revised highlighted

Title: Effect of several antioxidants on thawed ram spermatozoa submitted to 37 °C up to 4 h

Abridged title: Incubation of thawed ram spermatozoa with antioxidants

María Mata-Campuzano[1,2], Manuel Álvarez-Rodríguez[1,3], Mercedes Álvarez[1,3], Luis Anel[1,3], Paulino de Paz[1,2], J. Julián Garde[4], Felipe Martínez-Pastor[1,2]

[1] ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain.

[2] Molecular Biology (Cell Biology), University of León, Spain

[3] Animal Reproduction and Obstetrics, University of León, Spain

[4] Biology of Reproduction Group. National Wildlife Research Institute (IREC) (UCLM-CSIC-JCCM), Albacete, Spain.

Corresponding author:

Felipe Martínez-Pastor

INDEGSAL

University of León,

24071, León, Spain

e-mail: felipe.martinez@unileon.es

For Peer Review

Contents

Thawed ram spermatozoa were incubated at 37 °C in 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 analyzed at 2 and 4 h. Lipoperoxidation (MDA production), intracellular ROS and DNA status (TUNEL) were analyzed 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 presence of oxidative stress. NAC, rutin 1 mM and TEMPOL reduced lipoperoxidation in presence of oxidative stress. However, DHA did not affected lipoperoxidation. At 1 mM, DHA increased DNA damage in absence of oxidative stress. DHA effects could arise from spermatozoa having a low capacity for reducing it to ascorbic acid, and it may be tested in presence of other antioxidants or reducing power. Future research should focus in testing if 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.

Keywords: ram, spermatozoa, antioxidant, oxidative stress, DNA damage

For Peer Review

1
2
3
4 35
1. Introduction

36 Artificial insemination (AI) with frozen-thawed semen has been proposed as a valuable tool for genetic 37 improvement programs for sheep (Anel et al., 2006). This technique has still to gain a widespread use, 38 because of very variable and frequently low fertility with cervical AI, forcing the use of short-term 39 refrigerated semen or laparoscopic insemination. The main problem with the cervical AI consist in the 40 difficulty of perform a deep insemination, due to the sheep anatomy and to the convoluted shape of the 41 cervical channel, forcing to deposit the semen in the vagina or to perform shallow intracervical 42 inseminations (Kaabi et al., 2006; Druart et al., 2009). Moreover, cryopreservation impairs sperm quality 43 (Salamon and Maxwell, 2000), and possibly its ability to migrate to the oviduct, which explains the 44 requirement of laparoscopic AI to achieve acceptable results when using cryopreserved semen in sheep.

45 Antioxidants have been proposed for improving results of artificial reproductive techniques. Many 46 attempts have been tried in different species (Bilodeau et al., 2001; Roca et al., 2005; Fernandez-Santos 47 et al., 2007; Gadea et al., 2005; Fernández-Santos et al., 2009; Domínguez-Rebolledo et al., 2009), 48 although field trials have not been as successful as in vitro experiments (Foote et al., 2002; Mara et al., 49 2007). In this study, we have tested the effect of several antioxidants on ram sperm quality, following an 50 in vitro design that we used previously in red deer (Domínguez-Rebolledo et al., 2010). Such a test was 51 designed as a preliminary step to study the physiological changes of spermatozoa upon being submitted 52 to the antioxidants at 37 °C and to uncover toxic effects.

53 The antioxidants TEMPOL, N-acetyl-cysteine (NAC), rutin and deydroascorbic acid (DHA) were 54 used in our experiment. TEMPOL has been tried for the refrigerated storage of ram spermatozoa (Mara 55 et al., 2005), apparently improving the conservation of sperm quality and the in vitro fertility. However, 56 the base extenders used for TEMPOL and non-TEMPOL treatments were different, preventing a proper 57 comparison on the effect of this antioxidant. Furthermore, TEMPOL did not affected the motility of 58 cryopreserved bull spermatozoa (Foote et al., 2002), and it could not improve the kidding rate of goats 59 inseminated with refrigerated semen (Mara et al., 2007). However, this antioxidant seems promising, 60 because it has been defined as having a SOD-like activity (Mitchell et al., 1990).

