, rutin 1 mM decreased all kinetic parameters. However, when applied at 0.1 mM, there were no significant differences respect to Control (although mean values were lower). Tempol 1 mM reduced motility parameters too, except for ALH, but with a lower effect than rutin 1 mM. Tempol 0.1 mM only decreased TM. NAC decreased TM (interestingly, the effect was larger at 0.1 mM than at 1 mM, −16.0 ± 3.2 vs. − 11.3 ± 3.2). LIN, which was not significantly affected by incubation time in the Control, showed a significant decrease in both NAC concentrations. DHA 1 mM protected motility, in fact yielding a higher LIN than the Control (59.3 ± 2.8% vs. 53.3 ± 2.7%),

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Table 1
Sperm motility (mean ± SEM) in samples submitted to two and four h of incubation at 37 °C. Differences among incubation times (Control samples) are indicated with different letters ($P < 0.05$). Asterisks indicate significant differences among each antioxidant treatment and the Control within each incubation time (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Time	Antioxidant	mM	TM (%)	PM (%)	VAP (μm/sec)	Lin (%)	ALH (μm)
0	Control		92.9 ± 4.4	45.6 ± 6.1	101.9 ± 4.5	53.7 ± 4	4.8 ± 0.6
2	Control		81.2 ± 3.7	40.2 ± 3.3	80.1 ± 6.8	56.2 ± 2.4	3.6 ± 0.3
	DHA	0.1	83.7 ± 4.2	44.3 ± 5.5	81.5 ± 6.6	58.7 ± 4.4	3.3 ± 0.3
		1	82.5 ± 9.9	44.8 ± 4.4	89.5 ± 5.7	60.4 ± 3	3.4 ± 0.4
	NAC	0.1	77.3 ± 5.5	36.6 ± 2.9	74.6 ± 3.8	53.8 ± 2.3	3.5 ± 0.2
		1	74.8 ± 12.2	34.1 ± 6	77 ± 5.4	53.1 ± 1.8	3.7 ± 0.2
	RUT	0.1	75.6 ± 5	35.9 ± 3.2	75.2 ± 4.8	54.5 ± 2.3	3.5 ± 0.3
		1	63.2 ± 7.7	27.4 ± 2.7	59.2 ± 3.5	50.3 ± 1.8	3.1 ± 0.2
	TPL	0.1	75.4 ± 4.3	36.7 ± 4.7	74.8 ± 7	55.7 ± 2.5	3.4 ± 0.3
		1	75.3 ± 8.6	36 ± 5	70.2 ± 3.9	54.2 ± 2.8	3.3 ± 0.1
4	Control		79.1 ± 4.9	35.9 ± 3.2	66.5 ± 4.3	53.3 ± 2.7	3.3 ± 0.2
	DHA	0.1	70.5 ± 9.6	32.7 ± 5.2	65.2 ± 4.2	54.6 ± 4.1	3 ± 0.2
		1	84.1 ± 5.6	43.7 ± 4.5	78.2 ± 8.3	59.3 ± 2.8	3.1 ± 0.2
	NAC	0.1	63.1 ± 6.5	27 ± 4.5	58.6 ± 3.7	50 ± 3	3.1 ± 0.3
		1	67.8 ± 5.6	29 ± 4.2	62.5 ± 4.9	50.1 ± 2.5	3.2 ± 0.2
	RUT	0.1	71.9 ± 6.3	31.2 ± 3.2	65 ± 9.6	50.8 ± 1.6	3.5 ± 0.4
		1	54.1 ± 5.9	20.8 ± 3.4	51.5 ± 3.5	48.2 ± 1.2	2.9 ± 0.2
	TPL	0.1	70 ± 8.5	32.5 ± 5	64.1 ± 4.5	52.5 ± 2.7	3.3 ± 0.2
		1	64.6 ± 5.9	27.8 ± 4.4	62.3 ± 7.1	50.7 ± 3.4	3.2 ± 0.2

ALH, amplitude of the lateral displacement of the sperm head; LIN, linearity; PM, progressive motility; TM, total motility; VAP, velocity according to the smoothed path.

although at 0.1 mM TM and ALH were lower than for the Control ($P < 0.05$).

The cluster analysis yielded four clusters, whose characteristics are shown in the Table 2 being interpreted similarly to Martínez-Pastor, et al. [33]. Cluster 1 grouped progressive spermatozoa (high velocity and high linearity). The Cluster 2, with a high mean velocity, low linearity and highest ALH, grouped hyperactivated-like spermatozoa. Cluster 3 grouped slower spermatozoa, with linearity and ALH between those of Clusters 1 and 2, therefore being considered "moderately progressive". Cluster 4 grouped slow and nonlinear spermatozoa, likely exhausted ones. Figure 2 shows the changes in the proportions of each cluster over time and the effects of the different antioxidant treatments. In the controls, Cluster 1 increased from 0 to 2 h, decreasing at the 4 h endpoint. Cluster 2 decreased at 2 h. Clusters three and four increased with time. Rutin 1 mM impacted most the motility pattern, significantly decreasing the proportion of Clusters 1 and 2 at both sampling times, while increasing Cluster 4. Rutin 0.1 mM only decreased Cluster 1 at 4 h NAC 1 mM increased Cluster 2 at 2 h while decreasing Cluster 3. Tempol 1 mM increased the proportion of Cluster 4 at 2 h DHA 0.1 mM decreased Cluster 2 at 2 h, and at 1 mM it increased Cluster 1 at both times and decreased Cluster 3 at 4 h.

