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Supplementation of sperm media with zinc, D-aspartate and co-enzyme Q10 protects bull sperm against exogenous oxidative stress and improves their ability to support embryo development

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Summary

High levels of reactive oxygen species in the semen of infertile patients or spontaneously generated during *in vitro* sperm handling may impair sperm quality, fertilization and embryo developmental competence. We recently reported that zinc, D-aspartate and co-enzyme Q10, contained in the dietary supplement Genadis® (Merck Serono), have protective effects on human and bull sperm motility, lipid peroxidation and DNA fragmentation *in vitro*; furthermore, in bovine, treated spermatozoa had an improved ability to support embryo development. However, only a few studies have investigated the protective role of antioxidants during *in vitro* sperm handling in the presence of an exogenous oxidative stress. Herein, to simulate such conditions in an animal model, we induced exogenous oxidative stress on spermatozoa through the xanthine–xanthine oxidase system and investigated its effects on sperm function and subsequent embryo developmental competence in the presence of zinc, D-Asp and CoQ10 protection. The main results showed that exogenous oxidative stress decreased sperm motility, increased sperm DNA fragmentation, and reduced fertilization and blastocyst rates and quality. Pre-treatment with zinc, D-aspartate and co-enzyme Q10 before exogenous oxidative stress was able to prevent these effects. Supplementation of sperm culture media with zinc, D-aspartate and co-enzyme Q10 could protect sperm from oxidative stress damage during *in vitro* handling in assisted reproductive technologies.

Keywords: Antioxidants, DNA fragmentation, Embryo development, Oxidative stress, Spermatozoa

Introduction

Oxidative stress (OS) occurs when reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions and hydrogen peroxide, overcome the natural antioxidant defenses and cause cellular damage (Tremellen, 2008). ROS are byproducts of oxygen metabolism, and are physiologically produced via electron transfer chain system of the mitochondria. Low and controlled concentrations of ROS play an important role in sperm physiological processes such as maturation, hyperactivation, capacitation, zona pellucida binding, acrosome reaction, and sperm–oocyte fusion (Agarwal & Saleh, 2002; Agarwal & Allamaneni, 2004; De Lamirande & Lamothe, 2009). The principle physiological sources of ROS during sperm capacitation are the plasma membrane NADPH oxidase and the mitochondrial NADH-dependent oxidoreductase (Agarwal *et al.*, 2014). However, several studies have shown that increased levels of seminal ROS and decreased total antioxidant capacity were present in the semen of infertile men compared with fertile controls (Pasqualotto *et al.*, 2008; Agarwal & Sekhon, 2011). Main sources of ROS in semen

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of infertile men are leukocytes, and immature or abnormal spermatozoa (Henkel *et al.*, 2005).

Although high ROS levels have been reported to impair sperm motility, DNA integrity, and the ability to fertilize and support embryo development (Aitken *et al.*, 2010; Chen *et al.*, 2013; Talevi *et al.*, 2013; Agarwal *et al.*, 2014; Gualtieri *et al.*, 2014), it is still unclear whether OS can affect embryo outcome and live birth rate.

Moreover, several factors during routine sperm handling in assisted reproduction may cause excessive ROS production (Agarwal *et al.*, 2014). Exogenous factors such as visible light, centrifugation, cryopreservation, oxygen concentration, pH and temperature have been shown to contribute to ROS production during assisted reproduction (Thomson *et al.*, 2009; Zribi *et al.*, 2010; Agarwal *et al.*, 2014; Balasuriya *et al.*, 2014). In particular, we recently showed that routine human and bull sperm *in vitro* handling is a source of OS, and impairs sperm quality in terms of motility, lipid peroxidation and DNA fragmentation and such damage was prevented by the antioxidants zinc and co-enzyme Q10 (CoQ10), and the micronutrient D-aspartate (D-Asp), contained in the dietary supplement Genadis® (Merck Serono) (Talevi *et al.*, 2013; Gualtieri *et al.*, 2014). In addition, we demonstrated that spontaneous OS during routine handling of bull spermatozoa had negative consequences on embryo development that were completely prevented by sperm pre-treatment with Genadis®.

