REGULAR ARTICLE

STIMULATING AND PROTECTIVE EFFECTS OF VITAMIN E ON BOVINE SPERMATOZOA

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ABSTRACT

As spermatozoa are particularly sensitive towards oxidative damage, the search for a potential antioxidant substance to preserve and protect them under in vitro conditions has recently attracted the attention of the scientific community. The aim of this study was to assess the dose- and time-dependent in vitro effects of vitamin E on bovine spermatozoa during three different time periods (Time 0 h, 12 h and 24 h). Semen samples were collected from 15 adult breeding bulls, and diluted in physiological saline solution containing 0.5% ethanol (96%) together with 0, 10, 50, 100, 500 and 1000 µM of vitamin E. Spermatozoa motility was determined using the Sperm Vision™ and CASA (Computer Assisted Semen Analyzer) system. Cell viability was measured using the metabolic activity MTT assay, the nitroblue-tetrazolium (NBT) test was used to assess the intracellular superoxide formation. Spermatozoa motility differences between the control and groups A and B were the only one significant (P<0.05 and P<0.001, respectively) at 0 h, however, increased motility parameters were observed in all experimental groups after 12 h, with significant differences (P<0.001) after 24 h. The MTT assay indicated that none of the vitamin E concentrations had a negative or cytotoxic effect on the spermatozoa mitochondrial activity and furthermore showed a significantly (P<0.001) improved cell viability in groups B, C and D at 24 h. The NBT test showed that the addition of 500, 100 and 50 µM of vitamin E had an instant positive effect on
the spermatozoa protection against free radical production. This protection remained present with a significant impact at 12 h (P<0.001 and P<0.05, respectively). Furthermore, all vitamin E concentrations exhibited significant (P<0.05; P<0.001) protective effects on the spermatozoa free radical formation. The results indicate that the addition of vitamin E, especially in concentrations of 500 µM to 50 µM to the culture medium could be beneficial for the overall stimulation of spermatozoa activity and protection against possible in vitro oxidative stress development.

Keywords: vitamin E, spermatozoa, bulls, motility, CASA, MTT assay, NBT test

INTRODUCTION

Uncontrolled overproduction of reactive oxygen species (ROS) and the resulting oxidative stress (OS) development has become a serious problem in matters related to male fertility (Agarwal et al., 2003).

Oxidative stress is known to play a major role in the sperm malfunctions via induction of lipid peroxidation (LPO) to biomembranes (Arabi et al. 2001), which may result in a decrease in sperm motility and viability (de Lamirande and Gagnon, 1992) together with an increase in morphology defects, with deleterious effects on sperm capacitation and acrosome reaction, ultimately leading to infertility (Agarwal et al., 2003).

Studies have shown that antioxidants protect spermatozoa from ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation and provide an overall stimulation to the sperm cells (Agarwal et al., 2007). However, the majority of them are still uncontrolled, focus on healthy individuals or have indirect end-points of success. Several other studies are noted due to the quality of their study design, and demonstrate compelling evidence regarding efficacy of antioxidants towards improving semen parameters (Kefer et al., 2009).

Against ROS attacks, sperm cells are equipped with an antioxidant defense system, however an imbalance between the production of ROS and the available antioxidant defenses results in OS (Sikka, 1996). Therefore, antioxidants should be supplemented extracellularly under in vitro conditions (Bansal and Bilaspuri, 2009).

Vitamin E is believed to be the primary component of the antioxidant system of spermatozoa and is one of the major membrane protectants against ROS and LPO (Yousef et
al., 2003). It appears to be present in the first line of defense against the peroxidation of polyunsaturated fatty acids in the cellular membrane phospholipids because of its lipid solubility (Horton et al., 2002). It is a major chain-breaking antioxidant directly neutralizing superoxide anion, hydrogen peroxide and hydroxyl radical (Sharma and Agarwal, 1996).

The present in vitro study was aimed to find out the efficacy of vitamin E on bovine sperm motility, viability and free radical formation during a 24 hour in vitro cultivation.

MATERIAL AND METHODS

Bovine semen samples were obtained from 15 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The samples were obtained on a regular collection schedule using an artificial vagina. After collecting the samples were stored in the laboratory at room temperature (22–25°C).

Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italia) containing 0.5% ethanol (96% ethyl alcohol, EtOH; Merck Chemicals, Darmstadt, Germany), with various concentrations of vitamin E (α-tocopherol; Sigma-Aldrich, St. Louis, USA; A – 1000; B – 500; C – 100; D – 50; E – 10 μM/L) using a dilution ratio of 1:40. The samples were cultivated at room temperature (22–25°C). We compared the control (Ctrl) group (medium without vitamin E) with the experimental groups.

Motility analysis was carried out using the CASA (Computer Assisted Semen Analyzer) system equipped with the SpermVision™ program (MiniTub, Tiefenbach, Germany) and the Olympus BX 51 microscope (Olympus, Japan) at cultivation Times 0 h, 12 h and 24 h. Each sample was placed into the Makler Counting Chamber (depth 10 μm, Sefi-Medical Instruments, Israel) and the percentage of motile spermatozoa (motility>5μm/s; MOT) was evaluated. 1000–1500 cells were assessed in each analysis (Massányi et al., 2008).

Viability of the cells exposed to vitamin E in vitro was evaluated by the metabolic activity (MTT) assay (Mosmann, 1983; Knazicka et al., 2012). This colorimetric assay measures the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphényltetrazolium bromide (MTT; Sigma, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan can then be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to
vitamin E). Results from the analysis were collected during three repeated experiments at each concentration.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical (Esfandiari et al., 2003). This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride; Sigma, St. Louis, USA) and superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to vitamin E). Results from the analysis were collected during three repeated experiments at each concentration (Tvrdá et al., 2012).

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 with Dunnett's post test was used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Evaluation of the percentage of motile spermatozoa showed decreased values in all groups cultured for 24 hours (Table 1). Initial (time 0 h) spermatozoa motility was significantly (P<0.05) low in group A cultured with 1000 µM vitamin E (79.15±1.84%) and significantly high (P<0.001) in group B cultured with 500 µM vitamin E (91.93±0.97%), when compared to the control group, cultured without any vitamin E addition (85.05±1.94%). After 12 h, all of the experimental groups exhibited a higher spermatozoa motility in comparison with the control (70.53±1.08%) with a significant difference (P<0.05) in group B (77.86±1.31%). The highest stimulating effects of vitamin E on the spermatozoa motility was observed after 24 hours. All experimental concentrations of vitamin E managed to keep the spermatozoa motility above 60%, which was not observed in the control group (55.01±1.39%). A significant maintenance in spermatozoa motility was detected in groups B and C (64.89±1.76% and 64.62±2.19%, respectively; P<0.001), as well as in groups A and D (62.91±2.23% and 63.46±1.79%, respectively; P<0.001).
Table 1 Spermatozoa motility (MOT; %) in the absence (Ctrl) or presence (A-E) of vitamin E during different time periods (Mean±SEM; n=15)

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0 h</td>
<td>85.05±1.94</td>
<td>79.15±1.84*</td>
<td>91.93±0.97***</td>
<td>84.83±1.32</td>
<td>83.47±1.41</td>
<td>80.88±1.55</td>
</tr>
<tr>
<td>Time 12 h</td>
<td>70.53±1.08</td>
<td>72.95±1.93</td>
<td>77.86±1.31*</td>
<td>76.14±1.77</td>
<td>74.65±2.54</td>
<td>72.36±1.67</td>
</tr>
<tr>
<td>Time 24 h</td>
<td>55.01±1.39</td>
<td>62.91±2.23*</td>
<td>64.89±1.76***</td>
<td>64.62±2.19***</td>
<td>63.46±1.79*</td>
<td>60.92±2.31</td>
</tr>
</tbody>
</table>

