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Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa

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Abstract

Fe^{2+}/ascorbate, hydrogen peroxide (H_2O_2), and hypoxanthine/xanthine oxidase (XOD) are commonly used for inducing oxidative stress on spermatozoa. A comparative study of these agents was carried out on thawed spermatozoa from red deer. First, we tested a high, medium, and low concentration of each agent: 100, 10, and 1 μM Fe^{2+} (hydroxyl radical generator); 1 mM, 100, and 10 μM H_2O_2; and 100, 10, and 1 mU/ml XOD (superoxide and H_2O_2 generator), incubated at 37 °C for 180 min. Intracellular reactive oxygen species (ROS; H_2DCFDA) increased with dose and time, and, similarly, for the three systems at each concentration level. Motility and mitochondrial membrane potential ($\Delta\psi_m$) were considerably decreased by H_2O_2 (1 mM and 100 μM) and XOD (100 and 10 mU/ml). Only 1 mM H_2O_2 reduced viability. The antioxidant Trolox (10 μM) reduced intracellular ROS, but could not prevent the H_2O_2 or XOD effects. In a second experiment, we used YO-PRO-1 and M540 as apoptotic and membrane stability markers respectively. Only H_2O_2 increased the proportion of apoptotic and membrane-destabilized spermatozoa. Catalase added to XOD prevented $\Delta\psi_m$ loss, confirming that H_2O_2 was the causative agent, not superoxide. In a third experiment, caspase activation was tested using the (FAM-VAD-FMK) probe. Viable spermatozoa with activated caspases could be detected in untreated samples, and only H_2O_2 increased their proportion after 60 min. There were important differences between ROS generators, H_2O_2 being the most cytotoxic. Although H_2O_2 and XOD caused $\Delta\psi_m$ dissipation, this was not reflected in increasing apoptotic markers. ~~We hypothesize that a subpopulation of spermatozoa may lack apoptotic markers.~~

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Introduction

Oxidative stress has emerged as one of the major threats to sperm functionality, both *in vivo* and *in vitro*. Reactive oxygen species (ROS) have a fundamental role in sperm physiology (Aitken *et al*. 2004, O'Flaherty *et al*. 2006, de Lamirande & O'Flaherty 2008), but its excess can damage spermatozoa (Shen & Ong 2000, Aitken & Baker 2002, Agarwal & Saleh 2002, Agarwal *et al*. 2003). Concerning sperm work, ROS can be detrimental even within physiological levels, since they can trigger early capacitation and irreversible events such as acrosome reaction (Oehninger *et al*. 1995, Hsu *et al*. 1999, Satorre *et al*. 2007). Therefore, there is a great interest in protecting spermatozoa from ROS during manipulation and cryopreservation. In this attempt, researchers generally use external sources of ROS to study oxidative stress on spermatozoa. For instance, (hypo)xanthine/xanthine oxidase generates superoxide

radical and hydrogen peroxide (Aitken *et al*. 1993), whereas ferrous ion/ascorbate generates hydroxyl radical through the Haber–Weiss or Fenton reaction (Buettner & Jurkiewicz 1996). Hydrogen peroxide, a potent membrane-permeable oxidizing species, is often used directly (Oehninger *et al*. 1995, Hsu *et al*. 1999). However, few studies have aimed at comparing the effects of different oxidant systems in spermatozoa, although this is of great interest, since different systems yield different ROS at different rates, thus causing possible variations in the effects and interactions with antioxidants. Thus, Kovalski *et al*. (1992) showed that hydrogen peroxide and the hydroxyl radical, but not the superoxide radical, inhibited the motility of human spermatozoa. The differential action of hydrogen peroxide and superoxide radical on human spermatozoa motility was also found by other authors (Aitken *et al*. 1993, Bilodeau *et al*. 2002). Armstrong *et al*. (1999)

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characterized the ROS production of activated leukocytes and the hypoxanthine/xanthine oxidase (XOD) system, and their effect on sperm motility and energetic metabolism. These authors found that hydrogen peroxide was not only responsible for the loss of motility, but also for the loss of mitochondrial membrane potential. However, these effects were not found when superoxide or hydroxyl radical – contrary to Kovalski **Q1** *et al.* (1992) – was the predominant ROS.

~~Moreover,~~ research on oxidative stress has converged with research on apoptosis in spermatozoa. Apoptotic markers have been found in spermatozoa of several species, but their role on sperm physiology is still not completely understood (Sakkas *et al.* 2002, Cayli *et al.* 2004, Lachaud *et al.* 2004, Paasch *et al.* 2004*b*, Barroso *et al.* 2006, Marti *et al.* 2006, Angelopoulou *et al.* 2007, Martin *et al.* 2007, Moran *et al.* 2008, Ortega-Ferrusola *et al.* 2008). ~~Moreover,~~ the importance of ROS as apoptosis inducers and mediators has been demonstrated in somatic cells (Carmody & Cotter 2001, Ott *et al.* 2007). ~~Thus,~~ it has been suggested that high ROS levels in semen might be associated with the presence of apoptotic markers (Wang *et al.* 2003, Moustafa *et al.* 2004), although a causative effect has yet to be **Q1** demonstrated. In somatic cells, ROS (produced by the respiratory chain or externally) are among the inducers of type II apoptosis (also termed the intrinsic pathway; Carmody & Cotter 2001, Owuor & Kong 2002, Ott *et al.* **Q1** 2007), and it has been shown that targeting antioxidants to the mitochondria can prevent the loss of $\Delta\psi_m$ and apoptosis (Zhao *et al.* 2005). The intrinsic pathway is initiated in the mitochondria by the formation of the mitochondrial permeability transition pore, releasing components of the mitochondrial matrix, such as Ca^{2+} and cytochrome *c* (Ott *et al.* 2007). Cytochrome *c* induces the formation of the apoptosome, resulting in the activation of caspase 9, which subsequently activates caspase 3, a milestone event in apoptosis (Carmody & Cotter 2001, Owuor & Kong 2002). This intrinsic pathway of apoptosis may be present in spermatozoa, according to some studies (Paasch *et al.* 2004*a*, Wundrich *et al.* 2006, Bejarano *et al.* 2007, Martin *et al.* 2007). Previously (Martinez-Pastor *et al.* 2008*a*), we proposed that the loss of $\Delta\psi_m$ preceded plasmalemma destabilization as indicated by YO-PRO-1, suggesting an apoptotic-like phenomenon. Therefore, we expected that ROS treatments causing $\Delta\psi_m$ dissipation would elicit other apoptotic markers.

