Animal welfare, etológia és tartástechnológia

Animal welfare, ethology and housing systems

Volume 7 Issue 4

Különszám/Special Issue

Gödöllő
2011

HEAVY METALS AFFECTING ANTIOXIDANT STATUS IN BULL SEMINAL PLASMA – A COMPARATIVE STUDY

Tvrdá Eva, Kňažická Zuzana, Massányi Peter, Bárdos László[1], Kerti Annamária[1], Formicki Grzegorz[2] and Lukáč Norbert

Slovak University of Agriculture, Nitra, Slovak Republic
[1]Szent István University, Gödöllő, Hungary
[2]Pedagogical University of Cracow, Cracow, Poland
[3]University of Veterinary Medicine and Pharmacology, Kosice, Slovak Republic
norolukac@gmail.com

Abstract

Lead (Pb) and cadmium (Cd) are the two most abundant toxic metals in the environment, which have been associated with male reproductive toxicity. These metals have been shown to be associated with an overproduction of reactive oxygen species (ROS) and an impairment of antioxidant defensive capacity. The aim of this study was to evaluate, compare and assume relationships between lead and cadmium content, basic motility characteristics and selected antioxidant parameters (total antioxidant status, superoxide dismutase, and albumin) in the bovine seminal plasma. Semen samples from 30 breeding bulls were used in the study. Motility analysis was carried out using the Computer Assisted Sperm Analysis (CASA) system. Subsequently, the samples of seminal plasma were collected. Pb and Cd concentrations were determined by the voltametric method (ASV), antioxidant parameters were analyzed by UV/VIS spectrophotometry using commercial kits. The analysis showed that the average concentrations of the trace elements were 0.57±0.01µg/mL for Pb and 0.11±0.01 µg/mL for Cd. The correlation analysis revealed that both heavy metals were negatively correlated with motility ($r=-0.777$; $p<0.001$ for Pb and $r=-0.786$; $p<0.001$ for Cd), total antioxidant status ($r=-0.375$; $p>0.05$ and $r=-0.334$; $p>0.05$, respectively), superoxide dismutase ($r=-0.746$; $p<0.001$ and $r=-0.537$; $p<0.05$, respectively) as well as with albumin ($r=-0.609$; $p<0.01$ and $r=-0.699$; $p<0.001$, respectively). This study demonstrates that Pb and Cd are serious toxic elements, which are able to increase the risk of oxidative stress development and a subsequent decrease of semen quality.

Keywords: oxidative stress, lead, cadmium, total antioxidant status, superoxide dismutase, albumin, spermatozoa, bulls.

INTRODUCTION

The unrestricted developmental activities such as industrialization and urbanization carried out during the past few decades have given rise to serious problems of environmental pollution *(Azmat et al., 2005)*. Contamination of the food chain with heavy metals may negatively influence both the health status as well as animal production. Unfavourable effect on the animal health may depend on the kind of the element and its dose as well as on the utility orientation *(Slivkova et al., 2010)*.

Lead (Pb) and cadmium (Cd) are the two most abundant toxic metals in the environment. Their common sources are diverse including natural and anthropogenic processes such as combustion of coal and mineral oil, smelters, mining, as well as paint industries. *(Phillips et al., 2003, Patra et al. 2005, Patra et al. 2007)* Anthropogenic activities and vehicular emissions contribute to the entry of toxic metals to human and animal food chains *(Okada et al., 1997)*.

Pb and Cd do not have any detectable beneficial biological roles. On the contrary, their detrimental effects on physiological, biochemical, and behavioural dysfunctions in animals and humans have been documented by several investigators *(Ruff et al., 1997, Kaji et al., 2004, Kramarova et al, 2005)*. Higher levels affect the central and peripheral nervous systems *(Dressier et al., 1997)*, haemopoietic system *(De Silva et al., 1982)*, cardiovascular system (*Khalil-Manesh et al., 1993)*, kidneys and liver *(Kramarova et al., 2005)*. Pb and Cd contamination has also been associated with male reproductive toxicity in experimental animals and has the potential to produce adverse effects on fertility *(Rao et al., 2001)*.

