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Treatment with zinc, D-aspartate, and coenzyme Q10 protects bull sperm against damage and improves their ability to support embryo development

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ABSTRACT

Reactive oxygen species (ROS) are physiologically generated during mitochondrial respiration and are involved in several signaling mechanisms. However, under pathological conditions, the concentration of ROS may exceed the antioxidant scavenging systems and subsequently lead to cell damage. High ROS levels have been proven to be detrimental to spermatozoa and furthermore compromise sperm function through lipid peroxidation, protein damage, and DNA strand breakage. Although the oral administration of antioxidants has been demonstrated to improve the semen quality in subfertile men, it is still a matter of debate if it can positively influence fertilization outcome and embryo developmental competence. Studies carried out in suitable animal models could resolve these fundamental questions. Hence, the main aims of the present study were to evaluate: (1) the effects of zinc, D-aspartate, and coenzyme Q10, included in the dietary supplement Genadis (Merck Serono), on bull sperm motility and DNA fragmentation; and (2) whether treated spermatozoa have a superior competence in fertilization and in supporting the development of healthy embryos. Our data indicate that this treatment prevents the loss of sperm motility and the rise in sperm DNA fragmentation over time. Moreover, blastocyst rate was found to be significantly higher in oocytes fertilized by treated spermatozoa, and these blastocysts harbored a significantly lower percentage of apoptotic cells.

1. Introduction

Reactive oxygen species (ROS) are involved in several signaling mechanisms [1]. Although normally produced during mitochondrial respiration, at high levels they can interact with lipids, proteins, and DNA, leading to severe pathological conditions. Spermatozoa physiologically produce ROS that are involved in protein tyrosine phosphorylation during sperm capacitation [2]. High ROS levels are proven to be detrimental to gametes and compromise their function through lipid peroxidation, protein damage, and DNA strand breakage [3]. In some pathological conditions, the semen ROS levels exceed the sperm antioxidant defenses, and this can lead to a state of oxidative stress and subsequent infertility. Although, antioxidant therapy has been demonstrated to enhance semen quality in subfertile men and has been suggested to improve pregnancy rates [4,5], it is still a matter of debate if it can positively influence fertilization outcome and embryo developmental competence. According to Aitken et al. [3], sperm oxidative stress not only could impair the sperm fertilizing ability but also its competence to support a normal embryo

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development. Moreover, DNA damage in human spermatozoa has been correlated with increased miscarriage rates and morbidity in the offspring [3]. We recently demonstrated that the antioxidants zinc and coenzyme Q10 (CoQ10), and the micronutrient D-aspartate (D-Asp), contained in the dietary supplement Genadis (Merck Serono), have protective effects on human sperm motility, DNA fragmentation, and lipid peroxidation [6]. However, it is still unknown whether sperm exposure to these antioxidants *in vitro* can affect fertilization outcome and embryo developmental competence.

Due to ethical and methodological restrictions, the possibility to answer these questions directly in the human species is limited. Studies in animal models could provide insights into these fundamental questions. Hence, the main goals of the present study were to evaluate: (1) the effects of zinc, D-Asp, and CoQ10 on bull sperm motility and DNA fragmentation; and (2) whether pretreated spermatozoa have a higher competence in fertilization and in the development of healthy embryos.

2. Materials and methods

2.1. Chemicals

Zinc chloride, D-aspartic acid, coenzyme Q10, paraformaldehyde, Triton X-100, sodium citrate, Hoechst 33342, polyvinyl alcohol (PVA), M 199 (cod. 4530), gentamycin, amphotericine B, fetal calf serum (FCS), epidermal growth factor, HEPES sodium salt, heparin sodium salt (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).

2.2. Sperm preparation

Frozen bovine semen from seven ejaculates of seven bulls (0.5 mL straws; $\sim 40 \times 10^6$ spermatozoa per straw; motility after thawing $\geq$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 seconds and washed in 10 mL sperm TALP medium [7] by centrifugation at 170× *g* for 10 minutes. After resuspension in a fresh medium, the recovered spermatozoa were assessed for concentration and percent motility using a Makler chamber placed on a microscope stage heated to 39 °C as described below.