Considering these antecedents, and taking into account that most studies have been carried out on human spermatozoa, we aimed at analyzing the effects of different oxidant systems in small ruminants, using red deer spermatozoa. Red deer is a relatively new farming species, which offers new opportunities for some kind of studies, since the variability of some traits (fertility, for **Q1** instance) is still ~~larger~~ than that in other domestic species (Malo *et al.* 2005, Gomendio *et al.* 2006). ~~Moreover,~~

studies on deer can be translated to different contexts (Garde *et al.* 2006): farming, hunting, and conservation **Q1** (of endangered cervid species or populations). In this study, we analyzed the effect of incubating spermatozoa with different concentrations of the ferrous/ascorbate system (producing hydroxyl radicals), hydrogen peroxide, and XOD system (producing both superoxide and **Q1** hydrogen peroxide). A commonly used antioxidant (Trolox, an analogue of α-tocopherol) was tested against the oxidants to estimate which kind of effects could be abolished by a typical radical scavenger (Peña *et al.* 2003). Secondly, we evaluated the occurrence of apoptotic markers after incubation with these ROS generators, including the detection of activated caspases. According to previous studies, we hypothesized that part of the ROS effects may be mediated by apoptotic pathways, thus we tested the relationship between ROS presence, apoptotic markers, and other parameters of sperm quality.

Results

Experiment 1. Motility, viability, and mitochondrial activity of spermatozoa exposed to different ROS sources

The designs of the three experiments carried out in this study are illustrated in Fig. 1. The results of Experiment 1 **Q1** for each oxidant are summarized in Fig. 2 (Fe^{2+}/ **Q2** ascorbate), Fig. 3 (H_2O_2), and Fig. 4 (HX/XOD). The incubation at 37 °C with the ROS generators induced an increase in H_2DCFDA fluorescence, which was dependent on dose and incubation time, whereas control and Trolox did not show any significant variation. The concentrations of 1 mM and 100 µM H_2O_2 (HP1000 and HP100) caused a quick increase in fluorescence signal with respect to control ($P<0.001$) as soon as **Q1** 15 min after starting incubation. However, at 1 h and 3 h, fluorescence results were similar for the three ROS sources, XO100 yielding slightly lower values than FE100 and HP1000. The concentration of 10 mM Trolox delayed ROS increase, dropping fluorescence intensity near to the lowest oxidant concentration in all cases (even lower than 1 mM Fe^{2+}, $P<0.01$ at 3 h). In contrast to the observed ROS increase, lipid peroxidation was only evident at 1 h for HP1000 ($P<0.001$) and at 180 min for FE100 ($P<0.05$), HP1000, and XO100 ($P<0.001$). Again, Trolox reduced this increase, although it was still significantly different from control ($P<0.05$).

Motility decreased steadily with incubation time in the control samples (slope -0.15 ± 0.02, $P<0.001$, from an initial value of $51.25\%\pm2.96$). The oxidant treatments affected motility in a very different manner. Thus, FE100 and FE10 induced an initial drop (at 15 min) of -12.08 ± 2.88 ($P<0.01$) and -9.58 ± 2.88 ($P<0.05$) respectively, but these differences did not increase
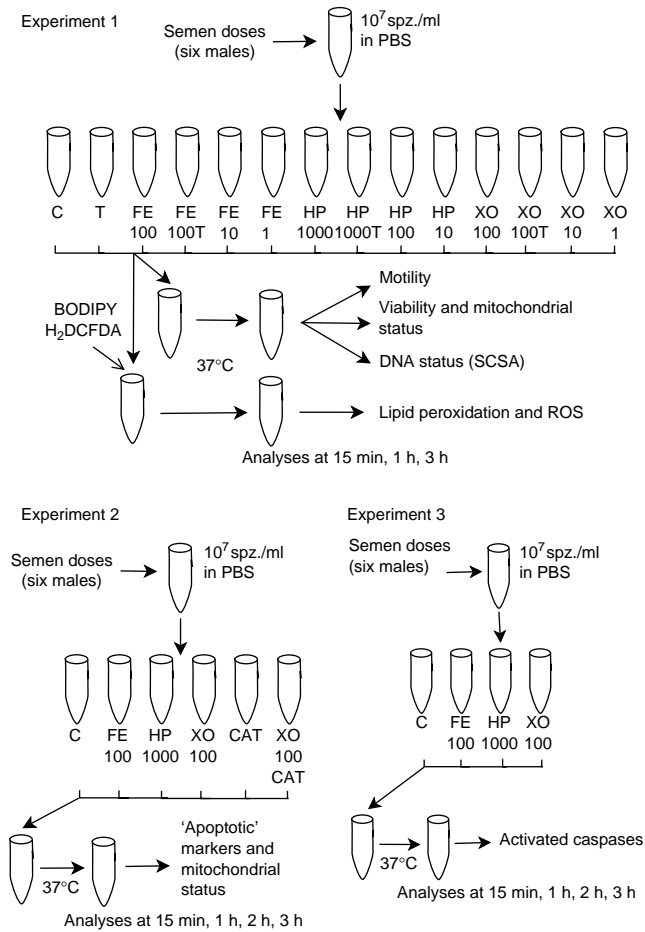
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Figure 1 Schematic designs of the three experiments carried out in the present study. C, control (no treatment); FE100, 100 μM Fe^{2+}; FE10, 10 μM Fe^{2+}; FE1, 1 μM Fe^{2+}; HP1000, 1 mM H$_2$O$_2$; HP100, 100 μM H$_2$O$_2$; HP10, 10 μM H$_2$O$_2$; XO100, 100 mU/ml XOD; XO10, 10 mU/ml XOD; XO1, 1 mU/ml XOD. Antioxidant supplementation is indicated by T (10 μM Trolox) and CAT (2500 U/ml catalase).