Of late, lead and cadmium-induced tissue damages have been attributed, at least in part, to toxicant-induced oxidative stress *(Patra, et al., 2001, Patra, et al., 2011)*. These metals have been shown to be associated with an overproduction of reactive oxygen species (ROS) and an impairment of antioxidant defensive capacity *(Stohs et al., 1995)*. There is growing evidence to suggest that oxidative stress (OS) is involved in many aspects of male infertility. Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by OS *(Alvarez et al., 1987)*. Spermatozoa are unable to repair the damage induced by excessive ROS because they lack the cytoplasmic enzyme systems that are required to accomplish this repair *(Aitken et al., 1995)*. High concentrations of polyunsaturated fatty acids in sperm cell membranes make spermatozoa more susceptible to lipid peroxidation than other cells *(Aitken et al., 1993)*. This combination of susceptibility along with a relative lack of vigorous intracellular defence mechanisms is exacerbated by the autogenous production of ROS by spermatozoa *(Duru et al., 2000)*. Inversely, the seminal plasma possesses a wide antioxidant system to scavenge ROS and prevent ROS related cellular damage. Antioxidants present in the seminal plasma are the most important form of protection available to spermatozoa against OS development *(Sikka et al., 1995, Sharma et al. 1996)*. The amounts of high molecular weight antioxidant enzymes such as superoxide dismutase, glutathione

peroxidase/reductase and catalase in semen have been measured in several studies *(Zini et al., 1993,* Kobayashi *et al. 2001)*. Additionally, low molecular weight scavengers from seminal plasma, such as albumin, uric acid, carotenoids and taurine appear to be more important than high molecular weight components *(Alvarez et al., 1989)*. Based on these facts, the aim of this study was to evaluate, compare and assume relationships between lead and cadmium content, basic motility characteristics and selected antioxidant parameters in the bovine seminal plasma. The antioxidant parameters were as follows:

- total antioxidant status (TAS) as the ability of all antioxidants in the sample to neutralize a prooxidant compound *in vitro*,
- superoxide dismutase (SOD) as a major antioxidant enzyme are produced by the organism,
- albumin (ALB) as an important non-enzymatic substance with antioxidant properties.

MATERIALS AND METHODS

Biological Material

Bovine semen samples were obtained from 30 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 Analysis

Spermatozoa motility 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) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5µm/s; MOT), percentage of progressive motile spermatozoa (motility > 20µm/s; PROG). 1000 cells were examined for each sample *(Massányi et al., 2008)*.

Samples Processing

The samples were centrifuged (15 min, 12000 rpm, 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.

Heavy Metal Analysis

For Pb and Cd detection the seminal plasma samples (at least 1 mL) were mineralized in the

laboratory. All material of 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. Pb and Cd concentration was determined by the voltametric method (ASV) using an EA9C potentiostat model equipped with working CGMDE electrode (MTM, Krakow, Poland), $AgCl_2$ and platinum electrodes. Concentrations are expressed as μg/mL.

Antioxidant Parameters Measurement

All of the measurements were based on a colorimetric reaction of the target substance and a subsequent UV/VIS spectrophotometric detection at a specific wavelength. TAS, SOD and ALB were measured using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc, USA).

The TAS Randox (Randox Laboratories, Crumlin, Great Britain) assay was based on an incubation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and H_2O_2 to produce the $ABTS^{®*+}$ radical cation. This had a relatively stable blue-green color, which was measured at 600 nm. SOD activity was analyzed with the Randox RANSOD assay (Randox Laboratories, Crumlin, Great Britain). This method employed xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. ALB concentration was measured using BioLa Test (PLIVA-Lachema, Brno, Czech republic) commercial kit. The measurement was based on the reaction between albumin and Bromocresol Green (BCG) at acid pH forming a complex, which was easy to detect photometrically at 578 nm.

Statistical Analysis

Statistical analysis of the results 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 error of mean (SEM). Pearson's correlation coefficient (two tailed) test was used to examine correlations between all the analyzed parameters.

RESULTS AND DISCUSSION

The results of the semen and seminal plasma evaluation in *Table 1* show that the total antioxidant status of the seminal plasma was 1.87±0.02 mmol/L, while the superoxide dismutase activity was 2.98±0.05 U/mL. The mean albumin concentration was recorded as 14.62±0.20 g/L. Average concentrations of the trace elements measured by the ASV method were 0.57±0.01μg/mL for lead and 0.11±0.01 μg/mL for cadmium.