Membrane status of Control spermatozoa was not affected by incubation time, while the addition of oxidative stress increased the proportion of membrane-damaged spermatozoa (PI+) at 2 h, remaining the same at 4 h (table in Fig. 3). This increase was caused mainly by the death of spermatozoa that already presented an increased membrane permeability at 0 h (YO–PRO - 1+/PI–). This subpopulation showed a reduction of −9.1 ± 1.6 at 2 h, whereas the subpopulation of viable spermatozoa (YO–PRO - 1-/PI–) was reduced by only −4.7 ± 1.3, at the same time. Figure 3 shows the effects of the antioxidant treatments on the YO–PRO-1/PI subpopulations. In general, the addition of antioxidants was detrimental for membrane status, although effects were generally small. At 2 h, the proportion of

Table 2
Clusters obtained from CASA data and average values of several kinetic parameters (mean ± SD). The characteristics of Cluster 2 resemble those of hyperactivated spermatozoa (high velocity and ALH, low linearity).

Control	0 h	4 h	4 h OXI
Ros	119.7 ± 58.8	245.6 ± 51.2	290.2 ± 52.3
MDA	15.3 ± 1.9	14.8 ± 1.5	20.8 ± 2.0
TUNEL+	5.5 ± 2.2	5.8 ± 2.9	28.1 ± 2.6

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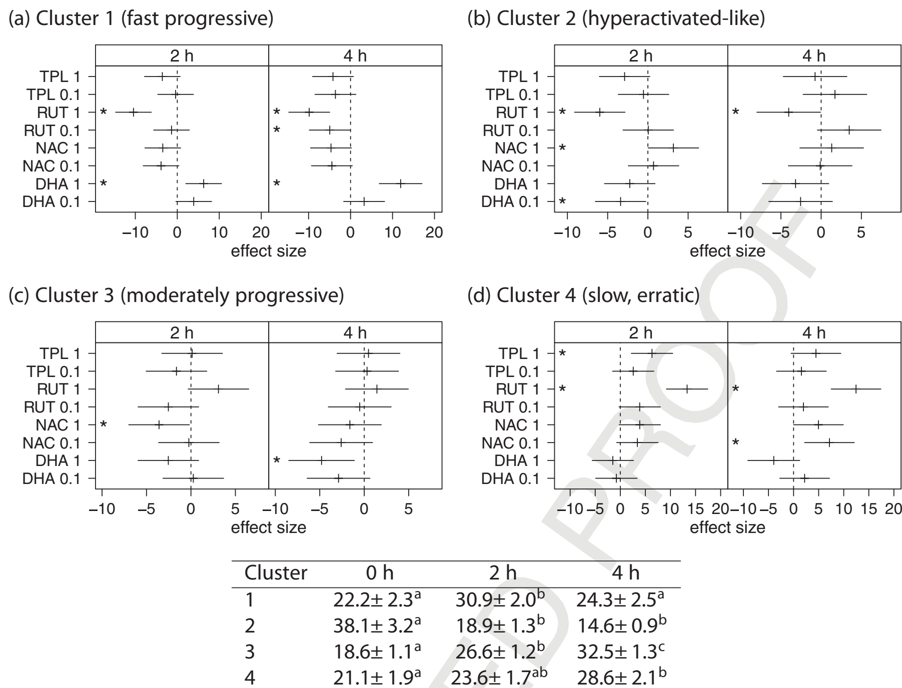

Cluster	0 h	2 h	4 h
1	22.2± 2.3^{a}	30.9± 2.0^{b}	24.3± 2.5^{a}
2	38.1± 3.2^{a}	18.9± 1.3^{b}	14.6± 0.9^{b}
3	18.6± 1.1^{a}	26.6± 1.2^{b}	32.5± 1.3^{c}
4	21.1± 1.9^{a}	23.6± 1.7^{ab}	28.6± 2.1^{b}

Fig. 2. Cluster analysis of CASA data. Four clusters were identified, one of them with characteristics resembling of hyperactivated spermatozoa. Graphs show the effects of the antioxidant treatments on cluster proportions at 2 h and 4 h of incubation. Mean and 95% CI are shown respective to Control (dotted line, no effect; asterisks mark significant effects): effects on the left of the dotted line imply a decrease of the parameter; effects on the right of the dotted line imply an increase of the parameter. The table shows mean ± SEM values for the Control at 0, two and four h (columns with different letters differ $P < 0.05$). DHA, Dehydroascorbic acid; NAC, N-acetyl-cysteine; RUT, rutin; TPL, TEMPOL.

viable spermatozoa was reduced significantly in the TEMPOL, DHA and rutin treatments at 1 mM, and in NAC 0.1 mM. At 4 h, only rutin and DHA at 1 mM had a significant effect. With the addition of oxidative stress, only rutin exerted a negative effect, at both times, while NAC 1 mM had a significant effect at 4 h. These changes were accompanied by some increases in the proportion of the YO–PRO - 1+/PI-subpopulation, which was slightly increased at 2 h by NAC 0.1 mM and at 4 h by TEMPOL 0.1 mM, whereas DHA 1 mM caused a decline in this population at this time. However, upon adding oxidative stress, this population grew when incubated with 1 mM of rutin or TEMPOL ($P < 0.05$), displaying this effect both at 2 and 4 h. Subfigure 3a shows that in this case the YO–PRO - 1+/PI+ subpopulation did not increase, thus, TEMPOL prevented in part its increase at 2 h, remaining similar to the Control.

