Journal of Microbiology, Biotechnology and Food Sciences

Tvrdá et al. 2012 : 2 (2) 536-547

REGULAR ARTICLE

RELATIONSHIPS BETWEEN IRON AND COPPER CONTENT, MOTILITY CHARACTERISTICS AND ANTIOXIDANT STATUS IN BOVINE SEMINAL PLASMA

Eva Tvrdá[*1], Zuzana Kňažická[1], Jana Lukáčová[1], Monika Schneidgenová[1], Peter Massányi[1], Zofia Goc[2], Robert Stawarz[2], Norbert Lukáč[1]*

Address: MSc. Eva Tvrdá,

[1] Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, phone number: 00421 37 641 4288 .

[2] Pedagogical University of Krakow, Faculty of Geography and Biology, Institute of Biology, Podbrzezie 3, 300 84 Krakow, Poland

*Corresponding author: evina.tvrda@gmail.com

ABSTRACT

To investigate the effects of iron (Fe) and copper (Cu) content on basic motility characteristics (motility, progressive motility) and total antioxidant status (TAS) in the bovine seminal plasma semen samples were collected from breeding bulls and used in the study. Motility analysis was carried out using the Computer Assisted Sperm Analysis (CASA) system. Subsequently, the samples were centrifuged and seminal plasma was collected. Fe and Cu concentrations were determined by flame absorption spectrophotometry (FAS), TAS was analyzed by UV/VIS spectrophotometry. The analysis showed that the average concentrations of the trace elements were 39.95±0.07 μg/mL for Fe and 1.55±0.22 μg/mL for Cu. The correlation analysis revealed that both metals were positively correlated with motility ($r=0.527$; $P<0.05$ for Fe and $r=0.571$; $P<0.05$ for Cu), progressive motility ($r=0.496$; $P<0.05$ for Fe and $r=0.513$; $P<0.05$ for Cu), as well as TAS ($r=0.694$; $P<0.01$ for Cu; $r=0.519$; $P<0.05$ for Fe). Moreover, the samples were categorized in three quality groups (Excellent, Good, Moderate) according to their motility values. The highest Cu and Fe concentrations together

with the best antioxidant characteristics were found in samples of excellent quality, moderate quality samples were described by the lowest Fe and Cu concentration and the worst antioxidant power. This study demonstrates that Cu and Fe are important for the preservation of sperm motility and antioxidant power, however this property is bound to their physiological amounts only.

Keywords: oxidative stress, iron, copper, total antioxidant status, spermatozoa, bulls

INTRODUCTION

Mammalian semen is known to contain a big variety of chemical elements **(Marzec-Wróblewska *et al.*, 2012)**, whose influence on spermatozoa viability has been extensively studied in animals as well as in humans **(Kanwal *et al.*, 2000; Massányi *et al.*, 2003a,b,c; 2004; 2005; 2008a; Eghbali *et al.*, 2008; Atig *et al.*, 2012)**. A large number of studies have proven that chemical elements are an essential component in the preservation of the fertilization capacity of spermatozoa. Some of them are essential for a proper sperm cell function (e. g. sodium, magnesium, calcium, potassium), others are required in narrow limits (zinc, manganese, copper, iron, cobalt, selenium) **(Massányi *et al.*, 2003a,b,c; 2004)**.

Copper (Cu) and iron (Fe) are essential micronutrients for all organisms because of their high redox potential, and importance as cofactors for a variety of metabolic proteins, such as cytochrome c oxidase and superoxide dismutase **(*Craig et al.*, 2009)**. However, disturbances in their regulative absorption mechanism and increased concentrations may have a negative impact on the sperm viability and morphology **(Massányi *et al.*, 2003a,b,c; 2004; Roychoudhury *et al.*, 2008; Knazicka *et al.*, 2012)**. Moreover, these transitional metals are implicated in a number of physiological, toxicological, and pathological processes due to their capacity to undergo changes of oxidation states involving electron transfer **(Okada, 1998)**.