Table 1. **Semen characteristics of bull semen samples (n=30)**

PARAMETER	MEAN±SEM
Lead (µg/mL)	0.57±0.01
Cadmium (µg/mL)	0.11±0.01
Total antioxidant status (mmol/L)	1.87±0.02
Superoxide dismutase (U/mL)	2.98±0.05
Albumin (g/L)	14.62±0.20
Motility (%)	89.99±0.39
Progressive motility (%)	87.68±0.37

Comparing these results with other authors we found out that according to *Massanyi et al. (2008)* the seminal lead concentration in bulls was 0.06±mg/kg, which was lower than in rams (0.35±0.68 mg/kg) and foxes (0.08±0.06 mg/kg), but higher than in stallion (0.05±0.05 mg/kg) or boars (0.02±0.03 mg /kg). Meanwhile, the total lead content of the seminal plasma of buffalo bulls in the study of *Eghbali et al. (2008)* was 0.026 ± 0.0008 mg/L.

Discussing the cadmium content in bovine semen, similar *in vivo* concentrations were detected by *Massanyi et al. (2003)* (0.100±0.14 mg/kg). When comparing the cadmium content in different species, the authors found out that the concentration of cadmium in the semen of all studied animals is very similar and no significant differences were found.

Pb and Cd are widespread, highly toxic environmental pollutants. Both accumulate in biological systems and because of a long biological half-life (10–30 years) in animals, they accumulate with advancing age *(Xu et al., 2001)*. According to *Eghbali et al. (2010)* lead intoxication during spermatogenesis can delay spermiation as well as release of immature spermatogenic cells in the tubules of testis. Low level of exposure may cause testicular atrophy, cellular degeneration, reduction in seminiferous tubule diameter and sperm count. Cadmium accumulates preferentially in male reproductive organs *(Xu et al., 2001)*. An *in vivo* study by *Xu et al. (2001)* demonstrates that Cd reduces rat sperm movements at a dose far below the dose affecting sperm production.

Recent research examining the etiology of Pb and Cd toxicity-induced oxidative stress reveals that the free radical production and lowering of inherent antioxidant reserves resulting from Pb and Cd toxicity are directly related to a fertility decrease *(Patra et al., 2011)*. Lead has two common valencies, Pb^{2+} and Pb^{4+}, and does not by itself catalyze free radical reactions. However, Pb^{2+} can apparently enter cells by passing though Ca^{2+} channels. Pb combines quickly with the sulfhydryl groups on proteins and, at high concentrations, can cause their depletion *(Liu et al., 2001)*. Cadmium is a nonredox metal, therefore it is unlikely to participate in Fenton-type reactions. However, Cd can compete with essential metals in protein-binding sites leading to the release of Fe^{2+} and Cu^{2+} ions causing an increased production of ROS and oxidative stress development *(Aitken et al., 1999)*. Cd depletes glutathione and protein-bound sulfhydryl groups, which results in an enhanced production of ROS such as the superoxide

ion, hydroxyl radicals and hydrogen peroxide *(Sikka et al., 2001)*.

In our study, the correlation analysis (*Table 2*) revealed a strong positive correlation between the cadmium and lead concentrations (r=0.713; p<0.001). Negative correlations were detected between both heavy metals and motility as well as antioxidant parameters. Lead together with cadmium negatively affected both motility (r=-0.777; p<0.001 for Pb and r=-0.786; p<0.001 for Cd) and progressive motility (r=-0.763; p<0.001 for Pb and r=-0.792; p<0.001 for Cd). The two metals had similar negative effects on the total antioxidant status (r=-0.375; p>0.05 and r=-0.334; p>0.05, respectively) and albumin (r=-0.609; p<0.01 and r=-0.699; p<0.001, respectively). Superoxide dismutase activity was negatively influenced by both lead and cadmium, however the deleterious effects of cadmium were more apparent (r=-0.746; p<0.001 compared to r=-0.537; p<0.05).