61 The other antioxidants have not been tested in ram spermatozoa before, although

5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

For Peer Review

N-acetyl-cysteine (NAC) has yielded good results in other species. NAC 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 reactive oxygen species (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. NAC 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 behavior, since 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 reactive oxygen species (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 familiy) (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, due 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.

1
2
3
4 88
2. Materials and Methods

2.1. 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.

2.2. 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, $\times$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: $\geq$0.5 mL; mass motility: $\geq$4; sperm concentration: $\geq 3000\times10^6$ mL^{-1}).

Semen was diluted with the same volume of freezing extender. The freezing extender (Anel et al.,

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

114 2003) consisted of a TTF medium (TES-Tris-fructose, 320 mOsm/kg, pH 7.2) supplemented with 10%
115 egg yolk and 4% glycerol. The sample was then refrigerated to 5 °C for two hours. Samples were packed
116 into 0.25-mL plastic straws and equilibrated for 1 h at 5 °C. Then, the straws were frozen using a
117 programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) using a rate of
118 -20 °C/min down to -100 °C. The straws were kept in liquid nitrogen containers. For each experimental
119 session, one straw per male was thawed in a water bath at 37 °C for 30 s. The contents of the four straws
120 were pooled and diluted with three volumes of TALP-Hepes. After centrifugation ($600 \times g$ for 5 min), the
121 supernatant was discarded, and the pellet was slowly resuspended in TALP-Hepes up to
122 30×10^6 cells/mL. The washed pool was assessed (motility, membrane and mitochondrial status, DNA,
123 lipoperoxidation and ROS) ten minutes after washing.

124 *2.3. Experimental design*

125 The experiments followed a factorial design. In all experiments, the washed pool was split among nine
126 tubes. Eight of them were supplemented with either 1/100 of the 100 mM solution (1 mM final) or the
127 10 mM solution (0.1 mM final) of each antioxidant: TEMPOL, N-acetyl-cysteine (NAC), rutin or
128 dehydroascorbic acid (DHA). The ninth tube was used as control (no antioxidant). All the experiments
129 were replicated seven times. Half of the volume of each tube was passed to another series of tubes, which
130 were submitted to oxidative stress by adding 1/100 of the oxidant solution (100 μM of $FeSO_4$ and
131 500 μM of sodium ascorbate). The tubes were incubated at 37 °C and analyzed at 2 h and 4 h.

132 *2.4. Sperm motility assessment*

133 The motility of the tubes without oxidative stress was assessed at 2 and 4 h. Sperm were diluted down to
134 $10–20 \times 10^6$ spermatozoa/mL and loaded into a Makler counting chamber (10 μm depth) at 37 °C. The
135 CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon;
136 Tokyo, Japan), equipped with a warming stage at 37 °C and a Basler A302fs digital camera (Basler
137 Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394
138 interface. Images were captured and analyzed using the Sperm Class Analyzer (SCA2002) software
139 (Microptic S.L.; Barcelona, Spain). Sampling was carried out using a $\times 10$ negative phase contrast

For Peer Review

objective (no intermediate magnification). Image sequences were saved and analyzed afterwards. The standard parameter settings were: 25 frames/s; 20 to 90 μm^2 for head area; 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).

2.5. 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 ($\Delta\psi_{\mathrm{m}}$) 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 analyzed by flow cytometry. CM-H_2DCFDA is retained within cells after being cleaved by cellular sterases. When it is oxidized, it fluoresces green, indicating presence of intracellular ROS.

DNA damage was assessed by TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling assay], as carried out previously (Domínguez-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 labeled 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 analyzed by flow cytometry. Positive and negative controls (incubation with DNase A and substituting water for the DNA labeling 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 Domínguez-Rebolledo et al. (2010). Samples were diluted with PBS to

For Peer Review

10×10^6 mL^{-1} 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 45 °C 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, Vermont, 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, Australia). The YO-PRO-1/MT stain was analyzed 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.

2.6. Statistical Analysis

Data were analyzed in the R statistical environment (R Development Core Team, 2011). To analyze the effects of time, antioxidant supplement and oxidant effect on sperm parameters, we used linear

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

194 mixed-effects models, with incubation time, antioxidant type and antioxidant concentration as fixed
195 effects. Replicate was the grouping factor in the random part of the models. Results are presented as
196 means and 95% confidence intervals (within parentheses). $P<0.05$ was considered significant.

