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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^{TM} 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 equiped with the SpermVision^{TM} 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-dimetylthiazol-2-yl)-2,5- diphenyltetrazolium 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 spectrophotometerically 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 spectrophotometerically 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

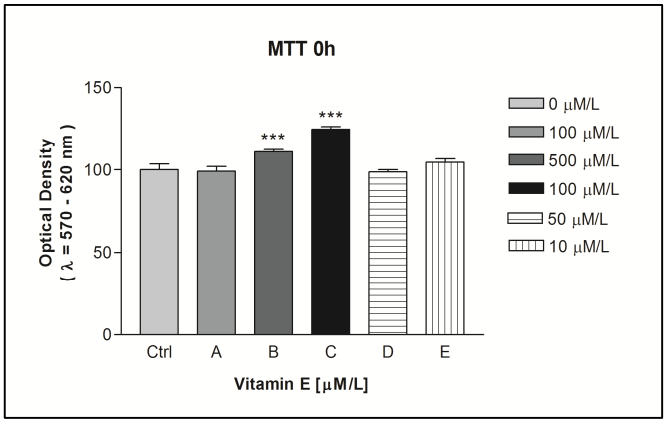
Evaluation of the percentage of motile spermatozoa showed decreased values in all groups cultured for 24 hours (Table 1). Initial (time 0 h) spermatozoa motility was significantly ($P<0.05$) low in group A cultured with 1000 μM vitamin E (79.15±1.84%) and significantly high ($P<0.001$) in group B cultured 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; $P<0.001$).

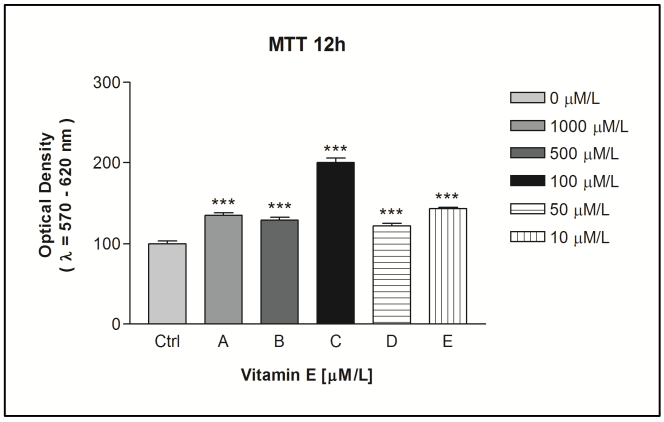
Table 1 Spermatozoa motility (MOT; %) in the absence (Ctrl) or presence (A-E) of vitamin E during different time periods (Mean±SEM; n=15)

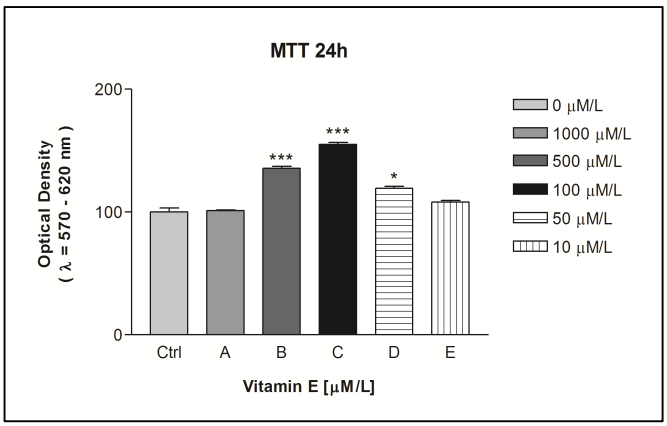
Ctrl	A	B	C	D	E
Time 0 h					
85.05±1.94	79.15±1.84*	91.93±0.97***	84.83±1.32	83.47±1.41	80.88±1.55
Time 12 h					
70.53±1.08	72.95±1.93	77.86±1.31*	76.14±1.77	74.65±2.54	72.36±1.67
Time 24 h					
55.01±1.39	62.91±2.23*	64.89±1.76***	64.62±2.19***	63.46±1.79*	60.92±2.31