2.3. Effects of zinc, D-Asp, and CoQ10 on sperm motility and kinetics

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

Sperm suspensions in sperm TALP were treated with 10 μg/mL zinc chloride, 500 μg/mL D-Asp, and 40 μg/mL CoQ10. In all experiments (n = 7), controls were added with identical concentrations of vehicle present in zinc-, D-Asp-, and CoQ10-treated samples (0.1% water, 0.08% chloroform). Samples were loaded on to a Makler chamber and analyzed on a heated stage at 39 °C every hour and until 5 hours after treatment at a Nikon TE 2000 inverted microscope connected to a Basler Vision Technology A312 FC camera with a positive phase contrast 10× objective. For each time point, at least 400 cells and four fields were acquired and analyzed. Progressive motility and kinetics, that is, curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP), were evaluated by Sperm Class analyzer (SCA Microptic S.L. Barcelona, Spain) with the following software setting: frame rates: 25 frames/s, number of frames: 10 frames/object, velocity limit for slow spermatozoa: 10 μm/s, velocity limit for medium spermatozoa, 25 μm/s, velocity limit for rapid spermatozoa 50 μm/s, minimal linearity 50%, and straightness for progressive fast spermatozoa 70%.

2.4. TUNEL assay

DNA fragmentation in spermatozoa and blastocysts (number of blastocysts = 185) was measured by the TUNEL assay (*in situ* cell death detection kit, fluorescein; Roche Diagnostics, Milan, Italy). Free 3′-OH ends of DNA were labeled with fluorescein isothiocyanate–conjugated dUTP (FITC-dUTP) by means of terminal deoxynucleotidyl transferase.

Control and treated sperm samples at 0, 1, 2, 3, 4, and 5 hours of incubation were centrifuged at 170× *g* for 10 minutes, fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature (rt), washed three times in PBS through centrifugation at 170× *g* for 10 minutes, smeared on glass slides, and then air-dried. Then, the samples were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 5 minutes at 4 °C and washed in PBS three times for 10 minutes as described above.

The blastocysts generated by control and treated spermatozoa were fixed in 4% paraformaldehyde in PBS for 4 hours at rt, washed three times for 10 minutes in PBS supplemented with 3 mg/mL polyvinyl alcohol (PBS-PVA), permeabilized as above for 30 minutes at 4 °C, and washed three times for 10 minutes in PBS-PVA.

The samples were then incubated in TUNEL reaction mixture according to the manufacturer's instructions for 1 hour at 37 °C in the dark. At the end of incubation, the samples were washed in PBS or PBS-PVA as above, labeled with 10 μg/mL Hoechst 33342 for 7 minutes at rt, washed again, mounted, and observed at 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, the negative controls were prepared by omission of terminal deoxynucleotidyl transferase in the reaction mixture, whereas positive controls were prepared by pretreatment with 1 mg/mL DNase I (Roche Diagnostics) for 10 minutes at rt. The percentages of TUNEL-positive spermatozoa were determined on at least 200 cells for each sample.

2.5. 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 hours.

Cumulus–oocyte complexes (COCs) were collected by aspiration of individual follicles with a 19-gauge needle. Cumulus–oocyte complexes (total number = 1568) were matured for 22 to −24 hours in M199 supplemented with 50 μg/mL gentamycin, 1 μg/mL amphotericine B, 10% FCS, and 10 ng/mL epidermal growth factor at 39 °C, 5% CO_2 in air, and 95% humidity.