***Table* 2. Correlations between lead and cadmium content, selected spermatozoa motility parameters and antioxidant parameters in bovine seminal plasma (n=30)**

	Pb	Cd	TAS	SOD	ALB	MOT	PROG
Pb	1						
Cd	0.713***	1					
TAS	-0.375	-0.334	1				
SOD	-0.537*	-0.746***	0.426	1			
ALB	-0.609**	-0.699***	0.559**	0.682***	1		
MOT	-0.777***	-0.786***	0.467*	0.569**	0.728***	1	
PROG	-0.763***	-0.792***	0.435*	0.539*	0.699***	0.992***	1

The correlation analysis was based on the value of the correlation coefficient: ±0.111 - ±0.333: low correlation; ±0.334 – ±0.666: moderate correlation; ±0.667 - ±0.999: high correlation. * - p<0.05; ** - p<0.01; *** - p<0.001

Antioxidants present in the seminal plasma are the most important form of protection available to spermatozoa against reactive oxygen species *(Mylroie et al., 1986)*. They provide a defence mechanism through several levels of protection, prevention, interception and repair. A growing body of evidence suggests that low seminal antioxidant capacity is related to male infertility *(El-Tohamy et al., 2010)*. A proper function of some enzymatic as well as non-enzymatic antioxidants is greatly influenced by heavy metals. SOD as a typical metal-enzyme has a prosthetic group that may be replaced by heavy metals leading to an inhibition of enzyme activity *(El-Tohamy et al., 2010)*. Pb and Cd are able to mimic and replace zinc in its sites, this suggests interactions of Pb and Cd with the copper, zinc and manganese SOD isoenzymes *(Keogh et al., 1992)*. Such interactions may be an explanation for the results obtained by *El-Tohamy and El-Nattat (2010)* and for our significantly negative correlations between the Pb and Cd content and SOD activity, as well as the decreasing activity of SOD according to an increasing concentration of Pb and Cd in the quality groups.

Furthermore, it is known that Pb or Cd can reduce the antioxidant defense systems of cells via depleting glutathione and albumin, containing sulfhydryl groups (-SH) at their site of the action *(Othman*

et al., 1998), which are a target for metals, which is possibly why a highly negative correlation was found also between the examined heavy metals and albumin concentration. Cd and Pb may also produce oxidative stress indirectly, depleting glutathione and albumin levels or via metal-induced displacement of redox metal ions *(Bae et al., 2001)*. On the other hand, according to *Othman and El Missiry (1998)* administration of selenium before Pb exposure produced a significant prophylactic action against Pb-induced oxidative stress by means of increasing SOD, glutathione reductase activity, glutathione and albumin content in male rats. In summary, impaired antioxidant defences can be a result of the inhibitory effects of lead on various enzymes, which in turn causes the cells to be more susceptible to oxidative insult. Pb and Cd are now recognized as two of the most important heavy metal contaminants in the environment. Since the two elements are often released simultaneously in the environment form a number of sources, adverse health effects caused by combined exposure to Pb and Cd has provoked a significant health concern. Lead and cadmium are the two most abundant toxic metals in the environment. The coexposure to these two toxic metals usually has a synergistic cytotoxicity *(Phillips et al, 2003, Patra et al, 2011*, *Bae et al., 2001)*, which explains the highly positive correlation between the two metals detected in our study. *Snow (1992)* suggests that the negative effects could be a result of several mechanisms, such as induction of cellular immunity and oxidative stress, the inhibition of DNA metabolism and repair, the formation of DNA and/or protein cross-links. Furthermore, a high positive correlation between seminal cadmium and lead in ram (r=0.976) and boar (r=0.973) was detected by *Massanyi et al. (2003)*.

CONCLUSION

Our results demonstrate that lead and cadmium are serious toxic elements, which are able to increase free radicals formation as well as antioxidants depletion, the risk of oxidative stress development followed by a decrease of semen quality parameters. The study suggest that even a weak enhancement of these elements in ejaculates might cause fertility disorders in animals and subsequently also in humans.

ACKNOWLEDGMENTS

This study was supported by the Scientific Grant of the Slovak Republic VEGA no. 1/0532/11 and by the Cultural and Educational Grant of the Slovak Republic KEGA no. 101-001SPU-4/2010.

REFERENCES

Aitken, R.J.; Paterson, M.; Fisher, H.; Buckingham, D.W.; Van Duin, M. (1995): Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J. Cell. Sci. 1995, *8*, 2017- 2025.

Aitken, R.J.; Harkiss, D.; Buckingham, D.W. (1993): Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol. Reprod. Dev. 1993, *35*, 302-315.