*** (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).

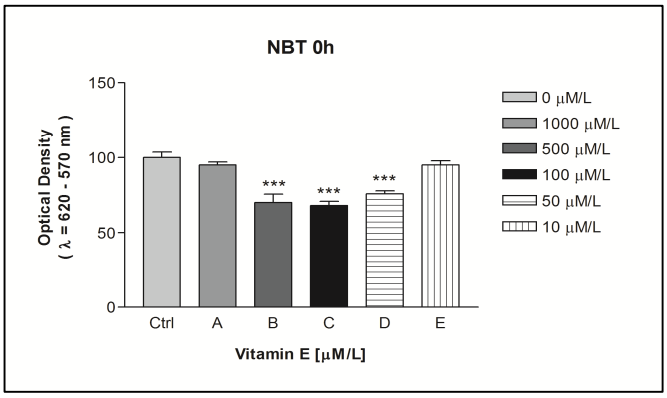


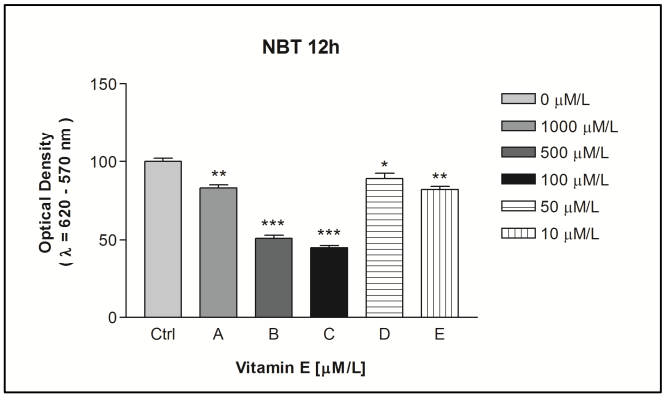


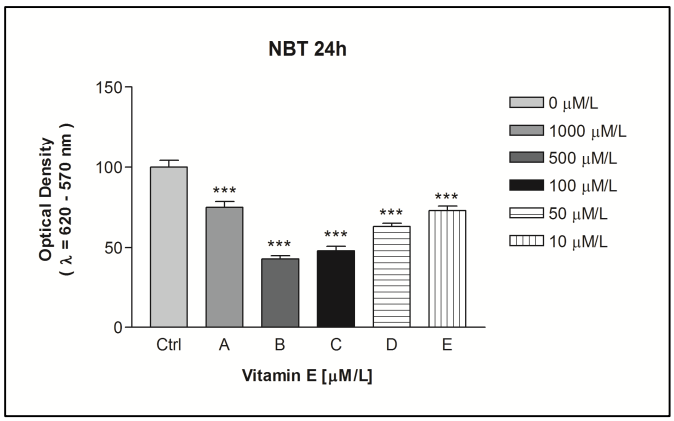


Figures 1, 2, 3 The effect of various doses of vitamin E on the viability of spermatozoa at 0, 12 and 24 h.
Each bar represents the mean (±SEM) optical density as the percentage of controls (n=15), which represent 100%.
The data were obtained from three independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

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 exibited 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).







Figures 4, 5, 6 The effect of various doses of vitamin E on the spermatozoa superoxide production at 0, 12 and 24 h.
Each bar represents the mean (±SEM) optical density as the percentage of controls (n=15), which represent 100 %.
The data were obtained from three independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

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, mantained 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 medias 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 agressive 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 ($P \geq 0.05$).

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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REFERENCES

AGARWAL, A. – PRABAKARAN, S.A. – SIKKA, S.C. 2007. Clinical relevance of oxidative stress in patients with male factor infertility: evidence-based analysis. In *AUA Update Series*, vol. 26, 2007, p. 1-12.

AITKEN, R.J. – CLARKSON, J.S. - FISHEL S. 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. In *Biology of Reproduction*, vol. 40, 1989, p. 183-197.