Sperm suspensions (1 mL at 2×10^6/mL) were treated for 3 hours with 10 μg/mL zinc chloride, 500 μg/mL D-Asp, and 40 μg/mL CoQ10 or with vehicle (control) in sperm TALP. At the end of treatment, sperm suspensions were diluted to 15 mL with sperm TALP and centrifuged at 170× *g* for 10 minutes, and the pellets (50 μL) were resuspended in 1 mL of IVF-TALP [7]. For fertilization, 50 *in vitro*–matured COCs in 250 μL IVF-TALP were inseminated with 250 μLof each sperm suspension (sperm final concentration, 1×10^6/mL) and added with heparin at a final concentration of 10 μg/mL. The cocktail final concentration in treated wells was: zinc chloride, 0.0166, D-Asp, 0.833, CoQ10 0.0666 μg/mL. To understand whether the residual concentration of zinc, D-Asp, and CoQ10 passively transported in the fertilization wells at insemination had an effect on embryo development, each experiment included three experimental COC groups: control, treated, and residue. In particular, one COC group was inseminated with treated and two groups with control spermatozoa. One served as vehicle control and the other, termed "residue", was supplemented with identical residual concentrations of the three molecules present in the treated COCs group (zinc chloride, 0.0166, D-Asp, 0.833, CoQ10 0.0666 μg/mL). After 18 to 20 hours of coincubation at 39 °C and 5% CO_2, the COCs were transferred in Hepes-TALP [7] and cumulus cells were removed by vortexing. Presumptive zygotes were collected, washed in synthetic oviduct fluid [8] supplemented with 5% FCS, and incubated in 700 μL of fresh SOF for 7 days at 39 °C, 5% CO_2, 5% O_2, and 90% N_2. The cleavage rates and percentages of embryos ≥8 cell (eight-cell embryos on cleaved embryos) were determined at Day 3 postinsemination (pi), whereas blastocyst rates (blastocysts on cleaved embryos) was determined at Day 8 pi. At that time, the blastocysts were fixed and labeled with TUNEL and Hoechst as described above to determine blastocyst mean cell number and percentages of TUNEL-positive cells.

2.6. Statistical analysis

TUNEL positivity in spermatozoa and blastocysts, and rates of cleavage, ≥8 cell embryos, and blastocysts were analyzed by Chi-square test. Sperm motility and kinetics were analyzed by the estimate model of ANOVA [9] followed by the Tukey's honestly significant difference test for pairwise comparisons when the overall significance was detected. Percent data were transformed into arcsine before statistical analysis.

3. Results

3.1. Sperm motility and kinetics

Experiments on sperm motility and kinetics (n = 7) indicated that zinc, D-Asp, and CoQ10 exert protective effects on bull spermatozoa. Data showed a significant decrease of total and progressive motility in control samples starting at 1 hour of incubation (Fig. 1). The decline of motility from 1 hour of incubation onward in control sperm suspensions can be ascribed to the typical behavior of frozen/thawed bull spermatozoa. In particular, initial total motility was 82.8 ± 10%, and progressive motility was 76.8 ± 12.2%. A significant decrease of both values was observed after 1 hour of incubation in control samples (total and progressive motility: 60.6 ± 14; 53.6 ± 14.5%; 0 vs. 1 hour, $P < 0.01$). Loss of total and progressive motility was significantly prevented by the treatment from 2 hours onward (Fig. 1).

Analysis of sperm kinetics (Fig. 2) showed that even though treated spermatozoa had a tendency to maintain kinetics values slightly higher compared with controls, such effects were not significant ($P > 0.05$).

3.2. Sperm DNA fragmentation

Experiments (n = 7) were addressed to evaluate the DNA fragmentation in the initial suspensions and after 1, 2, 3, 4, and 5 hours of incubation. Data (Fig 3A) showed that the percentage of spermatozoa with fragmented DNA in the control suspension significantly increased from 18.9% at 0 hour (Fig. 3B, C) to 27.2 and 34.5% at 3 and 5 hours of incubation, respectively (Fig. 3D, E) ($P < 0.01$), whereas