Therefore, in a typical clinical setting, high OS, due to both routine sperm handling *per se* and elevated ROS concentrations in semen of subfertile men, could be prevented or reduced through the supplementation of sperm culture media with antioxidants. Here, to simulate such condition in an animal model, we induced exogenous OS through the xanthine–xanthine oxidase system (X–XO) during handling of bull spermatozoa and investigated its effects on sperm function and subsequent embryo developmental competence in the presence or absence of Genadis® protection. Data showed that exogenous OS caused the loss of sperm motility, the increase of sperm DNA fragmentation and the impairment of the sperm's ability to support the development of healthy embryos; such negative effects could be prevented by Genadis® pre-treatment.

Materials and methods

Chemicals

Zinc chloride, D-aspartic acid, co-enzyme Q10, xanthine (code no. X7375), xanthine oxidase (code no. X4500), paraformaldehyde, Triton X-100, sodium citrate, Hoecht stain 33342, polyvinyl alcohol (PVA), M 199 (code no. 4530), gentamycin, amphotericin B, fetal calf serum (FCS), epidermal growth factor, HEPES sodium salt, heparin sodium salt (code no. H3393), and reagents and water (cell culture tested) for preparation of salines and culture media were from Sigma Chemical Company (Milan, Italy). *In situ* cell death detection kit, fluorescein, and DNase I were from Roche Diagnostics (Milan, Italy).

Sperm preparation

Frozen bovine semen from six ejaculates of six bulls (0.5 ml straws; 40 × 10^6 spermatozoa per straw; motility after thawing 70%), obtained from Inseme (San Giuliano Saliceta, Modena, Italy), was used in all experiments. Straws were thawed in a water bath at 38°C for 30 s and washed in 10 ml sperm TALP medium (Parrish *et al.*, 1989) by centrifugation at 170 *g* for 10 min. After resuspension in fresh medium, the recovered spermatozoa were assessed for concentration and percentage motility using a Makler chamber placed on a microscope stage heated to 39°C as described below.

Antioxidant pre-treatment and exogenous oxidative stress induction

The following stock solutions were prepared: 10 mg/ml zinc chloride in water, 50 mg/ml D-Asp in sperm TALP medium, 50 mg/ml CoQ10 in chloroform and 50 mg/ml xanthine in NaOH 1 M.

All experiments ($n = 4$) included three groups:

(1) Treated: sperm suspensions in sperm TALP were pre-treated 1 h with 10 µg/ml zinc chloride, 500 µg/ml D-Asp and 40 µg/ml CoQ10 and then added with 15 µg/ml xanthine–0.01 U/ml xanthine oxidase and incubated for 2 h.
(2) X–XO: parallel sperm suspensions were pre-treated 1 h with vehicles present in zinc-, D-Asp-, and CoQ10-pre-treated samples (0.1% water, 0.08% chloroform) and then added with 15 µg/ml xanthine–0.01 U/ml xanthine oxidase and incubated for 2 h.
(3) Control: sperm were pre-treated 1 h with vehicles present in zinc-, D-Asp-, and CoQ10-pre-treated samples (0.1% water, 0.08% chloroform) and then added with vehicle present in X–XO samples (0.1% NaOH) and incubated for 2 h.

Samples were loaded onto a Makler chamber and analyzed on a heated stage at 39°C every hour for 3 h using a Nikon TE 2000 inverted microscope connected to a Basler Vision Technology A312 FC camera with a positive phase contrast ×10 magnification objective. For each time point, at least 400 cells and four fields were acquired and analyzed. Progressive motility and kinetics, i.e. curvilinear velocity (VCL), straight-line

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velocity (VSL), and average path velocity (VAP), were evaluated by Sperm Class analyzer (SCA Microptic S.L. Barcelona, Spain) as previously reported (Gualtieri *et al.*, 2014).