ARABI, M. - ANAND, R.J.K. - KANWAR, U.K. 2001. Analysis of the impact of caeffine on membrane integrity, redox ratio and GST in human ejaculated sperm: Effectiveness of antioxidants. In *Andrology in the 21st Century* (Proceedings of VIIth International Congress of Andrology). Bologna: Medimond, 2001, p. 365-369.

ASKARI, H.A. - CHECK J.H. - PEYMER N. - BOLLENDORF A. 1994. Effect of natural antioxidants tocopherol and ascorbic acid in maintenance of sperm activity during freeze-thaw process. In *Archives of Andrology*, vol. 33, 1994, p. 11-15.

BAKER, H.W.G. – BRINDLE, J. – IRVINE, D.S. - AITKEN R.J. 1996. Protective effect of antioxidants on the impairment of sperm motility by activated polymorphonuclear leukocytes. In *Fertility and Sterility*, vol. 65, 1996, p. 411-419.

BANSAL, A.K. – BILASPURI, G.S. 2009. Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress. In *Animal Science Papers and Reports*, vol. 27, 2009, no. 1, p. 5-14.

DE LAMIRANDE, E. – GAGNON, C. 1992. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. In *Journal of Andrology*, vol. 13, 1992, p. 379-386.

ESFANDIARI, N. – SHARMA, R.K. – SHARMA, R.K. – SALEH, R.S. – THOMAS Jr., A.J. – AGARWAL, A. 2003. Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. In *Journal of Andrology*, vol. 24, 2003, no. 6, p. 862-870.

HORTON, H.R. – MORAN, L.H. – OCHS, R.S. – RAWN, J.D. – SCRIMGLOUR, K.G. 2005. Principles of Biochemistry. 4th Edition. New York: Worth Publishers, 2005, 896 p. ISBN 01-31-453-06-8.

KEFER, J.C. – AGARWAL, A. – SABANEGH, E. 2009. Role of antioxidants in the treatment of male infertility. In *International Journal of Urology*, vol. 16, 2009, p. 449-457.

KNAZICKA, Z. – TVRDA, E. – BARDOS, L. – LUKAC, N. 2012. Dose- and time-dependent effect of copper ions on the viability of bull spermatozoa in different media. In *Journal of Environmental Science and Health Part A*, vol. 47, 2012, p. 1294-1300.

MASSÁNYI, P. – CHRENEK, P. – LUKÁČ, N. – MAKAREVICH, A.V. – OSTRO, A. – ŽIVČÁK, J. – BULLA, J. 2008. Comparison of different evaluation chambers for analysis of rabbit spermatozoa motility using CASA system. In *Slovak Journal of Animal Science*, vol. 41, 2008, no. 2, p. 60-66.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and surrival: application to proliferation and cytotoxicity assays. In *Journal of Immunological Methods*, vol. 65, 1983, p. 55–63.

SHARMA, R.K. – AGARWAL, A. 1996. Role of reactive oxygen species in male infertility. In *Journal of Urology*, vol. 48, 1996, p. 835-850.

SIKKA S.C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. In *Frontiers in Bioscience*, vol. 1, 1996, p. 78-86.

SINCLAIR S. 2000. Male infertility: nutritional and environmental consideration. In *Alternative Medicine Review*, vol. 5, 2000, p. 28-38.

SLEBODZINSKA, B.E. – SLEBODZINSKI, A.B. – PIETRAS, B. – WIECZOREK, G. 1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. In *Biological Trace Element Research*, vol. 47, 1995, p. 69-74.

TVRDÁ, E. – KŇAŽICKÁ, Z. – LUKÁČOVÁ, J. – LUKÁČ, N. 2012. Stimulating and protective effects of resveratrol on bovine spermatozoa. In *MendelNet 2012* (Proceedings of the International Conference). Brno: Mendel University, 2012, p. 1036-1046.

VERMA, A. – KANVAR, K.C. 1999. Effect of vitamin E on human sperm motility and lipid peroxidation *in vitro*. In *Asian Journal of Andrology*, vol. 1, 1999, p. 151-154.

YOUSEF, M.I. – ABDALLAH, G.A. – KAMEL, K.I. 2003. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. In *Animal Reproduction Science*, vol. 76, 2003, p. 99-111.