Mitochondrial status as indicated by Mitotracker deep red was neither significantly affected by incubation time nor oxidative stress in the Control samples (table in Fig. 4). It was affected by some antioxidants, causing small significant decreases of the subpopulation of spermatozoa with active mitochondria (YO–PRO - 1-/MT+, subfigure 4a). This effect was shown at 2 h by TEMPOL, rutin and DHA 1 mM, and DHA 0.1 mM, and at 4 h by rutin and DHA 1 mM. Samples with oxidative stress only showed this effect with rutin 1 mM at 2 h and rutin 1 and 0.1 mM at 4 h. In several treatments, the proportion of viable spermatozoa with inactive mitochondria (YO–PRO-1-/MT-, subfigure 4b) increased significantly. This increase was concomitant with the decrease of the YO–PRO - 1-/MT+ subpopulation for DHA at 2 h and TEMPOL and DHA 1 mM at 4 h, and, in presence of oxidative stress, for DHA 1 mM at 2 h and rutin at 4 h. In samples with added oxidative stress, YO–PRO-1-/MT-spermatozoa increased for rutin 0.1 mM at 2 h and NAC 1 mM at 2 and 4 h, but without a significant decrease of YO–PRO - 1-/MT+ spermatozoa. These changes affected the relative proportion of spermatozoa with active mitochondria respect to the YO–PRO-1-population (subfigure 4c).

Unlike plasma membrane and mitochondrial status, the proportion of Control spermatozoa with damaged acrosomes was increased both by incubation time and oxidative stress, although this increase was only detected at 2 h, with no further increase at 4 h (table in Fig. 4). This percentage was slightly increased at 2 h by

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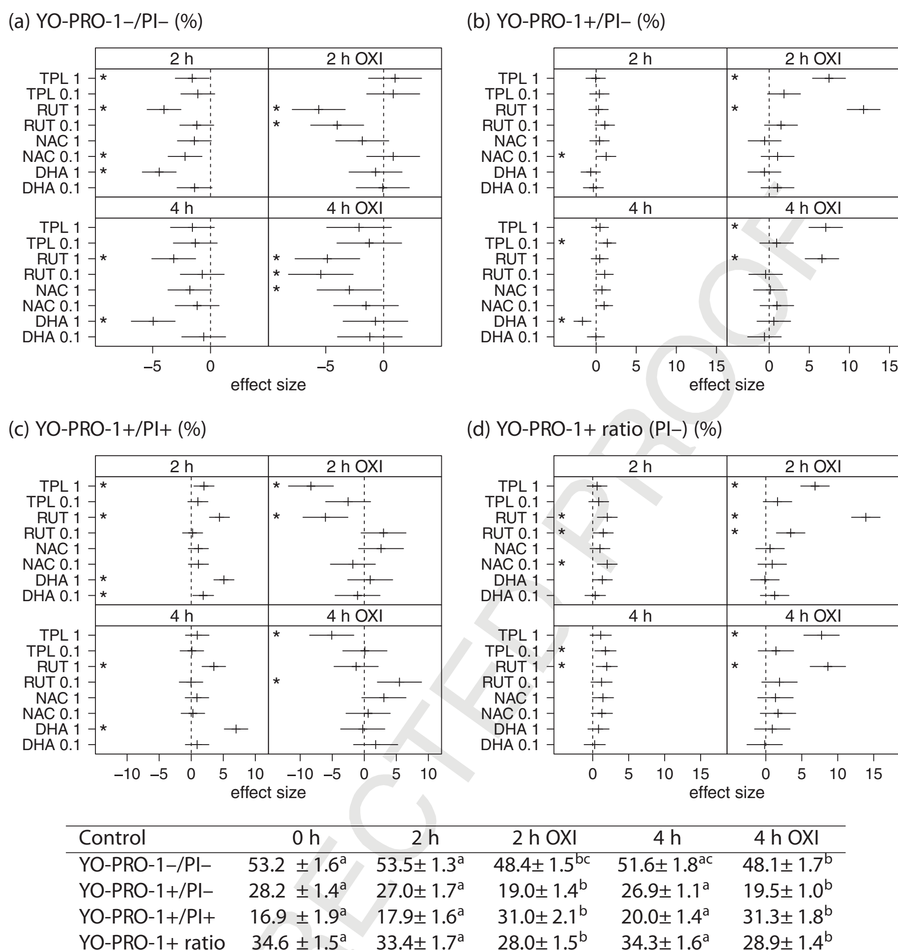