during the rest of the experiment (in fact, FE10 became not significantly different from control at 1 and 3 h). By contrast, sperm motility index (SMI) dramatically dropped in HP1000 (-48.67 ± 2.88 at 15 min, $P<0.001$), not showing motile spermatozoa afterwards. HP100 caused a lower initial inhibition (-15.00 ± 2.88 at 15 min, $P<0.05$). Nevertheless, motility was only $10.00\%\pm3.10$ at 1 h, and motility inhibition was total at 3 h. HP1 lowered motility at 15 min (-7.08 ± 2.88, $P<0.05$), but it was not significantly different from control afterwards. HX/XOD decreased motility in a similar manner, but more slowly than H$_2$O$_2$. All XOD concentrations inhibited motility at 1 and 3 h, but only XO100 affected motility significantly at 15 min (-17.50 ± 2.88, $P<0.001$). The effect of XO1 was relatively mild (-7.92 ± 2.53 at 3 h, $P<0.01$), whereas inhibition was almost total at 3 h with XO100 and XO10 ($P<0.001$). Trolox could not improve motility, neither in untreated samples nor when added to samples treated
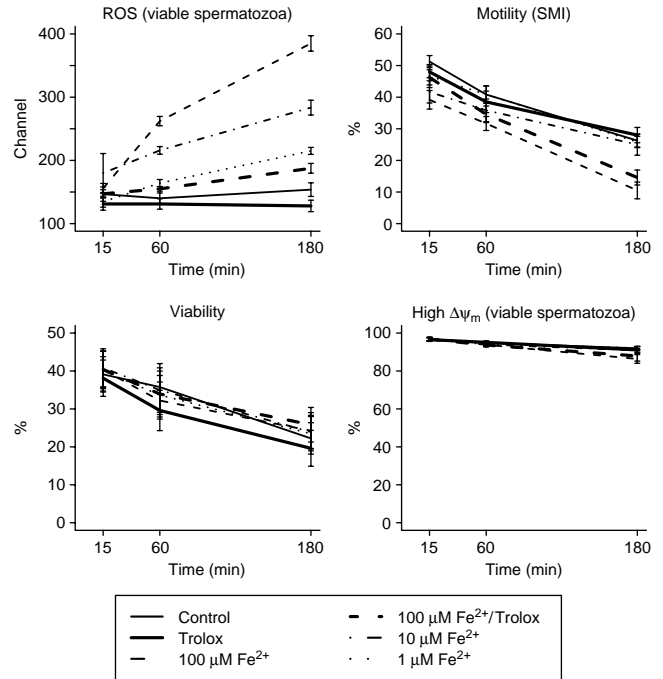

Figure 2 Sperm parameters in the presence of different concentrations of Fe^{2+} (plus 100 μM sodium ascorbate) and/or 10 μM Trolox. ROS presence (H$_2$DCFDA) is showed as the median fluorescence intensity of viable (PI$-$) spermatozoa, whereas the rest of the parameters are percentages of motile, viable or viable+high-$\Delta\psi_m$ spermatozoa. Model analyses are explained in the text. Data points are the mean$\pm$ SEM of six experiments.

with oxidants. Only in the case of FE100, it partially **Q1** prevented the motility decrease, and only at 15 min.

The most striking difference between the oxidant systems was shown in the evaluation of mitochondrial membrane potential ($\Delta\psi_m$). Spermatozoa subjected to incubation with any Fe^{2+} concentration maintained high $\Delta\psi_m$ while viable (all PI+ spermatozoa had low $\Delta\psi_m$), even after 180 min (control was $96.8\%\pm1.09$ at 15 min and $91.98\%\pm6.10$ at 3 h, not being different **Q1** from any Fe^{2+} treatment, $P>0.46$). By contrast, H$_2$O$_2$ and HX/XOD showed a high-efficiency dissipating $\Delta\psi_m$. At 60 min, both HP1000 and XO100 provoked a sharp drop in the percentage of viable spermatozoa with high $\Delta\psi_m$ (-33.18 ± 6.64 and -33.67 ± 6.64 from control respectively), abolishing it almost totally at 3 h. HP100 showed the same effect, but only at 3 h ($23.14\%\pm0.93$). XO10 and XO1 affected $\Delta\psi_m$, but only at 3 h ($59.31\%\pm$ 13.36 and $76.67\%\pm11.20$ respectively). Considering the median fluorescence intensity of Mitotracker (not **Q2** shown in Fig. 4), there was a significant decrease in H$_2$O$_2$ and HX/XOD treatments as soon as 15 min. This lower fluorescence was observed in the high-$\Delta\psi_m$ subpopulation at all times, even when it was not reflected in a significant drop of high-$\Delta\psi_m$ spermatozoa, ~~suggesting an effect in the sperm subpopulation capable~~ **Q1** ~~of maintaining active mitochondria.~~ By contrast, Fe^{2+}- treated samples had a median fluorescence intensity
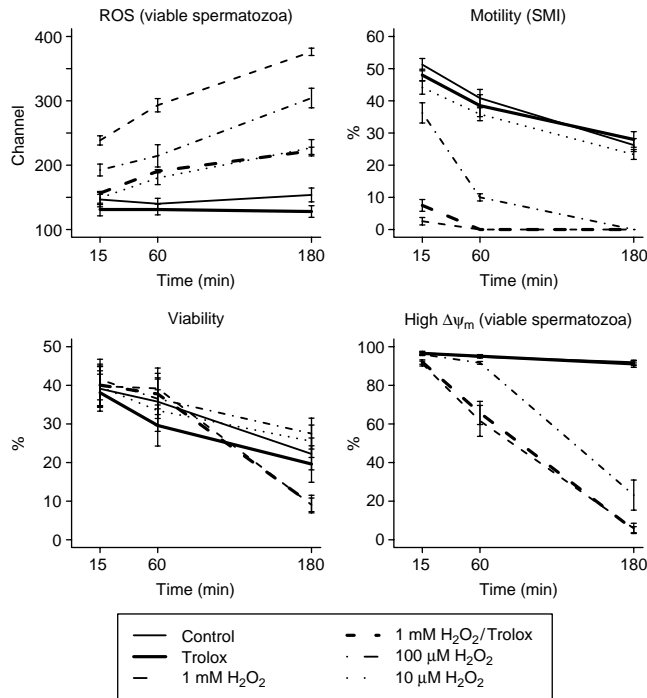
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Figure 3 Sperm parameters in the presence of different concentrations of H_2O_2 and/or of 10 μM Trolox. ROS presence (H_2DCFDA) is showed as the median fluorescence intensity of viable (PI−) spermatozoa, whereas the rest of the parameters are percentages of motile, viable or high-$\Delta\psi_m$ spermatozoa. Model analyses are explained in the text. Data points are the mean±S.E.M. of six experiments.
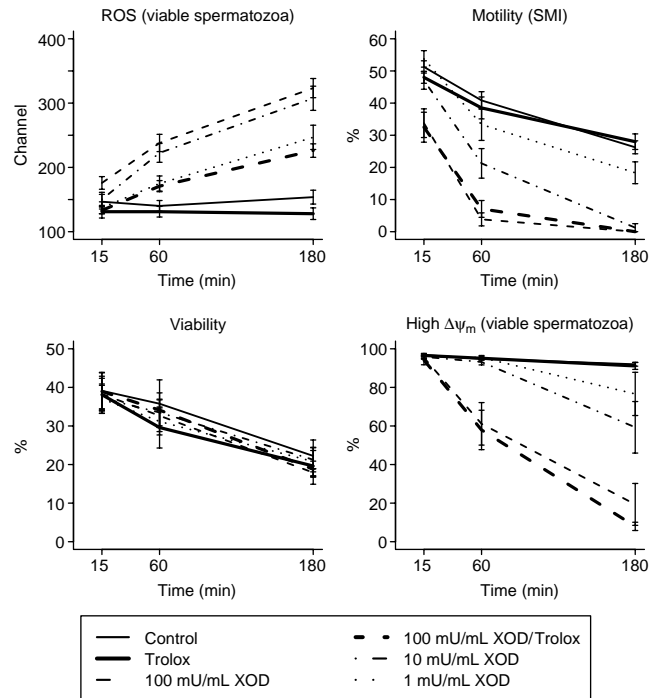
Figure 4 Sperm parameters in the presence of different concentrations of XOD (plus 100 μM hypoxanthine), and two combinations of 10 μM Trolox. ROS presence (H_2DCFDA) is showed as the median fluorescence intensity of viable (PI−) spermatozoa, whereas the rest of the parameters are percentages of motile, viable or high-$\Delta\psi_m$ spermatozoa. Model analyses are explained in the text. Data points are the mean±S.E.M. of six experiments.