Fe- and Cu-catalysis can cause reactive oxygen species (ROS) overproduction and subsequent oxidative stress (OS) development, commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level **(Tvrdá *et al.*, 2011)**. Oxidative damage is a result of such imbalance and includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage **(Lykkesfeldt and Svendsen, 2007)**. Increased levels of ROS have been correlated with a decreased sperm motility **(Eskenazi *et al.*, 2003)**, increased sperm DNA damage

(Armstrong *et al.*, 1999), sperm cellular membrane lipid peroxidation **(Aitken, 1995)** and decreased efficacy of oocyte–sperm fusion **(Agarwal *et al.*, 2007)**.

Large quantities of polyunsaturated fatty acids and low concentrations of antioxidant substances renders the sperm cells to be particularly sensitive to OS. Therefore, under normal conditions, the seminal plasma is an important protectant of spermatozoa against possible ROS formation and distribution, as it contains two major groups of antioxidant scavengers: the proteins and the low-molecular weight antioxidants (LMWA). The antioxidant proteins contain enzymes (superoxide dismutase, catalase, peroxidase and some supporting enzymes such as glucose-6 phosphate dehydrogenase and glutathione reductase) and the proper proteins (albumin, transferrin, caeruloplasmin and ferritin). The LMWA group contains a large number of compounds capable of preventing oxidative damage by direct and indirect interaction with ROS **(Rice-Evance and Miller, 1994)**. The efficiency of antioxidants is expressed as total antioxidant capacity (TAC) summarizing the overall activity of all types of antioxidants in living systems **(Ziyatdinova *et al.*, 2005)**.

Increased concentrations of Fe and Cu in natural ecosystems can be harmful, if not fatal, to the organism. Because of their persistence in the environment, it is important to examine the biological impacts of these metals, especially in cattle breed. Therefore, the aim of the study was to evaluate, compare and assume relationships between iron and copper content, basic motility characteristics and the total antioxidant status in bovine seminal plasma.

MATERIAL AND METHODS

30 bovine semen samples were obtained from adult breeding bulls (Slovak Biological Services, Nitra, Slovakia) on a regular collection schedule using an artificial vagina. After collecting the samples were stored in the laboratory at room temperature (22-25°C) for further analysis. Spermatozoa motility (percentage of motile spermatozoa; motility > 5μm/s; MOT) and progressive motility (percentage of progressive motile spermatozoa; motility > 20μm/s; PROG) analysis was carried out using the Computer Assisted Sperm Analysis (CASA) system – SpermVision (MiniTüb, Tiefenbach, Germany) with Olympus BX 51 phase contrast microscope (Olympus, Japan). Each sample was placed into Makler Counting Chamber (depth 10 μm, 37±1°C; Sefi–Medical Instruments, Haifa, Israel), motility and progressive motility parameters were evaluated. 1000 cells were examined for each sample **(Massanyi *et al.*, 2008b)**.

Subsequently, the samples were centrifuged (15 min, 10 090 x g, 4°C) to obtain the cell

sediment and seminal plasma fraction. The fractions were separated, seminal plasma was transferred into 1.5 mL tubes and kept frozen (-80°C) until analysis **(Tvrda et al., 2012)**.

For copper and iron detection, the blood plasma samples (at least 1 mL) were mineralized in the laboratory. The sample was placed in separate mineralization tubes and mineralized by adding 2 mL of HNO_3-$HCLO_4$ (4:1) mixture and heating it at 120 °C for 65 minutes in a thermostat-controlled digestion block. The resulting solution was diluted to 10 mL with demineralized water. Cu and Fe concentration was determined by the flame absorption spectrophotometry (FAS) with the Cole-Parmer 200A atomic absorption spectrophotometer (Cole-Parmer International, Court Vernon Hills, IL, USA). Concentrations are expressed as µg/mL.

The Total Antioxidant Status (TAS) assay is based on an incubation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS®*+. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration **(Miller *et al.*, 1993)**. TAS was measured using the TAS Randox commercial kit (Randox Laboratories, Crumlin, Great Britain) and Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc, USA).

