PROTECTIVE EFFECTS OF \( \alpha \)-TOCOPHEROL ON THE ACTIVITY AND ANTIOXIDANT PROFILE OF BOVINE SPERMATOZOA SUBJECTED TO FERROUS ASCORBATE-INDUCED OXIDATIVE STRESS

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Abstract


As spermatozoa are highly vulnerable to oxidative stress development, \textit{in vitro} antioxidants offer an additional line of defense to the male reproductive system against oxidative insults. \( \alpha \)-tocopherol (\( \alpha \)-TOC) is the most abundant form of vitamin E identified in the seminal plasma and spermatozoa membranes, being able to terminate numerous oxidative chain reactions causing substantial damage to biomolecules vital for sperm survival. This study was designed to shed more light on the \textit{in vitro} effects of \( \alpha \)-tocopherol with respect to the vitality and intracellular antioxidant profile of bovine spermatozoa subjected to ferrous ascorbate-induced oxidative stress. Spermatozoa were washed out from 50 bovine ejaculates, suspended in 2.9% sodium citrate and subjected to \( \alpha \)-TOC treatment (10, 50, 100 and 500 \( \mu \)mol/L) in the presence or absence of ferrous ascorbate (FeAA; 150 \( \mu \)mol/L \( \text{FeSO}_4 \) and 750 \( \mu \)mol/L ascorbic acid) during a 6h \textit{in vitro} culture. Spermatozoa motion parameters were assessed using the SpermVision™ computer-aided sperm analysis (CASA) system. Cell viability was examined with the metabolic activity (MTT) assay, and the nitroblue-tetrazolium (NBT) test was applied to quantify the intracellular superoxide formation. Cell lysates were prepared at the end of the experiments in order to assess the intracellular activity of superoxide dismutase (SOD), catalase (CAT), as well as glutathione (GSH) and malondialdehyde (MDA) concentrations. Treatment with FeAA reduced both spermatozoa motility parameters (\( P < 0.001 \)) as well as viability (\( P < 0.05 \) with respect to Time 0h; \( P < 0.01 \) in case of Time 2h and \( P < 0.001 \) in relation to Time 6h), decreased the antioxidant parameters of the samples (\( P < 0.001 \) in case of SOD; \( P < 0.01 \) with respect to CAT and GSH) but increased the superoxide production (\( P < 0.01 \) in case of Time 0h and \( P < 0.001 \) with respect to Times 2h and 6h) and lipid peroxidation (\( P < 0.001 \)). \( \alpha \)-TOC administration resulted in a preservation of the spermatozoa motility characteristics (\( P < 0.001 \) with respect to 500 \( \mu \)mol/L \( \alpha \)-TOC), viability (\( P < 0.001 \) in case of 500 \( \mu \)mol/L \( \alpha \)-TOC and \( P < 0.05 \) with respect to 100 \( \mu \)mol/L \( \alpha \)-TOC) and antioxidant profile (\( P < 0.01 \) related to the impact of 500 \( \mu \)mol/L \( \alpha \)-TOC on the SOD activity; \( P < 0.05 \) in relation to CAT; \( P < 0.01 \) with respect GSH; 100-500 \( \mu \)mol/L \( \alpha \)-TOC), with 500 \( \mu \)mol/L \( \alpha \)-TOC being the most effective. Our results suggest that \( \alpha \)-tocopherol possesses significant antioxidant properties that may prevent the deleterious effects caused by free radicals to spermatozoa, and extend the fertilization potential of male reproductive cells.

Keywords: \( \alpha \)-tocopherol, spermatozoa, oxidative stress, ferrous ascorbate, antioxidants, lipid peroxidation

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INTRODUCTION

A diversity of in vivo and in vitro studies have emphasized on a significant correlation between reactive oxygen species (ROS) overgeneration and male subfertility (Youset et al., 2005; Lavranos et al., 2012; Tvrda et al., 2012; 2013; Agarwal et al., 2014 a, b), and it is now widely accepted that oxidative stress (OS) plays a significant role in sperm structural or functional abnormalities, including DNA fragmentation, lipid peroxidation (LPO), enzymatic inactivation and apoptosis (Aitken et al., 2010; Aitken and De Iuliis, 2010). Oxidative damage to the spermatozoon is in turn associated with the loss of motility, alterations of spermatogenesis, acrosome reaction and sperm–oocyte fusion (Cocuzza et al., 2007), which may lead to poor fertilization rates or impaired fetal development (Baker and Aitken, 2005; Lewis and Aitken, 2005).