197 **3. Results**

198 Results are showed in figures 1–4 as means and 95% confidence intervals. Considering the effect of the
199 incubation on the control samples (without antioxidants), we noted a slight decrease on motility variables
200 at 2 h (Figure 1), which was not significant for total, progressive motility nor mean VCL. Nevertheless,
201 the mean ALH of the samples decreased significantly, from an initial value 3.20 (0.30) μm to
202 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
203 from 125.9 (11.4) μm/s to 94.4 (6.0) μm/s ($P<0.05$). ALH did not show significant changes respect to
204 2 h, with 2.23 (0.06) μm at 4 h. Viability and mitochondrial status (Figure 2), good indicators of the
205 overall integrity of spermatozoa, did not vary at 2 h [0 h: 42.4% (4.6) and 40.9% (4.5), respectively; 4 h:
206 43.5% (1.9) and 41.0% (2.0)]. At 4 h, the mean values decreased not significantly to 33.2% (2.84) and
207 31.23% (2.68), respectively. The variables related with oxidative stress, ROS and malondialdehyde
208 production (Figure 3), and DNA damage (TUNEL, Figure 4) did not vary with incubation [0 h: 11.3 (1.5)
209 MFI, 18.3 (2.6) nmol MDA/10^8 cells and 10.0% (5.1), respectively; 4 h: 13.1 (1.9) MFI, 15.6 (3.5)
210 nmol MDA/10^8 cells and 1.8% (0.42)]. Induced oxidative stress during the incubation caused an increase
211 in these parameters, which became significantly higher: 22.9 (2.7) MFI for ROS, 29.2 (3.6)
212 nmol MDA/10^8 cells and 34.7% (4.7) TUNEL+ spermatozoa.

213 The incubation of spermatozoa with antioxidants caused important changes in motility. Except for
214 DHA 0.1 mM and TEMPOL 0.1 mM, all treatments significantly affected total motility at 2 h, causing an
215 overall decrease of about 23 points (Subfigure 1a). At 4 h, total motility further decreased in all
216 treatments, except in DHA 0.1 mM (no significantly different to the control). This decrease in motility
217 was larger in DHA 1 mM, RUT 1 mM and TEMPOL 1 mM, with an average decrease of 25 points respect
218 to the control at 4 h. Progressive motility (Subfigure 1b) showed a similar trend at both times, whereas
219 VCL changes were less evident (Subfigure 1c). DHA 1 mM, NAC 1 mM, Rutin 0.1 mM and TEMPOL
220 1 mM induced a small decrease of mean VCL at 2 h (13 μm/s less on average), while at 4 h only DHA

For Peer Review

1 mM had a significant effect, decreasing mean VCL to 75.3 (4.5) μm/s. ALH (Subfigure 1d) 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 (Figure 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, 4 points less for 2 h and 5 points more for 4 h, on average.

The production of ROS (Subfigure 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 median fluorescence intensity of the samples. NAC 1 mM, rutin and TEMPOL were able to significantly reduce the MFI values even in 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. MDA production in absence of oxidative stress was similar to the control (Figure 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/10^8 cells for rutin and TEMPOL at 1 mM).

DNA damage (Figure 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). NAC 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.

4. Discussion

Many studies have tested the effects of antioxidants on spermatozoa, with variable results. While there is a general agreement that spermatozoa is highly vulnerable to the oxidative stress (Donnelly et al., 1999;

For Peer Review

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 presence of induced oxidative stress, and this efficiency was further demonstrated by indirect measures of oxidative stress such as MDA production and DNA fragmentation. NAC was not so effective in 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 phosphorilation , 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

For Peer Review

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 presence of Trolox (a soluble form of vitamin E) (Domínguez-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, since some studies have shown genotoxic effects in lymphocytes (Liu and Zheng, 2002). Flavonoids can act either as genotoxicants or antimutagens, due 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 to 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

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

304 et al., 2007).