similar to control. The presence of Trolox did not improve neither the percentage of high-$\Delta\psi_m$ spermatozoa nor the median fluorescence intensity.

Viability (membrane integrity) decreased in all treatments (slope -0.11 ± 0.02 for control). Only HP1000 at 180 min was significantly lower than control (-13.18 ± 3.05 from a control value of $22.23\%\pm3.65$, $P<0.001$), this decrease not being prevented by Trolox. Regarding chromatin status, we did not find any change from control. However, HDS was slightly higher for HX/XOD than for control ($0.77\%\pm0.13$) at 15 min ($2.82\%\pm0.26$)
Q1 and at 1 h ($1.98\%\pm0.33$), but not at 3 h. Thus, probably it is possible that these differences were not due to the effect of ROS on chromatin, but maybe to interferences of the HX/XOD treatment with the sperm chromatin structure assessment (SCSA) technique.

Experiment 2. Induction of apoptotic/necrotic changes

Labeling cells with the apoptotic marker YO-PRO-1 yielded three subpopulations: non-apoptotic (unstained), 'apoptotic' (YO-PRO-1+), and non-viable (membrane damaged, PI+; Martinez-Pastor *et al*. 2008a). As shown
Q2 in the Fig. 5, the proportion of non-apoptotic spermatozoa followed the same trend detected in Experiment 1 for viable spermatozoa, decreasing with time (slope -0.075 ± 0.01 for control, $P<0.001$), and there were

no differences between oxidants and control, except for H_2O_2 (slope -0.12 ± 0.01, $P<0.05$). ~~We defined an apoptotic index~~ as the proportion of YO-PRO-1+ spermatozoa within the population of PI− spermatozoa. The model showed that the apoptotic index increased in control (slope 0.07 ± 0.02, $P<0.01$), indicating that the decline in the YO-PRO-1− population was caused not only by membrane damage (PI+), but also by 'apoptotic' changes in the membrane, resulting in a higher number of 'viable apoptotic' spermatozoa (YO-PRO-1+/PI−). HX/XOD showed no difference with control, but H_2O_2 increased the slope of the model (0.14 ± 0.02, $P<0.001$), indicating an acceleration of the process. By contrast, Fe^{2+}-treated samples stayed at the same level during the
Q1 whole experiment. In this case, the reason was not that Fe^{2+} protected YO-PRO-1− spermatozoa, but rather that YO-PRO-1+ spermatozoa were stained by PI+ at a faster rate than in other treatments.

Another important difference between treatments
Q1 was shown by the Merocyanine 540 (M540) staining. Only a very low proportion of YO-PRO-1− spermatozoa fell in the high-M540 population (below 10% on average), except for H_2O_2-treated samples. In this case, the proportion of M540+/YO-PRO-1− spermatozoa increased up to $26.32\%\pm7.11$ at 2 h ($P<0.001$),
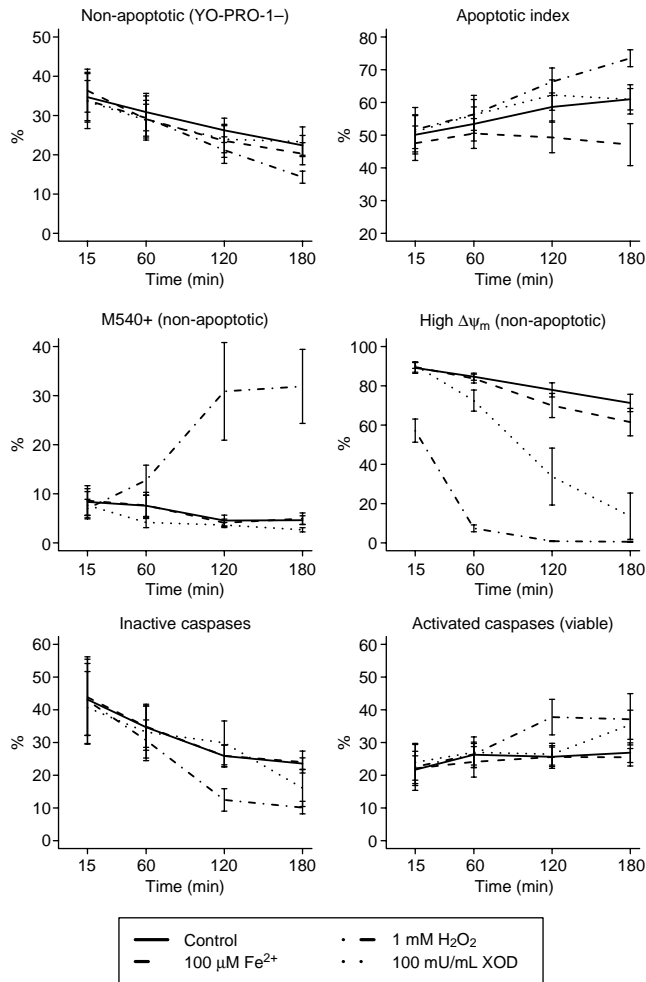
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Figure 5 Change in different parameters during the incubation of spermatozoa with different oxidative agents. The apoptotic index is the proportion of 'apoptotic' (YO-PRO-1+/PI− spermatozoa) taking the population of viable spermatozoa (PI−). For high $\Delta\psi_m$ and Merocyanine 580 (M580), only the 'non-apoptotic' population (YO-PRO-1−) was considered. For activated caspases, only the viable population (PI−) was considered. Model analyses are explained in the text. Data points are the mean±s.e.m. of six experiments.

without further increasing at 3 h. ~~In our experiment, Merocyanine 540 does not appear to be related to capacitation, but to decreasing membrane lipid packaging associated with membrane destabilization.~~ The results for $\Delta\psi_m$ were similar to those obtained in Experiment 1, although H_2O_2 was significantly different from control at 15 min (-31.97 ± 4.59, $P<0.001$).