Spermatozoa are exceptionally vulnerable to oxidative insults hence ex vivo antioxidants represent an additional line of defense to the male reproductive system against possible ROS sources, directly scavenge ROS, prevent oxidative damage to vital biomolecules, reduce cellular cryodamage or block premature sperm maturation (Agarwal et al., 2007; Gharghoslooz and Aitken, 2011). With the early observation that semen parameters could be improved using hydrophilic or lipophilic antioxidants, significant attention has been devoted to study their impact on male reproduction in health or disease.

Vitamin E (VIT E) is a term that encompasses a group of powerful lipid-soluble tocol (tocopherol) and tocotrienol derivatives qualitatively exhibiting the antioxidant activity is the flagship biological characteristic related to this lipophilic biomolecule (Wen and Geffen, 2006).

Vitamin E may be found in the seminal plasma and sperm plasma membranes (Agarwal et al., 2003). It is a chain-breaking antioxidant, able to terminate numerous oxidative chain reactions, particularly polyunsaturated fatty acid (PUFA) peroxidation, detrimental to spermatozoa survival during OS due to high PUFA concentrations in their membranes (Bolle et al., 2002).

A multitude of studies support the role for α-TOC in the pathogenesis of male infertility. α-TOC extracted from spermatozoa membranes was highly associated with the percentage of motile, living and morphologically normal sperm (Bolle et al., 2002). Furthermore, α-TOC levels were found to be significantly decreased in oligo- and azoospermic subjects when compared to normospermic controls (Bhardwaj et al., 2000). VIT E supplementation to males with seminal OS has led to a significant improvement in the in vitro ability of spermatozoa to bind to the zona pellucida of unfertilized oocytes (Wen and Geffen, 2006). At the same time, in vivo toxicology studies have suggested that VIT E may be efficient in alleviating testicular and epididymal damage as well as disruption of spermatogenesis caused by environmental pollutants or chemotherapeutics (Alam et al., 2012; Oda and El-Maddawy, 2012). Moreover, in vitro experiments have revealed favorable effects of α-TOC on the cryosurvival of human and animal sperm (Anghel et al., 2010; Pour et al., 2013), as well as its protective characteristics on male germ cells subjected to conditions with increased ROS-production (Bansal and Bilaspuri, 2009; Azawi et al., 2013). This preliminary body of evidence emphasizes on a significant potential of α-TOC in providing protection to male reproductive tissues and cells.

Therefore, the aim of this study was to shed more light on the in vitro antioxidant mechanisms of action of α-tocopherol against oxidative stress induced in bovine spermatozoa by exposure to ferrous ascorbate.

MATERIALS AND METHODS

Bovine ejaculates used for the following experiments were obtained in quintuplicates from 10 adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovak Republic) on a regular collection schedule using an artificial vagina. After collection, the samples were stored in the laboratory at room temperature (22–25 °C).

For the in vitro treatment we followed the protocol established by Bansal and Bilaspuri (2008; 2009). Fresh semen was centrifuged (800 × g) at 25 °C for 5 min, seminal plasma was removed, the sperm pellet was washed two times with 2.9 % sodium citrate (SC; pH 7.4; Centralchem, Bratislava, Slovak Republic), re-suspended in 2.9 % SC using a ratio of 1:20. To one tube (group A; Control 1; SC Control) were subjected to α-tocopherol ((±)-α-Tocopherol; Sigma-Aldrich; St. Louis, USA) and 750 μmol/L ascorbic acid (Centralchem), diluted in 2.9 % SC. The remaining six tubes (experimental portions) were subjected to α-tocopherol ((±)-α-Tocopherol; Sigma-Aldrich) treatment in the presence or absence of FeAA (see Tab. I). Specific α-TOC concentrations were chosen upon the data obtained from a previous standardization study on bovine spermatozoa (Tvrda et al., 2015). All sperm suspensions were incubated at 37 °C. At cultivation times of 0h, 2h and 6h, spermatozoa motility parameters, cell viability and intracellular superoxide production were assessed in each sample.