305 The effect of DHA was different to the other antioxidants tested. At 0.1 mM, it reduced
306 intracellular ROS in the absence of oxidative stress, while not affecting motility (except for a slight
307 decrease of ALH at 2 h). Nevertheless, its ROS scavenging effect seemed weak, not having effect in the
308 presence of oxidative stress, nor reducing lipoperoxidation and DNA damage. However, at 1 mM, DHA
309 not only had a strong effect on motility, but also affected sperm functionality, did not reduce intracellular
310 ROS nor lipoperoxidation, and induced a small increase in DNA damage in samples incubated without
311 oxidative stress. These paradoxical results could be explained considering that DHA needs to be reduced
312 to ascorbic acid upon entering the cell (KC et al., 2005) in order to contribute to the antioxidant pool.
313 However, the mammal spermatozoon has a very reduced cytoplasm, and seems to have a limited capacity
314 to regenerate antioxidants to its reduced form (Bilodeau et al., 2001). In these conditions, DHA may enter
315 in the sperm cytoplasm efficiently, but its usefulness as an antioxidant would be very limited due to its
316 slow reduction to ascorbic acid. Therefore, it may be acting as a pro-oxidant if applied at high
317 concentrations.

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

For Peer Review

5. Acknowledgements

This work has been supported by Junta de Castilla y León (LE019A10-2), by the Spanish Ministry of Science and Innovation (AGL2010-15758/GAN) and by INIA (RZ2010-00005-00-00). We thank ANCHE (National Association of Churra Breeders) for supporting this study with animals and personnel. M. Mata-Campuzano and Manuel Álvarez-Rodríguez were supported by FPI grants (Spanish Ministry of Science and Innovation). F. Martínez-Pastor was supported by the Juan de la Cierva program and by the Ramón y Cajal program (Ministry of Science and Innovation, Spain) was supported by the Juan de la Cierva program (Ministry of Science and Innovation, Spain). The authors thank Enrique del Olmo, José Luis Ros and Álvaro Domínguez (University of Castilla-La Mancha/IREC) for their support in flow cytometry analyses and lab work.

References

Aitken RJ, 2000: Possible redox regulation of sperm motility activation. Journal of Andrology **21**, 491–496.

Aitken RJ, Curry BJ, 2010: Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and dna damage in the germ line. Antioxid Redox Signal .

Aitken RJ, Harkiss D, Buckingham DW, 1993: Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol Reprod Dev **35**, 302–15.

Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M, 1995: Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. Journal of Cell Science **108 (Pt. 5)**, 2017–2025.

Aitken RJ, Sawyer D, 2003: The human spermatozoon–not waving but drowning. Adv Exp Med Biol **518**, 85–98.

Anderson D, Basaran N, Dobrzyńska MM, Basaran AA, Yu TW, 1997: Modulating effects of flavonoids

For Peer Review

353 on food mutagens in human blood and sperm samples in the comet assay. Teratog Carcinog Mutagen
354 **17**, 45–58.

355 Anderson D, Dobrzyńska MM, Başaran N, Başaran A, Yu TW, 1998: Flavonoids modulate comet assay
356 responses to food mutagens in human lymphocytes and sperm. Mutat Res **402**, 269–77.

357 Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, de Paz P, 2006: Improvement strategies
358 in ovine artificial insemination. Reprod Domest Anim **41 Suppl. 2**, 30–42.

359 Anel L, de Paz P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E, 2003:
360 Field and in vitro assay of three methods for freezing ram semen. Theriogenology **60**, 1293–308.

361 Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-López F, Guaiquil VH, Vera JC,
362 Concha II, 1998: Hexose transporter expression and function in mammalian spermatozoa: cellular
363 localization and transport of hexoses and vitamin c. J Cell Biochem **71**, 189–203.

364 Bilodeau JF, Blanchette S, Gagnon C, Sirard MA, 2001: Thiols prevent H_2O_2-mediated loss of sperm
365 motility in cryopreserved bull semen. Theriogenology **56**, 275–286.

366 Cemeli E, Baumgartner A, Anderson D, 2009: Antioxidants and the comet assay. Mutat Res **681**, 51–67.

367 Córdoba M, Mora N, Beconi MT, 2006: Respiratory burst and NAD(P)H oxidase activity are involved in
368 capacitation of cryopreserved bovine spermatozoa. Theriogenology **65**, 882–92.