Adding catalase to the sample treated with HX/XOD totally prevented $\Delta\psi_m$ dissipation and motility inhibition. ~~Thus, hydrogen peroxide was the causative agent of the mitochondrial depolarization and the loss of motility observed in the HX/XOD treatments, and not the superoxide radical.~~ The results for control, catalase, and catalase+HX/XOD treatments were very similar ($P>0.05$), not only for $\Delta\psi_m$, but also for membrane apoptotic markers (YO-PRO-1/PI).

Experiment 3. The presence of activated caspases

The analysis showed that all membrane-damaged spermatozoa (PI+) had activated caspases. The proportion of viable spermatozoa with inactive caspases (Fig. 5) decreased with incubation time in all cases (slope -0.12 ± 0.04 for control, $P<0.01$), and only H_2O_2 showed a lower proportion ($P<0.05$, except at 15 h). However, the proportion of viable spermatozoa with active caspases stagnated at $25.88\%\pm1.13$, only increasing with time for H_2O_2 ($P<0.05$). Nevertheless, the H_2O_2 values did not increase any further after 120 min, apparently reaching a plateau. HX/XOD showed an increase at 3 h, which did not reach significance from control ($P=0.08$). ~~This indicates that caspase activation could be stimulated by H_2O_2 treatment, at least in a subpopulation of spermatozoa.~~

Discussion

In this study, we compared the effects of the ROS generators Fe^{2+}/ascorbate, hydrogen peroxide, and XOD on red deer spermatozoa, as a model for small ruminants. Although the three systems differed greatly in their effects, they seemed to induce the same level of intracellular ROS. The 'low' concentrations increased noticeably the levels of ROS, but they did not lower sperm quality dramatically, contrary to the higher concentrations. This suggests that the concentrations in that magnitude could be used for increasing intracellular ROS while not reaching toxic levels, which might be useful in future studies. Nevertheless, two important facts should be taken into account on the nature of our samples while evaluating the results. First, cryopreserved samples were used. Cryopreservation not only reduces sperm quality, but also induces oxidative stress and decreases the antioxidants in semen (Aisen *et al.* 2005, Peris *et al.* 2007), therefore thawed spermatozoa could be more sensitive than fresh ones. Second, epididymal spermatozoa were used. The epididymal environment is not void of antioxidant systems (Vernet *et al.* 2004), but this protection is possibly lower than that conferred by seminal plasma, especially considering that pre-freezing extension is much higher when handling epididymal samples than when diluting semen. Therefore, one must be careful when extrapolating these results not only to other species, but also to fresh or ejaculated spermatozoa.

Despite increasing intracellular ROS, Fe^{2+}/ascorbate had little effects, whereas the 'medium' and 'high' concentrations of H_2O_2 and XOD greatly decreased sperm motility. Our results indicate that when combining HX/XOD and catalase, producing only the superoxide radical, motility was preserved. Thus, motility loss in red deer spermatozoa after oxidative stress seems to be caused by hydrogen peroxide, as previously reported in human semen (Aitken *et al.* 1993,

[Q1] Armstrong *et al.* 1999). In other studies (Kovalski *et al.* 1992, Chen *et al.* 1997), hydroxyl radical was found to affect motility similarly to hydrogen peroxide, but our results suggest otherwise. These contrasting results could be explained by either hydrogen peroxide interference or experimental differences (species, techniques, etc.). The concurrent high levels of ROS (H_2DCFDA fluorescence) yielded by Fe^{2+}/ascorbate, and its little effect on sperm motility or $\Delta\psi_m$ observed in this study, could be explained taking into account the short life of the hydroxyl radical and its relative impermeability to biological membranes. Thus, it might readily oxidize H_2DCFDA in the cytoplasm, but its action on intra-cellular targets may be limited.

Motility loss by hydrogen peroxide has been attributed to the inactivation of glycolytic enzymes, leading to energetic draining in the flagellum (Armstrong *et al.* 1999, Baumber *et al.* 2000). Our study seems to confirm that high doses of H_2O_2 cause an immediate inhibition of motility, which was almost total with 1 mM H_2O_2. The effect of xanthine oxidase was similar, but we observed a delay that might be explained considering that the enzymatic reaction could require some time to build up an equivalent amount of H_2O_2. It must be highlighted that mitochondrial activity was still high when motility had dropped. In another study (Martinez-Pastor *et al.* 2008*a*), we carried out an incubation without oxidants, noting that motility was much higher than the proportion of spermatozoa with high $\Delta\psi_m$. Thus, the motility of deer spermatozoa seems to be independent – at least in the short term – of mitochondrial activity, similarly to human spermatozoa (Armstrong *et al.* 1999). ATP produced by mitochondria would not be transported to the flagellum efficiently enough to compensate for the depletion after the inactivation of glycolysis by hydrogen peroxide. In fact, the decrease in viability shown 3 h after applying the higher H_2O_2 dose could be a long-term effect of the lack of mitochondrial activity, as suggested previously (Martinez-Pastor *et al.* 2008*a*). Armstrong *et al.* (1999) found that treating human semen with 5 mM H_2O_2 dropped the percentage of high-$\Delta\psi_m$ spermatozoa to 26% of control results in 30 min, whereas only 0.3 mM inhibited the motility of human spermatozoa in the same time. Similarly, Ramos & Wetzels (2001) found an almost total loss of motility after a 5 min incubation of human spermatozoa with 25 µM H_2O_2. This might imply that small ruminant spermatozoa would be much more resilient, since 10 mM H_2O_2 showed no significant effect after 180 min. Thus, the results obtained cannot be [Q1] extrapolated to very different species. Baumber *et al.* (2000), testing a wide range of H_2O_2 concentrations on equine semen, obtained the results that resembled ours, although 12.5 mM H_2O_2 decreased motility significantly with respect to control after 180 min. Interestingly, when we assessed DNA damage, we did not detect differences between treatments. In a previous experiment with bull semen (Fernandez-Santos *et al.* 2008, Martinez-Pastor

et al. 2008*b*), we detected increased DNA damage using SCSA, after subjecting thawed spermatozoa to oxidative [Q1] stress. The lack of differences in the present study could be due to species or treatment differences (6 h of incubation). Peris *et al.* (2007) reported that SCSA showed differences between different H_2O_2 treatments (0–300 µM) in ram semen, but only after 24 h incubation, and not after 4 h.