Statistical analysis was carried out using the statistical program GraphPad Prism 3.02 (Graphpad Software incorporated, San Diego, California, USA). Results are quoted as arithmetic mean±standard deviation (SD). Additionally, the samples were categorized in three groups of Excellent (Ex, > 95% motile; n=11), Good (Go; 90–95% motile; n=10) and Moderate quality (Mo; < 90% motile; n=9) according to their motility rates (Table 3) to a further assessment of the effects of Cu and Fe on the seminal antioxidant capacity. The comparison of semen parameters in the quality groups was carried out by the Tukey's Multiple Comparison Test. Pearson's correlation coefficient (two tailed) test was used to examine correlations between all the analyzed parameters. The level of significance for the comparison test as well as for the correlation analysis was set at *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$).

RESULTS AND DISCUSSION

Results of the semen and seminal plasma evaluation in Table 1 show that the average concentration of Fe measured by the FAS method was 39.95±0.07 µg/mL, while the Cu value was 1.55±0.22 µg/mL.

Table 1 Semen characteristics of bull semen and seminal plasma samples examined by the ASV method (Cu, Fe), UV/VIS spectrophotometry (TAS) and CASA (MOT, PROG) (n=30)

Parameter	Mean±SD
Iron (μg/mL)	39.95±0.07
Copper (μg/mL)	1.55±0.22
Total antioxidant status (mmol/L)	2.10±0.34
Motility (%)	93.53±3.41
Progressive motility (%)	88.41±3.31

The Fe concentration detected by **Eghbali *et al.* (2010)** in Water Buffalo bull seminal plasma was similar to our results: 40.68±0.75 mg/L. **Pesch *et al.* (2006)** reported that the iron content of equine seminal plasma was 1.9±0.106 μmol/L. **Massányi *et al.* (2003a,b,c)** compared the iron content in bull, ram, stallion, boar and fox, and reported that seminal iron concentration was significantly higher in the ram (40.32±10.81 mg/kg), bull (38.04±22.07 mg/kg), and fox (33.16±24.36 mg/kg) than that in the boar (16.14±10.35 mg/kg) and stallion (12.68 mg/kg). Moreover the authors add that the seminal copper concentration was significantly higher in ram (2.49 mg/kg) and fox (2.16 mg/kg) than that in bull (1.64 mg/kg), boar (1.64 mg/kg) and stallion (0.86 mg/kg). According to **Lukáč *et al.* (2009)** the concentration of copper in rabbit semen was 20.10±4.09 mg/kg.

The correlation analysis (Table 2) revealed positive correlations between TAS and copper ($r=0.694$; $P<0.01$) as well as between TAS and iron ($r=0.519$; $P<0.05$). According to these results it is apparent that iron and copper, in physiological amounts, do have beneficial effects on the antioxidant capacity of semen. Besides, both metals showed positive effects on motility ($r=0.527$; $P<0.05$ for iron and $r=0.571$; $P<0.05$ for copper) as well as progressive motility ($r=0.496$; $P<0.05$ for iron and $r=0.513$; $P<0.05$ for copper) of bovine spermatozoa.

Table 2 Correlations between iron, copper, selected spermatozoa motility parameters and antioxidant status in bovine seminal plasma evaluated by the Pearson's correlation coefficient test (n = 30)

	TAS	MOT	PROG	Fe	Cu
TAS	1	0.574**	0.502**	0.519*	0.694**
MOT	0.574**	1	0.990***	0.527*	0.571*
PROG	0.502**	0.990***	1	0.496*	0.513*
Fe	0.519*	0.527*	0.496*	1	0.728**
Cu	0.694**	0.571*	0.513*	0.728**	1

Legend: The correlation analysis was based on the value of the correlation coefficient: ±0.111 to ±0.333: low correlation; ±0.334 to ±0.666: moderate correlation; ±0.667 to ±0.999: high correlation. *P<0.05; **P<0.01; ***P<0.001. Fe – iron (μg/mL), Cu – copper (μg/mL), TAS – total antioxidant status (mmol/L), MOT – motility (%), PROG – progressive motility (%).