TUNEL assay

DNA fragmentation in spermatozoa and blastocysts (number of blastocysts = 90) was measured using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay. Free 3′-OH ends of DNA were labeled with fluorescein isothiocyanate–conjugated dUTP (FITC–dUTP) by means of TdT. Control and treated sperm samples at 0 and 3 h of incubation were centrifuged at 170 *g* for 10 min, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature (rt), washed three times in PBS through centrifugation at 170 *g* for 10 min, smeared onto glass slides, and then air dried. Then, the samples were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at 4°C and washed in PBS three times for 10 min as described above. The blastocysts generated by control, X–XO and treated spermatozoa were fixed in 4% paraformaldehyde in PBS for 4 h at rt, washed three times for 10 min in PBS supplemented with 3 mg/ml polyvinyl alcohol (PBS–PVA), permeabilized as above for 30 min at 4°C, and washed three times for 10 min in PBS–PVA. The samples were then incubated in TUNEL reaction mixture according to the manufacturer's instructions for 1 h at 37°C in the dark. At the end of incubation, the samples were washed in PBS or PBS–PVA as above, labelled with 10 μg/ml Hoechst 33342 for 7 min at rt, washed again, mounted, and observed on a Nikon TE 2000 fluorescence microscope. Images were acquired using a Nikon DS–cooled camera head DS-5Mc connected to a Nikon DS camera control unit DS-L1 using the same exposure conditions. In each experiment, negative controls were prepared by omission of terminal deoxynucleotidyl transferase in the reaction mixture, whereas positive controls were prepared by pre-treatment with 1 mg/ml DNase I for 10 min at room temperature. The percentages of TUNEL-positive spermatozoa were determined on at least 200 cells for each sample.

Oocyte collection, IVF, and embryo culture

Ovaries were collected from a local slaughterhouse and transported to the laboratory at 30°C within 2 to 3 h. Cumulus–oocyte complexes (COCs) were collected by aspiration of individual follicles with a 19-gauge needle. Cumulus–oocyte complexes (total number = 1846) were matured for 22–24 h in M199 medium supplemented with 50 μg/ml gentamycin, 1 μg/ml amphotericin B, 10% FCS, and 10 ng/ml epidermal growth factor at 39°C, in an atmosphere of 5% CO_2 in air, and 95% humidity.

At the end of treatment, the three sperm suspensions were diluted 12.5-fold with IVF-TALP, centrifuged at 170 *g* for 10 min, and the pellets (50 μl) were resuspended in 1 ml of IVF-TALP. For fertilization, groups of 50 *in vitro*-matured COCs in 250 μl IVF-TALP were inseminated with 250 μl of each sperm suspension (sperm final concentration, 1×10^6/ml) and added with heparin at a final concentration of 10 μg/ml. Overall, after insemination the components present in sperm culture medium during treatment were diluted 500-fold. Therefore, control and X–XO fertilization wells were added with the residual concentrations of the molecules present in the treated wells (Control: zinc chloride, 0.02 μg/ml, D-Asp, 1 μg/ml, CoQ10 0.08 μg/ml, xanthine 0.03 μg/ml, xanthine oxidase 0.00002 U/ml; X–XO: zinc chloride, 0.02 μg/ml, D-Asp, 1 μg/ml, CoQ10 0.08 μg/ml). After 18–20 h of coincubation at 39°C and 5% CO_2, the COCs were transferred into HEPES–TALP (Parrish *et al.*, 1989) and cumulus cells were removed by vortexing. Presumptive zygotes were collected, washed in synthetic oviduct fluid (Tervit *et al.*, 1972) supplemented with 5% FCS, and incubated in 700 μl of fresh SOF for 7 days at 39°C, in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The cleavage rates and percentages of embryos ≥8 cell (8-cell embryos on cleaved embryos) were determined at day 3 postinsemination (pi), whereas blastocyst rates (blastocysts on cleaved embryos) were determined at day 8 pi. At that time, the blastocysts were fixed and labelled with TUNEL and Hoechst stain as described above to determine blastocyst mean cell number and percentages of TUNEL-positive cells.

Statistical analysis

Sperm motility and kinetics were analyzed using ANOVA (SAS/STAT User's Guide, 1988) followed by the Tukey's honestly significant difference test for pairwise comparisons when the overall significance was detected. Per cent data were transformed into arcsine before statistical analysis. TUNEL positivity in spermatozoa and in blastocysts were represented as cumulative percentages and were analyzed by Fisher's exact test.