Control	0 h	2 h	2 h OXI	4 h	4 h OXI
YO-PRO-1–/PI–	53.2 ± 1.6[a]	53.5± 1.3[a]	48.4± 1.5[bc]	51.6± 1.8[ac]	48.1± 1.7[b]
YO-PRO-1+/PI–	28.2 ± 1.4[a]	27.0± 1.7[a]	19.0± 1.4[b]	26.9± 1.1[a]	19.5± 1.0[b]
YO-PRO-1+/PI+	16.9 ± 1.9[a]	17.9± 1.6[a]	31.0± 2.1[b]	20.0± 1.4[a]	31.3± 1.8[b]
YO-PRO-1+ ratio	34.6 ± 1.5[a]	33.4± 1.7[a]	28.0± 1.5[b]	34.3± 1.6[a]	28.9± 1.4[b]

Fig. 3. Effects of antioxidant treatments on sperm plasma membrane (YO–PRO-1/PI assessment) at 2 h and 4 h of incubation: (a) YO–PRO-1-/PI-spermatozoa (viable, intact plasmalemma, normal permeability); (b) YO–PRO - 1+/PI-spermatozoa (apoptotic-like, increased membrane permeability); (d) YO–PRO - 1+/PI+ spermatozoa (non-viable, damaged membrane); (c) ratio of the sperm subpopulation with increased membrane permeability (YO–PRO - 1+/PI–) respect to the whole PI-subpopulation (continuous membrane). Left and right panes show effects in absence and presence of oxidative stress, respectively. Mean and 95% CI are shown in respect to Control (dotted line, no effect; asterisks mark significant effects): effects on the left of the dotted line imply a decrease of the parameter; effects on the right of the dotted line imply an increase of the parameter. The table shows mean ± SEM values for the Control at 0, two and four h (columns with different letters differ $P < 0.05$). DHA, Dehydroascorbic acid; NAC, N-acetyl-cysteine; RUT, rutin; TPL, TEMPOL.

TEMPOL 1 mM, rutin and DHA 0.1 mM, and, in a higher degree, by DHA 1 mM (+7.5 ± 0.8). The effect of DHA 1 mM was even higher at 4 h (+9.2 ± 1.0), while NAC 1 mM slightly decreased the proportion of damaged acrosomes. In presence of oxidative stress and at 2 h, only DHA 1 mM increased this proportion (+4.7 ± 1.7), while TEMPOL 1 mM decreased it. At 4 h, the effect of DHA 1 mM was no significant, while rutin 0.1 mM increased acrosomal damage, and TEMPOL 0.1 and 1 mM decreased it.

Intracellular ROS, lipoperoxidation (as MDA production) and DNA damage (as the proportion of TUNEL+ spermatozoa) of the Control are shown in the table in Fig. 5. The presence of intracellular ROS increased significantly both with the 4 h incubation and with induced oxidative stress. However, lipoperoxidation and DNA damage were not significantly affected by incubation time, but they increased with oxidative stress. ROS production at 4 h (Subfigure 5a) was reduced by rutin 1 mM, but not by other treatments. In the samples with added oxidative stress, TEMPOL 1 mM and both rutin concentrations (specially 1 mM) reduced ROS production significantly respect to the Control. In the case of MDA levels (Subfigure 5b), NAC 1 mM and both concentrations of TEMPOL and rutin reduced them significantly. Adding oxidative stress, rutin 1 mM

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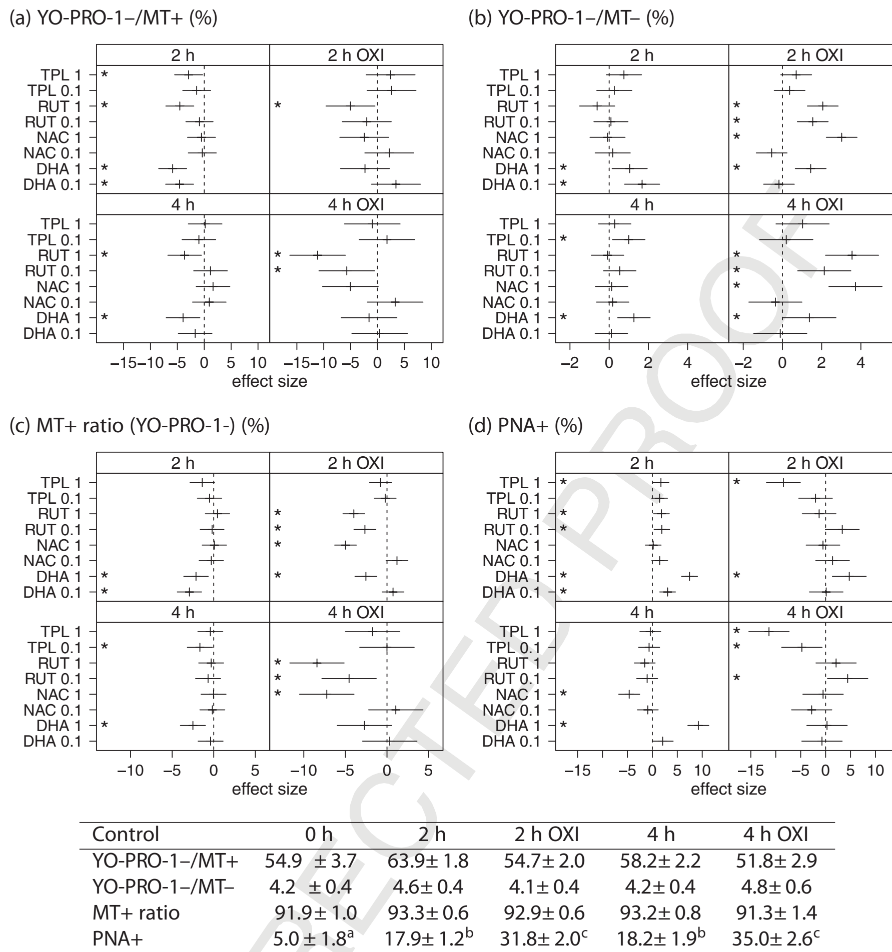