Iron and copper contribute only a small part of the entire diet; however, they are essential for normal growth, reproduction, and immunity. Iron and copper deficiencies belong to the most common nutritional problems existing today around the world and are correlated to impaired growth and reproductive performance **(Sharp, 2004)**.

Nevertheless, disturbances in the regulative absorption mechanism can appear due to pathological conditions or prolonged intake of high doses of both metals. Increased Fe concentration can bear negatively on the morphology of spermatozoa **(Massányi *et al.*, 2003a,b,c)**; significant differences in the Fe concentration between sperm of severely teratospermic subjects were reported in contrast to no differences in normozoospermic subjects **(Kwenang *et al.*, 1987)**. On the other hand, a study by **Eghbali *et al.* (2010)** shows that the mean iron concentration in seminal plasma was highly associated with sperm progressive motility, gross motility and viability. Their results showed that seminal plasma iron content is associated with the motility and viability of the spermatozoa after ejaculation.

Copper can also have negative impact on the morphology of spermatozoa **(Gamik *et al.*, 1990; Massányi *et al.*, 2003b, 2005)** and sperm motility **(Gamik *et al.*, 1990)**. The incubation of spermatozoa in the presence of Cu had a negative effect on some of motility parameters examined by computer-assisted semen analyser (CASA) in studies by **Roychoudhury *et al.* (2008)** and **Knazicka *et al.* (2012)** and caused decreased sperm motility or complete sperm immotility. On the other hand, positive influence of Cu on sperm concentration and counts was also noticed. Men with sperm concentrations above 40 million/mL showed higher Cu semen concentrations than men with azoospermia, displaying

sperm concentrations below 5 million/mL and between 10 and 20 million/mL **(Jackenhovel *et al.*, 1999)**. Positive correlation was noticed between Cu concentration in blood, sperm count in the ejaculate, and count of spermatozoa with progressive motility, and between Cu concentration in seminal plasma and the volume of ejaculate, motility of spermatozoa and number of spermatozoa with progressive motility **(Machal *et al.*, 2002)**.

Physiological amounts of Fe and Cu do have beneficial effect on the overall prooxidant-antioxidant balance, since both are directly involved in the antioxidant defense system. Cu together with zinc are involved in the antioxidant system via its involvement in superoxide dismutase (SOD) and ceruloplasmin. Cu-Zn SOD is responsible for dismutation of superoxide radicals to hydrogen peroxide in the cytosol **(Kankofer *et al.*, 2007)**. Iron is the integral part of catalase, one of the most active and powerful antioxidant enzymes, which breaks H_2O_2 down directly to oxygen and water. It has been demonstrated that as a result of iron deficiency the catalase activity of kidney and particularly of liver is significantly reduced **(Faixová *et al.*, 2006)**.

To have a better understanding of the stimulating effect of Cu and Fe on the seminal antioxidant capacity results, the samples were categorized in three groups of Excellent (Ex, >95 % motile; n=11), Good (Go; 90–95% motile; n=10) and Moderate quality (Mo; <90% motile; n=9) according to their motility rates (Table 3). Mean values for motility and progressive motility were significantly ($P<0.001$; $P<0.01$; $P<0.05$) different between the groups.

Table 3 Average values of observed parameters in the quality groups (mean±SD) and the Tukey's Multiple Comparison Test results.

	Ex (n=11)	**Go (n=10)**	**Mo (n=9)**
TAS	2.29±0.05	2.03±0.08^{*a}	2.01±0.06$^{**b, *c}$
MOT	97.22±2.50	93.63±3.03^{*a}	85.72±2.81^{**b,**c}
PROG	94.02±3.15	90.17±2.99^{*a}	82.38±4.05^{***b,**c}
Fe	45.05±0.10	35.65±0.12^{*a}	33.80±0.08^{**b}
Cu	1.85±0.07	1.53±0.05^{*a}	1.39±0.09^{**b}

Legend: ^{a} Ex vs. Go; ^{b}Ex vs.Me; ^{c}Go vs.Me. *P < 0.05; **P < 0.01; ***P < 0.001. Quality groups are based on the motility values: Ex – Excellent quality (>95% motile), Go - Good (90–95% motile), Mo - Moderate quality (<90% motile). TAS – total antioxidant status, MOT – motility, PROG – progressive motility, Fe – iron, Cu – copper.