Results

Sperm motility, kinetics and DNA fragmentation

To understand whether pre-treatment with antioxidants exerts protective effects on OS induced by X–XO, spermatozoa were pre-treated with zinc chloride

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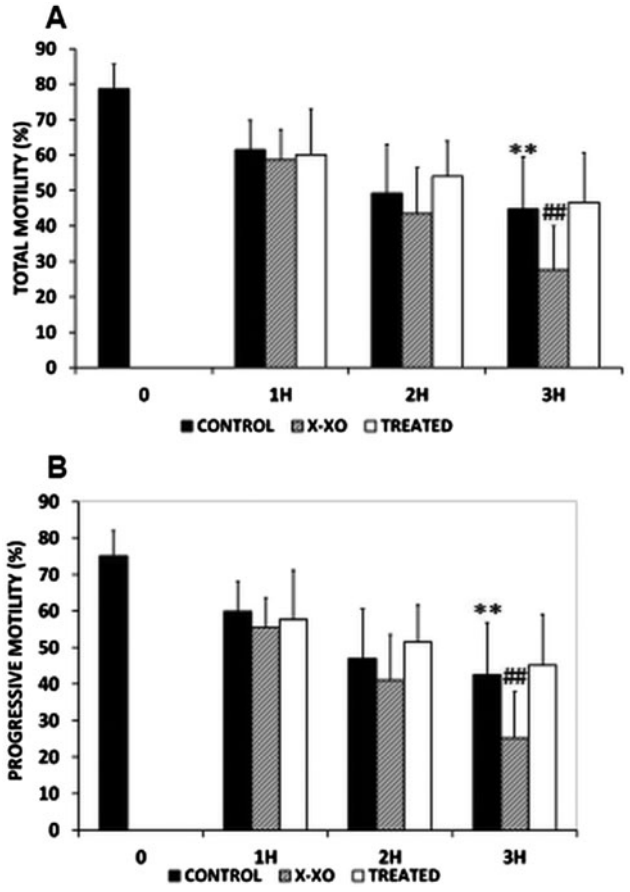

Figure 1 Effects of X–XO with and without zinc, D-Asp and CoQ10 pre-treatment on total (*A*) and progressive sperm motility (*B*). **Significant differences versus control at 0 h ($P < 0.01$); ##Significant differences versus corresponding control ($P < 0.01$).