Furthermore at 6h each sample was centrifuged at 800 × g at 25 °C for 10 min. The culture media
were removed and the resulting pellet was sonicated at 28 kHz for 30 sec on ice using RIPA buffer (Sigma-Aldrich) with protease inhibitor cocktail suitable for mammalian cell and tissue extracts (Sigma-Aldrich). Subsequently the samples were centrifuged at 11,828 × g, 4 °C for 15 min to purify the lysates from residual cell debris. The resulting supernatants involving the intracellular sperm content were stored at −80 °C for further assessment of the antioxidant profile.

Spermatozoa motion characteristics were assessed with the help of the Computer-aided sperm analysis (CASA) system using the SperrVision™ program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 μm, 37 °C; Scif Medical Instruments, Haifa, Israel) and at least 1000 cells were evaluated in each sample for the following features:

- Motility (MOT): percentage of motile spermatozoa (motility > 5 μm/s; %).
- Progressive motility (PROG): percentage of progressive motile spermatozoa (motility > 20 μm/s; %) (Tvrdá et al., 2015).

Cell viability was evaluated using the metabolic activity (MTT) assay. The MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, USA) was dissolved in PBS (Dulbecco's Phosphate Buffer Saline, Sigma-Aldrich) at 5 mg/mL. 10 μL of the solution was added to the cell culture. After 2h of incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO2), the cells and the formazan crystals were dissolved in 150 μL of acidified (0.08 M HCl; Centralchem) isopropanol. The optical density was determined at a wavelength of 570 nm against 620 nm as reference using a microplate ELISA reader (Anthos Multi Read 400, Austria). The data were expressed as percentage of the Control 1 (group A) set to 100 % (Knazicka et al., 2012).

The nitroblue-tetrazolium (NBT) test was used to study the intracellular formation of the superoxide radical, by counting the cells containing blue NBT formazan deposits, which originate due to a reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2'-bis(4-Nitrophenyl)-5,5'-diphenyl-3,3',5,5'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride; Sigma-Aldrich) by the superoxide radical. The NBT salt was dissolved in PBS (Dulbecco's Phosphate Buffer Saline, Sigma-Aldrich) containing 1.5 % DMSO (Dimethyl Sulfoxide, Sigma-Aldrich) to a final concentration of 1 mg/mL and added to the cells. After 1h of incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO2), the cells were washed twice with PBS [Phosphate Buffer Saline, Sigma-Aldrich, St. Louis, USA] and centrifuged at 300 × g for 10 min. Lastly, the cells and formazan crystals were dissolved in 2M KOH (Potassium Hydroxide; Centralchem) in DMSO. The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos Multi Read 400, Austria). The data were expressed in percentage of the Control 1 (group A) set to 100 % (Tvrdá et al., 2015).

Superoxide dismutase (SOD) activity was analyzed using the Randox RANSOD commercial kit (Randox Laboratories, Crumlin, Great Britain) employing xanthine and xanthine oxidase (XO) to generate superoxide radicals, which will react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity was then measured by the inhibition degree of the reaction at 505 nm using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). SOD activity is expressed as U/mg protein.

Catalase (CAT) activity was assessed by monitoring the decrease of hydrogen peroxide (H2O2) at 240 nm. The calculation was based on the rate of H2O2 decomposition, proportional to the reduction of the absorbance during 1 min measured with

### Table 1: Concentrations of α-tocopherol (α-TOC) used in the experiments

<table>
<thead>
<tr>
<th>Groups</th>
<th>α-TOC concentration [μmol/L]</th>
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<tbody>
<tr>
<td>1. Groups untreated with FeAA</td>
<td></td>
</tr>
<tr>
<td>A (Control 1; SC control)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>500</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>50</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
</tr>
<tr>
<td>2. Groups treated with FeAA</td>
<td></td>
</tr>
<tr>
<td>F (Control 2; FeAA control)</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>500</td>
</tr>
<tr>
<td>H</td>
<td>100</td>
</tr>
<tr>
<td>I</td>
<td>50</td>
</tr>
<tr>
<td>J</td>
<td>10</td>
</tr>
</tbody>
</table>

SC – sodium citrate; FeAA – ferrous ascorbate.
the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.). The values are expressed as U/mg protein.