The highest TAS were detected in the Ex group while the lowest value was recorded in the Mo group. Significant differences ($P<0.01$; $P<0.05$) were observed, especially when comparing the antioxidant status between the Ex and Mo group.

The highest concentrations of Fe and Cu were detected in the Ex group, meanwhile the lowest concentrations were found in the Mo group. Significant differences were observed when comparing the Ex and the Go group ($P<0.01$) as well as between the Ex and the Mo group ($P<0.05$).

This observation together with a positive correlation proves that physiological concentrations of both trace elements have stimulating effects on both motility parameters as well as antioxidant status of bovine spermatozoa. Additionally, a strong positive correlation between iron and copper ($r=0.728$; $P<0.01$) shows their synergic relationship, which has been already proved at numerous levels of human and animal metabolism. The best characterized link is provided by caeruloplasmin, a multi Cu-binding protein that is essential for the mobilization of Fe from storage tissues. Decreased Cu status has been shown to impair ferrioxidase activity, leading in a number of cases, to decreased tissue Fe release and the generation of anaemia that is responsive to dietary supplementation with Cu but not Fe. Furthermore, there is emerging evidence that a number of other components of the intestinal Fe transport pathway are also Cu sensitive **(Sharp, 2004)**.

CONCLUSION

It can be concluded that the iron and copper content of seminal plasma in bulls is important for the preservation of sperm motility, and viability after ejaculation, and their presence in the seminal plasma will help spermatozoa to maintain their functions. Furthermore, as both metals are important cofactors of the majority of antioxidant enzymes, their presence is required for a proper seminal prooxidant-antioxidant balance. However, this stimulating effect is conditional to their physiological amounts only. Higher concentrations of copper and iron could easily contribute to free radical formation and oxidative stress development. Therefore, it is important to systematically evaluate their content in tissues and body fluids in relation to antioxidant capacity, in order to prevent complications resulting from their bilateral roles in the organism.

Acknowledgments: This work was supported by the Scientific Agency of the Slovak Republic VEGA No. 1/0532/11 and by the Cultural and Educational Grant Agency of the Slovak Republic KEGA No. 101-001 SPU-4/2010.

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. 1995. Free radicals, lipid peroxidation, sperm function. In *Reproduction, Fertility and Development*, vol. 7, 1995, p. 659-668.

ARMSTRONG, J. S. – RAJASEKARAN, M. – CHAMULITRAT, W. – GATTI, P. – HELLSTROM, W. J. – SIKKA, S. C. 1999. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. In *Free Radicals in Biology and Medicine*, vol. 26, 1999, p. 869–880.

ATIG, F. – RAFFA, M. – BEN-ALI, H. – KERKENI, A. – SAAD, A. – MOUNIR, A. 2012. Impact of seminal trace element and glutathione levels on semen quality of Tunisian infertile men. In *BMC Urology*, vol. 12, 2012, p. 6-14.

CRAIG, P. M. – GALUS, M. – WOOD, CH. M. – McCLELLAND, G. B. 2009. Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*). In *Regulatory Physiology*, vol. 296, 2009, no. 2, p. 362-373.

EGHBALI, M. – ALAVI-SHOUSHTARI, S.M. – ASRI REZAII, S. 2008. Effects of copper and superoxide dismutase content of seminal plasma on buffalo semen characteristics. In *Pakistan Journal of Biological Sciences*, vol. 11, 2008, p. 1964-1968.

EGHBALI, M. - ALAVI-SHOUSHTARI, S. M. - ASRI-REZAEI, S. - KHADEM ANSARI, M. H. 2010. Effects of the seminal plasma iron and lead content on semen quality of Water Buffalo (*Bubalus bubalis*) bulls. In *Veterinary Research Forum*, vol. 1, 2010, no. 3, p. 142-148.