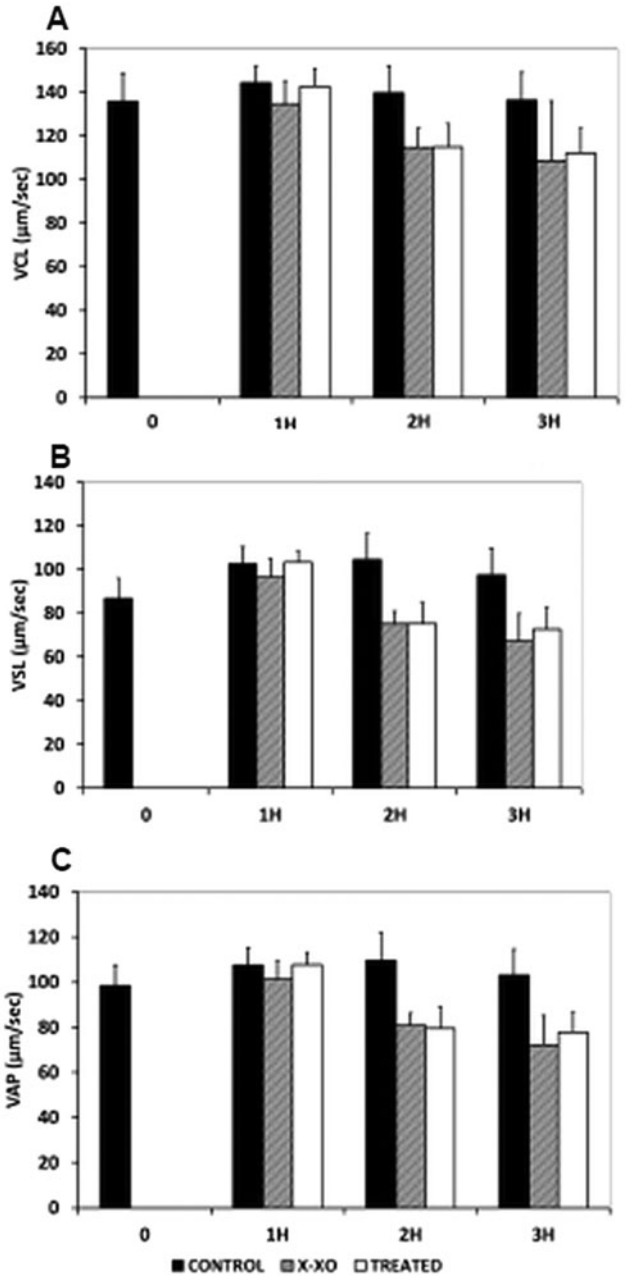

Figure 2 Effects of X–XO with and without zinc, D-Asp and CoQ10 pre-treatment on sperm kinetics. (*A*) Curvilinear velocity (VCL). (*B*) Straight-line velocity (VSL). (*C*) Average path velocity (VAP). Treatments versus corresponding controls, $P > 0.05$.

10 µg/ml, D-Asp 500 µg/ml and CoQ10 40 µg/ml for 1 h and then treated with 15 µg/ml xanthine-0.01 U/ml xanthine oxidase for 2 h. Data showed a significant decrease in total and progressive motility in the Control samples (Fig. 1). The decline of motility in Control sperm suspensions can be ascribed to the typical behaviour of frozen/thawed bull spermatozoa. In particular, in Control samples, motility (time 0: total, 78.6 ± 7.1%; progressive, 75.2 ± 6.8%) significantly and progressively decreased at 1 and 3 h (total, 44.8 ± 14.7; progressive, 42.5 ± 14.4%; 0 versus 3 h, $P < 0.01$).

Treatment with X–XO induced a high and significant decrease of total and progressive motility compared with the respective Control samples after 3 h of incubation (X–XO versus Control, 3 h: total 27.6 ± 12.4 versus 44.8 ± 14.7%; progressive 25.2 ± 12.8 versus 42.5 ± 14.4%; $P < 0.01$) (Fig. 1*A*, *B*). Interestingly, pre-treatment with zinc, D-Asp and CoQ10 prevented the drop in motility observed in X–XO samples (total and progressive motility: 46.7 ± 13.8; 45.2 ± 13.9; Treated 3 h versus X–XO 3 h, $P < 0.01$) (Fig. 1*A*, *B*). Although sperm kinetics values in X–XO and treated samples were slightly lower respect to controls, such effects were not significant ($P > 0.05$) (Fig. 2*A*–*C*).

Experiments were addressed to evaluate how DNA fragmentation in the initial suspensions was affected by exogenous OS with and without antioxidant protection after 3 h of incubation. Data (Fig. 3) showed that the percentage of spermatozoa with fragmented DNA in the control suspension significantly increased

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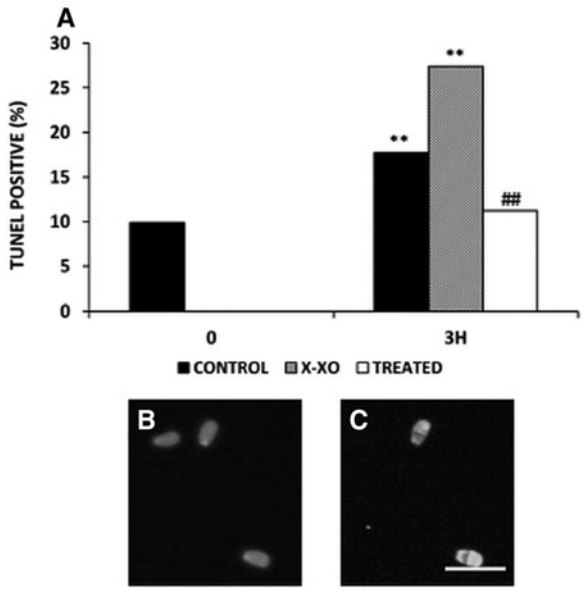