369 Córdoba M, Pintos L, Beconi MT, 2008: Variations in creatine kinase activity and reactive oxygen
370 species levels are involved in capacitation of bovine spermatozoa. Andrologia **40**, 370–6.

371 Córdoba M, Pintos LN, Beconi MT, 2007: Heparin and quercitin generate differential metabolic
372 pathways that involve aminotransferases and LDH-X dehydrogenase in cryopreserved bovine
373 spermatozoa. Theriogenology **67**, 648–54.

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

376 Deneke SM, 2000: Thiol-based antioxidants. Curr Top Cell Regul **36**, 151–80.

For Peer Review

377 Domínguez-Rebolledo AE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M,
378 Martínez-Pastor F, Garde JJ, 2010: Improving the effect of incubation and oxidative stress on thawed
379 spermatozoa from red deer by using different antioxidant treatments. Reprod Fertil Dev **22**, 856–70.

380 Domínguez-Rebolledo AE, Fernández-Santos MR, García-Alvarez O, Maroto-Morales A, Garde JJ,
381 Martínez-Pastor F, 2009: Washing increases the susceptibility to exogenous oxidative stress in red deer
382 spermatozoa. Theriogenology **72**, 1073–84.

383 Donnelly ET, McClure N, Lewis SE, 1999: The effect of ascorbate and alpha-tocopherol supplementation
384 in vitro on DNA integrity and hydrogen peroxide-induced dna damage in human spermatozoa.
385 Mutagenesis **14**, 505–12.

386 Donnelly ET, McClure N, Lewis SE, 2000: Glutathione and hypotaurine in vitro: effects on human sperm
387 motility, DNA integrity and production of reactive oxygen species. Mutagenesis **15**, 61–8.

388 Druart X, Cognié J, Baril G, Clément F, Dacheux JL, Gatti JL, 2009: In vivo imaging of in situ motility
389 of fresh and liquid stored ram spermatozoa in the ewe genital tract. Reproduction **138**, 45–53.

390 Fernández-Santos MR, Domínguez-Rebolledo AE, Esteso MC, Garde JJ, Martínez-Pastor F, 2009:
391 Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative
392 stress on motility and DNA integrity. Int J Androl **32**, 353–9.

393 Fernandez-Santos MR, Martinez-Pastor F, Garcia-Macias V, Esteso MC, Soler AJ, Paz P, Anel L, Garde
394 JJ, 2007: Sperm characteristics and dna integrity of Iberian red deer (*Cervus elaphus hispanicus*)
395 epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. J Androl
396 **28**, 294–305.

397 Foote RH, Brockett CC, Kaproth MT, 2002: Motility and fertility of bull sperm in whole milk extender
398 containing antioxidants. Anim Reprod Sci **71**, 13–23.

399 Gadea J, Gumbao D, Matas C, Romar R, 2005: Supplementation of the thawing media with reduced
400 glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after
401 cryopreservation. J Androl **26**, 749–756.

For Peer Review

402 Kaabi M, Alvarez M, Anel E, Chamorro CA, Boixo JC, de Paz P, Anel L, 2006: Influence of breed and
403 age on morphometry and depth of inseminating catheter penetration in the ewe cervix: a postmortem
404 study. Theriogenology **66**, 1876–1883.

405 KC S, Cárcamo JM, Golde DW, 2005: Vitamin C enters mitochondria via facilitative glucose transporter
406 1 (Glut1) and confers mitochondrial protection against oxidative injury. FASEB J **19**, 1657–67.

407 Liu GA, Zheng RL, 2002: Protection against damaged dna in the single cell by polyphenols. Pharmazie
408 **57**, 852–4.

409 Liu PY, Li K, Zhang J, Zhang DW, Lin HH, Yu XQ, 2010: Who is the king? the
410 alpha-hydroxy-beta-oxo-alpha,beta-enone moiety or the catechol B ring: relationship between the
411 structure of quercetin derivatives and their pro-oxidative abilities. Chem Biodivers **7**, 236–44.

412 Mara L, Accardo C, Pilichi S, Dattena M, Chessa F, Chessa B, Branca A, Cappai P, 2005: Benefits of
413 TEMPOL on ram semen motility and in vitro fertility: a preliminary study. Theriogenology **63**,
414 2243–53.