Control	0 h	2 h	2 h OXI	4 h	4 h OXI
YO-PRO-1–/MT+	54.9 ± 3.7	63.9± 1.8	54.7± 2.0	58.2± 2.2	51.8± 2.9
YO-PRO-1–/MT–	4.2 ± 0.4	4.6± 0.4	4.1± 0.4	4.2± 0.4	4.8± 0.6
MT+ ratio	91.9± 1.0	93.3± 0.6	92.9± 0.6	93.2± 0.8	91.3± 1.4
PNA+	5.0 ± 1.8[a]	17.9± 1.2[b]	31.8± 2.0[c]	18.2± 1.9[b]	35.0± 2.6[c]

Fig. 4. Effects of antioxidant treatments on mitochondria and acrosome (YO–PRO-1/Mitotracker deep red/PNA-FITC assessment) at 2 h and 4 h of incubation: (a) YO–PRO - 1-/MT+ spermatozoa (viable with active mitochondria); (b) YO–PRO-1-/MT-spermatozoa (viable with inactive mitochondria); (c) ratio of the sperm subpopulation with active mitochondria (YO–PRO - 1-/MT+) respect to the whole YO–PRO-1-subpopulation (viable spermatozoa); (d) PNA+ spermatozoa (damaged acrosomes). Left and right panes show effects in absence and presence of oxidative stress, respectively. Mean and 95% CI are shown respective to Control (dotted line, no effect; asterisks mark significant effects): effects on the left of the dotted line imply a decrease of the parameter; effects on the right of the dotted line imply an increase of the parameter. The table shows mean ± SEM values for the Control at 0, two and four h (columns with different letters differ $P < 0.05$). DHA: Dehydroascorbic acid; NAC: N-acetyl-cysteine; RUT: rutin; TPL: TEMPOL.

and both concentrations of TEMPOL and NAC significantly reduced MDA levels. The proportion of TUNEL+ spermatozoa was not reduced by any antioxidant treatment in the absence of oxidative stress (subfigure 5c), but DHA 1 mM increased it moderately (mean increase of 7.0 ± 3.2). Only with oxidative stress the antioxidants showed protective effects on sperm DNA, with TEMPOL 1 mM and both rutin concentrations showing the largest effects (TEMPOL 1 mM: −21.5 ± 3.3; rutin 1 mM: −19.8 ± 3.4; rutin 0.1 mM: −19.9 ± 3.4). In practice, these treatments abolished the DNA damage produced by the oxidative stress (table in Fig. 5). Tempol 0.1 mM and NAC 1 mM also reduced the proportion of TUNEL+ spermatozoa significantly, although only slightly above half the effect of the later treatments (TEMPOL 0.1 mM: −10.3 ± 3.3; NAC 1 mM: −11.8 ± 3.3).

The most relevant results are summarized in Table 3.

4. Discussion

The mammal spermatozoon is particularly vulnerable to oxidative stress, because of its lack of cytoplasm, composition of the plasma membrane (rich in polyunsaturated fat acids) and exposure to different environ-

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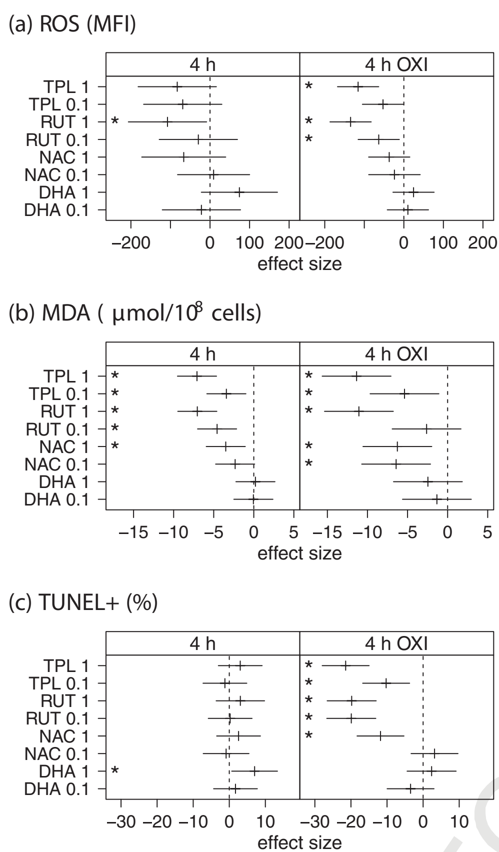