Figure 3 Effects of X–XO with and without zinc, D-Asp and CoQ10 pre-treatment on sperm DNA fragmentation. (*A*) Percentages of TUNEL-positive spermatozoa. (*B*, *C*) Representative micrographs of spermatozoa labeled with Hoechst (*B*) and TUNEL (*C*). Scale bar = 10 µm. **Significant differences versus control at 0 h ($P < 0.01$); ##Significant differences versus corresponding control ($P < 0.01$).

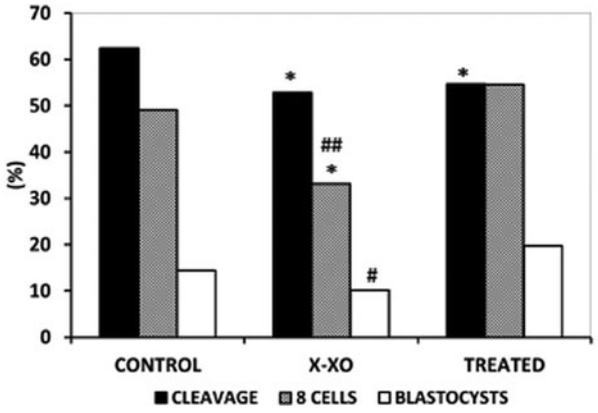

Figure 4 Effects of X–XO with and without zinc, D-Asp and CoQ10 pre-treatment on rates of cleavage and 8-cell embryos at day 3, and blastocyst rates at day 8. *Significant differences versus control ($P < 0.05$). # and ##Significant differences versus treated (#$P < 0.05$; ##$P < 0.01$).

from 9.9% at 0 h to 17.7% at 3 h of incubation ($P < 0.01$). Moreover, exogenous OS induced a marked increase of TUNEL-positive spermatozoa compared with the control samples at 3 h (X–XO versus control: 27.4% versus 17.7%, $P < 0.01$). Interestingly, pre-treatment with zinc, D-Asp and CoQ10 prevented the increase in sperm DNA fragmentation observed in both control and X–XO samples at 3 h (treated versus control: 11.2 versus 17.7%, $P < 0.01$; treated versus X–XO: 11.2 versus 27.4%, $P < 0.01$) (Fig. 3*A*).

In vitro fertilization and embryo culture

In vitro fertilization experiments were designed to understand whether exogenous OS with and without pre-protection with zinc, D-Asp, and CoQ10 influenced the sperm fertilization competence and its ability to support the preimplantation development.

As reported elsewhere (Gualtieri *et al.*, 2014) the low percentage of blastocyst development in control sample is due to spontaneous OS arising during the 3 h incubation of frozen–thawed sperm *in vitro*. Data indicated that treatment with X–XO significantly decreased cleavage rates respect to control, and pre-treatment with antioxidants did not prevent such a decrease (Fig. 4, Control, 62.4; X–XO, 52.8; Treated, 54.7%; $P < 0.05$). Furthermore, sperm exogenous OS decreased the rate of ≥8 cell stage embryos at day 3 and such an effect was prevented by pre-treatment with antioxidants (Fig. 4, Control, 49.1; X–XO 33.1; Treated, 54.6%; Control versus X–XO, $P < 0.05$; Control versus Treated, $P > 0.05$; Treated versus X–XO, $P < 0.01$). At day 8 pi, the oocytes fertilized with treated spermatozoa had a markedly higher competence to develop to the blastocyst stage compared with both control and X–XO spermatozoa (Fig. 4, Control, 14.4; X–XO 10.1; Treated, 19.7%; Control and Treated versus X–XO, $P < 0.05$).