Reduced glutathione (GSH) was determined by treating the sample with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid); Ellman's reagent; Sigma-Aldrich) which interacts with the thiol groups of GSH, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB-) and creating the NTB₂ dianion in water at neutral and alkaline pH. This ion has a yellow color and was quantified at 412 nm using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.). GSH concentration is expressed as mg/g protein.

Lipid peroxidation (LPO) expressed through malondialdehyde (MDA) concentration was assessed with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. Each sample was treated with 5 % sodium dodecyl sulfate, and subjected to 0.53 % thiobarbituric acid (TBA; Sigma-Aldrich) dissolved in 20 % acetic acid adjusted with NaOH (Centralchem) to pH 3.5, and subsequently boiled at 90–100 °C for 1 h. After boiling, the samples were placed on ice for 10 min and centrifuged at 1750 × g for 10 min. Supernatant was used to measure the end-product resulting from the reaction of MDA and TBA under high temperature and acidic conditions at 330–540 nm with the help of the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.) (Tvdá et al., 2013).

Protein concentration was assessed using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck®, Darmstadt, Germany). The measurement is based on the Biuret method, according to which copper sulfate react with proteins to form a violet blue color complex in alkaline solution, and the intensity of the color is directly proportional to the protein concentration when measured at 540 nm.

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA was used for specific statistical evaluations. Dunnett’s test was used as a follow up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at ** (P < 0.01); * (P < 0.05).

The comparative analysis was performed as follows:

- **Experimental groups subjected to FeAA treatment (groups G-J) were compared to the Control 1 (group A; SC Control) as well as to the Control 2 (group F; FeAA Control).**

### RESULTS AND DISCUSSION

Ferrous ascorbate was used to induce oxidative stress in order to examine the subsequent cellular damage to bovine spermatozoa. The CASA assessment showed a significant (P < 0.001) decrease of both motion parameters at 0h, 2h as well as 6h of the in vitro incubation caused as a consequence of FeAA supplementation (Tab. II and III). Meanwhile, α-TOC administration to FeAA-untreated samples led to significantly higher sperm motility parameters, particularly in the case of 100 and 500 μmol/L α-TOC (P < 0.001). Similarly, α-TOC supplementation to the FeAA groups has led to a significant improvement of the spermatozoa motility parameters (P < 0.001 with respect to 100 and 500 mol/L α-TOC and P < 0.05 in case of 50 mol/L α-TOC at Time 2h; P < 0.001 in relation to 100-500 μmol/L α-TOC, P < 0.01 with respect to 50 μmol/L α-TOC and P < 0.05 in case of 10 μmol/L α-TOC at Time 6h) when compared to the F group (Control 2), although none of the α-TOC concentrations was able to completely reverse the negative impact of FeAA on the spermatozoa motion characteristics (Tab. II and III).

In vivo α-tocopherol administration has been proven to significantly affect spermatogenesis, semen quality as well as quantity in boars (Brezezińska-Slebodzińska et al., 1997), mice (Hussein et al., 2009), rams (Yue et al., 2010), rabbits (Najjar et al., 2013) and roosters (Cerolini et al., 2006). All above listed studies agree that experimental feed or water enriched with VIT E may improve spermatozoa concentration, motility, vitality or morphology. Furthermore Mohammadi et al. (2013) showed that vitamin E administered intraperitoneally increased the expression of CatSper1 and CatSper 2, channel-like proteins, which are expressed exclusively in the testis and play a crucial role in sperm motility and male fertility.