ESKENAZI, B. – WYROBEK, A. J. – SLOTER, E. – KIDD, S. A. – MOORE, L. YOUNG, S. – MOORE, D. 2003. The association of age and semen quality in healthy men. In *Human Reproduction*, vol. 18, 2003, p. 447–454.

FAIXOVÁ Z. - FAIX Š. - MAKOVÁ Z. - VÁCZI P. - PROSBOVÁ M. 2006. Effect of divalent ions on ruminal enzyme activities in sheep. In *Acta Veterinaria (Beograd)*, vol. 56, 2006, no. 1, p. 17-23.

GAMIK, P. - BRE, J. - VRZGULA, L. - MESÁKO, P. 1990. Effect of experimental intoxication with copper from industrial emission on reproductive ability in rams. In *Reproduction of Domestic Animals*, vol. 25, 1990, p. 235-241.

JACKENHOVEL, F. - BALS-PRATSCH, M. - BERTRAM, H. P. - NIESCHLAG, E. 1999. Seminal lead and copper in infertile men. In *Andrologia*, vol. 22, 1999, vol. 503-511.

KANKOFER M. - LIPKO J. - ZDUNCZYK S. 2005. Total antioxidant capacity of bovine spontaneously released and retained placenta. In *Pathophysiology*, vol. 11, 2005, p. 215–219.

KANWAL, M.R. – REHMAN, N.U. – AHMAD, N. – SAMAD, H.A. – REHMAN, Z.U. – AKHTAR, N. ALI, S. 2000. Bulk cations and trace elements in the Nili-Ravi buffalo and crossbred cow bull semen. In *International Journal of Agriculture and Biology*, vol. 2, 2000, p. 302-305.

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. 9, 2012, p. 1294-1300.

KWENANG, A. - KROOS, M. J. - KOSTER, J. F. - VAN EIJK, H. G. 1987. Iron, ferritin and copper in seminal plasma. In *Human Reproduction*, vol. 2, 1987, p. 387-388.

LUKÁČ, N. – MASSÁNYI, P. - KROČKOVÁ, J. - NAĎ, P. - SLAMEČKA, J. - ONDRUŠKA, Ľ. - FORMICKI, G. – TRANDŽÍK, J. 2009. Relationship between trace element concentrations and spermatozoa quality in rabbit semen. In *Slovak Journal of Animal Science*, vol. 42, 2009, supplement 1, p. 46-50.

LYKKESFELDT, J. – SVENSEN, O. 2007. Oxidants and antioxidants in disease: oxidative stress in farm animals. In *The Veterinary Journal*, vol. 173, 2007, no. 3, p. 502-511.

MACHAL, L. - CHLADEK, G. - STRAKOVA, E. 2002. Copper, phosphorus and calcium in bovine blood and seminal plasma in relation to semen quality. In *Journal of Animal and Feed Sciences*, vol. 11, 2002, p. 425-435.

MARZEC-WRÓBLEWSKA, U. – KAMIŃSKI, P. - ŁaKOTA, P. 2012. Influence of chemical elements on mammalian spermatozoa. In *Folia Biologica (Praha)*, vol. 58, 2012, p. 7-12.

MASSÁNYI, P. - TRANDŽÍK, J. – NAĎ, P. – TOMAN, R. - SKALICKÁ, M. - KORÉNEKOVÁ, B. 2003a. Seminal concentrations of trace elements in various animals and their correlations. In *Asian Journal of Andrology*, vol. 5, 2003, no. 2, p. 101-104.

MASSÁNYI, P. - TRANDŽÍK, J. – NAĎ, P. - KORÉNEKOVÁ, B. - SKALICKÁ, M. – TOMAN, R. – LUKÁČ, N. – STRAPÁK, P. – HALO, M. – TURČAN, J. 2003b. Concentration of copper, iron, zinc, cadmium, lead and nickel in boar semen and relation to

the spermatozoa quality. In *Journal of Environmental Science and Health Part A*, vol. 38, 2003, p. 2643-2651.