*** (P<0.001); ** (P<0.01); * (P<0.05)

The MTT assay revealed a similar cell viability in all of the experimental groups with a significant (P<0.001) increase in the B and C group (111.00±1.41% and 124.80±1.56%, respectively) when compared to the control (100.00±3.54%; Figure 1). After 12 h the spermatozoa viability increased significantly (P<0.001) in all experimental groups as compared to the control (100.00±2.53%), with the group C assuring a two times higher cell viability (200.00±4.70%; Figure 2). After 24h, the spermatozoa viability remained significantly high in the groups B and C (135.00±2.53% and 155.09±1.61%, respectively; P<0.001) as well as in the group D (119.6±2.60%; P<0.05). Spermatozoa viability in groups A and E was very similar to the viability in the control group (100.00±3.23%; Figure 3).
The NBT test revealed that 500, 100 and 50 µM of vitamin E had an instant and significant (P<0.001) protective effect against superoxide production in the sperm cells, when compared to the control (Figure 4). The positive effect of the B and C group remained persistent and significant (P<0.001) over the course 12 h and was subsequently joined by all of the experimental groups (P<0.05 and P<0.01; Figure 5). After 24 h, all the experimental groups exhibited a long-term and significant (P<0.001) antioxidant protection of the sperm cells and prevention of the escalating intracellular superoxide production, with a special positive effect in case of the groups B (43.11±2.12%) and C (48.37±2.82%) when compared to the control group (100±4.15%; Figure 6).

In the present study, ROS-mediated damage to sperm cell with a subsequent loss of spermatozoa fertilizing potential could be reduced using various doses of vitamin E. ROS are...
generally produced under elevated oxygen tension, which may be induced when spermatozoa are transferred to and manipulated under in vitro conditions. Supplementation of antioxidants to semen can protect against the damaging effects of ROS on sperm motility and viability, and therefore may be of clinical value in assisted conception procedure (Baker et al., 1996).

Our results indicate that vitamin E improves the percentage of motile and viable spermatozoa under in vitro conditions. Normal ex vivo oxidative stress to spermatozoa with the treatment causes a significant reduction in their motility. Nevertheless, supplementing the culture medium with different doses of vitamin E, maintained and increased the motility from hour 0 to hour 24 of incubation. Thus, we may suggest that vitamin E is effective in preventing the rapid loss of motility that normally occurs during incubation of spermatozoa and maintains the motility under oxidative stress conditions.

This study shows that different doses of vitamin E improve the viability of cattle sperm. These results may be explained based on the fact that vitamin E protects the spermatozoa by preventing from mitochondrial oxidative DNA and membrane damages, thereby helping the sperm to overcome the oxidative attacks. Thus, by maintaining the cell integrity and optimum functioning of spermatozoa, vitamin E improves the per cent of sperm viability. Besides, supplementing culture media with vitamin E improved the sperm viability in humans (Askari et al., 1994) and in rabbits (Yousef et al., 2003).

All concentrations of vitamin E significantly reduced the intracellular production of superoxide radical. It may be explained by the fact that vitamin E reduces the oxidative damage in sperm cell membranes by disrupting the oxidative chain reaction. Thus, vitamin E promotes sperm membrane integrity and increases the protection of the sperm cells against the formation of the superoxide radical, which is considered to be the most aggressive ROS. Similar observations have been made on humans (Verma and Kanwar, 1999) and boars (Slebodzinska et al., 1995).

The present study shows the effectiveness of vitamin E in protecting spermatozoa motility and viability by decreasing the intracellular ROS protection. Similar observations have been made in humans (Aitken et al., 1989; Agarwal et al., 2004), boars (Slebodzinska et al., 1995) and rabbits (Yousef et al., 2003).

Vitamin E has been shown to inhibit the free-radical-induced damage to sensitive sperm cell membranes as it is a major chain-breaking antioxidant (Sinclair, 2000). The present results suggest that vitamin E may directly quench various free radicals generated during the in vitro generated OS. Thus, by scavenging these radicals, it breaks free-radical chain reaction
and forms a relatively stable complex such as tocopheroxyl radical. Similar suggestions concerning humans have been made by Verma and Kanwar (1999).

CONCLUSION

It may be concluded that all doses of vitamin E increased the percentage of motile and viable spermatozoa but decreased and prevented the intracellular overproduction of free radicals within the sperm mitochondrial membrane. The most effective concentrations of vitamin E seem to be 500 and 100 µM/L, even though differences among the selected doses were non-significant (P≥0.05).

Vitamin E seems to protect bovine spermatozoa against the damages caused by reactive oxygen species. Supplementing the semen samples with vitamin E could therefore be of scientific importance for extending the time of spermatozoa storage before further designated andrology experiments and clinical procedures, such as artificial insemination or in vitro fertilization techniques.

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