Control	0 h	4 h	4 h OXI
ROS	119.7 ± 58.8[a]	245.6± 51.2[b]	290.2± 52.3[c]
MDA	15.3 ± 1.9[a]	14.8± 1.5[a]	20.8± 2.0[b]
TUNEL+	5.5 ± 2.2[a]	5.8± 2.9[a]	28.1± 2.6[b]

Fig. 5. Effects of antioxidant treatments on oxidative damage at 4 h of incubation: (a) Intracellular ROS according to CM-H2DCFDA fluorescence (MFI: median fluorescence intensity, arbitrary units); (b) lipid peroxidation, as free malondialdehyde—MDA-production; (c) DNA damage as proportion of TUNEL+ spermatozoa. Left and right panes show effects in absence and presence of oxidative stress, respectively. Mean and 95% CI are shown in respect to Control (dotted line, no effect; asterisks mark significant effects): effects on the left of the dotted line imply a decrease of the parameter; effects on the right of the dotted line imply an increase of the parameter. The table shows mean ± SEM values for the Control at 0, and 4 h (columns with different letters differ $P < 0.05$). DHA: Dehydroascorbic acid; NAC: N-acetyl-cysteine; RUT: rutin; TPL: TEMPOL.

ments (especially when used in artificial reproductive techniques, ART) [13,34,35]. Therefore, antioxidants have been proposed as supplements to the media used for sperm processing and incubation in ART [36]. In this study, we have tested four antioxidants in red deer spermatozoa, to assess their suitability for practical use, and to investigate their effects on sperm physiology and functionality. This study follows a previous trial [9], in which lipoic acid, melatonin, trolox (a water-soluble form of α-tocopherol) and crocin (a saffron carotenoid) were tested, with different results. Contrarily to that study, in which trolox, crocin and melatonin showed many positive effects, the antioxidants of the present trial showed mixed results. In fact, we have detected many detrimental effects, which cast doubt about the practical use of some of them in red deer spermatozoa. However, some effects could be interesting for some applications, and deserve further testing.

Other authors have noted a lack of dramatic benefits when using antioxidants [21,37], and even reported loss of quality [5,13]. This lack of benefits could arise from the presence of other protective substances in extenders, low metabolic activity of the spermatozoa and relatively short exposure to aerobic conditions during sperm work. Moreover, sperm samples are usually processed and stored at low temperatures, decreasing the sperm metabolism and the rate of potentially detrimental reactions. Considering ruminants, it is possible that the response to oxidative stress could be different from other species. For instance, human spermatozoa is highly sensitive to the addition of hydrogen peroxide, quickly losing motility and viability upon exposure to 25 μMof H_2O_2 [38]. Contrarily, red deer epididymal spermatozoa seems to be more resistant to the effect of ROS [9], although some sperm parameters, such as motility, may be affected by relatively high H_2O_2 levels [39]. In our study, we used an incubation at 37 °C up to 4 h, looking for disclosing any detrimental effects helped by the increased sperm metabolism, which might be more evident in these conditions than during refrigerated or frozen storage.

The detrimental effects showed by TEMPOL contrast with the results obtained in previous studies in ram semen [19]. Nevertheless, it must be taken into account that we used epididymal spermatozoa, whereas these authors utilized ejaculated ones, which may account for those differences. Our group and others have noted that epididymal and ejaculated spermatozoa show some differences [40–42], which may root in different physiological regulation and, therefore, different response to ROS and antioxidants. Tempol is a stable piperidine nitroxide, whose effects have been compared with those of superoxide dismutase (SOD) [43], removing superoxide and other radicals. In fact, this antioxidant could convey advantages over SOD, since it can permeate membranes and act intracellularly, and it would not produce H_2O_2 [44]. The results obtained in ROS levels and MDA production showed indeed that

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Table 3
Summary table. The most relevant results are shown in a reduced form. Each parameter is shown when there is a significant effect respect to the control ($P < 0.05$); positive effects are shown underlined, and negative effects are shown plain. Abbreviations are shown at the bottom of the table.

Antioxidant	mM	2 h	4 h	2 h OXI	4 h OXI
DHA	0.1	MIT	Mot		
	1	Mi MIT	Mi mit DNA		
NAC	0.1	MI	Mot		LPO
	1	HYP	Mot LPO		LPO DNA
RUT	0.1		LPO	MI	Mi mit ros DNA
	1	Mot hyp mi MIT	Mot hyp mi MIT Ros LPO	Mi MIT	Mi mit ros LPO DNA
TPL	0.1		Mot LPO		LPO DNA
	1	Mi MIT	Mot LPO		Ros LPO DNA

DHA, dehydroascorbic acid; NAC, N-acetyl-cysteine; RUT, rutin; TPL, TEMPOL; MOT, motility; HYP, proportion of hyperactivated-like spermatozoa; MI, membrane integrity; MIT, active mitochondria; ros, ROS concentration; LPO, lipid peroxidation; DNA, DNA damage.