MASSÁNYI, P. - TRANDŽÍK, J. – NAĎ, P. – KORÉNEKOVÁ, B. - SKALICKÁ, M. – TOMAN, R. - LUKÁČ, N. – STRAPÁK, P. - HALLO, M. – TURČAN, J. 2003c. Concentration of copper, iron, zinc, cadmium, lead and nickel in boar semen and relation to the spermatozoa quality. In *Journal of Environmental Science and Health Part A,* vol. 38, 2003, p. 2634-2651.

MASSÁNYI, P. - TRANDŽÍK, J. – NAĎ, P. – KORÉNEKOVÁ, B. - SKALICKÁ, M. – TOMAN, R. - LUKÁČ, N. – HALLO, M. - STRAPÁK, P. 2004. Concentration of copper, iron, zinc, cadmium, lead and nickel in bull and ram semen and relation to the occurence of pathological spermatozoa. In *Journal of Environmental Science and Health Part A,* vol. 39, 2004, p. 3005-3014.

MASSÁNYI, P. - TRANDŽÍK, J. – NAĎ, P. – SKALICKÁ, M. - KORÉNEKOVÁ, B. – LUKÁČ, N. – FABIŠ, M. - TOMAN R. 2005. Seminal concentration of trace elements in fox and relationships to spermatozoa quality. In *Journal of Environmental Science and Health Part A*, vol. 40, 2005, p. 1097-1105.

MASSÁNYI, P. – WEIS, J. - LUKÁČ, N. – TRANDŽÍK, J. – BYSTRICKÁ, J. 2008a. Cadmium, zinc, copper, sodium and potassium concentrations in rooster and turkey semen and their correlation. In *Journal of Environmental Science and Health Part A*, vol. 43, 2008, p. 563-565.

MASSÁNYI, P. - CHRENEK, P. - LUKÁČ, N. - MAKAREVICH, A. V. - OSTRO, A. - ŽIVČÁK, J. - BULLA, J. 2008b. 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.

MILLER, N. J. – RICE-EVANS, C. – DAVIES, M. J. – GOPINATHAN, V. – MILNER, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. In *Clinical Science*, vol. 84, 1993, p. 407-412.

OKADA, S. 1998. Iron and carcinogenesis in laboratory animals and humans: a mechanistic consideration and a review of literature. In *International Journal of Clinical Oncology*, vol. 3, 1998, p. 191–203.

PESCH, S. – BERGMANN, M. – BOSTEDT, H. 2006. Determination of some enzymes and macro-and microelements in stallion seminal plasma and their correlations to semen quality. In *Theriogenology*, vol. 66, 2006, no. 2, p. 307-313.

RICE-EVANCE, C. – MILLER, N.J. 1994. Total antioxidant status in plasma and body

fluids. In *Methods in Enzymology*, vol. 234, 1994, p. 279-293.

ROYCHOUDHURY, S. – SLIVKOVÁ, J. – BULLA, J. – MASSÁNYI, P. 2008. Copper administration alerts fine parameters of spermatozoa motility *in vitro*. In *Folia Veterinaria*, vol. 52, 2008, p. 64-68.

SHARP P. 2004. The molecular basis of copper and iron interactions. In *Proceedings of the Nutrition Society*, vol. 63, 2004, p. 563–569.

TVRDÁ, E. – KŇAŽICKÁ, Z. – BÁRDOS, L. – MASSÁNYI, P. - LUKÁČ, N. 2011. Impact of oxidative stress on male fertility - a review. In *Acta Veterinaria Hungarica*, vol. 59, 2011, no. 4, p. 465-484.

TVRDA, E. - KNAZICKA, Z. - LUKAC, N. 2012. Selected heavy metals versus antioxidant parameters in bull seminal plasma – a comparative study. In *Journal of Environmental Science and Health Part A*, vol. 47, 2012, no. 9, p. 1261-1266.

ZIYATDINOVA, G.K. – GILMETDINOVA, D.M. – BUDINKOV, G.K. 2005. Reactions of superoxide anion radical with antioxidants and their use in voltammetry. In *Journal of Analytical Chemistry*, vol. 60, 2005, p. 56–59.