Aitken, R.J. (1999): The Amoroso lecture: the human spermatozoon-a cell in crisis? J. Reprod. Fertil. 1999, *115*, 1–7.

Alvarez, J.G.; Touchstone, J.C.; Blasco, L.; Storey, B.T. 1987): Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J. Androl. 1987, *8*, 338-348.

Alvarez, J.G.; Storey, B.T. (1989): Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 1989, *23*, 77–90.

Azmat, R.; Parveen, R.; Naqvi, I.I.; Shoukat, S. (2005): Effect of Cr (III) Combine with atrazine on protein, carbohydrate, amino acid and chlorophyll content in *Vigna radita* (L.) Wilczek. Int. J. Bio. & Biotechnol. 2005, *2*(2), 433-439.

Bae, D.S.; Gennings, C.; Carter, W.H.; Yang, R.S.H.; Campain, J.A. (2001): Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes. Toxicol. Sci. 2001, *63*(1), 132–142.

Bauer, R.O.; Demeter, I.; Hasemann, V.; Johansen, J.T. (1980): Structural properties of the zinc sites in Cu, Zn, superoxide dismutase: Perturbed angular correlation of gamma rays spectroscopy on the Cu-Cd superoxide dismutase derivative. Biochem. Biophys. Res. Commun. 1980, 94, 1296-1302.

De Silva, P. E. (1981): Determination of lead in plasma and studies on its relationship to lead in erythrocytes. Braz. J. Indig. Med. 1981, *38*, 209–217.

Dressier, J.; Kim, K.A.; Chakraborti, T.; Goldstein, G. (1999): Molecular mechanisms of lead neurotoxicity. Neurochem. Res. 1999, *24*(4), 595–600.

Duru, K.; Morshedi, N.; Oehninger, S. (2000): Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil. Steril. 2000, *74*, 1200–1207.

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. Vet. Res. For. 2010, *1*(3), 142 – 148.

El-Tohamy, M.M.; El-Nattat,W.S. (2010): Effect of antioxidant on lead-induced oxidative damage and reproductive dysfunction in male rabbits. J. Am. Sci. 2010, *6*(11), 613-622.

Halliwell, B.; Gutteridge, J.M.C. (1999): Free radicals in biology and medicine; 3rd Edition; Oxford University Press: Oxford, 1999; 584–591.

Kaji, T. (2004): Cell biology of heavy metal toxicity in vascular tissue. Yakugaku Zasshi. **2004**, *5*, 245-250.

Keogh, J.P. Lead. (1992): In: Hazardous Materials Toxicology. Clinical Principles of Environmental Health; Sullivan, J.B.; Krieger, G.R., Eds.; Williams and Wilkins: Baltimore, 1992; 834-844.

Khalil-Manesh, F.; Gonick, H.C.; Weiler, E.W.J.; Prins, B.; Weber, M.A.; Purdy, R.E. (1993): Lead-induced hypertension: possible role of endothelial factors. Am. J. Hypertens. 1993, *6*(9), 723–729.

Kobayashi, H.; Gil-Guzman, E.; Mahran, A.; Sharma, R.K.; Nelson, D.; Thomas, A.J.; Agarwal, A. (2001): Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J. Androl. 2001, *22*, 568–574.

Kramarova, M.; Massanyi, P.; Slamecka, J.; Tataruch, F.; Jancova, A.; Gasparik, J.; Fabis, M.; Kovacik, J.; Toman, R.; Galova, J.; Jurcik, R. (2005): Distribution of cadmium and lead in liver and kidneys of some wild animals in Slovakia. J. Environ. Sci. Health, Part A. 2005, *40*, 593-600.

Liu, J.; Shen, H.M.; Ong, C.N. (2001): Role of intracellular thiol depletion, mitochondrial dysfuction and reactive oxygen species in Salvia miltiorrhiza-induced apoptosis in human hepatoma HepG2 cells. Life Sci. 2001, *69*, 1833–1850.

Massanyi, P.; Trandzik, J.; Nad, P.; Toman, R.; Skalicka, M.; Korenekova, B. (2003): Seminal concentrations of trace elements in various animals and their correlations. Asian J. Androl. 2003, *5*(2), 101-104.