TEMPOL is an effective antioxidant. The 1-mM treatment was also very efficient scavenging free radicals and protecting DNA in presence of induced oxidative stress. The inhibition of motility observed in the TEMPOL samples could be caused either by a direct toxic effect or by the excessive removal of intracellular free radicals. It is well known that cells regulate their activity by means of a finely adjusted redox balance, using free radicals as second messengers in signal transduction cascades. Many studies have proved that similar mechanisms are present in spermatozoa, where free radicals modulate capacitation and other events [45]. The application of oxidative stress promotes capacitation and tyrosine phosphorylation [46,47], whereas the application of radical scavengers inhibits these processes. Is it possible that TEMPOL, being an efficient intracellular radical scavenger, were interfering with transduction cascades during the incubation of spermatozoa? It might be one explanation for the reduction of sperm motility at 4 h and other effects that we observed in TEMPOL-treated samples. Indeed, TEMPOL reduced in part the ROS-induced increase of damaged acrosomes at 2 and 4 h, which may be a hint of TEMPOL inhibiting capacitation-related events. Moreover, the effect of TEMPOL could vary depending on the treatments applied to the spermatozoa. Foote, et al. [21] indicated 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 instead.

Rutin elicited even a more pronounced inhibition of sperm motility at 1 mM, while showing a high ability to scavenge free radicals and to protect DNA. The protection of DNA against induced oxidative stress was not noted in previous studies with lymphocytes [48], but it was clearly showed in our study, where rutin abolished DNA damage even at 0.1 mM. Flavonoids can behave either as mutagens or antimutagens, depending on the conditions. For instance, several flavonoids, rutin among them, caused genotoxic effects in human lymphocytes and spermatozoa [49,50]. When they were combined with food mutagens they exacerbated genotoxic effects at low doses, but they showed antigenotoxic activity at high doses. Nevertheless, *in vivo* tests have discarded genotoxic effects for quercetin and other flavonoids [51]. Flavonoids (quercetin and kaemferol up to 500 μM) were capable to reduce the DNA damage on lymphocytes and spermatozoa when treated with estrogen-like compounds and hydrogen peroxide [24]. It seems that the concentrations of rutin used in this study favor the antioxidant effects of this molecule, but results may vary in other media or upon dilution of semen samples (e.g., AI).

Several studies have found a strong affinity of several flavonoids to the DNA [52]. The interaction of flavonoids with the DNA helix stabilizes the DNA structure, enables the flavonoid to react with free radicals and to repair oxidized bases or the sugar backbone [53]. However, this is a double-edged property, since the close proximity of the flavonoid phenyl rings to the base pairs can equally increment the prooxidant effect. According to our results, rutin protected sperm DNA against oxidative stress, possibly because of the mechanisms described in the aforementioned studies. Plasma membrane limits the intracellular concentration of flavonoids [54], explaining the similar effects of 0.1 and 1 mM, and possibly stabilizing it to such a concentration that favors its antioxidant activity.

Taking into account the results for DNA status, lipoperoxidation levels and intracellular ROS, the effects of rutin in motility and other physiological parameters are unlikely to be caused by a prooxidant effect.

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Like in the interpretation of TEMPOL results, its negative effects may be due to an excessive removal of intracellular ROS, interfering with intracellular signaling. The inhibition of motility affected clearly the subpopulation structure, with a decrease in the proportion of the fast subpopulations (Clusters 1 and 2) and a concomitant increase of the slow subpopulation (Cluster 4). These results suggest that rutin at high concentrations may inhibit not only sperm metabolism, but also signaling routes regulating flagellar beating [55]. This is supported by the fact that motility inhibition was much larger than the detrimental effect on membrane integrity or mitochondrial activity. Moreover, quercetin, another flavonoid, has been identified as a specific inhibitor of plasma membrane calcium-ATPase, inducing an increase of intracellular calcium and modulatory effects on sperm capacitation [56]. Rutin, although less active than quercetin, has similar effects [57], and thus part of our results could be a consequence of the direct interaction of rutin with the signal transduction system of the spermatozoa.

Contrarily to the other antioxidants, NAC at 1 mm exerted smaller negative effects than at 0.1 mm. This is a rather paradoxical result, which could be interpreted as the consequence of negative effects being superseded by the higher antioxidant protection of NAC 1 mm. Although NAC did not have a significant effect on reducing ROS, at 1 mm was effective both reducing MDA levels and TUNEL+ spermatozoa, not achieving rutin or TEMPOL levels, though. Moreover, although NAC decreased the proportion of motile spermatozoa at 4 h, at 2 h it slightly increased the proportion of Cluster 2 (hyperactivated-like) while decreasing the proportion of Cluster 3 (moderately progressive). At 2 h, there was a decrease in the proportion of Cluster 2 in the control, accompanied by increases in Clusters 1 and 3. This may indicate a modulating effect in regulation pathways, and different from other treatments, such as rutin 1 mm. Thiol antioxidants have interesting properties [58], making them good candidates for antioxidant supplementation of sperm media. Nevertheless, the use of these antioxidants has resulted in mixed results. Cysteine, NAC and GSH at 0.5 and 1 mm maintained the motility of thawed bull semen up to 6 h in absence of oxidative stress [26]. In the same study, 1 mm, but not 0.5 mm, of these antioxidants protected motility in presence of oxidative stress (100 μm H_2O_2). However, Foote, et al. [21] found that, although GSH supplementation of bull semen helped to maintain the progressive motility 12 h after thawing, it did not improve non-return rates.