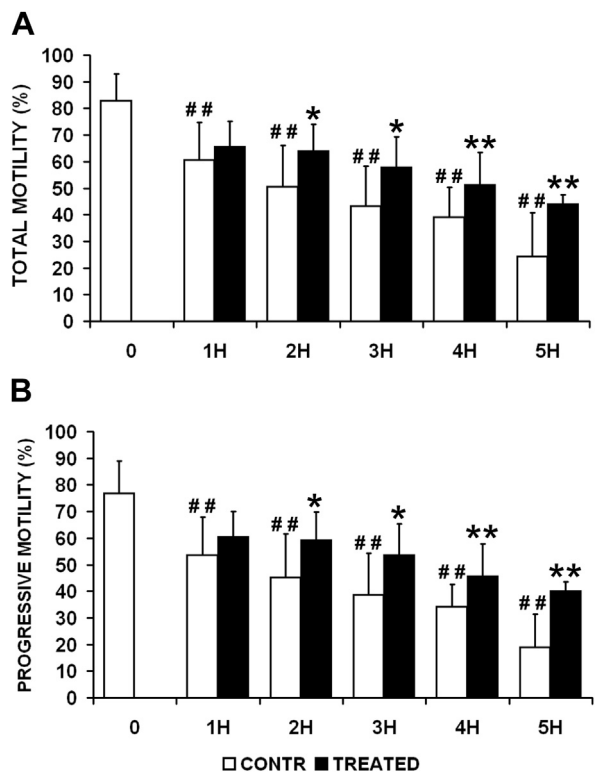

Fig. 1. Effects of zinc, D-Asp, and CoQ10 on (A) total sperm motility (B) and progressive sperm motility. ##Significant differences versus control at 0 hours ($P < 0.01$); *and **significant differences versus corresponding control (* = $P < 0.05$; ** = $P < 0.01$).

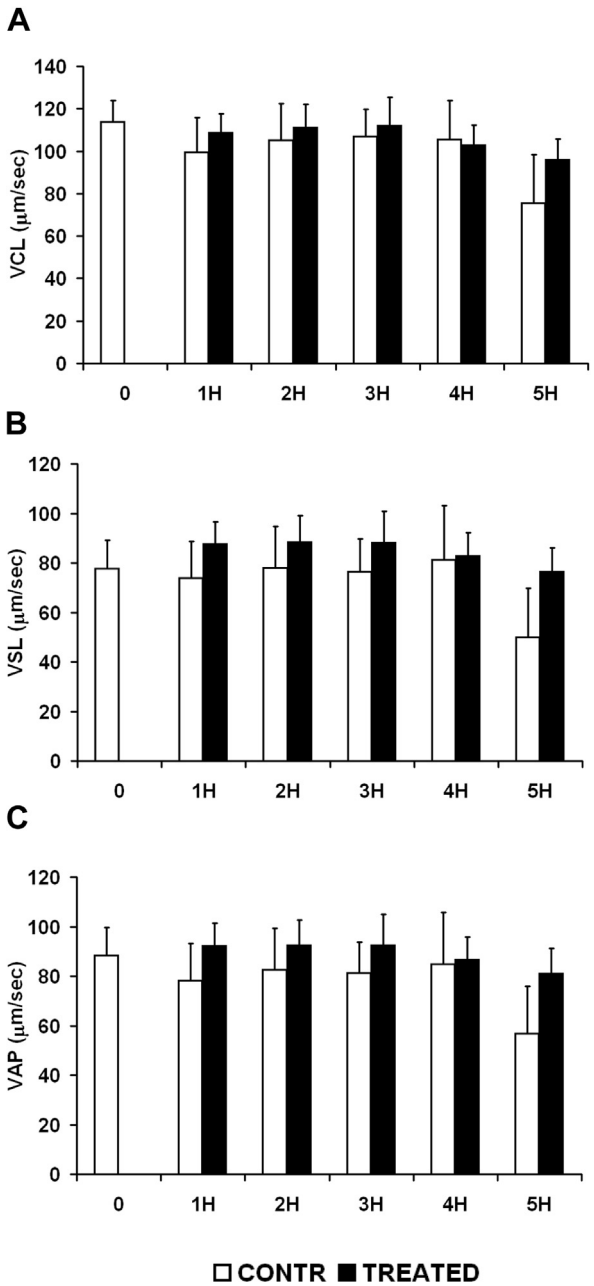

Fig. 2. Effects of zinc, D-Asp, and CoQ10 on sperm kinetics. (A) Curvilinear velocity (VCL). (B) Straight line velocity (VSL). (C) Average path velocity (VAP). Treatments versus corresponding controls, $P > 0.05$.