Traditionally, VIT E is called an anti-sterility vitamin (Pant et al., 2001), associated with normal functions of the male reproductive system (Momeni and Eskandiar, 2012). Marin-Guzman et al. (1997) reported that VIT E deficiency in boars adversely affected sperm motility compared to control animals given supplemental vitamin E. On the other hand, a double-blind randomized placebo crossover controlled trial executed by Kessopoulou et al. (1995) showed no significant effect on the conventional semen parameters after a 3-month treatment with VIT E alone. A different study emphasizes that antioxidant treatment combining high doses of vitamin C and E for an 8-week period does not improve any conventional semen parameter or a 24h
II: Spermatozoa motility [%] affected by four doses of α-tocopherol (α-TOC), untreated or treated with ferrous ascorbate (FeAA)

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Groups untreated with FeAA</th>
<th>Groups treated with FeAA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>MOT 0h [%]</td>
<td></td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>84.89±0.91</td>
<td>90.90±1.02</td>
</tr>
<tr>
<td>MOT 2h [%]</td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>61.24±2.64</td>
<td>78.83±3.71</td>
</tr>
<tr>
<td>MOT 6h [%]</td>
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<tr>
<td>X±S.E.</td>
<td>42.34±2.12</td>
<td>58.14±2.94</td>
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</tbody>
</table>

III: Spermatozoa progressive motility [%] affected by four doses of α-tocopherol (α-TOC), untreated or treated with ferrous ascorbate (FeAA)

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Groups untreated with FeAA</th>
<th>Groups treated with FeAA</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>PROG 0h [%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±S.E.</td>
<td>66.51±2.01</td>
<td>75.12±1.36</td>
</tr>
<tr>
<td>PROG 2h [%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X±S.E.</td>
<td>37.28±3.96</td>
<td>49.74±3.63</td>
</tr>
<tr>
<td>PROG 6h [%]</td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>33.10±3.07</td>
<td>44.16±2.38</td>
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IV: Markers of oxidative balance in bovine spermatozoa affected by four doses of α-tocopherol (α-TOC), untreated or treated with ferrous ascorbate (FeAA)

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Groups untreated with FeAA</th>
<th>Groups treated with FeAA</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>SOD [U/mg prot]</td>
<td></td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>0.32±0.04</td>
<td>0.36±0.02</td>
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<tr>
<td>CAT [U/mg prot]</td>
<td></td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>8.72±1.44</td>
<td>10.97±1.90</td>
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<tr>
<td>GSH [mg/g prot]</td>
<td></td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>7.57±1.18</td>
<td>8.38±1.61</td>
</tr>
<tr>
<td>MDA [μmol/g prot]</td>
<td></td>
<td></td>
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<tr>
<td>X±S.E.</td>
<td>3.27±0.17</td>
<td>2.25±0.13</td>
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X – Mean; S.E. – Standard Error; * - P < 0.05; ** – P < 0.01; *** – P < 0.001; ^ – vs. Control 1 (group A); † – vs. Control 2 (group F); A – 0 μmol/L α-TOC/SC (Control 1); B – 500 μmol/L α-TOC/SC; C – 100 μmol/L α-TOC/SC; D – 50 μmol/L α-TOC/SC; E – 10 μmol/L α-TOC/SC; F – 0 μmol/L α-TOC/FeAA (Control 2); G – 500 μmol/L α-TOC/FeAA; H – 100 μmol/L α-TOC/FeAA; I – 50 μmol/L-TOC/FeAA; J – 10 μmol/L α-TOC/FeAA.
sperm survival rate in patients with asthenospermia or moderate oligospermia (Rolf et al., 1999).

Compatible to the alterations of the sperm motility and progressive motility, a decrease of spermatozoa viability was recorded after FeAA administration, with significant differences at all three time periods of analysis (P < 0.05 with respect to Time 0h; P < 0.01 in case of Time 2h and P < 0.001 in relation to Time 6h; Fig. 1).

Inversely, administration of particularly higher α-TOC concentrations was able to promote the mitochondrial viability of spermatozoa not exposed to FeAA treatment (100-500 μmol/L α-TOC; P < 0.01 with respect to time 2h; P < 0.05 in case of Time 6h), while the same concentrations had the ability to at least partially prevent the decrease of mitochondrial activity in the experimental groups subjected to FeAA supplementation (P < 0.001 with respect to 100–500 μmol/L α-TOC at Time 6h; Fig. 1).

An increase in the mitochondrial activity of spermatozoa in the presence of α-TOC may be explained by previous suggestions from rabbit studies (Yousef, 2005) concluding that vitamin E prevents oxidative insults to the sperm DNA and membranes, thereby boosting the sperm metabolism to overcome oxidative insults. Thus, by maintaining the cellular integrity and mitochondrial activity of spermatozoa, in vitro α-TOC administration may improve the sperm vitality intimately connected to the motion performance.