Blastocyst's mean cell number and DNA fragmentation

Blastocyst's developmental competence was determined through assessment of mean cell number and percentages of DNA fragmented cells. Data indicated that exogenous OS did not affect blastocyst's mean cell number (Control, 102.8 ± 34.9, Treated, 104.7 ± 39.5, X–XO, 104.7 ± 42.7), whereas it increased the percentages of blastocyst's DNA fragmented cells with respect to both control and treated samples (Fig. 5) (X–XO, 10.6; Control, 7.5; Treated, 7.8%; X–XO versus Control and Treated, $P < 0.01$).

Discussion

Oxidative stress is a well recognized cause of male infertility (Agarwal *et al.*, 2014). Although oral antioxidant therapy exerts beneficial effects on semen quality, the consequences of sperm OS and antioxidant therapy *in vivo* and *in vitro* on reproductive outcome are still being debated (Gharagozloo & Aitken, 2011; Showell *et al.*, 2014). We recently demonstrated that a spontaneous endogenous OS arises during the *in vitro* handling of human and bull spermatozoa and can be prevented through medium supplementation

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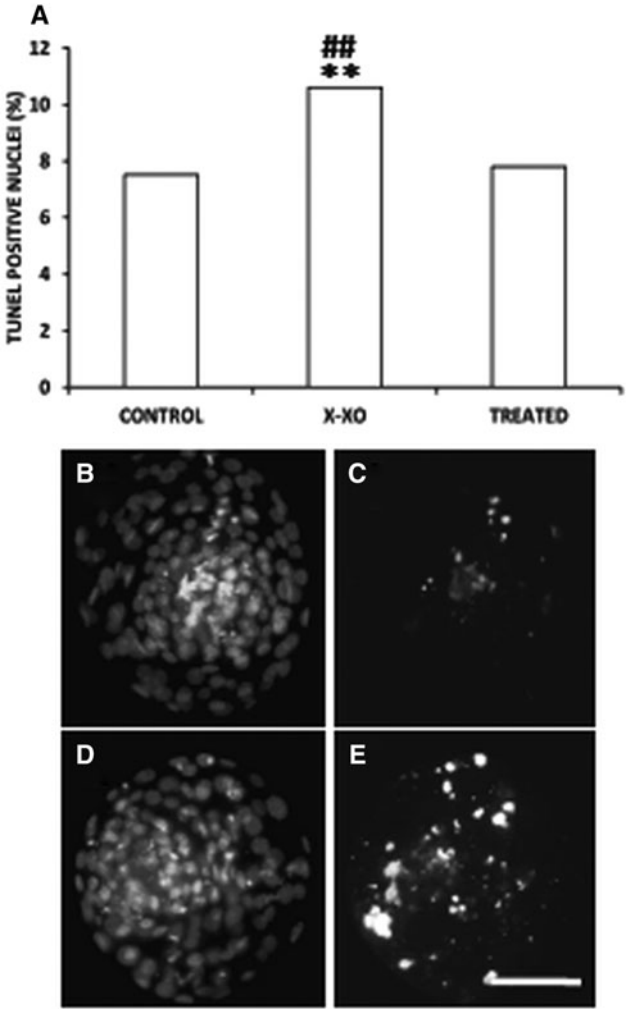

Figure 5 Effects of X–XO with and without zinc, D-Asp and CoQ10 pre-treatment on blastocyst's DNA fragmentation. (*A*) Percentages of TUNEL-positive cells in blastocysts developed from oocytes fertilized with control, X–XO or treated spermatozoa. (*B–E*) Representative micrographs of two blastocysts labeled with Hoechst (*B*, *D*) and TUNEL (*C*, *E*). Scale bar = μm. **Significant differences versus control ($P < 0,01$). ##Significant differences versus treated ($P < 0.01$).