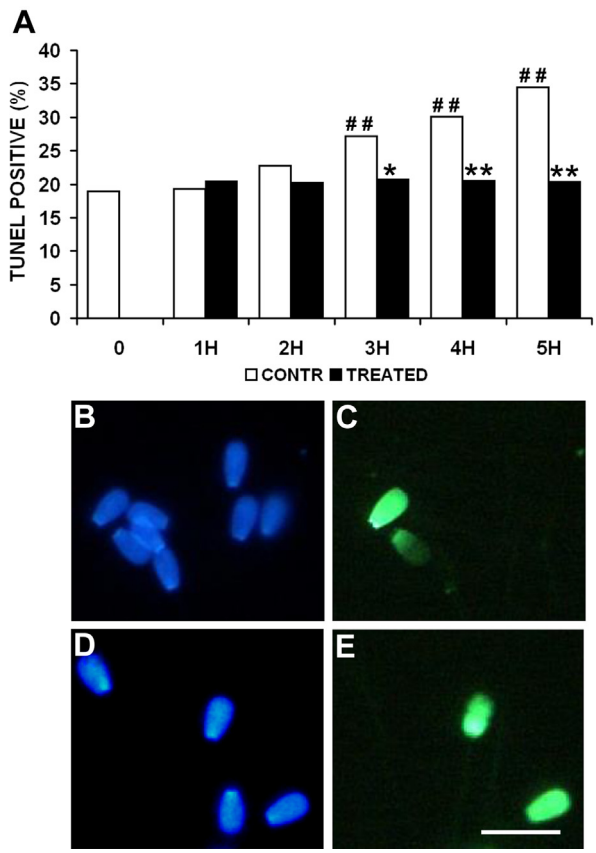

Fig. 3. Effects of zinc, D-Asp, and CoQ10 on sperm DNA fragmentation. (A) Percentages of TUNEL-positive spermatozoa in suspensions treated with zinc, D-Asp, and CoQ10 or vehicle. (B–E) Representative micrographs of spermatozoa labeled with Hoechst (B, D) and TUNEL (C, E) in the treated (B, C) and control (D, E) suspension at 5 hours. (B–E) bar, 10 μm. ##Significant differences versus control at 0 hour ($P < 0.01$). * and **Significant differences versus corresponding control (* = $P < 0.05$; ** = $P < 0.01$).

such an increase was significantly prevented by the treatment from 3 hours onward (Fig. 3A; control vs. treatment: 3 hours, $P < 0.05$; 5 hours, $P < 0.01$).

3.3. In vitro fertilization and embryo culture

In vitro fertilization experiments (n = 7) were designed to understand whether sperm treatment with zinc, D-Asp, and CoQ10 improved the sperm fertilization competence and its ability to support the preimplantation development. Data indicated that cleavage rates of oocytes inseminated with treated and control spermatozoa were not significantly different (Fig. 4, control 68.7%, treated 67.3%, residue 64.5%). In sharp contrast, the number of ≥8 cell stage embryos at Day 3 developed from oocytes inseminated with treated spermatozoa was significantly increased compared with embryos generated from control spermatozoa (Fig. 4; control 37.1%, treated 51.7%, residue 33.2%, $P < 0.01$). At Day 8 pi, the oocytes inseminated with treated spermatozoa had a markedly higher competence to develop to the blastocyst stage compared with both control and residue wells (Fig. 4, control 13.9%, treated 28.3%, residue 10.8%, $P < 0.01$).

3.4. Blastocyst mean cell number and DNA fragmentation

Blastocyst's mean cell number and percentage of nuclei with fragmented DNA are two well-recognized markers of blastocyst's developmental competence. Our data (n = 7) indicated that even though the mean cell number of blastocysts was not significantly different in control, treated, and residue wells (control, 126.4 ± 35.54, treated, 116.1 ± 15.79, residue, 110.5 ± 22.12), the

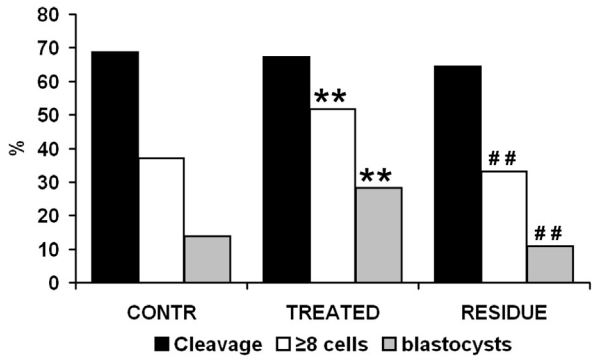

Fig. 4. Effects of sperm pretreatment with zinc, D-Asp, and CoQ10 on the rates of cleavage and eight-cell embryos at Day 3, and blastocyst rates at Day 8. ##Significant differences versus control at 0 hour ($P < 0.01$). * and **Significant differences versus corresponding control (* = $P < 0.05$; ** = $P < 0.01$).

percentages of blastomeres with fragmented DNA (Fig. 5A) was significantly reduced in treated blastocysts (Fig. 5B, C) compared with control blastocysts (Fig. 5D, E) (treated vs. control, 6.9 vs. 9.6%; $P < 0.01$). No significant difference was found between treated and residue blastocysts (6.9 vs. 7.9%).