The antioxidant Trolox seemed to successfully decrease the intracellular ROS level of the 'high' concentration (although not down to control levels), but it could not prevent the negative effects of hydrogen peroxide and HX/XOD, except lowering membrane lipoperoxidation. It is possible that Trolox could be particularly efficient in protecting lipids and other molecules from oxidation (such as H_2DCFDA), but it failed in protecting other kind of targets, such as [Q1] sulphydryl groups on proteins. We did not test other antioxidants (except catalase, in Experiment 2), but other studies have suggested that glutathione, for instance, had a protective effect on motility when H_2O_2 was present (Baumber *et al.* 2000), and studies on other cell types have identified glutathione and other compounds as mitochondrial protectors (Fernandez-Gomez *et al.* 2005, Hardeland 2005). In a previous study, we found that catalase could improve deer spermatozoa cryopreservation better than other antioxidants (Fernandez-Santos *et al.* 2007*b*), which agrees with our findings in this work (nevertheless, we did not assess apoptotic markers). Nonetheless, we have to take into account that we chose Trolox in this experiment because of its ubiquity among other studies, and because we wanted a 'generic', instead of a specific (superoxide dismutase, catalase, and DMSO), antioxidant.

We hypothesized that ROS and antioxidants could influence the occurrence of apoptotic markers in red deer spermatozoa through $\Delta\psi_m$ dissipation, as shown previously in human semen (Paasch *et al.* 2004*a*, Wundrich *et al.* 2006, Bejarano *et al.* 2007, Martin *et al.* 2007), and we showed that the loss of $\Delta\psi_m$ may induct changes conducing to YO-PRO-1 labeling [Q1] (Martinez-Pastor *et al.* 2008*a*). In the present study, 1 mM and 100 µM H_2O_2 caused a dramatic loss of $\Delta\psi_m$, but this was not accompanied by a similar increase of other apoptotic markers, either based on membrane destabilization (apoptotic index, YO-PRO-1) or on the detection of activated caspases (FLICA™). Although hydrogen peroxide increased the apoptotic index and the proportion of viable spermatozoa with activated caspases (with respect to control), suggesting some degree of caspase activation, this increase should have been much larger (nearly affecting most spermatozoa at 60 min) if the loss of $\Delta\psi_m$ effectively initiated apoptotic pathways. Furthermore, HX/XOD, which yielded H_2O_2, could not elicit a significant increase in these para-meters. Nevertheless, in Experiment 2, there was a delay of $\Delta\psi_m$ dissipation by HX/XOD when compared with

hydrogen peroxide, thus its effect could have been similar if longer times had been tried. Unfortunately, we could not test caspase activation when we combined HX/XOD and catalase, to study the superoxide effect specifically. However, we did not note differences concerning YO-PRO-1/PI labeling. Considering the similarities among the apoptotic index in Experiment 2 and caspase activation in Experiment 3, it seems that caspase activation might not occur with the superoxide radical alone.

As indicated in the introduction, the presence of functional apoptotic pathways is a controversial issue in spermatozoa. Nevertheless, apoptotic markers can be detected, and the possibility that they might have an effect on sperm fate should not be discarded (Paasch *et al*. 2004*a*, Wundrich *et al*. 2006, Bejarano *et al*. 2007, **Q1** Martin *et al*. 2007). Based on our results, it is possible that H_2O_2 would induce at least some elements of apoptotic pathways, but only in a reduced sub-population of spermatozoa, as suggested previously (Weng *et al*. 2002). The plateau reached after 60 min by the proportion of viable spermatozoa with activated caspases seems to support this idea, suggesting that a large proportion of spermatozoa were unable to show apoptotic markers in response to oxidative stress by H_2O_2. Another plateau was found when plotting the percentage of YO-PRO-1$-$ spermatozoa stained by Merocyanine 540, supporting the previous suggestion (Martinez-Pastor *et al*. 2008*a*) that YO-PRO-1 staining might be preceded by another kind of membrane changes. Muratori *et al*. (2004) indicated that Mero-cyanine 540 would stain cells with early membrane **Q1** degeneration. In our study, it is likely that Merocyanine 540 was related to decreasing membrane lipid packaging associated with membrane destabilization, but it cannot be confirmed in this study.

These findings suggest – as a hypothesis for future studies – that apoptotic markers (such as caspase activation and YO-PRO-1 staining) would occur only in a subpopulation of spermatozoa. Previous studies seem to support this idea. Paasch *et al*. (2004*a*) and Grunewald *et al*. (2005) detected an increase in activated caspases after applying betulinic acid (an activator of the apoptotic intrinsic pathway) ~~from~~ human **Q1** spermatozoa. Recently, Moran *et al*. (2008) showed that both betulinic acid and nitric oxide increased YO-PRO-1$+$/PI$-$ spermatozoa in boar semen. These increases (either activated caspases or YO-PRO-1$+$/PI$-$) ~~did only affect~~ part of spermatozoa, just like as we detected in the H_2O_2 treatment. The confirmation of this kind of subpopulations would represent a breakthrough finding, since many separation and evaluation methods for spermatozoa rely on the detection of apoptotic markers (such as phosphatidylserine externalization; Grunewald *et al*. 2006). Alternatively, if these pathways were **Q1** involved in sperm DNA damage (by the activation of endogenous endonucleases; Gorczyca *et al*. 1993,

Lamond *et al*. 2003), caspase inhibitors (Eley *et al*. 2005, Marti *et al*. 2008) or mitochondrial protectors (Lamond *et al*. 2003, Fernandez-Gomez *et al*. 2005, Hardeland 2005) might improve artificial reproductive techniques. In this study, we did not find an increase in DNA damage as shown by the SCSA, but other techniques (such as oxidized bases detection; Shen & **Q1** Ong 2000) might be more sensitive. Nevertheless, Moran *et al*. (2008) found that nitric oxide caused caspase activation in almost all viable spermatozoa, whereas, contrary to findings in human sperm, betulinic acid could not increase activated caspases. This indicates that the activation of apoptotic markers, and **Q1** its consequences, may depend on species and inductors.

In conclusion, our results show clear differences among ROS generators. Hydrogen peroxide was cyto-toxic to red deer spermatozoa, in agreement with results **Q1** in other species. This does not mean that superoxide and hydroxyl radicals had no effect, only that their effect was not evident on the studied traits. Taking into account that oxidant levels were chosen according to similar H_2DCFDA fluorescence levels, factors such as half-life or membrane permeability only partly explain the observed differences. In fact, superoxide and hydroxyl radicals may trigger physiological pathways, disqualify-ing spermatozoa for further use. Moreover, only H_2O_2 seemed to cause an increase in apoptotic markers, and it seems that sperm subpopulations differing on its ability to display these markers may exist. Another interesting observation is the presence of activated caspases in red deer thawed spermatozoa, even in the absence of treatment. These results suggest future lines of work to improve sperm work in red deer and, possibly, small ruminants.