Some of our results on TEMPOL and NAC differ from previous studies. For instance, it has been reported that 2 mM TEMPOL improved fertility of refrigerated semen in ram [19] (but not in goat [20]), and Foote, et al. [21] reported no motility decrease when freezing bull spermatozoa with an egg yolk-Tris extender supplemented with TEMPOL. It is possible that TEMPOL exerts negative effects at physiological temperatures, but not at low temperatures. Moreover, in the IVF and insemination trials [19,20], sperm media is highly diluted or removed, and therefore spermatozoa do not remain in contact with the antioxidant for a long time at physiological temperatures. Thus, taking into account the good results of TEMPOL protecting DNA, as a lipoperoxidation blocker and scavenging ROS, this antioxidant might be tested for red deer spermatozoa cryopreservation, but taking care of removing it after thawing. Similarly, several studies have reported that NAC (0.5 and 1 mM) improved the motility of refrigerated dog semen [28] and cryopreserved bull semen [26], and Oeda, et al. [27] reported a small improvement of motility in fresh human semen (6.3 mM). There are differences among studies, most probably because of interspecific or experimental differences. For instance, NAC had no effect on the motility of refrigerated stallion semen [29], but it was used at only 200 μM, whereas it improved dog semen motility being used at 1.5 mM [59]. Moreover, 1.5 mM NAC was not able to decrease basal ROS levels of dog spermatozoa [28,59], and in our study neither one nor 0.1 mM reduced ROS, even in presence of oxidative stress. However, NAC, both at 6.3 and 31.3 mM (but not at 0.6 mM), reduced ROS in fresh human spermatozoa. The fact that higher NAC concentrations improved results in all these studies, including ours, enable us to suggest that future cryopreservation trials with red deer spermatozoa should include concentrations above 1 mM. Nevertheless, Michael, et al. [28], after refrigerating dog semen for 72 h, obtained no improvement with 2.5 mM, and there was a decrease of motility with 5 mM, announcing toxic effects at relatively high concentrations.

The results of DHA are paradoxical, and have some resemblance to those obtained testing 1 mM crocin in our previous study by Domínguez-Rebolledo, et al. [9]. Similarly, crocin improved motility during a 4 h incubation and, in absence of oxidative stress, increased the proportion of damaged acrosomes. However, there are some important differences: 1 mM DHA improved motility slightly, increasing kinematic parameters, while the effect of crocin was more dramatic, maintaining the proportion of motile spermatozoa above Control lev-

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els; despite of a slight decrease of average values, the effect of crocin on mitochondrial and membrane parameters –in absence of oxidative stress-was not significant; crocin prevented acrosomal damage in presence of induced oxidative stress; and, more importantly, despite of enhancing lipid peroxidation, it protected sperm DNA in presence of oxidative stress. In this study, we found that DHA 1 mM not only stimulated motility, but also affected the subpopulation patter, increasing Cluster 1 (fast and progressive spermatozoa), at the expense of Cluster 3 (less rapid and linear).

Therefore, it seems that DHA behaved more as a prooxidant than as an antioxidant. DHA has been studied in other cell types [10,11], observing that it is reduced to ascorbic acid intracellularly, entering in the antioxidant pool of the cell. However, the ability of spermatozoa to reduce DHA to ascorbic acid might be limited, and other studies have shown analogous deficiencies on spermatozoa. For instance, bull spermatozoa was unable to regenerate GSSG to GSH efficiently [26], unless provided with external reducing power in the form of NADPH. We could be witnessing a similar problem here, with DHA accumulating intracellularly and altering the redox balance of the spermatozoa because of its deficient reduction. Although no clear effects could be detected in the ROS assessment, the increased acrosomal damage, stimulated motility and increased DNA damage in absence of oxidative stress provide indirect evidence of possible prooxidant activity. Nevertheless, we would expect that DHA modified the subpopulation pattern increasing the hyperactivated-like spermatozoa because of ROS increase, but we found an increase in the highly progressive spermatozoa.

In conclusion, the results of this study are mixed. On the one hand, DHA, it behaved as a prooxidant. Despite some motility-stimulating activity, the possible induction of DNA damage prevents against its use with spermatozoa. By contrast, taking into account previous studies in other species, TEMPOL and NAC could be used for supplementing extenders for red deer spermatozoa, because of its ability to suppress lipid peroxidation and to protect sperm DNA. Rutin, despite its good results protecting sperm DNA, clearly altered sperm physiology in a dose-dependent manner. It may be due to excessive ROS scavenging and even direct interference in transduction pathways. In this study, we have assessed these antioxidants in conditions pursuing to amplify any effects on the spermatozoa. The modification of motile subpopulations pattern further support that the antioxidants modulate or interfere with regulation pathways. Taking into account this information, the next step must be testing these antioxidants in the cryopreservation of red deer epididymal spermatozoa, using our results here to help to design new experiments. The steps of a cryopreservation protocol take place usually at low temperatures, and therefore the detrimental effect of these antioxidants may be minimized if added to the semen before cooling or at 5 °C, and removing the extender or inseminating immediately after thawing. Moreover, the possible effects of these substances in capacitation and other pathways (as suggested by the modifications of the motility subpopulation pattern) should be studied with more specific techniques [60] to acquire valuable knowledge of sperm physiology.

Acknowledgments

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