4. Discussion

Oxidative stress has a clearly recognized role in male infertility [10], and several studies have been focused on the role of antioxidant therapy on semen quality and reproductive outcome [4,5]. Although antioxidant therapy may exert positive effects on semen quality, there is still a lack of conclusive evidences that this therapy leads to increased pregnancy rates [11]. Another significant source of sperm oxidative stress arises from sperm handling and cryopreservation procedures during assisted reproduction [12,13]. The effects of antioxidants on human spermatozoa have also been studied *in vitro*, and positive data were reported on the protection against loss of motility, increased lipid peroxidation, and DNA fragmentation [6; reviewed in 11]. We recently demonstrated that the antioxidants zinc, CoQ10, and the micronutrient D-Asp, contained in the dietary supplement Genadis (Merck Serono), have protective effects on human sperm motility, DNA fragmentation, and lipid peroxidation [6]. However, it was not possible to directly test whether spermatozoa treated *in vitro* had a higher competence in fertilization or in supporting embryo development in human samples.

In the present study, experiments aimed to understand the effects of treatment on sperm motility and kinetics demonstrated that total and progressive motility of frozen/thawed bull spermatozoa significantly decrease starting at 1 hour of incubation under control conditions. Such a decrease was significantly prevented from 2 hours onward in parallel treated sperm aliquots. Overall, the data demonstrated that zinc, D-Asp, and CoQ10, at the concentrations previously found to exert protective effects on human spermatozoa, were also effective on bull spermatozoa. Furthermore, findings suggested that frozen/thawed bull spermatozoa undergo an oxidative stress during

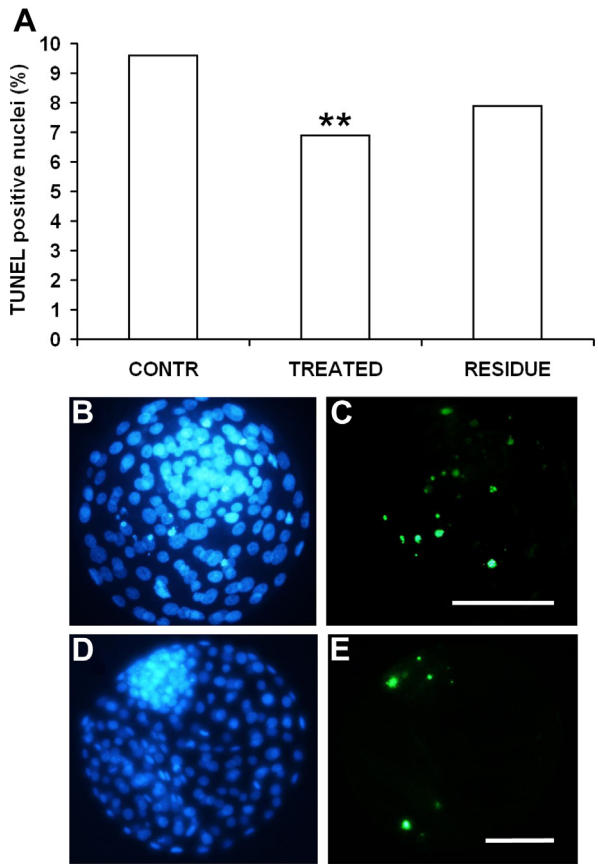

Fig. 5. Effects of sperm pretreatment with zinc, D-Asp, and CoQ10 on blastocyst's DNA fragmentation. (A) Percentages of TUNEL-positive blastomeres in blastocysts developed from oocytes fertilized with control or treated spermatozoa under control, treated, or residue condition. (B–E) Representative micrographs of blastocysts labeled with Hoechst (B, D) and TUNEL (C, E). (B–E) bar 100 μm. ##Significant differences versus control at 0 hour ($P < 0.01$). * and **Significant differences versus corresponding control (* = $P < 0.05$; ** = $P < 0.01$).

incubation *in vitro*. Because, no exogenous oxidants were used in this study, results are in agreement with other reports showing that both sperm cryopreservation and *in vitro* handling can be a significant source of oxidative stress [12,13]. This can be accounted to several factors like the removal of seminal plasma containing high concentrations of ROS scavengers through sperm washing, the incubation at relatively high temperatures compared with the scrotal temperatures, or the freezing and thawing process itself. Moreover, the treatment also protected bull spermatozoa against DNA fragmentation. These data are in agreement with the generally accepted view that the primary cause of post testicular sperm chromatin damage is ROS-induced oxidative stress [14].