Materials and Methods

Reagents and media

Fluorescence probes and the Vybrant FAM Poly Caspases Assay Kit were purchased from Invitrogen. Acridine orange (chromatographically purified) was purchased from Poly-sciences Inc. (Warrington, PA, USA). Flow cytometry equip-ment, software, and consumables (including the sheath fluid) were purchased from BD Biosciences (San José, CA, USA). The rest of the chemicals were acquired from Sigma. Chemicals were of reagent grade or higher. PBS was prepared in milli-Q water: 136.9 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 7 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, 1.5 mM KH_2PO_4, and 1 g/l PVA (290 mOsm/kg; pH 7.2). Hypoxanthine was prepared at 200 mM in 0.1 M NaOH. Stock solutions of the antioxidant and oxidants were as follows: Trolox (vitamin E), 1 mM in ethanol; H_2O_2, 100 mM in water; XOD, 10 UI/ml in water; Fe^2/ascorbate, 10 mM $FeSO_4$ and 100 mM sodium ascorbate in water. From each oxidant solution, we prepared 1/10 and 1/100 solutions in water. Stock solutions of the fluorescence probes were as follows: propidium iodide, 7.5 mM in water; SYBR-14, 1 mM in DMSO; Mitotracker

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Deep Red, 1 mM in DMSO; BODIPY C_{11} 665/676, 0.1 mM in DMSO; H_2DCFDA, 0.5 mM in DMSO. All solutions were kept at $-20\,°C$ and in the dark until needed, except Trolox and the oxidant working solutions, which were prepared the same day. Preparation and staining of samples for flow cytometry analysis were performed by flow cytometer PBS (BD FACSFlow; BD Biosciences).

Semen 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–October). Hunting was in accordance with the harvest plan of the game reserves, following Spanish Harvest Regulation (Law 2/93 of Castilla-La Mancha), which conforms to European Union Regulations. These operations were carried out as part of a project approved by the ethical committee of the University of Castilla-La Mancha, and which adheres to the Guidelines for the Care and Use of Animals. Spermatozoa were collected from the cauda epididymis within 3 h postmortem, and diluted to 200×10^6 spermatozoa/ml in Triladyl (Minitüb, Q3 Tifenbach, Germany) with 20% egg yolk and 6% glycerol. After 2 h of equilibration at $5\,°C$, samples were loaded into 0.25 ml plastic straws (IMV, L'Aigle Cedex, France) and frozen in nitrogen vapor (4 cm above liquid nitrogen) for 10 min. The straws remained for a minimum period of 1 year in liquid nitrogen. Thawing was carried out by immersing straws in a water bath at $37\,°C$ for 30 s.

Experiment 1

This experiment evaluated the effect of oxidative stress on sperm motility, viability, mitochondrial membrane potential $(\Delta\psi_m)$, intracellular ROS, and membrane lipid peroxidation. It was replicated using samples from six different males. Thawed semen was resuspended to 10^7 spermatozoa/ml with PBS. The sperm solution was then split among 14 aliquots in microtubes. One of them was left untreated as control (C), and another one was supplemented with 10 µM Trolox (T). The other aliquots were subjected to oxidative stress using three concentrations of Q1 each oxidant system. For Fe^{2+}/ascorbate: 100 µM Fe^{2+} (FE100), 10 µM Fe^{2+} (FE10), and 1 µM Fe^{2+} (FE1); for H_2O_2: 1 mM (HP1000), 100 µM (HP100), and 10 µM (HP10); and for HX/XOD: 100 mU/ml XOD (XO100), 10 mU/ml XOD (XO10), and 1 mU/ml XOD (XO1). We replicated the highest concentration of each oxidant, supplementing this replication Q1 with 10 µM Trolox (FE100T, HP1000T, and XO100T). The aliquots with XOD received 2 mM hypoxanthine and 1 mM EDTA. All the treatments were split into two aliquots. One of them received 0.5 µM H_2DCFDA and 0.2 µM BODIPY C_{11} 665/676 (for the detection of intracellular ROS and membrane lipoperoxidation respectively). The microtubes were incubated at $37\,°C$ in the dark and analyzed at 15, 60, and 180 min after starting the incubation. The three concentrations for each system were selected after a preliminary assay using H_2DCFDA, in order to obtain similar 'low', 'medium', and 'high' production of ROS in each system.

Motility was subjectively assessed. A 5 µl drop was placed on a prewarmed slide, covered with a coverslip and examined using a phase contrast microscope (Nikon Eclipse 80i; negative Q1 contrast optics), with a warming stage at $37\,°C$. We calculated a (SMI; Fernandez-Santos *et al.* 2007a), assessing the percentage of motile sperm (MI) and the quality of movement (QM; from 0, no motility to 5, fast and lineal motility), and using the formula $SMI=(MI+20\times QM)\times0.5$. The same skilled evaluator was in charge of assessing all samples.

Sperm viability and $\Delta\psi_m$ (SYBR-14/propidium iodide (PI) and Mitotracker Deep Red), and intracellular ROS and membrane lipid peroxidation (H_2DCFDA and BODIPY C_{11} 665/676) were assessed by flow cytometry. For the analysis of viability and $\Delta\psi_m$, we diluted the spermatozoa down to 10^6/ml in flow cytometry PBS with 10 µM SYBR-14, 24 µM PI, Q1 and 100 nM Mitotracker Deep Red. For the analysis of intracellular ROS and membrane lipid peroxidation, the pre-labeled samples were diluted down to 10^6/ml in flow cytometry PBS with 5 µM PI. In both stainings, the samples were left 15 min in the dark before running them through a flow cytometer.

Q4 Moreover, an aliquot of each sample was diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH 7.4) to 2×10^6/ml in cryotubes for chromatin assessment (SCSA). The cryotubes were flash-frozen in liquid nitrogen and stored at $-80\,°C$ until analysis (see below).