The decrease of spermatozoa motility and viability after FeAA treatment was accompanied by an increase in the superoxide production, quantified by the NBT assay. Compared to the FeAA-absent Control 1, the superoxide overproduction became significant practically the moment FeAA was added to the spermatozoa culture medium (P < 0.01; Time 0h) and the superoxide production increased further over the time of in vitro incubation (P < 0.001; Fig. 2).

In the meantime, the highest α-TOC concentration was able to significantly decrease the superoxide production probably occurring as a result of a routine in vitro spermatozoa cultivation, a phenomenon we noted at 2h (P < 0.05) as well as at 6h (P < 0.01) of the experiment. On the other hand, when quantifying the antioxidant potential of α-TOC after FeAA supplementation, only the highest concentrations (100–500 μmol/L α-TOC) were able to substantially reduce the superoxide production, with significant differences at 2h (P < 0.05 in case of 500 μmol/L α-TOC) and 6h (P < 0.01 with respect to 500 μmol/L α-TOC and P < 0.001 in case of 100 μmol/L α-TOC; Fig. 2).

α-TOC supplementation has led to a reduction of the intracellular superoxide production under normal as well as oxidative conditions. This observation may be linked to the ROS-quenching capacity of α-TOC within spermatozoa membranes and organelles by disrupting oxidative chain reactions. Thus α-tocopherol may increase the antioxidant protection of male reproductive cells against superoxide generation, which is considered to be the first step in oxidative damage to a biological system. At the same time, our results suggest that α-TOC may directly quench different ROS generated during the in vitro induced OS. By exhibiting its scavenging activities, α-TOC may break down diverse ongoing oxidative reactions, creating relatively stable complexes such as the tocopheroxyl radical. Similar conclusions have been drawn in studies examining bull (Tvrda et al., 2015) and boar (Brezeziska-Slebodzińska et al., 1995) male fertility.

The assessment of the antioxidant profile revealed that FeAA treatment led to a significant decrease of all antioxidant markers (P < 0.001 in case of SOD; P < 0.01 with respect to CAT and GSH; Tab. IV). Inversely, a significant increase of MDA (P < 0.001) as a byproduct of LPO was detected after FeAA administration using the TBARS assay (Tab. IV). Although no significant differences were recorded with respect to the effect of α-TOC on any enzymatic or non-enzymatic under physiological in vitro conditions, its antioxidant effects became notable in the FeAA-treated experimental groups. 100 and 500 μmol/L α-TOC significantly improved the activity of both SOD (P < 0.01 and P < 0.05 respectively) and CAT (P < 0.05). Moreover, α-TOC concentrations as low as 50 μmol/L were able to significantly restore the GSH concentration when compared to the F group (P < 0.01 with respect to 100–500 μmol/L α-TOC and P < 0.05 in relation to 50 μmol/L α-TOC, Tab. IV). A typical α-TOC property as a LPO-preventing and membrane protecting agent was confirmed by the TBARS data showing that all α-TOC concentration were able to significantly reduce the MDA production accelerated by FeAA (P < 0.001). Up to a 3-fold decrease of MDA was recorded in the case of 100 and 500 μmol/L α-TOC (Tab. IV).

Data presented in Table IV suggest that α-TOC supplementation to sperm culture media could reduce oxidative damage in the intracellular milieu of spermatozoa caused by lipid peroxidation. Under normal circumstances, crucial sperm structures are a primary target of ROS due to high PUFA concentrations (Doran and Halestrap, 2000). α-TOC may play a major protective role against oxidative stress by preventing the production of lipid peroxides and by scavenging ROS in biological membranes, as shown by our TBARS analysis and in accordance with reports by Takanami et al. (2000) and Sen Gupta et al. (2004).

In addition, our results agree with Thuwanuta et al. (2011) studying ram semen cooled gradually and preserved at 5 °C for five days, and Brezezińska-Slebodzińska et al. (1995) examining boar semen subjected to FeAA treatment.