415 Mara L, Dattena M, Pilichi S, Sanna D, Branca A, Cappai P, 2007: Effect of different diluents on goat
416 semen fertility. Anim Reprod Sci **102**, 152–7.

417 Martinez-Pastor F, Fernandez-Santos MR, del Olmo E, Dominguez-Rebolledo AE, Esteso MC, Montoro
418 V, Garde JJ, 2008: Mitochondrial activity and forward scatter vary in necrotic, apoptotic and
419 membrane-intact spermatozoan subpopulations. Reprod Fertil Dev **20**, 547–556.

420 Michael A, Alexopoulos C, Pontiki E, Hadjipavlou-Litina D, Saratsis P, Boscos C, 2007: Effect of
421 antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine
422 spermatozoa. Theriogenology **68**, 204–12.

423 Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, Boscos CM,
424 2009: Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen
425 species of chilled canine spermatozoa. Anim Reprod Sci **112**, 119–35.

426 Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, Boscos CM,

For Peer Review

427 2010: Effect of n-acetyl-l-cysteine supplementation in semen extenders on semen quality and reactive
428 oxygen species of chilled canine spermatozoa. Reprod Domest Anim **45**, 201–7.

429 Mitchell JB, Samuni A, Krishna MC, DeGraff WG, Ahn MS, Samuni U, Russo A, 1990: Biologically
430 active metal-independent superoxide dismutase mimics. Biochemistry **29**, 2802–7.

431 Oeda T, Henkel R, Ohmori H, Schill WB, 1997: Scavenging effect of n-acetyl-l-cysteine against reactive
432 oxygen species in human semen: a possible therapeutic modality for male factor infertility?
433 Andrologia **29**, 125–31.

434 O'Flaherty C, de Lamirande E, Gagnon C, 2006: Reactive oxygen species modulate independent protein
435 phosphorylation pathways during human sperm capacitation. Free Radic Biol Med **40**, 1045–55.

436 Pagl R, Aurich JE, Müller-Schlösser F, Kankofer M, Aurich C, 2006: Comparison of an extender
437 containing defined milk protein fractions with a skim milk-based extender for storage of equine semen
438 at 5 degrees C. Theriogenology **66**, 1115–22.

439 R Development Core Team, 2011: *R: A Language and Environment for Statistical Computing*. R
440 Foundation for Statistical Computing, Vienna, Austria.

441 Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, Vazquez JM, Martinez EA, 2005:
442 Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase
443 and/or catalase. J Androl **26**, 15–24.

444 Salamon S, Maxwell WM, 2000: Storage of ram semen. Anim Reprod Sci **62**, 77–111.

445 Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R, Smith JF, 1998: Studies on aromatic
446 amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. Anim Reprod Sci
447 **51**, 275–87.

448 Utesch D, Feige K, Dasenbrock J, Broschard TH, Harwood M, Danielewska-Nikiel B, Lines TC, 2008:
449 Evaluation of the potential in vivo genotoxicity of quercetin. Mutation Research **654**, 38–44.

For Peer Review

FIGURE LEGENDS

Figure 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% C.I. 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).

Figure 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 is the proportion of YO-PRO-1– events, and active mitochondria is the proportion of YO-PRO-1–/Mitotracker Deep Red+ events. Mean and 95% C.I. 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).

Figure 3.

Effects of antioxidant treatments on ROS (reactive oxygen species) 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% C.I. 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).

Figure 4.