with Genadis® (Talevi *et al.*, 2013; Gualtieri *et al.*, 2014). Moreover, using the bovine as an animal model to investigate the effects of sperm OS on embryo development, we demonstrated that it exerts a paternal effect, reducing the rate of on time embryos (≥8 cells) at day 3, and yielding a decreased rate of blastocysts characterized by a higher number of DNA fragmented cells. Such negative paternal effects were prevented in parallel sperm protected by antioxidants *in vitro* (Gualtieri *et al.*, 2014). Herein, to simulate a high OS environment in an animal model we induced an exogenous OS on sperm and investigated its effects on sperm function and subsequent embryo developmental competence in the presence of Genadis® protection. To this end we chose the X–XO system that has been demonstrated to affect sperm quality, in terms of motility, velocity, lipid peroxidation and DNA integrity in a time- and dose-dependent manner in several species (Aitken *et al.*, 1993; Hagedorn *et al.*, 2012; Burruel *et al.*, 2013; Shaliutina-Kolešová *et al.*, 2014, 2015; Gazo *et al.*, 2015). Data showed that treatment with X–XO decreased sperm motility and kinetics, and increased sperm DNA fragmentation from 2 h of incubation onwards. Although pre-treatment with Genadis® followed by exogenous OS did not prevent the decrease of sperm kinetics values, it was able to avoid the loss of motility and the rise of DNA fragmentation. To our knowledge, this study is the first to address the effects of *in vitro* pre-treatment with zinc, D-Asp and CoQ10 on exogenous OS induced by X–XO in bull spermatozoa. Our data confirm recent findings that bull sperm exogenous OS *in vitro* induced by hydrogen peroxide affects sperm motility, kinetics and chromatin integrity (De Castro *et al.*, 2016) and demonstrate that prior protection *in vitro* with Genadis® is able to prevent the deleterious effects of exogenous OS on spermatozoa.

Herein, IVF and embryo culture experiments demonstrated that *in vitro* exogenous OS affected the sperm competence to fertilize and promote embryo development, reducing cleavage and ≥8-cell embryo rates at day 3, and blastocyst rates at day 8 and such decreases were prevented by *in vitro* protection with Genadis®. Moreover, although the difference was not statistically significant, the blastocyst rates of oocytes inseminated with spermatozoa pre-protected before exogenous OS was higher compared with those derived from oocytes inseminated with control non-stressed spermatozoa. This finding indicates that, at least under our experimental conditions, pre-treatment with Genadis® is able to prevent the deleterious effects of both endogenous and exogenous sperm OS on embryo development. Different studies have correlated sperm OS and/or DNA damage to cleavage and blastocyst rates. In the mouse, sperm chromatin fragmentation causes a delay of both paternal DNA replication in the zygote and embryonic development (Gawecka *et al.*, 2013). As regards the outcome of sperm exogenous OS on embryo development, ICSI of macaque sperm stressed with X–XO has been shown to result in developmental arrest before the 8-cell stage, blocked embryos being characterized by alteration of the first and second mitosis and presence of micronuclei and DNA fragmentation (Burruel *et al.*, 2013, 2014). Conversely, in agreement with present findings, treatment of bull spermatozoa with increasing concentrations of hydrogen peroxide caused a reduction in the number of 8–16-cell embryos and blastocyst formation (De Castro *et al.*, 2016). Although, in these and our study, exogenous OS was not carried out under identical conditions, it could be

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hypothesized that bull spermatozoa are more resistant to OS compared with macaque sperm. As macaque sperm OS have a deleterious paternal effect on embryo development before paternal gene expression, it has been suggested that centrosomal contribution to the mitotic spindle could be a potential cause (Burruel *et al.*, 2013, 2014). In our study, the reduction in 8-cell embryos at day 3 could reflect a similar paternal effect on embryo development. However, even though at a reduced rate, blastocysts develop from those embryos and harboured a significantly higher proportion of DNA fragmented nuclei compared with blastocysts derived from both control and pre-protected/stressed spermatozoa. This finding is in agreement with what occurs during bull sperm handling in the absence of exogenous OS (Gualtieri *et al.*, 2014) and with data from Simões *et al.*, (2013) on the influence of sperm susceptibility to OS on blastocyst DNA fragmentation and *in vitro* embryo production.

Overall, our results showed that *in vitro* pre-treatment with Genadis® exerts a protective effect on sperm exogenous OS and the ability of stressed spermatozoa to support embryo development. Supplementation of media with zinc, D-Asp and CoQ10, during routine sperm handling in assisted reproduction, could represent a valuable strategy to minimize the deleterious effects of sperm OS on embryo development.

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