Massányi, P.; Chrenek, P.; Lukáč, N.; Makarevich, A.V.; Ostro, A.; Živčak, J.; Bulla, J. (2008): Comparison of different evaluation chambers for analysis of rabbit spermatozoa moility using CASA system. Slovak J. Anim. Sci. 2008, *41*(2), 60-66.

Mylroie, A.A.; Collins, H.; Umbles, C.; Kyle, J. (1986): Erythrocyte superoxide dismutase activity and other parameters of copper status in rats ingesting lead acetate. Toxicol. Appl. Pharm. 1986, 82, 512-520.

Okada, I.A.; Sakuma, A.M.; Maid, F.D.; Dovidemskas, S.; Zenebon, O. (1997): Evaluation of lead and cadmium in milk due to environmental contamination in Paraiba valley region of South Estern Brazil. Raissade-Saude-Publica. 1997, *31*, 140–143.

Othman, I.; El Missiry, M.A. (1998): Role of selenium against lead toxicity in male rats. J. Biochem. Mol. Toxicol. 1998, *12*(6), 345–349.

Patra, R.C.; Swarup, D.; Dwivedi, S. K. (2001): Antioxidant effects of α tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology. 2001, *162*(2), 81–88.

Patra, R.C.; Swarup, D.; Naresh, R.; Kumar, P.; Shekhar, P.; Ranjan, R. (2005): Cadmium level in blood and milk from animals reared around different polluting sources in India. Bull. Environ. Contam. Toxicol. 2005, *74*(6), 1092–1097.

Patra, R.C.; Swarup, D.; Naresh R.; Kumar, P.; Nandi, D.; Shekhar, P.; Roy, S.; Ali, S.L. (2007): Tail hair as an indicator of environmental exposure of cows to lead and cadmium in different industrial areas. Ecotoxicol. Environ. Saf. 2007, *66*(1), 127–131.

Patra, R.C.; Rautray, A.K.; Swarup, D. (2011): Oxidative stress in lead and cadmium Toxicity and its amelioration. Vet. Med. Int. 2011, Article in press. doi:10.4061/2011/457327.

Phillips, C.; Gyori, Z.; Kovacs, B. (2003): The effect of adding cadmium and lead alone or in combination to the diet of pigs on their growth, carcase composition and reproduction. J. Sci. Food Agr. 2003, *83*(13), 1357–1365.

Pruski, A.M.; Dixon, D.R. (2002): Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. Aquat. Toxicol. 2002, *57*,127–137.

Rao, M.V.; Sharma, P.S. (2001): Protective effect of vitamin E against mercuric chloride reproductive toxicity in male mice. Reprod. Toxicol. 2001, *15*, 705-712.

Ruff, H.A.; Markowitz, M.E.; Bijur, P.E.; Rosen, J.F. (1996): Relationships among blood lead levels, iron deficiency, and cognitive development in two-year-old children. Environ. Health Perspect. 1996, *104*(2), 180–185.

Sikka, S.C.; Rajasekaran, M.; Hellstrom, W.J. (1995): Role of oxidative stress and antioxidants in male infertility. J. Androl. 1995, *16*, 464-468.

Sikka, S.C. (2001): Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem. 2001, *8*, 851–862.

Sharma, R. K.; Agarwal, A. (1996): Role of reactive oxygen species in male infertility. Urology. 1996, *48*, 835–850.

Slivkova, J.; Massanyi, P.; Pizzi, F.; Trandzik, J.; Roychoudhury, S.; Lukac, N.; Dankova, M.; Almasiova, V. (2010): In vitro toxicity of mercuric chloride on rabbit spermatozoa motility and cell membrane integrity. J. Environ. Sci. Health, Part A. 2010, *45*, 767 – 774.

Snow, E.T. (1992): Metal carcinogenesis: mechanistic implications. Pharmacol. Ther. 1992, *53*, 31–65.

Stohs, S.J.; Baggihi, D. (1995): Oxidative mechanisms in the toxicity of metal ions. Free Radic. Med. 1995, *18*, 321–336.

Zini, A.; De Lamirande, E.; Gagnon, C. (1993): Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. Int. J. Androl. 1993, *16*, 183–188.

Xu, L.C.; Wang, S.Y.; Yang, X.F.; Wang, S.R. (2001): Effects of cadmium on rat sperm motility evaluated by computer assisted sperm analysis. Biomed. Environ. Sci. 2001, *14*(4), 312–317.