Moreover, α-TOC has shown to modulate the activities of antioxidant enzymes, which are known to act as primary defense against oxidative damage to the spermatozoa structural integrity and functional activity. Subudhi et al. (2008) showed that VIT E is efficient in protecting mitochondria from
Viability of bovine spermatozoa affected by four doses of α-tocopherol (α-TOC), untreated or co-treated with ferrous ascorbate (FeAA). Each bar represents mean (±SEM) optical density as the percentage of the Control 1 (group A; SC control), which was set to 100% and the data are expressed as a % of the Control 1 value. The data were obtained from five independent experiments. The level of significance was set at * - P < 0.05; ** - P < 0.01; *** - P < 0.001. A – vs. Control 1 (group A); F – vs. Control 2 (group F). A – 0 µmol/L α-TOC/SC (Control 1); B – 500 µmol/L α-TOC/SC; C – 100 µmol/L α-TOC/SC; D – 50 µmol/L α-TOC/SC; E – 10 µmol/L α-TOC/SC; F – 0 µmol/L α-TOC/FeAA (Control 2); G – 500 µmol/L α-TOC/FeAA; H – 100 µmol/L α-TOC/FeAA; I – 50 µmol/L α-TOC/FeAA; J – 10 µmol/L α-TOC/FeAA.
Intracellular superoxide production of bovine spermatozoa affected by four doses of $\alpha$-tocopherol ($\alpha$-TOC), untreated or co-treated with ferrous ascorbate (FeAA).

Each bar represents mean (±SEM) optical density as the percentage of the Control 1 (group A; SC control), which was set to 100% and the data are expressed as a % of the Control 1 value. The data were obtained from five independent experiments. The level of significance was set at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ A – vs. Control 1 (group A); F – vs. Control 2 (group F). A – 0 µmol/L $\alpha$-TOC/SC (Control 1); B – 500 µmol/L $\alpha$-TOC/SC; C – 100 µmol/L $\alpha$-TOC/SC; D – 50 µmol/L $\alpha$-TOC/SC; E – 10 µmol/L $\alpha$-TOC/SC; F – 0 µmol/L $\alpha$-TOC/FeAA (Control 2); G – 500 µmol/L $\alpha$-TOC/FeAA; H – 100 µmol/L $\alpha$-TOC/FeAA; I – 50 µmol/L $\alpha$-TOC/FeAA; J – 10 µmol/L $\alpha$-TOC/FeAA.
oxidative stress generated by l-thyroxine. However, Sahoo et al. (2008) argue that VIT E significantly increased the SOD activity, as seen in our study, but decreased the mitochondrial GSH-PX activity in the testis of T4-treated rats. These results suggest that the mechanisms of action of VIT E on oxidative damage to the testicular mitochondria may be very complex and further research needs to be done. Meanwhile, co-administration of α-tocopherol and FeAA in our experiments has led to a dose-dependent increase in the SOD and CAT activities, compatible to the observations reported by Sönmez et al. (2007) studying homocysteine-treated rats.

Glutathione and glutathione-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as ROS. In this study α-TOC supplementation led to a significant improvement of GSH availability, most likely due to its antioxidant protection provided to the sulfhydryl groups abundant in the GSH molecule, which are responsible for ROS quenching, while at the same time exhibiting a particular vulnerability to oxidative overload (Oda and El-Maddawy, 2012).

Finally, our results indicate that α-TOC improves the percentage of motile and viable spermatozoa under in vitro conditions hence we may suggest that this biomolecule is effective in preventing the rapid loss of motility that normally occurs during an in vitro sperm culture, and furthermore stabilizes the metabolic and motion performance under oxidative stress conditions.

CONCLUSION

In conclusion, the present study provides more specific evidence that α-tocopherol is able to significantly prevent the decline of spermatozoa vitality, functional activity and antioxidant capacity as a consequence of FeAA-associated ROS overproduction. α-tocopherol concentrations of 100–500 μmol/L were particularly effective in protecting spermatozoa from damage caused by oxidative in vitro conditions through protection against lipid peroxidation as well as stabilization of enzymatic antioxidants in the reproductive cell. Accordingly, α-tocopherol administration may be a suitable strategy for a therapeutic intervention in order to preserve sperm activity and to prevent oxidative damage and its deleterious effects on the sperm architecture or behavior.

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