In vitro fertilization experiments demonstrated that even though the sperm fertilization competence was not affected by the treatment, the rates of ≥8 cell embryos at Day 3 were significantly increased. Discordant findings were reported in studies that evaluated the influence of semen endogenous oxidative stress [15], treatment with prooxidants [16], antioxidants [17], and X- or gamma-ray irradiation [18], on bull sperm chromatin integrity and

IVF outcome. In fact, increasing sperm oxidative stress or decreasing antioxidant concentrations were associated with a reduction in cleavage rates [15,17], whereas, in agreement with the present findings, increasing doses of gamma irradiation on spermatozoa had no effects on cleavage rates [18]. Such a discordance could be due to a different extent of sperm damage and/or the heterogeneity of antioxidants tested.

Early cleavage and the developmental rate at which an embryo reaches the 8-cell stage are important measurements for future development and successful outcomes [19]. In particular, in cattle, a paternal effect on developmental kinetics and onset of the first S-phase has been reported [20–22], whereas in the mouse, sperm chromatin fragmentation causes a delay of both paternal DNA replication in the zygote and embryonic development [23]. Herein, the high and significant difference in the rate of 8-cell stage embryos between oocytes inseminated with treated or control spermatozoa can be explained by a delayed or arrested cleavage caused by the paternal DNA repair in the zygote.

Under our experimental conditions, the improved ability of treated spermatozoa to promote the development of 8-cell embryos at Day 3 was associated to an increased blastocyst rate at Day 8. This finding demonstrates that treatment with antioxidants improves the sperm ability to support embryo development. Data are in agreement with the observation that early cleaving human and bovine zygotes are more likely to reach the blastocyst stage [24–27]. On the basis of these findings, it can be speculated that similar paternal effects reported on blastocyst yield [28,29] could depend on a different oxidative stress condition among bulls used for *in vitro* embryo production.

It is well known that the quality of a blastocyst affects its ability to implant and develop to term. All mammalian species show the highest level of spontaneous apoptotic processes at the blastocyst stage. Within certain levels, apoptosis in the blastocyst is an important feature of the preimplantation period with a protective function as it is thought to be fundamental for the removal of genetically abnormal and mutated blastomeres [30]. Compared with their *in vivo* counterparts, bovine *in vitro*–produced blastocysts have an higher rate of TUNEL-positive cells ranging from 4% to 9% [30]. Our findings, which were recently reported in a preliminary form [31], demonstrated that there is a relationship between oxidative damage in the spermatozoon and apoptosis in the blastocyst. Moreover, these data are in agreement with a recent study on the influence of sperm susceptibility to oxidative stress on blastocyst DNA fragmentation and *in vitro* embryo production [15].

Overall, our data could be interpreted according to the following scenario. Oxidative stress can damage sperm DNA at different extents within an ejaculate and this could gradually affect the development of embryos. Spermatozoa with intact DNA are able to support the development of good quality blastocysts. A low level of sperm DNA damage might be repaired in the zygote delaying cleavage and promoting the development of blastocysts with a higher number of apoptotic cells. Finally, spermatozoa with a higher DNA damage would still be able to promote fertilization and cleavage, but the latter could be arrested before reaching the blastocyst stage. Of course, the most dangerous condition would be a sperm DNA damage compatible with embryo implantation and development to term, but that could adversely affect the health of the offspring [reviewed in 3].

In conclusion, our findings emphasize that it is mandatory to avoid the oxidative stress associated to sperm cryopreservation and handling in assisted reproduction. The supplementation of sperm media with an appropriate cocktail of antioxidants could improve the developmental competence of embryos produced *in vitro* and their ability to implant and give rise to healthy newborns.

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