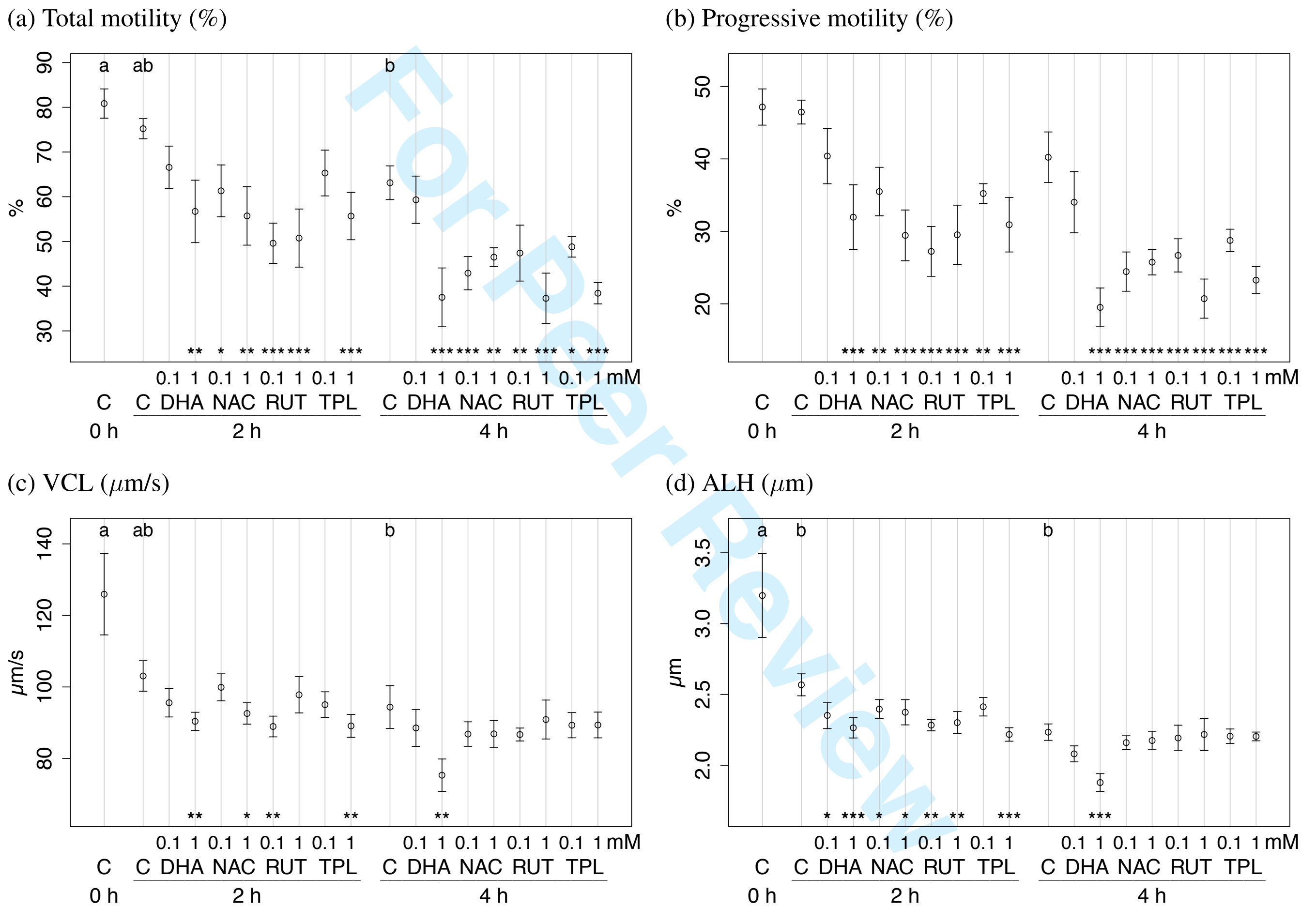
For Peer Review

Effects of antioxidant treatments on TUNEL results (DNA damage), after 4 h of incubation without or with oxidative stress. Mean and 95% C.I. 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 (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