Experiment 2

This experiment evaluated the expression of apoptosis/necrosis markers after exposure to oxidative stress. It was replicated using samples from six different males. Thawed semen was resuspended to 10^7 spermatozoa/ml with PBS. The sperm solution was then split among four aliquots in microtubes. One of them was left untreated as control, and the other was Q1 subjected to oxidative stress: Fe^{2+}/ascorbate (100 µM Fe^{2+}), H_2O_2 (1 mM), and HX/XOD (100 µM XOD, 2 mM hypoxanthine, and 1 mM EDTA). The microtubes were incubated at $37\,°C$. At 15 min, 1 h, 2 h, and 3 h, the samples were diluted down to 10^6/ml in flow cytometry PBS with 5 µM Hoechst 33342, 100 nM YO-PRO-1, 1 µM Merocyanine 540, 10 µM PI, and 100 nM Mitotracker Deep Red. After 15 min in the dark, the samples were run through a flow cytometer. YO-PRO-1 is a probe capable of staining early apoptotic cells, with intact plasmalemma (not stained by PI, for instance), but showing increased permeability (typical of early apoptotic cells; Martinez-Pastor *et al.* 2008a). Merocyanine 540 is a lipophilic probe that increases its fluorescence as membrane lipid packaging decreases, being useful for assessing membrane stability and, possibly, capacitation-related events (Fernandez-Santos *et al.* 2007a).

To confirm that H_2O_2 was the causative agent of $\Delta\psi_m$ dissipation, two more treatments were added: 2500 U/ml catalase and HX/XOD plus 2500 U/ml catalase. Thus, the H_2O_2 resulting from XOD or superoxide dismutation was removed. Incubation and analysis were performed in the same Q1 way as for the other treatments of the experiments.

Experiment 3

In this experiment, we assessed the presence of activated caspases after exposure to ROS. The samples were prepared in the same manner as in Experiment 2 and incubated at 37 °C. At 15 min, 1 h, 2 h, and 3 h, the samples were processed to evaluate caspase activation using the Vybrant FAM Poly Caspases Assay Kit. This kit is based on the fluorescent caspase inhibitor (FAM-VAD-FMK), which binds to active caspases, thus labeling apoptotic cells. Briefly, the aliquots were diluted down to 10^6/ml in 300 μl of PBS with 10 μl of 30X FLICA™ reagent (fluorescent caspase inhibitor) in flow cytometry tubes. The tubes were incubated for 1 h at 37 °C in the dark and then washed twice with the washing buffer provided by the kit (300 *g*, 5 min). After the second centrifugation, the washing buffer was replaced by 400 μl of the flow cytometer sheath fluid with 5 μM Hoechst 33342 and 10 μM PI. After 15 min in the dark, the samples were run through a flow cytometer.

Flow cytometer configuration

For flow cytometry, a Becton Dickinson LSR-I model (BD Biosciences) was used. ~~We used A 488 nm Ar-ion laser~~ for exciting SYBR-14, YO-PRO-1, FLICA™, PI, Merocyanine 580, and H_2DCFDA, and a 633 nm He–Ne laser for exciting Mitotracker Deep Red and BODIPY C_{11} 665/676. Fluorescence from SYBR-14, YO-PRO-1, FLICA, Merocyanine 540, and H_2DCFDA was read with the FL1 photodetector (530/28BP filter). Fluorescence from Merocyanine 540 was read with the FL2 photodetector (575/25BP filter). Fluorescence from PI was read with the FL3 photodetector (670LP filter). Fluorescence from TO-PRO-1, Mitotracker Deep Red, and BODIPY C_{11} 665/676 was read with the FL6 photodetector (670/40BP filter). FSC/SSC signals were used to discriminate spermatozoa from debris. When Hoechst 33342 was used, it was excited using a 350 nm He–Cd laser, and its fluorescence was read with the FL5 photodetector (424/44BP filter). The FL5 signal was used to improve the ~~discrimination of spermatozoa/debris. The acquisition was that~~ fluorescence captures were controlled using the Cell Quest Pro 3.1 software (BD Biosciences). All the parameters were read using logarithmic amplification. For each sample, ~~we acquired~~ 10 000 spermatozoa ~~from each sample~~, saving the data in FCS v. 2 files. The analysis of the flow cytometry data was carried out using WinMDI v. 2.8 (The Scripps Research Institute, La Jolla, CA, USA).

Sperm chromatin structure assessment

The SCSA technique (Evenson *et al*. 2002) is based on the metachromatic stain acridine orange, which fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). The samples flash-frozen in TNE were thawed on crushed ice and 200 μl were transferred to a flow cytometry tube. Then, ~~we added~~ 400 μl of cold acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100; pH 1.2). Exactly 30 s after adding the acid-detergent solution, ~~we added~~ 1.2 ml of cold staining solution (6 μg/ml of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA,

and 150 mM NaCl; pH 6). After 3 min, the sample was run through the flow cytometer. Acridine orange was excited using the Ar-ion laser (488 nm). The red fluorescence was detected using the FL3 photodetector (670LP) and the green fluorescence using the FL1 photodetector (530/28BP). In both cases, linear scales were used. The cytometer was previously equilibrated using a tube with 0.4 ml of acid-detergent solution and 1.2 ml of staining solution. This tube was run regularly between samples to diminish changes due to flow variations (Boe-Hansen *et al*. 2005). The data acquisition was controlled using the Cell Quest Pro 3.1 software, acquiring 5000 spermatozoa, and the data were processed using WinMDI v. 2.8. We calculated the DNA fragmentation index (DFI) for each spermatozoa as the ratio of red fluorescence with respect to total fluorescence (red + green), expressed as a percentage. The processing of DFI data was performed by the R statistical environment (R Development Core Team 2007). From the DFI values, ~~we obtained~~ the SD of DFI (SD-DFI), as well as the percentage of spermatozoa with high fragmentation index (%DFI) and the percentage of spermatozoa with high DNA stainability (HDS), defined as those events with green fluorescence above channel 600.

Data processing and statistical analysis

The R statistical environment was used to process flow cytometry data and to perform subsequent statistical analysis. Our hypotheses were tested by using mixed-effects linear models, treating the replication as a random effect. Despite individual differences, the samples from different males behaved similarly, and an interference of male with treatment was discarded. Pairwise comparisons were carried out using contrasts and Holm's correction for multiple comparisons. Numerical results were expressed as mean ± S.E.M.

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Q1   We have made a change to this sentence. Please review our edit.

Q2   Please check and approve the citation of figures.

Q3   Please confirm the edit of 'Tryladyl' to 'Triladyl'.

Q4   Please provide expansion of the acronym 'TNE'.

Q5   A declaration of interest statement reporting no conflict of interest has been inserted. Please confirm whether the statement is accurate.

Q6   Please provide the volume and page range details for references 'Bejarano *et al.* (2007), Fernandez-Santos *et al.* (2008) and Martinez-Pastor *et al.* (2008*b*)'.

Q7   Please provide complete details for reference 'Owuor & Kong (2002)'.