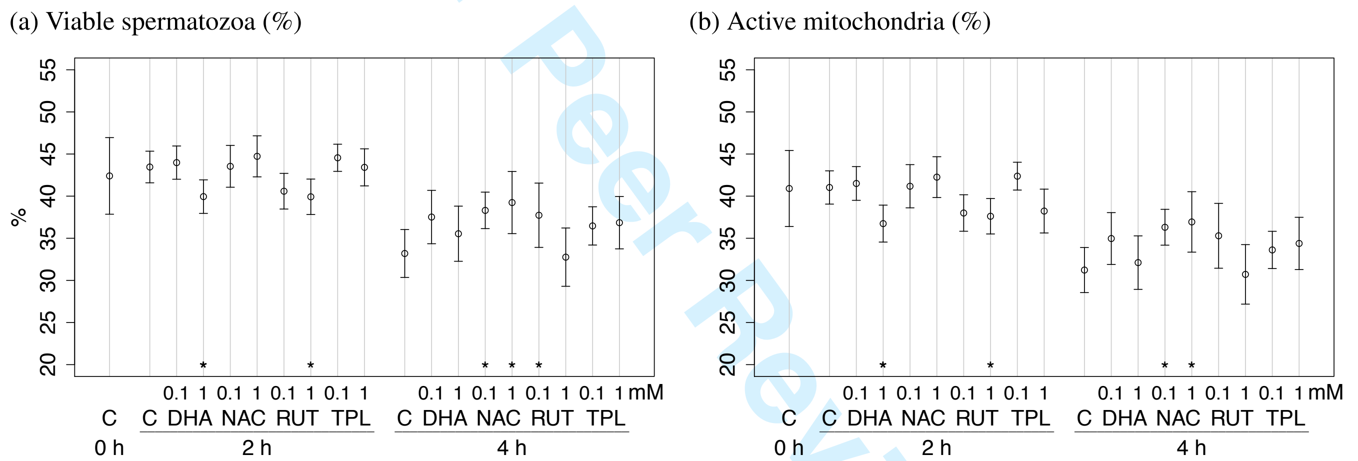
FIGURE 1



For Peer Review

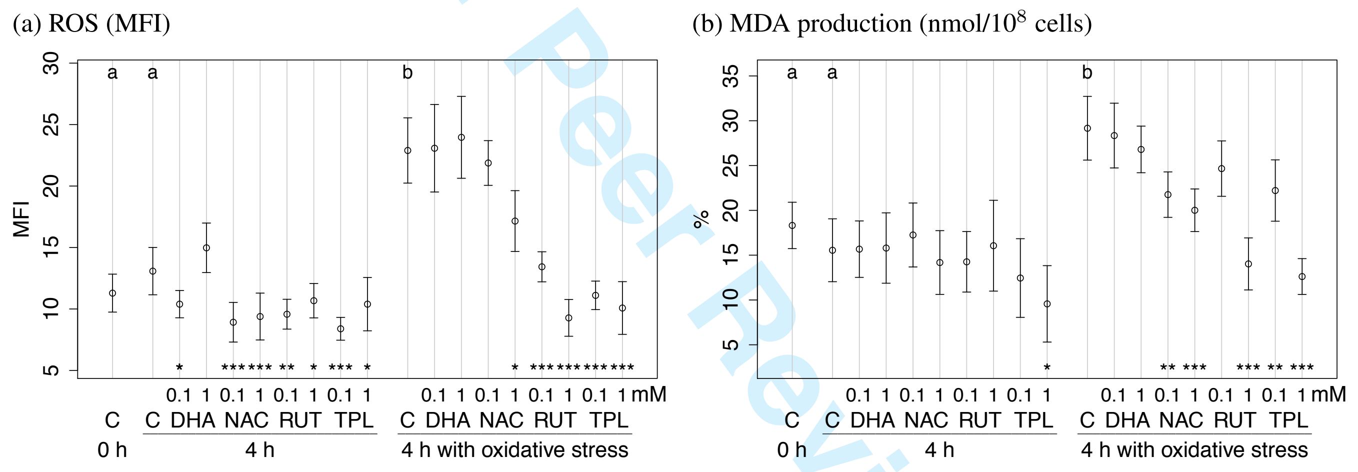
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

FIGURE 2



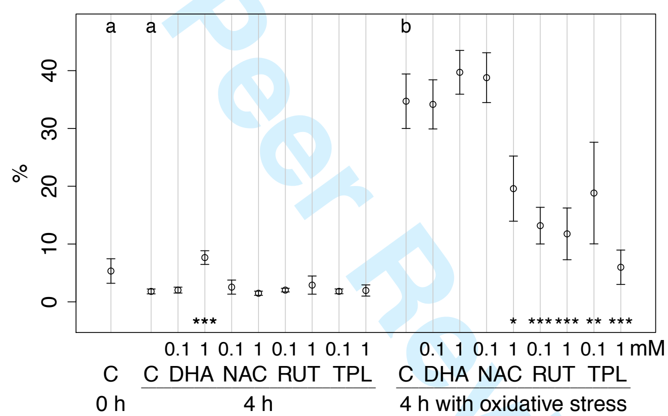
For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22 FIGURE 3
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23 FIGURE 4
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



For Peer Review